**HABILITATION À DIRIGER DES RECHERCHES**

**Discipline : Sciences de la Vie**

**Année universitaire : 2019/2020**

# *Communication chez les insectes : signaux, adaptation, spécialisation*

Présenté et soutenu publiquement par

**Christophe Lucas**

Le mercredi 18 décembre 2019

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**JURY :**
***(Par ordre alphabétique)***

| | | | |
|---|---|---|---|
| Mme Anne-Geneviève | BAGNERES-URBANY | Directeur de Recherche CNRS | Université de Montpellier |
| M. Damien | CHARABIDZE | Maître de Conférences-HDR | Université de Lille |
| Mme Emmanuelle | JACQUIN-JOLY | Directeur de Recherche INRA | Sorbonne Université |
| M. Claudio | LAZZARI | Professeur des universités | Université de Tours |
| M. Joël | MEUNIER | Chargé de Recherche-HDR | Université de Tours |
| M. Thibaud | MONNIN | Directeur de Recherche CNRS | Sorbonne Université |
| M. Yves | ROISIN | Professeur | Université libre de Bruxelles |
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« Ces merveilles m'étaient connues par la lecture ; mais voir, de ses propres yeux voir, et du même coup expérimenter un peu, c'est bien autre chose. »

Jean-Henri Fabre (1823-1915)

# Remerciements

*Voici la section la plus informelle, celle où l'on peut tout dire, en ayant l'impression que personne ne la lira, un peu comme un carnet intime. En réalité, les méta-analyses montrent que c'est la partie la plus lue de ce type de mémoire (Lucas, Pers. Com.). Alors, toi qui passes par-là, voici la longue liste de mes remerciements, car la recherche est avant tout une histoire de rencontres, d'opportunités, de déconfits et de réussites, c'est-à-dire une histoire d'aventure, une histoire de personnes sans qui rien ne serait.*

*Mon épopée scientifique commence avec mon DEA (les plus jeunes d'entre vous peuvent allez chercher ce que cela signifie dans le glossaire), où Max Goyffon m'avait convaincu d'étudier le monde des animaux venimeux au Muséum. Lors de mon stage, les tarentules de Marie-Louise Célérier m'ont ouvert les portes de Jussieu. Marie-Louise dont le dynamisme m'a donné envie de continuer en thèse. C'est là où mon intérêt pour les analyses chimiques et pour la chromatographie a commencé.*

*De mes "connaissances" en chimie, Dominique Fresneau m'a proposé un sujet de thèse, lors d'une rencontre mythique au Quick, pour parler lipophorine et sauce burger. Projet qui s'est réalisé à Orsay dans le laboratoire de Jean-Marc Jallon, en étroite collaboration avec mon mentor à jamais, l'indétrônable sage, Dang Ba Pho. Au moment où j'écris ces lignes, j'apprends le décès de Jean-Marc, une triste nouvelle qui me laisse sans voix. Il faut savoir que 5 ans après ma thèse, Jean-Marc m'appelait tous les 6 mois pour discuter projet et me pousser à avancer, en considérant que c'était de son devoir de directeur de thèse. Je leur renouvelle toute mon amitié et mon respect, sans eux rien n'aurait pu commencer.*

*Puis au détour d'un séminaire interne, j'ai croisé Marla Sokolowsky, une femme extraordinaire mêlant connaissance, gentillesse, vivacité d'esprit. Elle m'a accueilli à Toronto pour un post-doc dont j'avais assuré le financement pour un an grâce à la formidable fondation Fyssen. J'y suis resté 4 ans, grâce à un renouvellement exceptionnellement fournit par la fondation, et surtout grâce à l'indéfectible confiance de Marla en mes projets. C'est durant cette période que Joel Levine m'a intégré dans ses projets fous. Ce visionnaire qui voulait chambouler nos connaissances sur la communication chez la droso. Ces nombreuses communications dans nature et science lui donnèrent raison. A Toronto, j'ai rencontré des gens formidables qui ont permis au petit "Français" de s'intégrer dans cet immense pays qu'est le Canada, où Tony le vendeur de télé du coin de la rue te prête sa voiture si*

*tu en as besoin, alors que l'on ne s'est vu qu'une fois... Je pense bien sûr à Karla, Reza, Craig, Sam (tu les as fini ces livres ?), Amsale, John, Scott (tes primers déchirent) et mes étudiants Bianco (encore merci pour ta carte d'anniversaire annuelle), Jennifer, Julia. Un remerciement particulier pour Benjamin PC Smith, qui a toujours été présent, surtout dans les moments difficiles comme un vrai ami et qui m'a accueilli chez lui en Suisse lors de mes péripéties européennes. Mon buddy de bureau Ken Dawson-Scully: I keep special cans at home for you, whenever wherever!*

*Puis vient mon passage à Lausanne grâce à Laurent Keller qui m'a débauché du Canada en m'invitant à rentrer en Europe pour poursuivre ma carrière. Lausanne avec son ambiance scientifique et humaine hors du commun, où j'ai fait la rencontre de Romain, Michael, Dietrich, Alex, Valérie, let's go FIPE!*

*Merci à la direction de l'IRBI, au directeur actuel David Giron toujours présent pour un conseil et à Christelle Suppo Magal qui a été très bonne conseillère ces dernières années.*

*Des remerciements particuliers pour Anne-Geneviève Bagnères et Martine Hossaert avec leurs très nombreux conseils lors de ma candidature au CNRS. Ainsi que la directrice de l'INEE Stéphanie Thiebault et le président de la section 29 Fabrice Vavre pour leur suivi attentionné.*

*Je remercie les membres du jury qui ont montré beaucoup de bienveillance dans ce processus d'écriture.*

*Il y a trop de monde à L'IRBI pour vous citer tous, alors je remercie évidemment tous les membres de notre jeune et dynamique équipe ESORE. Evidemment, à l'homme de terrain Simon et son omniprésence au labo. A l'incontournable Carole qui me suit dans toutes mes idées d'expérience et à Séverine pour son aide précieuse. A Karine et Annie, toujours présentes pour m'aider lorsque je cherche du matériel pour bricoler un truc ! A mon ancienne collègue de bureau Elfie et sa gentillesse. Miguel et Thibaut pour leur bonne humeur et leur soutien. Aux supers "précaires", comme ils se nomment eux-mêmes, Sophie, Anthony, Caroline (plus qu'une semaine !) et l'infatigable Fanny avec son indéfectible moral positif et son dynamisme dans nos longues conversations scientifiques.*

*Merci tout particulier à Charlotte qui est toujours présente pour discuter et qui sais tendre l'oreille pour m'écouter. Au jeune Joël de Lausanne et au nouveau collègue de bureau. A nos discussions scientifiques, politiques, culturelles, à nos*

*longues redéfinitions des interactions humaines. Si un jour tu cherches une pelle de jardin, saches que tu pourras prendre celle qui se trouve dans mon garage.*

*Mes voisins du haut du côteau, pour m'aider à me souvenir qu'il n'y a pas que la science dans la vie, il y a le sport aussi.*

*Parce que le plus important dans la vie c'est la famille, je remercie ma mère qui a toujours trouvé un petit quelque chose de spécial en moi et qui a su le cultiver (je suis sûr que tu trouves que cette police d'écriture déchire!), mon père qui n'a pas besoin de me dire qu'il est fier de moi car je le lis dans ses yeux, à mon grand frère qui a toujours ouvert la voie, à pépé, mamita et mémé qui ne pouvaient/peuvent pas voir un insecte sans penser à moi, à Bô pôpô et Martiiiiine qui m'ont toujours fait confiance pour mener la barque à bon port, à ma GGteam Gautier et Grégoire, qui ont réussi, grâce à leurs questions sans détours et toujours justes, à m'inspirer tout au long de cette écriture. Pour reprendre un dialogue avec ma famille : "...Finalement l'HDR, c'est un bilan de ce que j'ai fait en science depuis que je suis chercheur... C'est comme la crise de la cinquantaine quoi ?!? oui mais scientifique...".*

*Comment définir ce qui ne peut, comment parler de la personne avec qui j'ai partagé 25 ans... déjà. Aucun mot ne pourrait parvenir à définir ce que je ressens en vivant à tes côtés, malgré les aléas de la vie et les difficultés, je sais que je pourrai toujours compter sur toi. Alors, comme le dit une chanson bientôt célèbre : Do you remember, when you told me... and here we are...*

# Sommaire

# *I – Activité de Recherche, d'Enseignement et d'Administration*

# 1. Curriculum Vitæ

## Renseignements Personnels


## Christophe Lucas

Français, Marié, 2 Enfants, 46 ans

Institut de Recherche sur la Biologie de l'Insecte
UMR 7261 CNRS – Université de Tours
Avenue Monge, Parc Grandmont
37200 Tours, France

Tél :+33 (0) 247-367-372
Fax : +33 (0) 247-366-966

christophe.lucas@univ-tours.fr
www.univ-tours.fr/m-christophe-lucas


## Cursus professionnel et universitaire

Oct. 2010- ***Chercheur CNRS*** (CR1, Section 29), Université de Tours, France
Ecologie chimique, Ecologie comportementale, Génétique comportementale, Adaptation

2010 ***Chercheur FNS Senior***, University of Lausanne, Suisse
Génétique comportementale chez la fourmi *Solenopsis invicta*

2007-2009 ***Premier assistant***, University of Lausanne (Keller L), Suisse
Structure sociale et expression génétique de la fourmi invasive *Solenopsis invicta*

2003-2006 ***Chercheur Post-doctorant***, University of Toronto (Sokolowski MB), Canada
Mécanismes moléculaires de la plasticité des comportements sociaux chez la fourmi *Pheidole pallidula*

1998-2002 ***Doctorat ès sciences***, Université de Paris-Sud (Jallon JM & Fresneau D)
Bases chimiques et comportementales de la reconnaissance intra- et inter-spécifique chez les fourmis Ponérines *Pachycondyla* (mention très honorable)

1997 ***Master 2***, Muséum & Université Paris VI (Celerier ML & Rholam M)
Purification et caractérisation des composants toxiques des venins de mygales du genre *Brachypelma*

1996 ***Master 1*** *Biologie des Populations et des Ecosystèmes*, Université Paris VI

1993-1995 ***Licence*** *Biologie des Organismes,* Université Paris VI & XII

# 2. Activité de recherche

## Direction de la recherche

### **Direction scientifique du plateau d'écologie chimique** (depuis 2015)

Plateforme commune aux 3 équipes du laboratoire IRBI (Université de Tours).
Personnel technique : 1 ingénieur de recherche CNRS.
Machines de la plateforme : GC-TQ, GC-MS, 3 GC-FID, HPLC, LC-TOF, Electrophorèse capillaire
Responsable de l'achat de matériel de haute précision (>200k€) : GC-TQ, LC-TOF

### Crédits de recherche obtenus

**En tant que porteur**

| | |
|---|---|
| 2019-21 | Bourse de l'école doctorale SSBCV de l'Université de Tours (50%, 36 mois, 77k€) |
| 2017-20 | Financement de recherche APR d'Intérêt Régional, Région Centre (36 mois, 210k€) |
| 2014 | APR Post-doctorat de l'Université de Tours (12 mois, 29k€) |
| 2012 | APR Recherche d'Initiative Académique, PRES-CVLU Région Centre (138k€)<br>Achat d'équipement lourd : Spectromètre de masse triple quadripolaire (GC-MS-MS) |
| 2012 | Financement d'équipement, INEE – CNRS (50k€)<br>Plateau robotisé injection/extraction Gerstel avec injecteur large volume cryo-focalisé. |
| 2009 | Subside de recherche de la Société Académique Vaudoise (12 mois, 8k€) |
| 2001 | Financement de collaboration internationale du CNRS avec l'Univ. de Tel-Aviv (10k€) |

**En tant que collaborateur**

| | |
|---|---|
| 2018-21 | Financement de recherche, Europe H2020 - ADEME (10%, 36 mois, 1 349k€) |
| 2017-20 | Financement de recherche, DFG-Allemagne (10%, 36 mois, 250k€) |
| 2017 | Financement de recherche, Région Aquitaine (15%, 18 mois, 61k€) |
| 2016 | Financement du Centre Scientifique et Technique du Bâtiment (50%, 6 mois, 10k€) |
| 2015-16 | Partenariat Hubert Curien Franco-Allemand, ambassade de France (50%, 24 mois, 18k€) |
| 2015 | Bourse de mobilité de l'Université de Tours (50%, 12 mois, 2,5k€), collaboration avec l'Université de Freiburg (Allemagne). |
| 2014 | Financement de soutien de projet inter-équipe de l'IRBI (50%, 6 mois, 5k€) |
| 2014-18 | ANR JCJC FertiliWasp (20%, 48 mois, 264k€) |
| 2013-16 | Bourse de l'école doctorale SSBCV de l'Université de Tours (50%, 36 mois, 73k€) |

**Distinctions**

2005 Prime d'excellence des post-doctorants français à l'étranger, Ministère (5k€)

**Bourses d'études**

2008 Fondation du 450ème Anniversaire, Université de Lausanne (1,5k€)

2005-06 Program "Behaviour, Genes and Environment", CIHR funding Canada (12k€)

2003-05 Bourse d'études Post-doctorales de la Fondation Fyssen (36 mois, 45k€)

2001 Bourse de l'Union Internationale pour l'Étude des Insectes Sociaux (1k€)

2000 Bourse de mobilité internationale étudiante de l'Université d'Orsay (1,5k€)

## Responsabilités scientifiques

### Evaluation de projet de recherche

2018 Expert Actions de Recherche Concertée (Belgique)

2016 Kentucky Science & Engineering Foundation (USA)

2014-19 F.R.S.- FNRS (Belgique) : 8 projets

2011 Agence Nationale de la Recherche (ANR) : 2 projets

2011 Expert externe à mi-parcours de l'ANR "ADAPTANTHROP" du Laboratoire Evolution, Génomes et Spéciation (LEGS, Gif-sur-Yvette)

### Evaluation d'articles scientifiques pour des journaux

Animal Behaviour / Apidologie / Behavioral Ecology / Behavioral Ecology and Sociobiology / Behavioural Processes / Bulletin of Entomological Research / Biochemical Systematics and Ecology / Biological Journal of the Linnean Society / Current Opinion in Insect Science / Environmental Entomology / Insectes Sociaux / Integrative Zoology / Journal of Pest Science / Molecular Ecology / Myrmecological News / Oecologia / PeerJ / Physiological Entomology / PlosOne / The Canadian Entomologist / The Science of Nature

### Conseiller scientifique

2018-19 Responsable d'une formation pour les communes et les professionnels du bâtiment – "Le risque termite, de la biologie des espèces à la réglementation", avec CNRS Formation Entreprise et FREDON-CVL

2018-19 Responsable d'une formation pour les enseignants (1er et 2ème degrés) – "Le génie des insectes sociaux", avec la maison pour la science Centre-Val de Loire

2018-19 Parrain du salon des sciences de Vendôme – "Sciences et citoyenneté"

2016-18 Formation professionnelle des formateurs d'enseignants de l'Education Nationale, académie Orléans-Tours – Conseiller "Utilisation des outils numériques (Web 2.0) dans la formation disciplinaire numérique LVE"

2008-09 Formation professionnelle des enseignants du secondaire de l'Education Nationale, académie de Grenoble – Conseiller scientifique "Mémorisation active et comportement"

## Champs d'étude

Ecologie chimique, Ecologie comportementale, Génétique comportementale, Adaptation

### Mots-clefs

Communication chimique, comportement, expression génique, polyéthisme, polyphénisme, gène *foraging*, conflits sociaux, reproduction, sociétés d'insectes, espèces invasives.

### Modèles biologiques

Insectes sociaux (termite, fourmi, guêpe), forficule, drosophile, nasonia.

### Outils techniques

**Ecologie chimique** : chromatographie en phase gazeuse – GC (certificat), spectrométrie de masse – GC-MS/GC-TQ (certificat), chromatographie en couche mince – TLC, chromatographie en phase liquide – HPLC, techniques d'extraction (SPME, SBSE, SHS, DHS, DMDS)

**Biologie moléculaire** : quantitative RT-PCR (certificat), *in situ*, ARN interférant, immunoessaies, microarray, clonage, Northern blot, Western blot

**Omique** : W4M, Web Apollo, Galaxy, Script Linux

**Comportement** : video-tracking, reproductions croisées, cross-fostering

**Biochimie** : Radioactivité (certificat d'aptitude), enzymologie, biosynthèse

**Microscopie** : confocal (certificat), électronique à balayage – MEB

**Bio-informatique** : Script Bash (certificat), Galaxy (certificat), WebApollo

**Informatique** : R-project (certificat), Adobe suite (certificat), Office suite, Windows, Linux

## Collaborations scientifiques

Collaborations récentes et passées, classées par ordre alphabétique.

1. Arango Rachel, United States Department of Agriculture (Etats-Unis)
   How the foraging gene modulates an invasive termite behavioral spectrum
2. Ayali Amir, University of Tel Aviv (Israël)
   Foraging behavior of the desert cricket *Schistocerca gregaria*
3. Cadène Martine, CNRS d'Orléans (France)
   Identification des protéines impliquées dans les interactions chez les termites
4. Casacci Luca, Univerisity of Turin (Italie)
   How a fungus (*Rickia wasmannii*) influences behavior and recognition system of *Myrmica scabrinodis* ant
5. Delabie Jacques HC, UESC/CEPLAC (Brésil)
   Diversité cytogénétique des fourmis du genre Pachycondyla
6. Griffith Leslie, University of Brandeis (Etats-Unis)
   Learning and memory processes involved in courtship behavior of flies
7. Grozinger Christina, Pensylvania State University (Etats-Unis)
   Molecular mechanism of the division of labor in ants
8. Hefetz Abraham, University of Tel Aviv (Israël)
   Circulation of recognition cues through social interaction in ants
9. Heinze Jürgen, Universität Regensburg (Allemagne)
   Trade of sexuals among colonies in an ant with local mating (*Cardiocondyla elegans*)
10. Kölliker Mathias, Universität Basel, (Suisse)
    Chemical cues involved in parent-descendants' interactions in *Forficula auricularia*
11. Kutnik Magdalena, FCBA Institut Technologique, (France)
    Traitements naturels vis-à-vis des termites
12. Lécureuil Charlotte, Université de Tours (France)
    Conséquences de stress environnementaux sur la fertilité mâle de la guêpe parasitoïde *Nasonia vitripennis*
13. Levine Joel, University of Toronto (Canada)
    Reproduction mediators of Drosophila flies
14. Lorenzi Maria Christina, Université Paris 13 (France)
    Coevolutiary arms race between wasps and their social parasites
15. Meunier Joël, Université de Tours (France)
    Choix des partenaires sexuels chez le forficule européen
16. McMahon Dino, Freie Universität Berlin, (Allemagne)
    Proximate cues involved in termite canibalism

17. Ruano Díaz Francisca, Universidad de Granada, (Espagne)
    Recognition signals of aphid-ant mutualism
18. Suttie Ed, Building Research Establishment, (Angleterre)
    Innovating in forest-based bioeconomy: Delivering fingertip knowledge to enable service life performance specification
19. Vargo Ed, Texas A&M University (Etats-Unis)
    Dispersion, foraging and colonization behavior of the invasive termite *Reticulitermes flavipes*

## Animations scientifiques

# Animation du GDR Médiation chimique dans l'environnement - Ecologie Chimique

**Responsable de l'axe "Communications" (2019) et membre du comité scientifique du GDR3658 "Médiation chimique dans l'environnement - Ecologie Chimique" depuis 2014.**

Les connaissances scientifiques ne valent que si elles sont partagées par tous. Cependant, elles sont également en constante évolution, ce qui nécessite des moyens pour les maintenir à jour et un accès à tous de ces actualisations permanentes. Il est donc primordial de mettre en place une véritable politique de transfert de nos connaissances afin de diffuser le plus largement possible nos objectifs et nos découvertes. L'axe 9 " communication – enseignement " du GDR MediatEC, qui est un axe de partage et de diffusion scientifique, présente donc un triple objectif de réalisation d'une veille scientifique, de coordination des collaborations entre les membres du GDR et de transmission des actions et des connaissances de notre GDR afin de lui donner la plus grande visibilité possible.

L'aspect Communication de l'axe 9 consistera en la mise en place d'informations sur les nouvelles publications (ouvrages, articles majeurs, conférences) ou sur les sites web présentant des intérêts pour la thématique de l'écologie chimique et ce en impliquant l'ensemble des membres spécialistes du GDR via l'utilisation d'un site web participatif. Il aura également pour rôle de stimuler et de simplifier la communication entre les membres via la mise en place de l'utilisation de mots clés associés aux pages personnelles des participants au GDR. Ainsi l'ensemble des domaines scientifiques, des modèles d'études, des techniques expérimentales et des méthodes d'analyses utilisées seront répertoriés, permettant de retrouver tous les membres du réseau de façon rapide et efficace. Nous stimulerons aussi l'utilisation d'un forum sur le site web du GDR (ou sur la page dédiée de ResearchGate) pour permettre de façon directe et informelle des discussions sur des sujets précis ou des conseils d'utilisateurs concernant des techniques expérimentales ou des analyses particulières. Cet axe de communication scientifique pourra également assurer l'animation de tables rondes aux cours des journées du GDR afin de stimuler l'émergence de nouvelles théories ou de nouvelles idées d'expériences et de faciliter la collaboration et la rédaction de projets

communs entre les membres de notre GDR. Nous faciliterons également toutes les opérations de communication des membres du GDR (aide à la rédaction de communiqués de presse, contacts avec les journalistes, interviews radio-télé, vulgarisation). Enfin, nous rendrons notre GDR plus visible en incitant la participation de ces membres à différents événements à destination du grand public (fête de la science, assises de la biodiversité) ou vers des publics plus spécialisés (écoles thématiques, colloques nationaux et congrès internationaux). Nous réaliserons aussi une page dédiée sur ResearchGate pour regrouper l'ensemble de l'activité scientifique de notre GDR (https://www.researchgate.net/project/GDR-MediatEC-Mediation-chimique-dans-lenvironnement-Ecologie-Chimique).

L'aspect Enseignement de cet axe 9 s'appuiera sur la réalisation d'un MOOC sur l'écologie chimique afin de sensibiliser davantage les étudiants à cette discipline et de les inciter à se former dans ce domaine de recherche à part entière. Afin d'accompagner cette démarche, une formation diplômante de niveau master 2 sera également proposée à distance. Elle permettra ainsi aux étudiants francophones d'acquérir savoirs et compétences et offrira à nos laboratoires des personnels formés en écologie chimique. S'appuyant sur la seule formation diplômante actuellement proposée en écologie chimique en France, ouverte en 2015 à l'université de Bretagne-Sud, ce master 2 sera la vitrine des actions menées en écologie chimique, notamment au sein du GDR.

Mots-clés : Animation scientifique, diffusion scientifique, veille scientifique, MOOC, Master

Lien : https://www.gdr-mediatec.cnrs.fr

## Organisation de congrès

| | |
|---|---|
| 2015-16 | 18ème *Colloque Biologie de l'Insecte* (Tours) |
| 2014-15 | Direction du comité organisateur du 28ème congrès de l'*Union Internationale pour l'Étude des Insectes Sociaux - Section Française* (Tours) |
| 2013- | Plusieurs congrès (Paris, Banyuls, Marseille, Montpellier, Rennes) du GDR 3658 Médiation chimique dans l'environnement - Ecologie Chimique |
| 2012 | 1ére *Ecole Thématique d'Ecologie Chimique* du CNRS (Fréjus) |

## Animation de symposiums

| | |
|---|---|
| 2019 | 30ème congrès de l'UIEIS-SF (27-30 août, Avignon) |
| 2017 | 29ème congrès de l'UIEIS-SF (23-25 août, Paris) |
| 2016 | 18ème Colloque Biologie de l'Insecte (27-29 juin, Tours) |
| 2015 | 28ème congrès de l'UIEIS-SF (26-28 août, Tours) |

2011 Omiques et écologie chimique, Prospectives nationales "Ecologie Chimique" CNRS, INEE (29 juin, Paris)

2011 L'écologie chimique et les omiques, Prospectives nationales "Ecologie Chimique" CNRS, INEE (3-4 mai, Paris)

2011 Interactions plantes-animaux, GDR 2827 Ecologie chimique (24-26 oct., Dijon)

## Animation grand public

2015- Fête de la Science – Insectes sociaux (Tours)

2011- Journées Portes Ouvertes (Université de Tours)

2010 Fête de la Science – Insectes sociaux, cité des sciences de La Villette (Paris)

2009-10 GÈNial Darwin – Les Jours du gène sous les feux de l'évolution, U. de Lausanne (Suisse)

## Animation scolaire

2019 1ère S, La chimie des fourmis, lycée Eugène Ionesco (Issy-les-Moulineaux)

2019 TPE CPGE-BCPST, La communication des fourmis, lycée Champollion (Grenoble)

2019 CE2/CM1, La vie grégaire et sociale, école Yvonne Chollet (Vendôme)

2018 1ére S, La communication chimique, lycée Institut Notre Dame (Saint-Germain-en-Laye)

2018 TPE 2ème Classe Prépa BCPST, Les phéromones de fourmis, lycée Camille Guérin (Poitiers)

2017 TPE 1ère SSVT, Stratégies des fourmis en cas d'attaque/défense ? lycée Ronsard (Vendôme)

2016 TPE 1ère SSVT, Communication chimique chez les fourmis, lycée Vaucanson (Tours)

2015 TPE BCPST2, Impact de l'environnement sur des fourmilières, lycée du Chesnoy (Montargis)

2015 TPE Classe prépa SSVT, Etude d'une phéromone de fourmi, lycée Descartes (Tours)

2013 TPE 1ère SSVT, Les phéromones des fourmis, lycée Saint-Gatien (Joué-lès-Tours)

2011 Les Petits Débrouillards, Découvertes autour de la forêt, centre de loisirs de La Rabière (Joué-lès-Tours)

## Appartenance à des sociétés scientifiques

2014- GDR 3658 Médiation chimique dans l'environnement - Ecologie Chimique (www.gdr-mediatec.cnrs.fr)

2012- i5k Genome Sequencing Initiative (www.arthropodgenomes.org/wiki/i5K)

2010-14 International Society of Chemical Ecology – ISCE (http://chemecol.org)

2010-11 GDR 2827 Ecologie chimique (2004-2011)

2009-10 Société Académique Vaudoise – SAV (www.s-a-v.org)

1999- Union Internationale pour l'Etude des Insectes Sociaux - Section Française (http://insectes-sociaux.org)

# 3. Activité d'enseignement

Tout au long de mon parcours, j'ai effectué un total de 288h de cours magistraux et 176h TD dans 4 universités différentes. J'ai aussi récemment monté 2 formations à destination des enseignants et des professionnels.

## Enseignements universitaires

8. **Communication chimique chez les insectes**
   10h Cours Magistraux & 8h TD | 2016-19
   *Master 2 - Ecologie comportementale, évolution & biodiversité, Université de Tours.*
   Programme : Principes, Contextes évolutifs, Biosynthèses, Reproduction, Fertilité, Plante-insecte, Proie-prédateur, Mimétisme chimique, Socialité, Méthodes d'analyse

7. **Web 2.0 et veille bibliographique**
   22h Cours Magistraux & 16h TD | 2016-18
   *Licence 3 - Méthodes et Techniques en Analyse Sensorielle, Université de Tours.*
   *Master 2 - Qualité et Environnement en Productions animales, Université de Tours.*
   Programme : Analyse des besoins, Circulation "push & pull", Flux, Agrégateur, Réseaux sociaux, Portails personnalisables, Widgets, Logiciels de gestion bibliographique.

6. **Biologie, Evolution et Zoologie**
   60h TP | 2006-09
   *Licence 1 - Biologie, Université de Lausanne.*
   Programme : Diversité du vivant, Annélides, Mollusques, Insectes, Mammifères.

5. **Biologie moléculaire**
   90h TP | 2006-09
   *Licence 1 - Biologie, Université de Lausanne.*
   Programme : Extraction, PCR, Gel de séparation, Séquençage, Outils d'analyse informatique.

4. **Evolution et Ecologie**
   16h Expert examen | 2006-09
   *Licence 3 - Biologie, Université de Lausanne.*
   Programme : Ecologie évolutive, Systèmes adaptatifs.

3. **Les sociétés d'insectes**
   4h Cours Magistraux | 2006
   *Master 2 - Signalisation cellulaire et neurosciences, Université de Paris-Sud.*
   Programme : Biologie et écologie des sociétés d'insectes.

2. **Neuroanatomie** (responsable de l'UE)
   36h Cours Magistraux | 1998-2001
   *Master 1 - Biologie Cellulaire et Physiologie, Université de Médecine Paris XIII.*

Programme : Données embryologiques, Système nerveux central, Système nerveux Périphérique, Nerfs crâniens.

1. **Neurophysiologie du Comportement** (responsable de l'UE)
   200h Cours Magistraux | 1997-2002
   *Master 1 - Biologie Cellulaire et Physiologie, Université de Médecine Paris XIII.*
   Programme : Somesthésie, Rythmes du cerveau, Sommeil et vigilance, Conditionnement, Apprentissage et mémoire, Motivations sexuelles, Motivations alimentaires, Comportements antagonistes.

## Autres enseignements

1. **Formation "Le génie des insectes sociaux"** (responsable de la formation)
   12h Cours Magistraux | 2019
   *Enseignants du 1er et 2nd degrés, Maison pour la science Centre-Val de Loire.*
   Programme : (i) Acquérir des connaissances sur les insectes sociaux : caractéristiques, classification au sein du règne animal, place dans le cycle biologique des forêts, anatomie des différentes castes (reproducteurs, soldats, ouvriers, larves). (ii) Etudier les spécificités des termites (cycle biologique, bonnes pratiques pour éviter les infestations et les disséminations). (iii) Expérimenter un protocole issu d'une démarche scientifique en rapport avec le comportement des insectes.

2. **Formation "Le risque termite, de la biologie des espèces à la réglementation "** (responsable)
   20h Cours Magistraux & 36h TD | 2019-20
   *Professionnels (entreprises, agents de l'Etat, artisans du bâtiment), CNRS formation entreprises et FREDON-CVL.*
   Programme : (i) Acquérir des notions de base sur la biologie des termites. (ii) Savoir rechercher et identifier les termites sur le terrain, reconnaître leurs dégâts. (iii) Connaître les bonnes pratiques pour éviter les infestations et les disséminations. (iv) Acquérir des notions de réglementation et de lutte contre ces insectes.

## Jury de doctorants et de masters

### Evaluation de doctorants

| | |
|---|---|
| 2018 | Comité de pilotage, thèse de Violette Chiara (CRCA, Toulouse) – Dir. Jeanson R |
| 2018-20 | Comité de pilotage, thèse de Quentin Fouché (UTML, Lille) – Dir. Charabidze D |
| 2018-20 | Mentor, thèse d'Anaïs Chanson (Guyane) – Dir. Duplais C |
| 2017-19 | Mentor, thèse de Mathilde Vidal (Regensburg, Allemagne) – Dir. Heinze J |

2014 Comité de pilotage, thèse de Kevin Berthelot (CRCA, Toulouse) – Dir. Jeanson R

2012-13 Examinateur et président du comité de pilotage, thèse de Floriane Chardonnet (LEGS, Gif-sur-Yvette) – Dir. Kaiser-Arnauld L

### Evaluation de master

2018-19 Jury Master 1 & 2 - Ecologie Comportementale, évolution et biodiversité (Tours)

## Activités d'encadrements

L'ensemble de mes encadrements directs ou en collaboration se résume à un total de 34 étudiants et 2 post-doctorants.

### Post-doctorats

2018-20 Fanny Ruhland (Post-doctorat, Rennes)
Caste determination in termites: what is the role of social interactions?

2014-15 Sabrine Attia (Post-doctorat, Tunisie)
Identification of new sexual pheromones of an invasive species of termite

### Thèses

2019-21 Louis Pailler (Thèse, HDR Meunier J)
Reproductive differentiation in termites: study of the chemical signals and the behaviors involved

2013-17 Lou Brossette (Thèse, HDR Bagnères AG, Montpellier)
Interactions sociales et stratégies de fondation chez deux termites européens invasif et natif

### Ingénieurs

2016 Marius Bredon (collab. Lécureuil C, Tours)
Marqueurs génétiques de la fonction de reproduction chez le termite invasif *R. flavipes*.

2005-06 Bianco Marco (Toronto, Canada)
Foraging and defense behavior of *Pheidole pallidula*.

2005-06 Julia Schonfeld (Toronto, Canada)
Immunoassays and confocal imaging of the brain of Drosophila.

2005 Jennifer McGaw (Toronto, Canada)
Behavioral polymorphism of the ant *P. pallidula*.

## Master 2 & équivalents

2020 Alexis Groseiller (Orléans)
Stratégies de fourragement et contraintes environnementales chez les termites

2019 Timothée Toussaint (Perpignan)
Médiation chimique et reproduction au sein des sociétés de termite

2019 Samuel Desvignes (collab. Miguel Pineirua Menendez, Saint-Etienne)
Caractérisation physique et implication biologique de la communication vibratoire chez les termites

2018 Marion Moulin (Tours)
Rôle des signaux chimiques dans l'organisation sociale des termites

2018 Amandine Loyant (collab. Lécureuil C, Tours)
Un stress chimique peut-il perturber la reconnaissance sexuelle chez un hyménoptère parasitoïde *Nasonia vitripennis* ?

2017 Sophie van Meyel (collab. Lécureuil C, Poitiers)
Impact d'un stress chimique sur la reconnaissance sexuelle d'un hyménoptère parasitoïde *Nasonia vitripennis* ?

2017 Florian Königseder (collab. Schrempf A, Allemagne)
Trade of sexuals among colonies in an ant with local mating

2016 Rached Botsy (collab. CSTB, Bagnères AG, La Réunion)
Etude des émissions volatiles associées à la présence des termites.

2016 Anaïs Chanson (collab. Mercier JL, Saint-Etienne)
Etude des stratégies de reproduction chez la fourmi *Cardiocondyla elegans* : rôle du transport des sexuées par les ouvrières.

2015 Marius Bredon (collab. Lécureuil C, Tours)
Caractérisation moléculaire de la fonction de reproduction chez le termite *Reticulitermes flavipes*.

2012 Lauriane Lefloch (Tours)
Facteurs environnementaux et système de castes des termites.

2003-04 Julia Schonfeld (Honors thesis, Toronto, Canada)
Cloning of the *foraging* gene of the desert locust *Schistocerca gregaria*.

## Master 1 & Licence

2019 Rémi Metayer (L3, Orléans)
Attractivité des volatiles issus des environnements confinés des termites.

2018 Matthieu De Lamarre (M1, ENS de Lyon)
Caractérisation des facteurs environnementaux biotiques et abiotiques du comportement "body shaking" chez les termites.

2018 Marine Fillaud (M1, Tours)
Différenciation des reproducteurs et biais de sexe ratio chez *Reticulitermes flavipes*.

2017 Marina Choppin (M1, Tours)
Contrôle social et chimique de la différenciation des reproducteurs chez le termite *Reticulitermes flavipes*.

2015-16 Florian Königseder (M1, Collab. Schrempf A, Allemagne)
Trade of sexuals among colonies in an ant with local mating.

2015-16 Larissa Kalb (L3, Collab. Schrempf A, Allemagne)
Chemical cues involved in the trading of sexuals in the ant *Cardiocondyla elegans*.

2015 Solène Blanchard (M1, Poitiers)
Comportement de fondation des différents types de reproducteurs de deux espèces de termites : *Reticulitermes grassei* et *R. flavipes*.

2015 Anthony Millot (M1, Tours)
Fitness des reproducteurs primaires de deux espèces de termites (endémique et invasive).

2014 Solène Blanchard (L3, Poitiers)
Comportement des reproducteurs ailés de deux espèces de termites : *Reticulitermes grassei* et *R. flavipes*.

2013 Jérôme Mabilleau (M1, Tours)
Interactions néoténiques-ouvriers chez deux espèces de termites *Reticulitermes grassei* et *R. flavipes*.

2011 Valentin Julien (M1, Tours)
Comportement agressif des différentes formes sociales de la fourmi de feu *Solenopsis invicta*.

2002 Aurelien Drouart & Bertrand Jacquemin (M1, Paris-Sud)
Rôle des lipides dans la reconnaissance coloniale.

2002 Herve Dressaud (M1, Paris-Sud)
Reconnaissance des apparentés chez les fourmis.

2001-02 Sophie Circosta (M1, Paris-Sud)
Biosynthèse des lipides dans la glande post-pharyngienne.

2001 Benoit Jahyny (M1, Paris-Sud)
Spéciation et biométrie chez *Pachycondyla villosa*.

2000 Cécile Berthouly (M1, Paris-Sud)
Mise en place d'un nouveau test éthologique chez les fourmis.

# 4. Activité d'administration

## Comités de gestion

| | |
|---|---|
| 2015- | Direction scientifique du plateau d'écologie chimique de l'IRBI |
| 2014- | Responsable de l'axe "Communications" et membre du comité scientifique GDR3658 "Médiation chimique dans l'environnement - Ecologie Chimique". |
| 2012- | Comité d'organisation des séminaires du laboratoire IRBI (Tours) |
| 2012- | Commission Scientifique Disciplinaire Paritaire (élu, sections 67-68, Tours) |
| 2012-18 | Conseil de gestion du laboratoire IRBI (élu, UMR CNRS 7261, Tours) |
| 2012-14 | Commission des finances du laboratoire IRBI (Tours) |
| 2012 | Chargé de mission sur l'amélioration de la gouvernance au sein du labo IRBI (Tours) |
| 2009-10 | Création d'un pôle inter-département d'analyses chimiques entre la police scientifique et le Département d'Écologie et d'Évolution (Lausanne, Suisse) |
| 1998-2002 | Responsable informatique du laboratoire NAMC (hardware, software, réseaux, achats, installation, maintenance…) |

## Webmaster

| | |
|---|---|
| 2014- | GDR 3658 Médiation chimique dans l'environnement - Ecologie Chimique (gdr-mediatec.cnrs.fr) |
| 2012- | Union Internationale pour l'Étude des Insectes Sociaux - Section Française (insectes-sociaux.org) |
| 2012 | Ecole Thématique d'Ecologie Chimique (CNRS, co-responsable) |
| 2010- | Institut de Recherche sur la Biologie de l'Insecte (irbi.univ-tours.fr) |

# 5. Publications & Communications

Les statistiques bibliographiques détaillées sont disponibles en ligne sur google scholar

## Publications avec comité de lecture

(Impact Factor moyen 4.32 ; h-index de 14 ; i10-index de 16)
Les astérisques signifient que les auteurs ont contribué de façon équivalente.

24. Ruhland F, Moulin M, Choppin M, Meunier J, **Lucas C** (Submitted)
Reproductives and eggs trigger worker vibration at a short-term scale in a subterranean termite.

23. Brossette L, Meunier J, Dupont S, Bagnères AG, **Lucas C** (2019)
Unbalanced biparental care during colony foundation in two subterranean termites.
***Ecology and Evolution*** 9 (1): 192-200 (IF: 2.34)

22. **Lucas C**, Brossette L, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2018)
When predator odour makes groups stronger: effects on behavioral and chemical adaptations in two termite species.
***Ecological Entomology*** 43 (4): 513-524 (IF: 2.24)

21. Brossette L, Bagnères AG, Millot A, Blanchard S, Dupont S, **Lucas C** (2017)
Termite's royal cradle: does colony foundation success differ between two subterranean species?
***Insectes Sociaux*** 64 (4): 515-523 (IF: 1.70)

20. Csata E*, Timuș N*, Witek M*, Casacci LP*, **Lucas C**, Bagnères AG, Sztencel-Jabłonka A, Barbero F, Bonelli S, Rákosy L, Markó B (2017)
Lock-picks: fungal infection facilitates the intrusion of strangers into ant colonies.
***Scientific Reports*** 7: 46323 (IF: 4.26)

19. Elia M, Blancato G, Picchi L, **Lucas C**, Bagnères AG*, Lorenzi MC* (2017)
Nest signature changes throughout colony cycle and after social parasite invasion in social wasps.
***PLoS ONE*** (12): e0190018 (IF: 2.81)

18. **Lucas C**, Nicolas M, Keller L (2015)
Expression of *foraging* and *Gp-9* are associated with social organization in the fire ant *Solenopsis invicta*.
***Insect Molecular Biology*** 24 (1): 93-104 (IF: 2.98)

17. Chirault M, **Lucas C**, Goubault M, Chevrier C, Bressac C, Lécureuil C (2015)
A combined approach to heat stress effect on male fertility in *Nasonia vitripennis*: from the physiological consequences on spermatogenesis to the reproductive adjustment of females mated with stressed males.

***PLoS ONE*** 10(3): e0120656 (IF: 3.53)

16. Manfredini F, **Lucas C**, Nicolas M, Keller L, Shoemaker DW, Grozinger C (2014)
Molecular and social regulation of worker division of labor in fire ants.
***Molecular Ecology*** 23 (3): 660-672 (IF: 6.49)

15. Wong JWY*, Meunier J*, **Lucas C**, Kölliker M (2014)
Paternal signature in kin recognition cues of a social insect: Concealed in juveniles, revealed in adults.
***Proceedings of the Royal Society of London B*** 281 (1793): 20141236 (IF: 5.29)

14. Wong JWY, **Lucas C**, Kölliker M (2014)
Cues of maternal condition influence offspring selfishness.
***PLoS ONE*** 9 (1): e87214 (IF: 3.53)

13. Meunier J, Delémont O, **Lucas C** (2011)
Recognition in ants: social origin matters.
***PLoS ONE*** 6 (5): e19347 (IF: 4.09)

12. **Lucas C**, Kornfein R, Chatterjee M, Schonfeld J, Geva N, Sokolowski MB, Ayali, A (2010)
The locust *foraging* gene.
***Archives of Insect Biochemistry and Physiology*** 74 (1): 52-66 (IF: 1.56)

11. **Lucas C**, Hugson NB, Sokolowski MB (2010)
Job switching in ants: role of a kinase.
***Communicative & Integrative Biology*** 3 (1): 6-8

10. **Lucas C**, Sokolowski MB (2009)
Molecular basis for changes in behavioral state in ant social behaviors.
***Proceedings of the National Academy of Sciences of the United States of America*** 106 (15): 6351-6356 (IF: 9.43)
media coverage: The New York Times (press), Nature Education (dispatch), Rogers TV (TV news), CBC radio, 24 heures (press), Uniscope (press), The Bulletin (press), UTM Press, The journal of experimental biology (dispatch)

9. Krupp J, Kent C, Billeter JC, So T, Azanchi R, Smith BP, Schonfeld JA, **Lucas C**, Levine JD (2008)
Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*.
***Current Biology*** 18 (18): 1373-1383 (IF: 10.78)
media coverage: Current Biology (dispatch)

8. Ejima A, Smith BPC, **Lucas C**, van der Goes van Naters W, Miller CJ, Carlson JR, Levine JD, Griffith LC (2007)
Generalization of courtship learning in Drosophila is mediated by cis-Vaccenyl Acetate.
***Current Biology*** 17 (7): 599-605 (IF: 10.54)

7. **Lucas C**, Pho DB, Jallon JM, Fresneau D (2005)
Role of cuticular hydrocarbons in the chemical recognition between ant species in the *Pachycondyla villosa* species complex.
***Journal of Insect Physiology*** 51 (10): 1148-1157 (IF: 2.04)

6. Ejima A, Smith BPC, **Lucas C**, Levine JD, Griffith LC (2005)
Sequential learning of pheromonal cues modulates memory consolidation in trainer-specific associative courtship conditioning.
***Current Biology*** 15 (3): 194-206 (IF: 11.73)
media coverage: Current Biology (dispatch)

5. **Lucas C**, Pho DB, Fresneau D, Jallon JM (2004)
Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*.
***Journal of Insect Physiology*** 50 (7): 595-607 (IF: 1.55)

4. Soroker V, **Lucas C**, Simon T, Fresneau D, Durand JL, Hefetz A (2003)
Hydrocarbon distribution and colony odour homogenisation in *Pachycondyla apicalis*.
***Insectes Sociaux*** 50 (3): 212-217 (IF: 1.57)

3. **Lucas C**, Fresneau D, Kolmer K, Heinze J, Delabie JHC, Pho DB (2002)
A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae)
***Biological Journal of the Linnean Society*** 75 (2): 249-259 (IF: 2.31)

## Ouvrages & chapitres d'ouvrage

3. Baudino S, **Lucas C**, Smadja C (2017)
Les Omiques en écologie chimique.
In: Ecologie Chimique, *ISTE*, ISBN: 978-1-78405-186-0, 135-157

2. Baudino S, **Lucas C**, Smadja C (2016)
Omics in chemical ecology.
In: Chemical Ecology, *ISTE-Wiley*, ISBN: 978-1-84821-924-3, 117-137

1. **Lucas C**, Charpentier M (2012)
Odeurs en sociétés.
In: Ecologie Chimique - Le Langage de la Nature, Collective Eds. *CNRS (INEE) - Le cherche midi*, ISBN 978-2-7491-2772-9, 72-85
media coverage: France inter (radio), RFI (radio), Biofutur (press)

## Publications grand public & autres ouvrages

5. **Lucas C**, Ruhland F (2018)
   Les termites s'invitent en région Centre-Val de Loire.
   *Microscoop, le journal du CNRS en délégation* HS: 16-17

4. Smadja C, **Lucas C**, Caissard JC (2012)
   L'écologie chimique et les omiques.
   In: *Les Cahiers Prospectives - Prospective Ecologie Chimique*, CNRS eds. Prospective de l'Institut Ecologie et Environnement (INEE). Paris, CNRS, 16-23

3. Darrouzet E, **Lucas C** (2011)
   Les termites, un monde de castes.
   *Microscoop, le journal du CNRS en délégation* 63: 14-15

2. **Lucas C** (2002)
   Etude des bases chimiques et comportementales de la formation du "visa" colonial chez les Ponérines du genre *Pachycondyla*.
   Thèse de doctorat en *Biologie du comportement*, Université Paris-Sud, Orsay, 158 p.

1. **Lucas C** (1997)
   Purification et caractérisation des composants toxiques des venins de mygales du genre *Brachypelma*.
   DEA (master 2), Muséum National d'Histoire Naturelle, Paris, 32 p.

## Communications

20 congrès internationaux (8 communications orales ; 12 affichées)
26 congrès nationaux (18 communications orales ; 8 affichées)

# Conférences invitées

### Conférences scientifiques

2. International termite meeting, Fort Lauderdale, Florida, USA (3-8 juin 2019)
   Signals involved in social interactions in insects: what is known about non-volatile cues?

1. International Society of Chemical Ecology, 26$^{th}$ Annual Meeting, Tours (31 juil. - 4 août 2010)
   Genomics in chemical communication

### Conférences grand public

8. Cobaty internationales - Association de professionnels du bâtiment, Tours (20 mai 2019)
   Les termites en région Centre-Val de Loire

7. Sciences et citoyenneté, conférence-débat grand public, Vendôme (9-10 mai 2019)
La science est - elle garante de progrès pour l'homme ? L'exemple des insectes sociaux

6. Mardis de la science, conférence grand public, Chartres (23 mai 2018)
Les termites : ces insectes sociaux qui s'invitent chez vous

5. Sciences et citoyenneté, conférence-débat grand public, Vendôme (17-18 mai 2018)
Sciences, citoyenneté et termites

4. Rencontre Jeunes/Chercheurs, conférence grand public, Tours (20 avril 2018)
Le génie des insectes sociaux : termites, fourmis, abeilles, frelons

3. Cycle déTours, conférence grand public, Tours (17 avril 2018)
Les termites : ces insectes sociaux qui s'invitent chez vous

2. Université Paris 13 Sorbonne Paris Cité, conférence publique, Villetaneuse (9 oct. 2017)
How insects switch jobs? Behavioral polymorphisms and gene expression

1. Parc Culturel Galéa, conférence grand public, Corse (350 personnes ; 24 avril 2016)
Les insectes sociaux

## Congrès

### Congrès internationaux

20. Ruhland F, Moulin M, Choppin M, Meunier J, **Lucas C** (2019)
Body-shaking: a vibratory indicator of reproductives and eggs presence in a subterranean termite (poster)
*Association for the Study of Animal Behaviour* (26-28 mai, Konstanz, Allemagne)

19. Suttie E, Brischke C, Frühwald Hansson E, Fortino S, Sandak J, Kutnik M, Alfredsen G, **Lucas C**, Stirling R (2019)
Performance based specification of wood – Introducing project CLICKdesign. (talk)
*International Research Group on Wood Protection, 50th Annual Meeting* (12-16 mai, Quebec, Canada)

18. **Lucas C**, Brossette L, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2018)
When predator odor makes groups stronger: effects on behavioral and chemical adaptations in two termite species. (poster)
*XI European Congress of Entomology* (2-6 juil., Naples, Italy)

17. **Lucas C**, Brossette L, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2018)
Effects of predator odor on behavioral and chemical adaptations in two termite species. (poster)
*International Society of Chemical Ecology, 34th Annual Meeting* (12-18 août, Budapest, Hungary)

16. **Lucas C**, Nicolas M, Bredon M, Brossette L, Dupont S, Bagnères AG, Lécureuil C, Keller L (2016)
Is there any link between foraging, defense and dispersion behaviors? Evidence from the candidate gene *foraging*. (talk)
*International Union for the Study of Social Insects, 6th European section* (8-12 août, Helsinki, Finland)

15. Brossette L, Bagnères AG, Dupont S, **Lucas C** (2016)
Journey to the heart of termites' family life: An uncommon inner insight into nascent colonies. (talk)
*International Union for the Study of Social Insects, 6th European section* (8-12 août, Helsinki, Finland)

14. Elia M, Lorenzi MC, Christidès JP, **Lucas C**, Bagnères AG (2016)
Hosts & parasites: chemical communication and signal interception in two polistes social wasps. (talk)
*International Society of Chemical Ecology, 32th Annual Meeting* (4-8 juil., Iguassu, Brazil)

13. Elia M, Lorenzi MC, Christidès JP, **Lucas C**, Bagnères AG (2015)
Social parasites change host-nest odors in social wasps. (poster)
*International Society of Chemical Ecology, 31th Annual Meeting* (29 juin - 3 juil., Stockholm, Sweden)

12. **Lucas C**, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2014)
Predator's odor influences aggressive behavior of Reticulitermes termites in competition tests. (poster)
*International Union for the Study of Social Insects, 17th World section* (13-18 juil., Cairns, Australia)

11. Brossette L, Dupont S, Bagnères AG*, **Lucas C*** (2014)
Reproductive caste differentiation dynamics of two termite species (*Reticulitermes sp.*): A multimodal approach based on behavior. (*Authors contributed equally) (poster)
*European PhD Network in Insect Science, 5th Annual Meeting* (7-8 juin, Orosei, Italy)

10. Chirault M, **Lucas C**, Goubault M, Ménoret C, Chevrier C, Bressac C, Lécureuil C (2013)
Heat stress during male development alters the offspring sex ratio in *Nasonia vitripennis*. (poster)
*European PhD Network in Insect Science, 4th Annual Meeting* (19-22 sept., Paluzza, Italy)

9. Lefloch L, Bagnères AG, Dupont S, Christidès JP, **Lucas C** (2012)
Do environmental factors influence social organization traits in Reticulitermes termites? (poster)
*International Union for the Study of Social Insects, 5th European section* (26-30 août, Montecatini, Italy)

8. Lefloch L, Bagnères AG, Christidès JP, Dupont S, **Lucas C** (2012)
Chemical signature overtime & behaviors of 2 termite species in contact with alien hydrocarbons. (poster)
*International Society of Chemical Ecology, 28th Annual Meeting* (22-26 juil., Vilnius, Lithuania)

7. Gilliard E, Christidès JP, Mercier JL, **Lucas C**, Bagnères AG (2011)
Interactions between ants and termite soldiers: does fighting influence the chemical signatures? (poster)
*International Society of Chemical Ecology, 27th Annual Meeting* (24-28 juil., Burnaby, British Columbia, Canada)

6. **Lucas C**, Keller L, Sokolowski MB (2010)
How ants switch jobs - Role of the foraging gene. (poster)
*International Union for the Study of Social Insects, 16th World section* (8-4 août, Copenhagen, Denmark)

5. **Lucas C**, Sokolowski MB (2010)
Molecular basis of foraging and defense behaviors in the ant *Pheidole pallidula*. (talk)
*Biology10* (11-12 fév., Neuchatel, Switzerland)

4. **Lucas C**, Sokolowski MB (2009)
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*International Union for the Study of Social Insects, 4th European section* (30 août - 4 sept., La Roche en Ardenne, Belgium)

3. Ayali A, **Lucas C**, Hershtik L, Schonfeld J, Kinamon S, Sokolowski M (2004)
The role of cGMP-dependent protein kinase and the *foraging* gene in locust density-dependent Phase polymorphism. (talk)
*7th International Congress of Neuroethology* (8-13 août, Nyborg, Denmark)

2. **Lucas C**, Berthouly C, Fresneau D, Pho DB, Jallon JM (2001)
Distinction of specific behaviours in nestmate recognition with a new ethological test. (poster)
*International Union for the Study of Social Insects, 2nd European section* (25-28 sept., Berlin, Germany)

1. Escoubas P, Celerier ML, **Lucas C**, Rholam M, Nakajima T (1997)
MALDI-TOF MS fingerprinting of tarantula venoms: taxonomic and pharmacological applications. (talk)
*12th World Congress on animal, plant and microbial toxins* (21-26 sept., Cuernavaca, Mexico)

**Congrès nationaux**

26. Brossette L, Meunier J, Dupont S, Bagnères AG, **Lucas C** (2019)
Unbalanced biparental care during colony foundation in two subterranean termites. (talk)
*International Union for the Study of Social Insects, 30th French section* (28-30 août, Avignon)

25. Loyant A, Guerin V, Khalil A, **Lucas C**, Lecureuil C (2018)
The effects of bisphenol A on sexual recognition in a Hymenoptera parasitoid: *Nasonia vitripennis*. (talk)
*Hormones and behaviour in arthropods workshop (05-06 juin, Tours)*

24. Choppin M, **Lucas C** (2017)
Social control on reproductive differentiation of termites (*Reticulitermes flavipes*). (talk)
*GDR MediatEC 3658, 4ème journée du GDR d'écologie chimique* (2-4 nov., Montpellier)

23. Brossette L, Bagnères AG, **Lucas C** (2017)
Voyage au cœur de la vie familiale des termites. (poster)
*Biotechnocentre, 30ème Colloque annuel* (12-13 oct., Domaine de Seillac)

22. Choppin M, **Lucas C** (2017)
Social control on reproductive differentiation of termites (*Reticulitermes flavipes*). (talk)
*International Union for the Study of Social Insects, 29th French section* (23-25 août, Paris)

21. Brossette L, Bagnères AG, **Lucas C** (2017)
Social interactions and foundation success in two termite species, invasive and native. (talk)
*International Union for the Study of Social Insects, 29th French section* (23-25 août, Paris)

20. **Lucas C**, Brossette L, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2016)
Predator odor influences social cohesion in a subterranean termite. (talk)
*GDR MediatEC 3658, 3ème journée du GDR d'écologie chimique* (28-30 oct., Marseille)

19. Bagnères AG, Perdereau E, Dupont S, Baudouin G, **Lucas C**, Darrouzet E, Brossette L, Leniaud L, Bankhead-Dronnet S, Zimmermann M, Vargo EL, Dedeine F (2016)
Retour à l'envoyeur : une espèce américaine de termite découverte en Autriche, donnée en France comme endémique pendant des décennies, finalement bien américaine… (talk)
*GDR InvaBio 3647, 3ème journée du GDR Invasions Biologiques* (25-27 oct., Marseille)

18. Brossette L, Bagnères AG, Dupont S, **Lucas C** (2016)
Journey to the heart of termites' family life: An uncommon inner insight into nascent colonies. (talk)
*Colloque Biologie de l'Insecte, 18ème congrès* (27-29 juin, Tours)

17. **Lucas C**, Nicolas M, Bredon M, Brossette L, Dupont S, Bagnères AG, Lécureuil C, Keller L (2016)
Why does the foraging gene represent a major player regulating behavior in social insects? (talk)
*Colloque Biologie de l'Insecte, 18ème congrès* (27-29 juin, Tours)

16. **Lucas C**, Attia S, Bagnères AG (2015)
Phéromones du couple royal du termite invasif *Reticulitermes flavipes*. (talk)
*GDR MediatEC 3658, 2ème journée du GDR d'écologie chimique* (28-30 oct. Banyuls-sur-Mer)

15. Brossette L, Bagnères AG, Dupont S, Millot A, Blanchard S, **Lucas C** (2015)
La parentalité chez les termites : Comment varie l'investissement du couple royal dans le succès reproducteur entre deux espèces native et invasive ? (talk)
*Biotechnocentre, 28ème Colloque annuel* (15-16 oct., Domaine de Seillac)

14. Attia S, Bagnères AG, Christidès JP, Foster S, Dupont S, **Lucas C** (2015)
New specific chemicals of the invasive termite *Reticulitermes flavipes*. (talk)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

13. Brossette L, Bagnères AG, Dupont S, **Lucas C** (2015)
Termite cocooning: How royal couple investment in reproductive success varies between two native and invasive species? (talk)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

12. Bredon M, Lécureuil C, **Lucas C** (2015)
Des marqueurs génétiques trahissent l'activité reproductrice des ouvriers chez le termite invasif *Reticulitermes flavipes*. (poster)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

11. Limousin D, Baudouin G, Dupont S, Andrieux T, Lhuillier V, Brossette L, **Lucas C**, Bagnères AG, Dedeine F (2015)
Etude de l'AQS (Asexual Queen Succession) et du sexe ratio parmi les essaimants chez les termites *Reticulitermes flavipes* et *R. grassei*. (talk)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

10. Elia M, Lorenzi MC, Christidès JP, **Lucas C**, Bagnères AG (2015)
Host-nest odor variation due to wasp social parasites. (poster)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

9. Attia S, Bagnères AG, Christidès JP, Dupont S, **Lucas C** (2015)
Volatile molecules of termites: a comparison of different chemical techniques for identification and analysis. (poster)
*International Union for the Study of Social Insects, 28th French section* (26-28 août, Tours)

8. Brossette L, Dupont S, Bagnères AG, **Lucas C** (2015)
Royal couple investment on foundation success of two termites' species (*Retculitermes sp.*). (poster)
*Ecology & Behaviour, 11th Annual Meeting* (18-21 mai, Toulouse)

7. **Lucas C**, Lefloch L, Dupont S, Christidès JP, Bagnères AG (2013)
Odors and social organization in termites (*Reticulitermes flavipes* & *R. grassei*). (talk)
*International Union for the Study of Social Insects, 27th French section* (28-30 août, Villetaneuse)

6. Baudouin G, Perdereau E, Dupont S, **Lucas C**, Dedeine F, Bagnères AG (2013)
Organisation sociale d'un termite invasif *R. flavipes* en France et originaire de Louisiane. (poster)
*International Union for the Study of Social Insects, 27th French section* (28-30 août, Villetaneuse)

5. **Lucas C**, Keller L, Sokolowski MB (2011)
How ants switch jobs - Role of the foraging gene. (poster)
*Ecole chercheur, Génomique et diversité des caractères à déterminisme complexe* (15-20 mai, La Colle-sur-Loup)

4. **Lucas C**, Keller L, Sokolowski MB (2011)
How ants switch jobs - Role of the foraging gene. (poster)
*International Union for the Study of Social Insects, 26th French section* (12-15 avril, Banyuls-sur-Mer)

3. Meunier J, Delemont O, **Lucas C** (2010)
Recognition in ants: social origin matters. (talk)
*16ème Colloque Biologie de l'Insecte* (18-20 oct., Lyon)

2. **Lucas C**, Sokolowski MB (2009)
Molecular basis of foraging and defense behaviours in the ant *Pheidole pallidula*. (talk)
*20th International Congress of Zoology* (26-29 août, Paris)

1. **Lucas C**, Fresneau D, Pho DB, Kolmer K, Heinze J, Pompolo SG, Delabie JHC, Jallon JM (1999)
Chemical characterization in *Pachycondyla villosa* group: is there only one species? (talk)
*International Union for the Study of Social Insects, 18th French section* (1-3 sept., Tours)

## Séminaires invités

### Laboratoires à l'étranger

5. Univ. Regensburg (Germany), "Evolution, Behavior and Genetics" (Schrempf A), 2015
4. Univ. Regensburg, (Germany) "Evolution, Behavior and Genetics" (Heinze J), 2012
3. Univ. Mainz, (Germany) "Evolutionary Biology Group" (Meunier J / Foitzik S), 2012
2. Univ. Würzburg, (Germany) "Neurobiology of Social Organization" (Kleineidam C), 2008
1. Univ. Lausanne, (Switzerland) "Department of Ecology and Evolution" (Keller L), 2006

### Laboratoires en France

8. Univ. Rennes 1, "ECOsystèmes, BIOdiversité, Evolution" (Atlan A), 2011
7. Univ. Paris-Sud Orsay, "Ecologie, Systématique et Evolution" (Gireau T), 2011
6. CNRS Gif-sur-Yvette, "Laboratoire Evolution, Génomes et Spéciation" (Capy P), 2009
5. Univ. Tours, "Insectes Sociaux & Écologie Chimique" (Bagnères AG), 2008
4. INRA Versailles, "Physiologie de l'Insecte Signalisation & Communication" (Anton S), 2008
3. Univ. Toulouse, "Centre de Recherches sur la Cognition Animale" (Giurfa M), 2003
2. Univ. Tours, "Insectes Sociaux & Écologie Chimique" (Bagnères AG), 2003
1. CNRS Gif-sur-Yvette, "Populations, Génétique et Evolution" (Cariou ML), 2001

## Couvertures médiatiques

### TV

Rogers TV - Canadian news TV, 13 avril 2009 (Canada)
Arte TV documentaire – Phéromones, 1 mars 2002 (France)

**Radio**

France inter - La tête au carré, 14 fév. 2013 (France)
RFI – Autour de la question, 14 janv. 2013 (France)
CBC radio - Quirks & Quarks, 4 avril 2009 *(*Canada)

**Presse scientifique – Highlights/Dispatch**

Current Biology, 23 fév. 2010, 20 (4): R147-R149
The journal of experimental biology, 1 juil. 2009, 212 (13): iv
Current Biology, 8 août 2005, 15 (3): R88-R90

**Presse généraliste**

La Nouvelle République, 26 août 2015 (France)
Biofutur, 1 déc. 2012 (France)
Uniscope, 24 mai 2009 (Switzerland)
24 heures, 24 avril 2009 (Switzerland)
The Bulletin, 14 avril 2009 (Canada)
The New York Times, 31 mars 2009 (USA)

**Presse internet**

Structural Timber Magazine, 23 août 2019 (Angleterre)
La Nouvelle République, 7 juin 2011 (France)
Nature Education, 7 avril 2009 (USA)
The Bulletin, 31 mars 2009 *(*Canada)
The New York Times, 30 mars 2009 (USA)
UTM Press, 30 mars 2009 *(*Canada)

# *II – Mémoire*

# Introduction

Les organismes vivants ne sont pas isolés dans leurs environnements. Ils côtoient des semblables, des partenaires, des prédateurs et des compétiteurs dans un milieu où ils doivent s'orienter, s'organiser, se substanter pour y survivre. Ainsi les êtres vivants interagissent tous les uns avec les autres et de ces interactions complexes émergent une force évolutive. Comprendre ces relations qui sont au centre de l'adaptation des espèces à leur environnement est une question centrale en biologie évolutive. Force majeure de l'évolution, cette adaptation dépend d'un système de communication qui permet aux individus d'obtenir des informations sur ce qui les entourent. Une relation dynamique d'échange de messages forme la nature même de la nécessité de communication, entre un émetteur et un receveur, entre une source et un récepteur. C'est sur cette base d'informations provenant de sources biotiques et abiotiques, que les individus modifient leurs comportements (Lynch & Walsh, 1998; Rutter, 2006). Ainsi, un polymorphisme comportemental émerge, influençant par la même les autres organismes et les autres facteurs abiotiques avoisinants. L'individu lui-même émetteur de ce comportement va modifier sa physiologie, l'expression de ses gènes, voire sa morphologie. Cette double modification, des facteurs externes et internes de l'individu définit la dynamique "environnement – gène – comportement".

Les systèmes de communication ont donc un rôle central dans les interactions écologiques (interactions individu–environnement), s'appuyant sur des signaux de communication émis dans l'environnement, ils agissent sur l'expression génique des individus et modifient leurs réponses comportementales (Fig. 1). Les systèmes de communication regroupent l'ensemble des éléments de base que sont les émetteurs, les récepteurs, les messages, les canaux (visuel, chimique, vibro-acoustique) véhiculant le message jusqu'aux receveurs (Brossut, 1996). Nous avons donc les individus acteurs de la communication (émetteurs, receveurs) ; l'information contenue dans le message qui est dépendante du canal utilisé pour diffuser ce message et qui compte plusieurs types de modalité liés aux caractéristiques physiques intrinsèques des canaux (signaux vibratoires : sons/lumière, signaux tactiles, signaux chimiques : odorat/goût) (Tanzarella, 2005) ; et le décryptage du message qui va être perçu par des récepteurs spécialisés (cellule sensorielle localisée ou non dans un organe) et qui sera intégré au niveau du système nerveux central et périphérique (Burrows, 1996). Ceci inclus donc un code de communication et un contexte environnemental avec éventuellement une réponse feedback vers l'émetteur. Le modèle de Jakobson, utilisé dans les processus linguistiques, résume assez bien les différentes composantes des systèmes de communication (Jakobson, 1960). Le comportement est la résultante observable des systèmes de communication, il représente donc l'objet principal quantifiable de mes travaux, tout en y étant intimement lié.

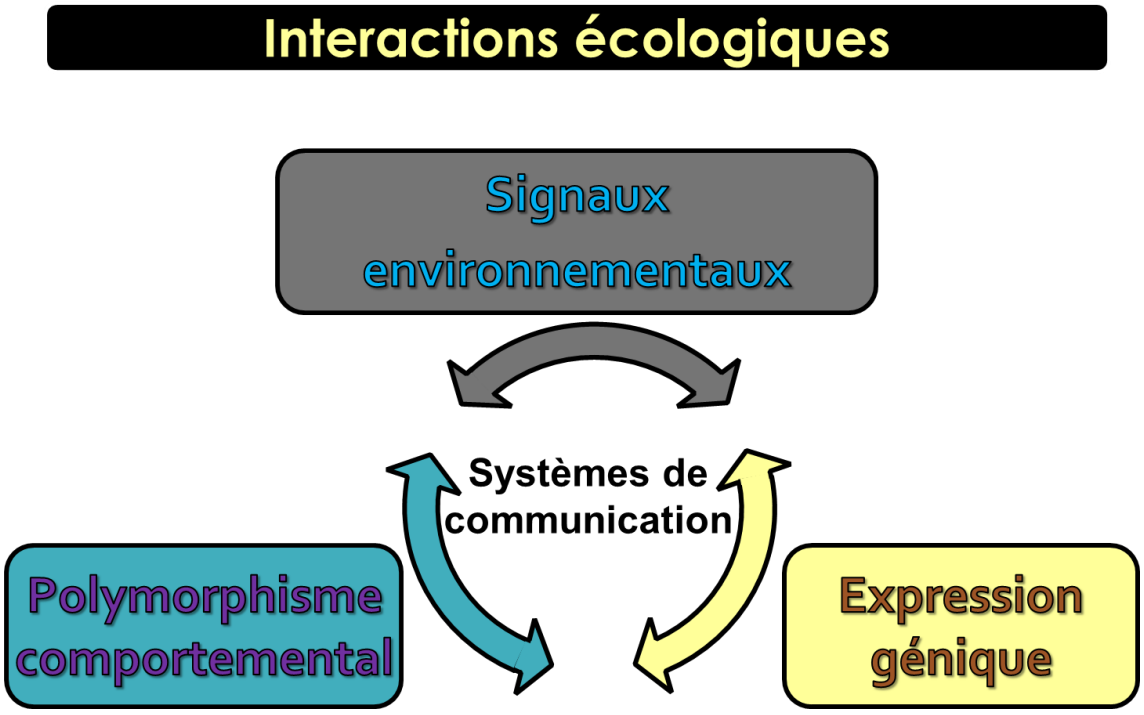


**Figure 1.** Les systèmes de communication sont au centre de la dynamique "environnement – gène – comportement".

Connectée avec les notions de communication, la notion de signal se définit comme toute action ou structure qui modifie le comportement d'un autre organisme, qui a évolué à cause de son effet, et qui est efficace parce que la réponse du receveur a aussi évolué dans ce sens (Maynard Smith & Harper, 2003; Davies *et al.*, 2012). La notion de signal est à différencier de la notion d'indice. Cette dernière est définit comme toute information de l'environnement, biotique ou abiotique, qui peut être utilisée par un individu pour le guider dans une future action (Hasson, 1994). L'exemple type d'indice est le poids d'un individu, qui peut influencer le comportement d'un compétiteur lors de rencontres antagonistes dyadiques. Pour autant, le poids n'a pas évolué parce qu'il a un effet sur le compétiteur (Riechert, 1978). Que ce soient les signaux émis par un émetteur ou les indices présents dans l'environnement, tous deux peuvent agir sur la réponse comportementale du receveur.

La perception et la communication des signaux environnementaux peuvent être réalisées à longue ou à courte distance en fonction du type même du signal et de la nature du milieu de transmission. Les signaux vibratoires (visuels et acoustiques) étant généralement des signaux à plus longue portée comparés aux signaux chimiques (goût et odorat). La nature du milieu de transmission impacte de façon importante la portée du signal, comme par exemple les milieux aqueux qui diminuent la diffusion des signaux visuels tandis qu'ils accélèrent les signaux acoustiques. La communication chimique est la plus répandue dans le monde animal et particulièrement chez les insectes (Wyatt, 2003). Elle participe en effet au fonctionnement de nombreux comportements

primordiaux pour la survie de l'individu comme la recherche de partenaire sexuel (Jallon, 1984; Antony *et al.*, 1985; Singer, 1998), la localisation d'un lieu propice à la ponte, les interactions proies-prédateurs, la fertilité (Monnin *et al.*, 1998; Cuvillier-Hot *et al.*, 2002; Heinze *et al.*, 2002), la défense, la reconnaissance des apparentés (Dettner & Liepert, 1994; Vander Meer & Morel, 1998; Lenoir *et al.*, 1999), l'isolation reproductive (Blomquist *et al.*, 1987; Cobb & Jallon, 1990; Chase *et al.*, 1992) ou encore la recherche de nourriture (Howard, 1993; Blomquist & Bagnères, 2010). Les récepteurs du goût et de l'odorat présentent des différences structurelles et ne sont pas localisées au même endroit afin d'optimiser les contacts avec leurs substrats (Salesse & Gervais, 2013). C'est là qu'intervient la notion de volatilité du signal ou plus généralement sa distance de transmission. Elle va dépendre à la fois des propriétés physico-chimiques du signal avec l'élément qui le transporte et aussi de sa taille. Le principe de volatilité est donc à mettre en parallèle avec la distance de perception en plus des qualités intrinsèques du signal (Fig. 2). Une fois la cible atteinte, le signal ne déclenche pas forcément de réponse immédiate, il existe des seuils de concentration spécifiques aux récepteurs de ces signaux qui peuvent aboutir à l'évolution de canaux de communication spécialisés avec une coadaptation "molécule-récepteur-transmission" (Córdoba-Aguilar *et al.*, 2018).

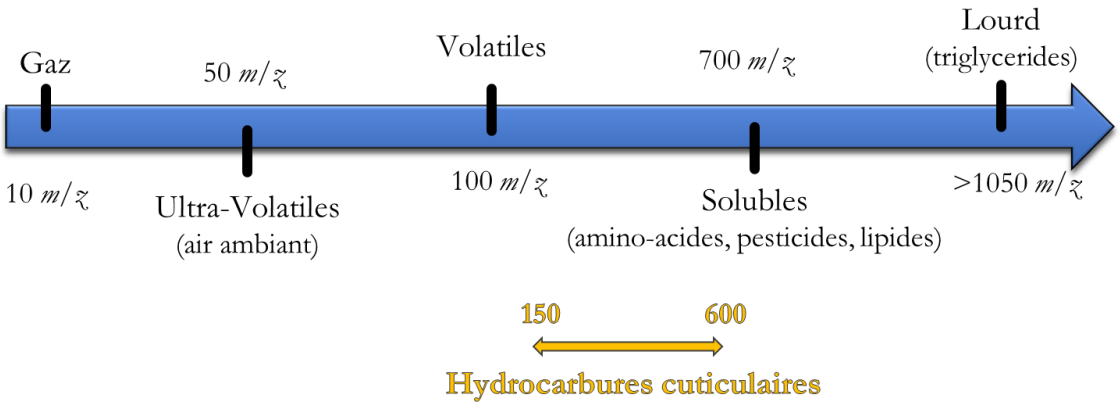


**Figure 2.** Gamme de volatilité des composés chimiques avec l'exemple des hydrocarbures cuticulaires.

Les composés sémiochimiques sont des substances émises par les organismes vivants dans l'environnement, qui vont déclencher une réponse comportementale ou physiologique chez l'individu receveur. Ces médiateurs peuvent agir sur les membres de la même espèce ou sur des espèces différentes, on parle alors respectivement de phéromones ou de médiateurs allélochimiques (Fig. 3) (Karlson & Lüscher, 1959; Brossut, 1996). Ces composés peuvent être nouvellement synthétisés par des glandes spécialisées ou encore dérivés de l'alimentation par transformation chimique des micro-organismes contenus dans le tractus digestif. C'est le cas de certaines phéromones des scolytes qui proviennent de l'oxydation des terpènes du bois dont ils se nourrissent (Huber *et al.*, 2007), ou encore les profils hydrocarbonés des drosophiles qui sont sous l'influence de leurs microorganismes (Lizé *et al.*, 2014). Par exemple, les hydrocarbures cuticulaires peuvent changer au cours du cycle de vie en

fonction de l'âge (Panek *et al.*, 2001) ou en fonction de la tâche comportementale de l'individu (Greene & Gordon, 2003). Ils peuvent être influencés par l'environnement, comme le substrat du nid (Heinze *et al.*, 1996; Bos *et al.*, 2011), les conditions nutritives (Liang & Silverman, 2000; Steiger *et al.*, 2007), ou les interactions sociales avec les conspécifiques par des échanges actifs ou passifs (Soroker *et al.*, 2003; Meunier *et al.*, 2011; Lucas *et al.*, 2018).

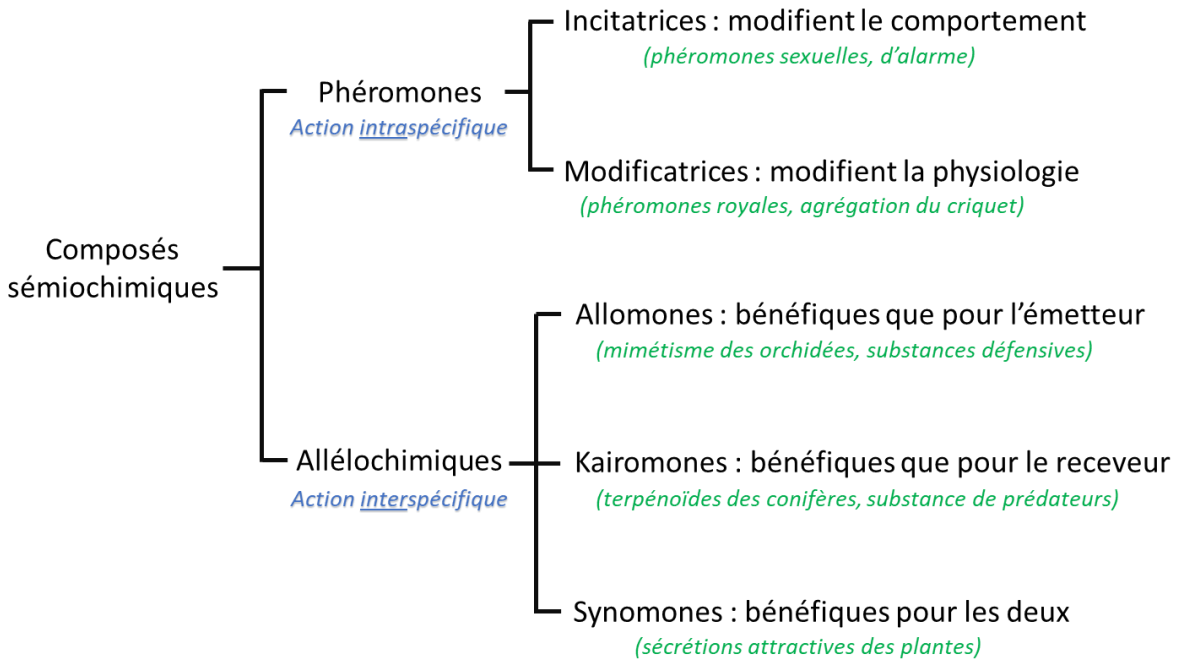


**Figure 3.** Catégories des médiateurs chimiques (adapté de Brossut, 1996).

La pluralité des signaux environnementaux est à mettre en parallèle avec la pluralité existante des systèmes biologiques. L'ordre des insectes est le plus riche du règne animal avec sa variabilité sans fin de morphologies et sa diversité comportementale. Avec plus de 1,3 millions d'espèces décrites, ils constituent 85% de la biodiversité animale en nombre d'espèces, dont la moitié de leur biomasse est représentée par les insectes sociaux. Leurs facilités de manipulation expérimentale et leur temps de génération souvent court en font de bons modèles d'étude. A cela s'ajoute une diversité de niveaux d'interaction, allant de systèmes solitaires à des systèmes à socialité dite vraie (eusociaux) caractérisés par des relations complexes d'interactions sociales obligatoires pour la survie des individus constituant ces sociétés (Wilson, 1971). La vie au sein de ces sociétés, regroupant parfois plusieurs millions d'individus, est dépendante d'un système de communication qui doit faciliter la transmission de l'information tout en l'enrichissant de la diversité des membres de la colonie. En effet, un signal est plus complexe qu'un simple lien d'action-réponse entre deux individus dans lequel l'individu receveur présente une réponse optimale appropriée à ce que l'individu émetteur attendrait. Ainsi, lorsque deux individus interagissent de façon répétée, la réponse individuelle à un signal peut dépendre de la mémorisation de la réponse comportementale de l'autre individu lors d'interactions

précédentes, la réponse est donc non strictement linéaire (Maynard Smith & Harper, 2003). La richesse des relations au sein des sociétés d'insectes apporte donc une complexification dont l'étude permet d'approfondir nos connaissances sur l'impact des systèmes de communication sur l'organisation sociale, mais aussi sur l'exploitation optimum des ressources, la diversité morphologique, le polyéthisme, l'organisation spatiale et temporelle, le vieillissement, le routage, la biologie de l'invasion, les services systémiques, inspirant au passage la robotique et les techniques de construction. L'étude de la communication des insectes, et particulièrement des sociétés d'insectes, est donc riche d'enseignement.

Les questions qui se posent sur les systèmes de communication sont donc innombrables. Mes recherches visent à apporter des réponses à une partie d'entre elles : Comment les signaux de l'environnement sont-ils générés, perçus, analysés et quelles réponses comportementales s'en trouvent modifiées ? Comment les interactions intra- et interspécifiques influencent-elles les comportements en participant au changement d'expression de certains gènes ? En intégrant les outils de l'écologie chimique et de la génétique moléculaire, mes recherches se situent à la croisée de l'écologie comportementale et de la génétique comportementale. Pour répondre à ces questions, j'étudie plusieurs modèles d'insectes, sociaux ou solitaires : termites, fourmis, guêpes, forficules, drosophiles et nasonia. Chacune de ces espèces a ses particularités et ses avantages qui sont utiles pour mener à bien mes recherches.

Dans l'exposé qui suit, je résume mes travaux sur la communication chez les insectes à travers l'étude des signaux de communication, la formation de ces signaux, l'adaptation des espèces à leurs environnements en utilisant les différents signaux perçus et le fonctionnement du polymorphisme comportemental via des canaux de communication spécialisés. Evidemment, par définition la recherche se développe au fil des découvertes et des opportunités, nous amenant parfois sur des chemins inattendus. Afin de faciliter la lecture d'une quinzaine d'années de recherche, j'utilise un cadre de lecture autours de la communication bien que certains travaux pourraient être lus autrement. Ainsi à travers le prisme de mes travaux, nous allons découvrir comment un signal de communication est produit, centralisé puis circule entre les individus pour finir par modifier leurs comportements, leurs capacités d'adaptation face aux contraintes environnementales jusqu'à changer leurs propres devenir en modifiant l'expression de leurs gènes et leurs phénotypes.

# 1. Signaux de communication

## 1.1 GENESE D'UN SIGNAL : L'EXEMPLE DES HYDROCARBURES CUTICULAIRES

Parmi les différents facteurs environnementaux (biotique et abiotique) porteurs d'informations qui peuvent influencer les comportements, on compte bien sûr l'environnement social. C'est évidemment une des composantes essentielles des interactions écologiques (interactions individu–environnement), qui comme nous allons le voir, agit sur la nature et la formation des hydrocarbures cuticulaires. En effet, les interactions sociales chez la plupart des insectes sont régulées par un système de communication d'origine chimique qui codifie les informations sociales et qui induit des réponses comportementales. Déchiffrer l'information que représentent les signaux chimiques est indispensable pour comprendre les mécanismes de communication des insectes et leur évolution. Afin de comprendre un tel système, j'ai étudié le rôle et la nature des médiateurs chimiques entre autres dans les sociétés d'insectes.

### 1.1.1 Les médiateurs chimiques dans les sociétés d'insectes

Les insectes sociaux représentent un exemple marquant d'utilisation de signaux chimiques pour la reconnaissance des apparentés (Howard, 1993; Blomquist & Bagnères, 2010). Puisque la socialité est basée sur la coopération, il est important de s'assurer que le fruit de leur travail aille au profit des individus apparentés au sein de la même communauté, pour éviter l'exploitation des ressources et pour limiter l'érosion de l'apparentement entre les membres du même groupe (Hamilton, 1964; Crozier & Pamilo, 1996). Ainsi, de nombreux mécanismes de reconnaissance et de discrimination des individus non-apparentés existent chez les insectes sociaux. L'un de ces mécanismes est la mise en place d'une odeur coloniale caractéristique des membres de la colonie. Cette odeur coloniale est la signature chimique qui regroupe l'ensemble des signaux non seulement coloniaux mais aussi individuels et qui est propre à chaque espèce. Les individus n'ayant pas le signal de reconnaissance spécifique et colonial sont écartés voire éliminés.

Lorsque deux individus se rencontrent, ils comparent immédiatement leur odeur via de nombreux contacts antennaires. Chez les insectes sociaux, cette odeur est généralement constituée d'hydrocarbures présents sur la cuticule (exosquelette des insectes). Afin d'homogénéiser l'odeur coloniale en fonction des individus constituant la colonie, plusieurs mécanismes de toilettage (social et individuel) s'opèrent entre les membres de la colonie. Ainsi, l'odeur coloniale, formée à partir de l'ensemble des odeurs individuelles, plus ou moins variables d'un individu à l'autre, se retrouve homogénéisée, globalisée au cours du temps. Etudier la mise en place, l'évolution et la perception de ces signaux est primordial pour comprendre l'existence et le maintien de la socialité chez les insectes.

Parmi l’ensemble des signaux chimiques identifiables sur la cuticule ou produits par les glandes sécrétrices, quels sont ceux qui participent à la tolérance des "semblables" vis à vis des "différents" ? Et quels sont ceux qui déclenchent un comportement agoniste ?

Nous avons tenté de répondre à ces questions en utilisant comme matériel biologique une espèce de fourmis primitives : *Pachycondyla villosa* (Lucas *et al.,* 2002). Avant toute chose, il fallait d'abord caractériser les signatures cuticulaires de colonies prélevées dans un même site écologique. L'analyse de ces hydrocarbures (HCs) cuticulaires a montré un polymorphisme chimique extrêmement marqué entre colonies. Ces différences beaucoup plus marquées que celles classiquement trouvées entre colonies, pouvaient suggérer que ces colonies appartenaient, en fait, à des espèces différentes. L'analyse d'autres critères morphologiques (géométrie du pétiole par Microscopie Electronique à Balayage), biochimiques (isozymes) et cytogénétiques (caryotypes) nous ont conduits aux mêmes conclusions : l'existence d'un complexe de trois espèces. Ainsi, une espèce jusqu'ici classée en synonymie taxonomique avec *Pachycondyla villosa*, nommée *P. inversa*, s'avère être une espèce "vraie" et une autre espèce jusqu'alors inconnue, provisoirement nommée *P. subversa*, a pu être caractérisée (Lucas *et al.,* 2002). Cette espèce a depuis été décrite et renommée *Neoponera curvinod*is (Mackay & Mackay, 2010). D'ailleurs ce phénomène d'espèces cryptiques que l’on pensait alors ponctuel du complexe d’espèces *P. villosa* s’est avéré finalement assez fréquent (Santos *et al.,* 2018) et de nouveaux complexes ont été découverts depuis avec notamment *N. apicalis* (Wild, 2005; Ferreira *et al.,* 2010) et *P. chinensis* (Yashiro *et al.,* 2010).

Une des conclusions intéressantes de ces travaux est le maintien d'un "*villosa* morph" constitué d'au moins trois espèces de fourmis et de deux espèces d'araignées. Le tout constituant ce que l'on nomme un cercle de mimétisme müllerien, lorsqu'il est constituée d'espèces nocives, ou batésienc lorsqu'il regroupe des espèces considérées comme inoffensives (Mclver & Stonedahl, 1993). Ces araignées considérées comme "inoffensives", appartiennent à deux familles différentes : *Sphecotypus niger* (Clubionidae) et *Zuniga sp.* (Salticidae), ce qui pose un problème évolutif intéressant (Fig. 4). En effet, la myrmécomorphie n'est pas un phénomène rare dans les régions néotropicales, pourtant dans le genre Pachycondyla, seul le "*villosa* morph" apparait être un modèle pour les araignées (Delabie, 1999). Il est probable que ce "*villosa* morph" permette à l'ensemble des espèces le constituant, individuellement faiblement compétitives et non dominantes, d'apparaître omniprésentes et numériquement abondantes, de façon à devenir dissuasives à l'encontre des différents prédateurs. Il est d'ailleurs assez curieux d'observer ces araignées qui vont jusqu'à mimer les antennes des fourmis en relevant leurs premières paires de pattes en l'air. L'analyse des profils chimiques pourrait nous renseigner sur le peu d'agressivité que reçoivent ces araignées lorsque les fourmis les rencontrent, malheureusement il est difficile d'obtenir des spécimens en quantité suffisante.

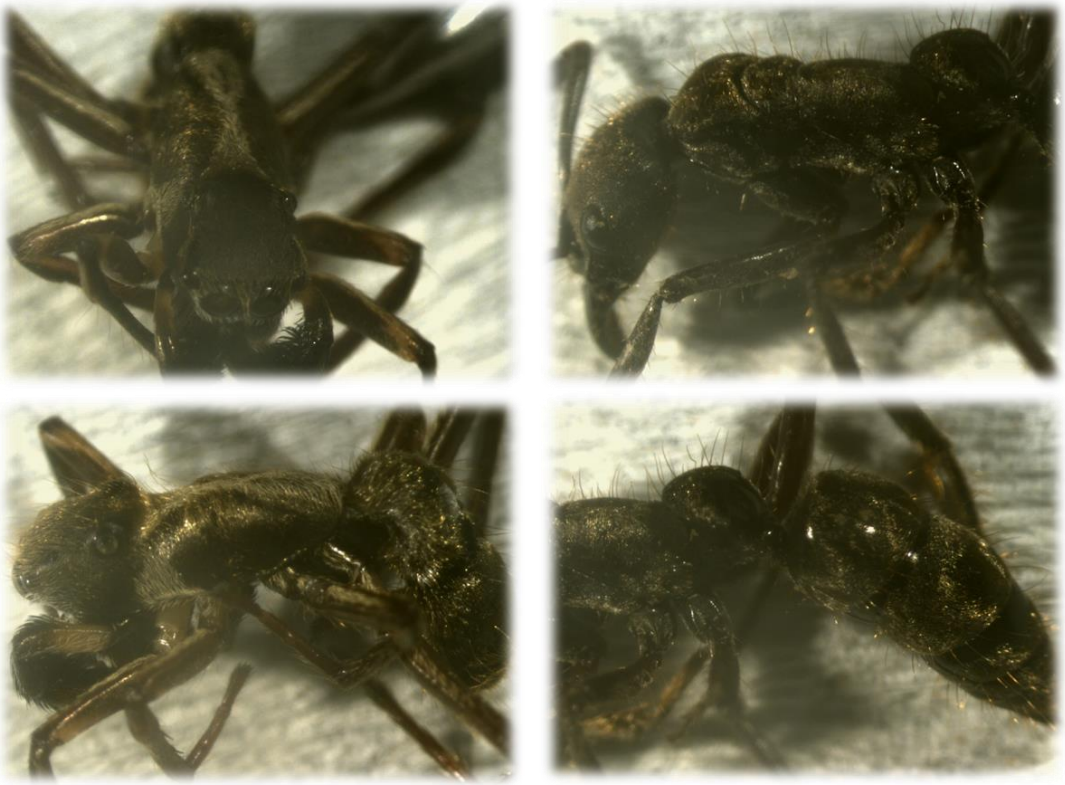


**Figure 4.** Photos de l'araignée *Sphecotypus niger* à gauche et la fourmi *Pachycondyla villosa* à droite (aimablement fournies par JHC Delabie).

L'analyse chimique des trois espèces ainsi découvertes a apporté des informations sur la signature coloniale. Mais parmi les différents composants chimiques de ces signatures spécifiques, quels sont donc ceux qui jouent un rôle dans le contrôle de la tolérance/intolérance ? Les hydrocarbures cuticulaires sont connus pour être impliqués dans la reconnaissance des nestmates (membres de la colonie) (Wagner *et al.*, 2000). Mais au sein d'une signature chimique, il existe de nombreux composés de natures et de structures différentes (Gibbs & Pomonis, 1995; Pennanec'h *et al.*, 1997). Afin de tester le rôle des principales familles chimiques d'HCs cuticulaires, dans un premier temps, nous les avons isolés de la cuticule des trois espèces. Grâce à la chromatographie en couche mince et à l'utilisation de tamis moléculaire de 5Å, nous avons séparé plusieurs fractions basées sur leurs structures chimiques : les non-hydrocarbonés, les alcanes, les insaturés (alcènes/alcynes mélangés) et les HCs branchés. Par la suite, nous avons testé ces différentes familles en les présentant à des individus provenant de colonies et d'espèces différentes dans un test comportemental d'immobilisation que nous avions mis au point. Pour la première fois, ce test permettait de mesurer l'agressivité des individus dans différentes conditions, face à un autre individu ou face à un extrait chimique sans contact physique directe, bien souvent mortel. Deux items comportementaux sont ressortis comme étant caractéristiques de la détection d'odeurs étrangères : l'ouverture mandibulaire et le retrait antennaire (Fig. 5). Lors de ces tests, nous avons montré que les

3 espèces développaient des comportements agressifs spécifiques directement corrélés à la divergence chimique de leurs HCs cuticulaires. Ainsi, plus les différences entre signatures chimiques étaient marquées plus l'agressivité était forte. De plus, cela démontrait pour la première fois que la fraction "non-hydrocarbonée" cuticulaire n'avait pas d'impact sur l'agressivité. Enfin, seule la fraction contenant les hydrocarbures branchés (méthyle, diméthyle, triméthyle) déclenchait une réponse comportementale agressive (Lucas *et al.*, 2005).

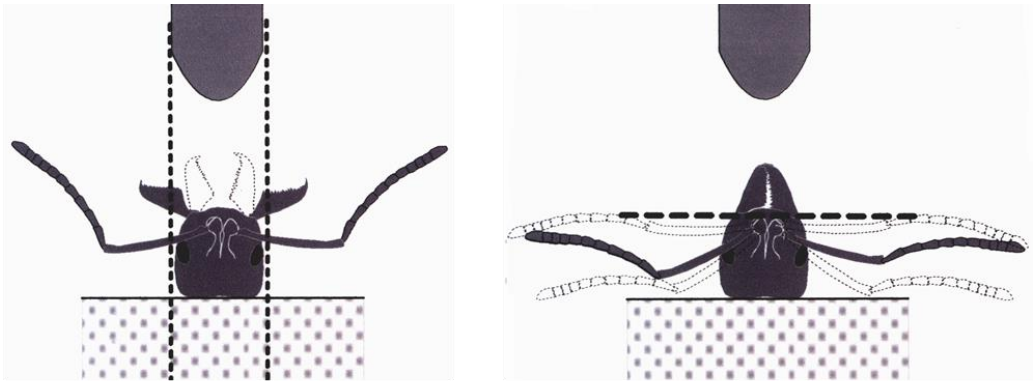


**Figure 5.** (**A**) Ouverture mandibulaire, (**B**) retrait antennaire (Lucas *et al.*, 2005).

Ces résultats couplés à des analyses sur la composition de la glande post-pharyngienne (Lucas *et al.*, 2002), ont permis de mieux connaître le processus de formation de l'odeur coloniale, en précisant le schéma de circulation "interne" (physiologique et biochimique) et "externe" (comportemental et social) des hydrocarbures impliqués dans les mécanismes de reconnaissance des apparentés (Soroker *et al.*, 2003; Lucas *et al.*, 2004), comme présenté ci-dessous.

### 1.1.2 Le "visa" colonial : production commune d'origine individuelle

Comme nous venons de le voir, les hydrocarbures cuticulaires jouent un rôle clé dans la communication chimique, aussi bien chez les insectes solitaires que sociaux (Howard & Blomquist, 2005). On peut se demander comment ces signaux chimiques sont produits et comment un signal collectif peut émerger. Tout d'abord, il faut savoir qu'à l'échelle de l'individu, les HCs sont biosynthétisés au niveau des corps lipidiques associés à des cellules sous épidermiques modifiées (œnocytes) (Krupp *et al.*, 2008; Makki *et al.*, 2014). Puis, ils sont transportés par une lipoprotéine circulante dans l'hémolymphe, la lipophorine, jusqu'à leurs lieux de destination. Il existe donc bien une production individuelle des HCs or chez les insectes sociaux cette production individuelle reflète aussi une odeur commune coloniale.

#### *1.1.2.1 Circulation "interne" des hydrocarbures*

La formation de l'odeur coloniale des fourmis appelée "visa" colonial (ou gestalt) fait intervenir trois acteurs dans le schéma de circulation des HCs (Lucas, 2002) : (i) la lipophorine qui est une protéine ubiquitaire chez les insectes servant de transporteur dans l'hémolymphe des HCs synthétisés (Schal *et al.*, 2001) ; (ii) la glande post-pharyngienne (PPG) qui est une glande contenant des HCs. Cette glande est spécifique aux fourmis mais des glandes équivalentes ont été décrites chez les autres insectes sociaux (Billen & Morgan, 1998) ; et (iii) la cuticule qui est l'exosquelette des insectes, support des signaux de reconnaissance (Gibbs, 1998b).

L'analyse comparée des HCs de ces 3 facteurs a apporté des informations nouvelles sur les mécanismes impliqués dans la reconnaissance des apparentés (Lucas *et al.*, 2004). En effet, l'analyse comparative des profils hydrocarbonés, par chromatographie en phase gazeuse couplée à la spectrométrie de masse, montre que la cuticule, la PPG et la lipophorine ne présentent aucune différence qualitative. En revanche, de nettes différences quantitatives sont présentes entre la cuticule et la lipophorine d'une part et la PPG d'autre part. Les HCs présents en proportion similaire sur la cuticule et dans la PPG diffèrent dans leurs proportions d'une colonie à l'autre et sont par conséquent les plus susceptibles de participer de façon prépondérante à la reconnaissance des apparentés. Ainsi, les *n*-alcanes et les monométhylalcanes externes (méthyle en positions carbonnées 2 à 6) sont en très faible concentration, voire quasiment absents de la PPG. Sachant que chez plusieurs espèces de fourmis, les HCs de la PPG et de la cuticule induisent une réponse comportementale identique (Soroker *et al.*, 1994; Lahav *et al.*, 1999), il est probable que l'absence de ces HCs signifie qu'ils ne sont pas impliqués dans la reconnaissance des apparentés. Les *n*-alcanes et les monométhylalcanes externes ne seraient donc pas porteurs d'informations spécifiques et coloniales chez les espèces de *Pachycondyla* étudiées. C'est ce que nous avons démontré par la suite avec des tests comportementaux, au moins en ce qui concerne les *n*-alcanes (Lucas *et al.*, 2005).

En ce qui concerne les HCs absents de la PPG, leurs propriétés physico-chimiques montrent qu'ils sont de bons candidats pour protéger les insectes contre la dessiccation. Ainsi sur la cuticule, les HCs importants pour la reconnaissance des apparentés et ceux indispensables pour la lutte contre la dessiccation coexistent dans un juste équilibre. Au regard des propriétés physicochimiques des différentes familles d'HCs, on arrive à la conclusion que les HCs impliqués dans la lutte contre la dessiccation possèdent une plus haute température de fusion et sont par conséquent moins volatiles, tandis que ceux impliqués dans la communication chimique sont plus volatiles (Gibbs, 1998a; Young *et al.*, 2000). Par exemple, on peut facilement opposer les alcanes aux alcènes qui avec leurs insaturations ont des propriétés évidemment plus volatiles. On peut aussi séparer les HCs branchés en fonction de la position des groupements méthylés, avec d'un côté les méthylations externes et de l'autre les méthylations internes, la charnière se situant aux alentours du carbone en position 6 (Gibbs & Pomonis, 1995).

Finalement, l'ensemble du mélange hydrocarboné influence la température de fusion générale de la cuticule et doit permettre aux individus de s'adapter aux conditions climatiques, à

l'environnement et/ou à la situation sociale de l'individu. La PPG peut donc être considérée comme une réponse évolutive au problème posé par la présence sur la cuticule des 2 grands types d'HCs. Les hautes températures sont des facteurs environnementaux dangereux pour la survie des individus à moyen terme qui nécessite la présence d'HCs lourds peu volatiles, tandis que les molécules plus volatiles sont idéales en tant que signaux de reconnaissance et leur absence sur la cuticule entrainerait l'agressivité des congénères ce qui représente un danger continu, immédiat, bien souvent fatal (Fig. 6). La PPG représente donc un réservoir de grande capacité, facilement accessible et efficace pour reconstituer l'odeur coloniale dans les situations nécessitant un renforcement immédiat de signaux coloniaux, permettant une grande flexibilité comportementale quelles que soient les situations rencontrées.

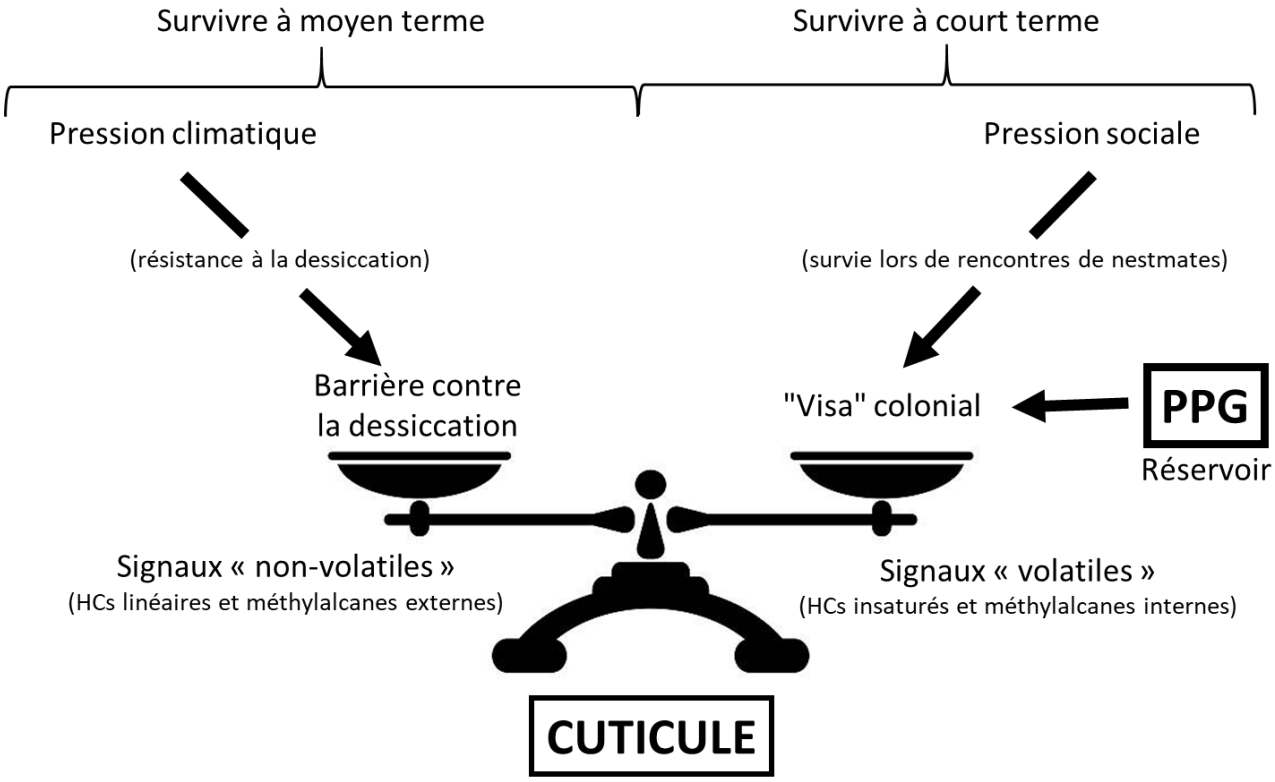


**Figure 6.** Trade-off entre la résistance à la dessiccation et la survie lors de rencontres d'autres membres de la colonie.

#### *1.1.2.2 Circulation "externe" des hydrocarbures*

Chez les insectes sociaux, l'élaboration d'un signal collectif en tant que signature coloniale suppose une circulation des HCs à l'intérieur de chaque individu comme nous venons de le voir, mais aussi entre tous les individus appartenant à la même colonie. Pour ce faire, nous avons étudié le schéma de circulation "externe" des HCs ainsi que la façon dont ces HCs sont homogénéisés, confirmant le rôle important joué par la PPG.

Les HCs nouvellement biosynthétisés sont transportés jusqu'à la cuticule par la lipophorine comme cela a été démontré chez la mouche (Pho *et al.*, 1996; Schal *et al.*, 2001). Puis ils sont déposés sur la cuticule et sont sensiblement accumulés au niveau des brosses basitarsales (Hefetz *et al.*, 2001). En effet, les brosses basitarsales sont constituées de spatules dont la surface représente environ 10 $mm^2$, soit un quart de la surface totale du thorax et des 2 paires de pattes arrière (Soroker *et al.*, 2003). De plus, le comportement de toilettage des pattes avant entre les mandibules voit sa fréquence augmentée lors de rencontres intercoloniales. Une expérience empêchant le contact des brosses basitarsales avec la bouche a montré une augmentation de l'accumulation d'HCs sur les brosses. Ce qui signifie que les HCs sont d'origine interne et que le contact avec la bouche permet à la fourmi de décharger ces HCs présents sur les pattes vers la bouche. Nous avons fait des expériences complémentaires en utilisant des marqueurs radioactifs pour confirmer ces observations et pour montrer une accumulation des HCs dans la PPG. Pour ce faire, nous avons injecté des précurseurs radioactifs de la chaîne de biosynthèse des HCs (1 µCi d'acétate de sodium [1–$^{14}$C]) à dix ouvrières au niveau de l'abdomen. Après dissection des différentes parties, la répartition de la radioactivité, a permis de retrouver les HCs nouvellement synthétisés au niveau de la cuticule, des brosses basitarsales, de la PPG et du jabot.

Afin de préciser la circulation des HCs, nous avons fait des expériences avec une fourmi marquée radioactivement dit "donneuse" que nous avons réintégrée dans un groupe de 10 nestmates dites "receveuses" après 24h d'isolement. Ces 10 individus receveurs acquièrent progressivement les HCs radioactifs nouvellement synthétisés par le biais d'interactions sociales avec le "donneur". Au bout de 5 à 10 jours, l'homogénéisation de la radioactivité totale est atteinte. Cela démontre l'existence d'échanges actifs lors des toilettages sociaux (allo-grooming) ou d'échanges passifs lors de contacts physiques (Soroker *et al.*, 2003). Les HCs passent aussi de la cuticule à la PPG lors de toilettages individuels (self-grooming). D'autres expériences ont montré que les HCs pouvaient être aussi échangés durant les trophallaxies (Lenoir *et al.*, 2001). L'analyse comparée des différentes espèces présentant des degrés distincts de toilettages (self-grooming, allo-grooming et trophallaxie) montre que la rapidité d'échange des HCs est supérieure chez les espèces qui font de la trophallaxie et des toilettages mutuels intensifs. Chez les espèces plus primitives telles que celles du genre *Pachycondyla*, les HCs sont principalement échangés par contacts corporels puisque ces espèces ne font pas de trophallaxie et présentent un taux de toilettage mutuel faible. La complexification des toilettages sociaux au cours de l'évolution conduit donc à une fluidification de la transmission des signaux de communication (Soroker *et al.*, 2003).

Il faut savoir que la PPG ne synthétise pas directement d'HCs comme le montrent deux expériences indépendantes d'incubation de glandes dans des milieux contenant des précurseurs radioactifs. Ces résultats "non significatifs" n'ont pu être malheureusement publiés (Lucas *et al.*, unpublished, Hefetz *et al.*, pers. com.). La PPG va donc stocker les HCs cuticulaires de l'individu mais aussi ceux de ses congénères. L'ensemble du mélange homogénéisé des HCs de la PPG est ensuite redistribué sur toute la cuticule de chaque individu constituant ainsi une odeur coloniale. La PPG apparaît donc comme un intermédiaire indispensable pour la formation du "visa" colonial. Une question restant en suspens porte sur les mécanismes permettant l'accumulation dans la PPG de

seulement certains HCs, alors que la source de production contient les mêmes HCs que la PPG mais en quantité beaucoup plus grande comme le montrent nos travaux sur la lipophorine (Lucas *et al.*, 2004).

### 1.1.3 Production et rythme circadien

Les HCs sont issus d'une production interne à partir de l'acétate et de certains acides aminés par la voie de biosynthèse des acides gras (Fig. 7). Elle met en jeu une série d'enzymes afin d'allonger la chaine hydrocarbonée et aussi d'ajouter des insaturations (Dallerac *et al.*, 2000; Kent *et al.*, 2008; Bousquet *et al.*, 2012; Dembeck *et al.*, 2015; Wicker-Thomas *et al.*, 2015). Chez la drosophile, des analyses génétiques ont montré l'implication de 24 gènes candidats régulant la biosynthèse des HCs dont la perturbation entraine des altérations du profil chimique chez les mâles et les femelles (Dembeck *et al.*, 2015). La bibliographie s'accordait sur le fait que la production des HCs devait se faire dans les œnocytes (Chertemps *et al.*, 2006), des cellules sous épidermiques modifiées qui semblaient montrer des activités de synthèse lipidique, à cause de leurs intrications avec le corps gras (Diehl, 1975; Romer, 1980). Des fractions de tissus abdominaux enrichis en œnocytes montraient des activités de synthèse chez *Blatella germanica* (Fan *et al.*, 2003).

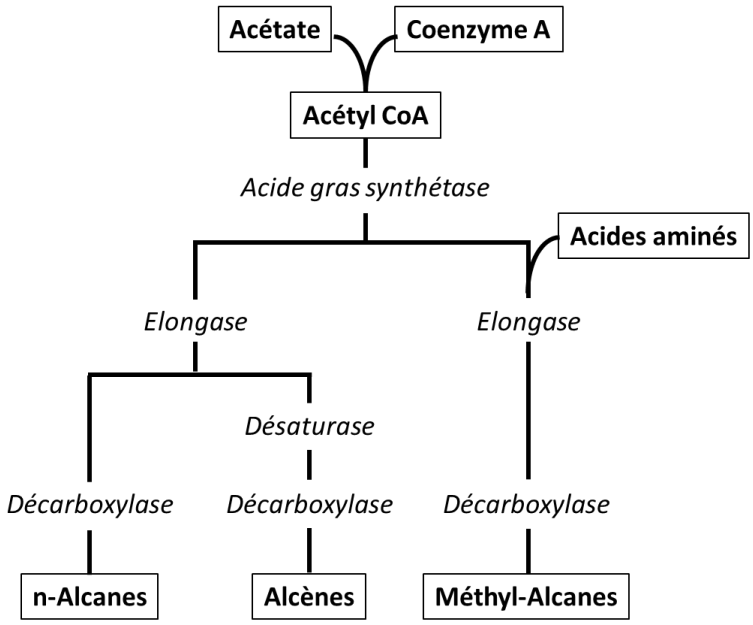


**Figure 7.** Chaine enzymatique simplifiée de la biosynthèse des hydrocarbures (D'après Kent *et al.*, 2008).

En travaillant sur l'origine de la production des HCs chez la drosophile, nous avons trouvé l'existence d'un rythme circadien interne qui est propre aux œnocytes. A l'aide de marqueurs spécifiques cellulaires et d'images en microscopie électronique à balayage, les œnocytes ont été

localisés précisément. Cela a permis des dissections fines des cellules pour analyser l'expression des gènes *timeless*, *period* et *clock* par qPCR ainsi que *desat1*, un gène impliqué dans la production phéromonale (Fig. 8). Dans le même temps, nous avons analysé les profils hydrocarbonés des mêmes individus par GC-MS. Les résultats montrent que les œnocytes possèdent une horloge biologique périphérique qui leur est propre. De plus, cette activité cellulaire est corrélée avec l'accumulation des hydrocarbures sur la cuticule en fonction des pics d'activité du rythme circadien (Krupp *et al.*, 2008). Nos travaux renforcent donc l'hypothèse selon laquelle les HCs sont produits par les œnocytes.

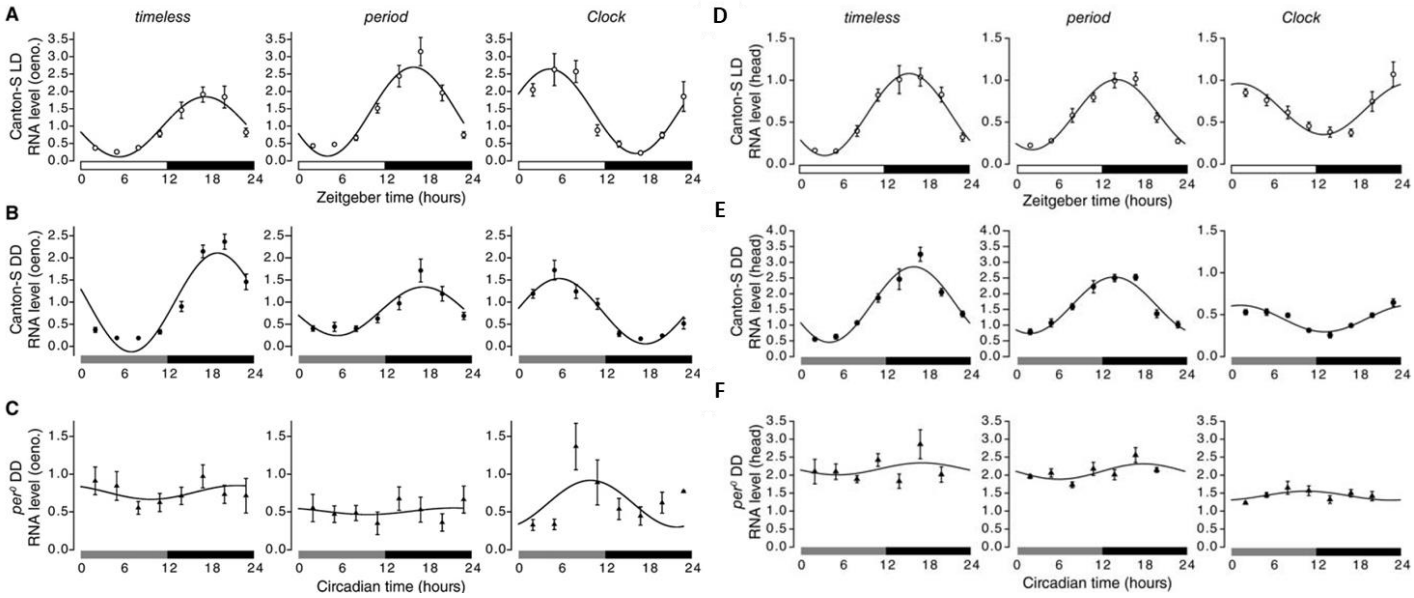


**Figure 8.** Oenocytes (**A**, **B**, **C**) and head (**D**, **E**, **F**) contain a per-dependent clock. Total RNA was isolated from dissected oenocytes (**A**, **B**, **C**) and head (**D**, **E**, **F**) preparations of the same animals. The temporal patterns of *tim*, *per*, and *Clk* RNA expression in wild-type and $per^0$ oenocytes as determined by quantitative RT-PCR. These patterns indicate a functional clock in the oenocytes for the wild-type in LD (**A**) and DD (**B**). The functional pattern is disrupted for $per^0$ in DD (**C**). In heads isolated from flies under LD (**D**) and DD (**E**) conditions, the temporal patterns of expression are significantly sinusoidal for *tim*, *per*, *Clk*. The temporal expression patterns of these genes are disrupted in $per^0$ heads isolated from flies under DD conditions (**F**). The phase relationships between the clock genes in heads in DD and LD conditions are stable, however the amplitude of expression is greater for *tim* and *per* in DD as compared to LD. Note that the phases of *tim*, *per* and *Clk* expression are slightly delayed in oenocytes relative to heads in both LD and DD (compare **D**, **E**, **F** with **A**, **B**, **C**).
Best-fit cosine curves (solid lines) are fitted to RNA expression values ± SEM. Each time point represents the average of three replicate qPCR reactions from three independent experiments. Canton-S (wild type fly strand); LD (Light/Dark) open circles; DD (Dark/Dark) filled circles; $per^0$ (mutant individuals missing circadian clock) DD, filled triangles (Krupp *et al.*, 2008).

Cette rythmicité de production phéromonale va permettre aux individus de synchroniser leurs comportements. En effet, il ne suffit pas seulement de savoir comment réagir avec ses congénères, grâce à la perception de phéromones spécifiques, mais il faut aussi savoir quand interagir (Dunlap *et al.*, 2004). A la suite de nos travaux, d'autres expériences complémentaires ont été développées par la même équipe de recherche, démontrant l'implication directe des œnocytes dans la production des HCs grâce à des expériences d'élimination génétique ciblées des cellules. Ils ont aussi montré que l'ajout d'un seul HC (7Z,11Z-heptacosadiène) était suffisant pour restaurer la barrière spécifique des femelles mutantes dépourvues d'HCs. Ils ont donc identifié un mécanisme commun de la reconnaissance sexuelle et spécifique dans lequel les HCs jouent un rôle clé (Billeter *et al.*, 2009).

## 1.2 APPRENTISSAGE ET MEMORISATION DES SIGNAUX

Lorsqu'un signal de l'environnement a été perçu et que celui-ci est critique pour la survie de l'individu, il est nécessaire de stocker cette information afin de maximiser les futures réponses comportementales. Plus l'apprentissage est rapide et plus la mémorisation est forte, meilleures seront les capacités d'adaptation de l'individu à son environnement. En effet, choisir le bon partenaire sexuel est une décision critique dans la vie d'un organisme se reproduisant par la voie sexuée (Lefevre *et al.*, 2016). Le choix du partenaire n'est pas seulement dirigé par la qualité du potentiel partenaire mais aussi par sa compatibilité génétique, ainsi que divers facteurs biotiques (Andersson, 1994; Davies *et al.*, 2012). Beaucoup d'organismes ont développé des processus pour s'orienter vers le bon choix mais peu d'études ont à ce jour été menées sur les mécanismes mis en jeu. Ces notions d'apprentissage et de mémorisation, je les ai étudiées à travers le comportement sexuel de la drosophile.

Le comportement sexuel des mâles de *Drosophila melanogaster* est composé d'une succession de mouvements stéréotypés stimulés par des signaux provenant de la femelle (Sokolowski, 2001). Ces signaux sont principalement d'ordre chimique, essentiellement hydrocarbonés, même si des signaux vibratoires sont utilisés (Rybak *et al.*, 2002). Ils influencent les différentes phases complexes qui constituent le comportement de cour de la Drosophile. Si un mâle est exposé à une femelle vierge, celle-ci fera l'objet de tentatives d'accouplement. Cependant, si la femelle est déjà fécondée, celle-ci le rejettera. Il faut savoir que normalement les mâles sont attirés uniquement par les femelles matures vierges et que les femelles ayant déjà copulé inhibent totalement le comportement de cour des mâles vers ces femelles quel que soit leur état de maturité. Par la suite, le mâle ayant été rejeté diminuera ses tentatives d'accouplement avec toute autre femelle déjà fécondée. Nous avons donc utilisé ce procédé expérimental pour disséquer les mécanismes de l'apprentissage et de la mémorisation dans le comportement sexuel de la drosophile, et ainsi comprendre le rôle joué par les HCs.

Nos travaux montrent qu'il est possible d'utiliser des femelles décapitées qui vont être systématiquement l'objet de tentatives d'accouplement de la part des mâles. Ces femelles vont survivre pendant plusieurs heures mais les tentatives des mâles vont systématiquement être vouées à l'échec. Les mâles apprendront à éviter ce type de femelle dont ils ont été rejetés même s'il s'agit de

femelles qui sont normalement l'objet de tentatives d'accouplement. Ainsi, si des mâles ont été pré-exposés à des femelles décapitées matures, les mâles éviteront les femelles matures mais pas les femelles immatures et vice-versa (Fig. 9, à noter qu'un "memory index" égal à 1 signifie qu'il n'y a pas eu de mémorisation).

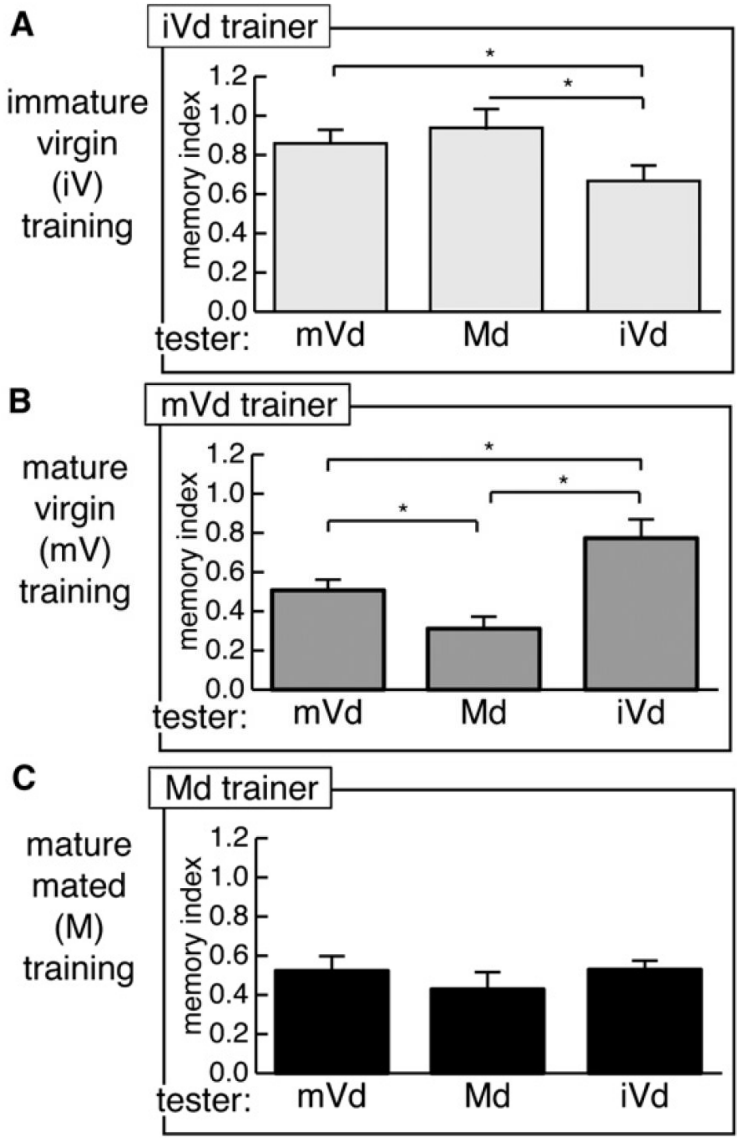


**Figure 9.** Courtship suppression learned with a mated female trainer is generalized to all types of females.

(**A**) Training with an immature decapitated virgin female produces suppression only when the tester is also an immature female.

(**B**) Training with a mature decapitated female produces suppression of courtship with mature (virgin and mated) testers.

(**C**) Training with a decapitated mated female produces a generalized suppression to all female testers. Data were analyzed as described in Experimental Procedures; *p<0.05 for the indicated comparison. Trainer and tester types are abbreviated as follows: Md, decapitated mated female; mVd, decapitated mature virgin; iVd, decapitated immature virgin. Histogram bars for data in which males were trained with mated females are black; with mature virgins, dark gray; immature virgins, light gray (Ejima *et al.*, 2007).

De plus, si l'on pré-expose des mâles uniquement à l'odeur de femelle sans la présence physique de la femelle, par la suite, lors de rencontres avec une nouvelle femelle, ces mâles apprendront à éviter les femelles qui présentent la même odeur (Fig. 10). En effet, les mâles ont été mis en présence avec une odeur de femelle mais il n'y a pas eu de succès d'accouplement associé. Par la même, cela démontre que l'apprentissage des mâles à discriminer les femelles est principalement basé sur la reconnaissance des profils hydrocarbonés des femelles (Ejima *et al.*, 2005). D'ailleurs, les mâles dont les antennes et les maxillaires ont été ablatés ne sont pas capables de copuler avec les femelles.

L'analyse des HCs cuticulaires des femelles matures et immatures a montré l'existence de différences importantes. Sur les 85 composés analysés, 63 sont communs aux deux types de femelles et 22 sont différents. Si l'on regarde plus en détails ces composés, les différences qualitatives et quantitatives ne portent pas sur les HCs linéaires et les monométhylalcanes externes (carbone 2 à 6). Il est donc probable que ces types d'hydrocarbures ne sont pas impliqués dans cet apprentissage du comportement sexuel de la mouche. En revanche, les hydrocarbures insaturés, beaucoup plus volatiles, montrent d'importantes différences quantitatives et une plus grande proportion d'insaturés de haut poids moléculaire chez les femelles immatures. Ces hydrocarbures sont donc probablement utilisés par les mâles pour discriminer les femelles vierges immatures des femelles vierges matures, ce qui corrobore nos travaux précédents chez les fourmis (Soroker *et al.*, 2003; Lucas *et al.*, 2004; 2005). Nous avons aussi utilisé des mâles mutants qui ont montré l'implication de la voie de signalisation métabolique de l'AMPc (Adénosine MonoPhosphate cyclique) (Ejima *et al.*, 2005).

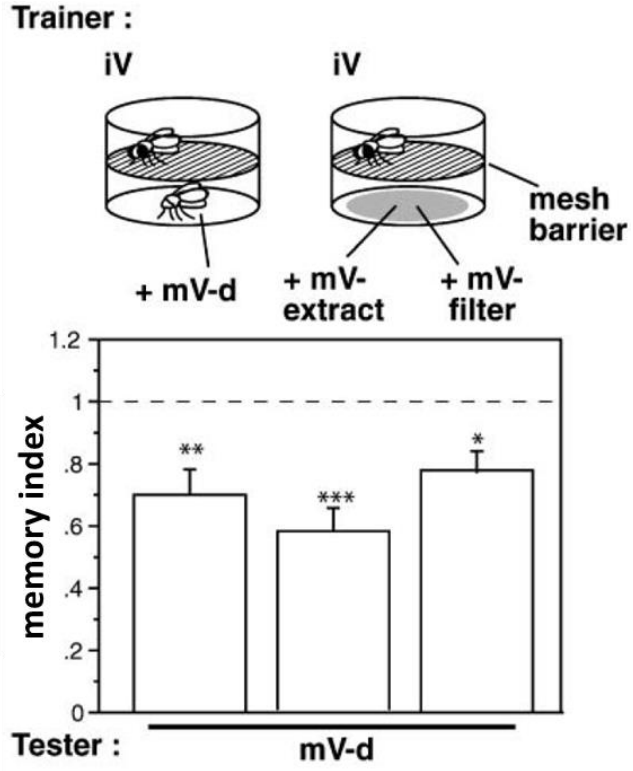


**Figure 10.** The conditioned stimulus for trainer-specific learning is a volatile odorant. Males were trained for 1h. Trainer condition is indicated above the panel, and tester type is indicated below each set of bars (mV, mature virgin; iV, immature virgin; -d, decapitated). Data are presented as means SEM.

Males can sense trainer-specific pheromones without contacting them. Males were trained in a two-part chamber separated by a mesh. The courtship object was placed in the same chamber as the male, and the conditioned stimulus (either a mature virgin or a pheromone filter that had been in contact with a mature virgin or that contained a hexane extract equivalent to 1.25 mature virgins) was placed in the lower chamber. Volatile conditioned stimulus was an effective cue (*p 0.1,**p 0.05, or ***p 0.001, for comparison with the null hypothesis) (Ejima *et al.*, 2005).

La suppression de la mémoire acquise par l'apprentissage de nouveaux signaux démontre que la dynamique de consolidation de la mémoire est plastique chez la drosophile, ce qui est essentiel pour la navigation au sein d'un environnement naturel riche. En effet, nos expériences montrent que la mémorisation est sensible à l'ordre de présentation dans le temps des différentes odeurs et que celles-ci nécessitent un délai dans le temps pour pouvoir être mémorisées (Fig. 11).

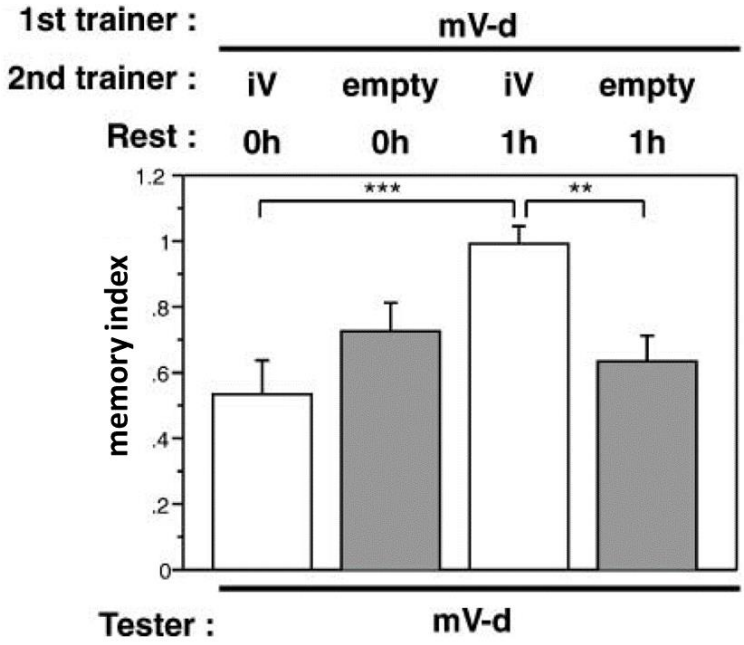


**Figure 11.** Multiple associations and cue interactions in trainer-specific learning. Cue interactions were investigated by concurrent and sequential presentation of multiple conditioned stimuli. Trainer conditions are indicated above each panel, and tester type is indicated below each panel (mV, mature virgin; iV, immature virgin; -d, decapitated). Data are presented as means SEM. Sequential training modulates memory retention. Males received two training sessions. In the first they were trained for 1h with a mature virgin. The second training session consisted of 30 min with an immature virgin or an empty chamber. Males were tested immediately after the second training session or after a 1h delay. Males who spent the second session in an empty chamber formed memory against mature virgins, and this memory was evident even if testing was delayed. Males that received a second training session with an immature virgin had memory when tested with immature virgins, even after a 1h delay (data not shown). When tested with a mature virgin, however, these males had normal memory immediately after training, but by 1h after the second session memory was gone (***p 0.001 for comparison of memory tests for double-trained males 1h after the second session with double-trained males tested immediately after training and **p 0.05 for comparison with males receiving an empty-chamber second session and a 1h delay before testing) (Ejima *et al.*, 2005).

Dans une autre série d'expériences, nous avons montré que cet apprentissage est principalement dû à l'existence d'une phéromone répulsive le (Z)-11-octadecenyl acetate (cis-vaccényle acétate, cVA). Le cVA est un composé majeur du profil cuticulaire des mâles et il est absent chez les femelles vierges. Pendant l'accouplement, les mâles le transmettent aux femelles avec le sperme et quelques peptides. Lorsque les mâles détectent la présence de cette phéromone, ils développent une suppression généralisée du comportement de cour. Nous avons donc utilisé cette phéromone pour aller encore plus loin dans les mécanismes neuronaux. En réalité, il y avait une polémique autour du cVA, certains travaux donnaient un rôle attractif (Bartelt *et al.*, 1985; Xu *et al.*, 2005) tandis que d'autres le considéraient comme un anti-aphrodisiaque (Jallon *et al.*, 1981; Mane *et al.*, 1983; Zawistowski & Richmond, 1986), bien que cette dernière fût soumise à caution (Vander Meer *et al.*, 1986; Scott & Richmond, 1987). Plusieurs récepteurs étaient en fait connus (Krieger & Fleischer, 2018), notre étude ayant permis de montrer que les récepteurs Or65a sont bien les responsables de la réponse répulsive, et de suggérer que les récepteurs Or67d sont impliqués dans l'aspect attractif (Ejima *et al.*, 2007). Dans tous les cas, le double effet du cVA à la fois attractif et répulsif semble être contexte dépendant ce qui amène à l'hypothèse que le transfert du cVA par les mâles, fait partie d'une stratégie pour

diminuer la compétition intraspécifique lorsqu'il est associé à l'odeur d'une femelle, alors que si le cVA est en présence de nourriture seulement, cela pourrait être un signal utilisé par les mâles pour localiser une source de nourriture et de potentielles femelles matures. L'effet aversif du cVA a par la suite été davantage documenté par l'utilisation d'individus mutants (Billeter *et al.*, 2009).

## 1.3 INFLUENCE SOCIALE SUR LA NATURE D'UN SIGNAL

Nous venons de voir comment un signal est produit, partagé et mémorisé. Les insectes sont donc capables de discriminer les individus sur la base de leurs signaux chimiques (van Zweden & D'Ettorre, 2010 ; et citations précédentes). Maintenant, nous allons voir comment un signal peut être influencé par l'environnement social.

### 1.3.1 L'origine sociale

Les interactions sociales sont aussi des facteurs environnementaux qui peuvent moduler le comportement de chaque individu telle que l'activité locomotrice (Levine *et al.*, 2002; Wyatt, 2003), l'agressivité (Wang *et al.*, 2008) ou encore la reproduction (Ferveur, 1997; 2005; Svetec *et al.*, 2005; Fujii *et al.*, 2007). Chez les insectes sociaux, la structure sociale de la colonie, c'est-à-dire le nombre de reines présentes en son sein, est connue pour influencer la capacité des membres de la colonie à discriminer les nestmates des individus étrangers. En effet, dans les colonies polygynes, l'agressivité est généralement moindre que dans les colonies monogynes. La présence de multiples reines au sein de la colonie, élargit la base génétique qui produit les signaux chimiques de reconnaissance. Ceci augmente donc la diversité chimique et les erreurs de reconnaissance des apparentés, diminuant par voie de conséquence l'agressivité envers les congénères conspécifiques (Vander Meer & Morel, 1998), même si le lien n'est pas toujours claire entre diversité génétique et richesse chimique (Martin *et al.*, 2009).

L'influence de la structure sociale a été étudiée chez plusieurs espèces de fourmis montrant que les ouvrières sont capables de reconnaitre l'origine sociale des reines et peuvent montrer à leur encontre une tolérance ou au contraire de l'agressivité en fonction des espèces, tel que chez *Solenopsis invicta* (Ross & Keller, 1998), *Messor barbatus* (Provost & Cerdan, 1990) ou encore *Formica selysi* (Rosset *et al.*, 2007; Meunier *et al.*, 2010). En revanche, la question se posait de savoir si les œufs pouvaient eux-aussi renfermer une information sur l'origine sociale de la reine, les œufs étant de ce fait acceptés ou rejetés. En effet, les mécanismes d'élimination des œufs étrangers sont clairement importants pour maintenir l'intégrité de la colonie, sans cela la descendance de reines infiltrées pourrait mettre en péril l'intégrité de la colonie en diminuant l'apparentement entre nestmates et par conséquent en diminuant la fitness inclusive de l'ensemble des membres de la colonie (Crozier & Pamilo, 1996; Holzer *et al.*, 2008). C'est ce que l'on a étudié chez la fourmi *Formica selysi*, en

regardant l'acceptation par les ouvrières des œufs en fonction de leur origine sociale et en recherchant les liens entre les profils chimiques et l'origine sociale des œufs et des ouvrières testées (Meunier *et al.*, 2011).

Un groupe de 30 œufs provenant de colonies étrangères monogynes ou polygynes ou encore de la même colonie a été ajouté à un groupe de 100 ouvrières. La survie des œufs à 24h après l'introduction montre que les œufs étrangers issus de colonies monogynes survivent moins que ceux des colonies polygynes quelle que soit l'origine sociale des ouvrières (Fig. 12). La survie des œufs est aussi la même entre œufs étrangers polygynes et œufs nestmates. En revanche, les contrôles avec des œufs issus de nestmates de colonies monogynes ou polygynes, ne montrent pas de différence de survie, indiquant qu'il n'y a pas de différence de viabilité intrinsèque des œufs en fonction de leur origine sociale. Les résultats surprenants des tests comportementaux montrent donc que les ouvrières rejettent systématiquement les œufs monogynes tandis qu'elles acceptent les œufs polygynes. Ceci va à l'encontre de ce qui a été décrit chez d'autres espèces d'hyménoptères sociaux (Ratnieks & Visscher, 1989; Ratnieks & Boomsma, 1995; Martin *et al.*, 2002; Endler *et al.*, 2004), bien que pondéré par Helanterä and Sundström (2007), et contraste avec ce qui a été trouvé lors d'introduction d'ouvrières étrangères (Rosset & Chapuisat, 2006).

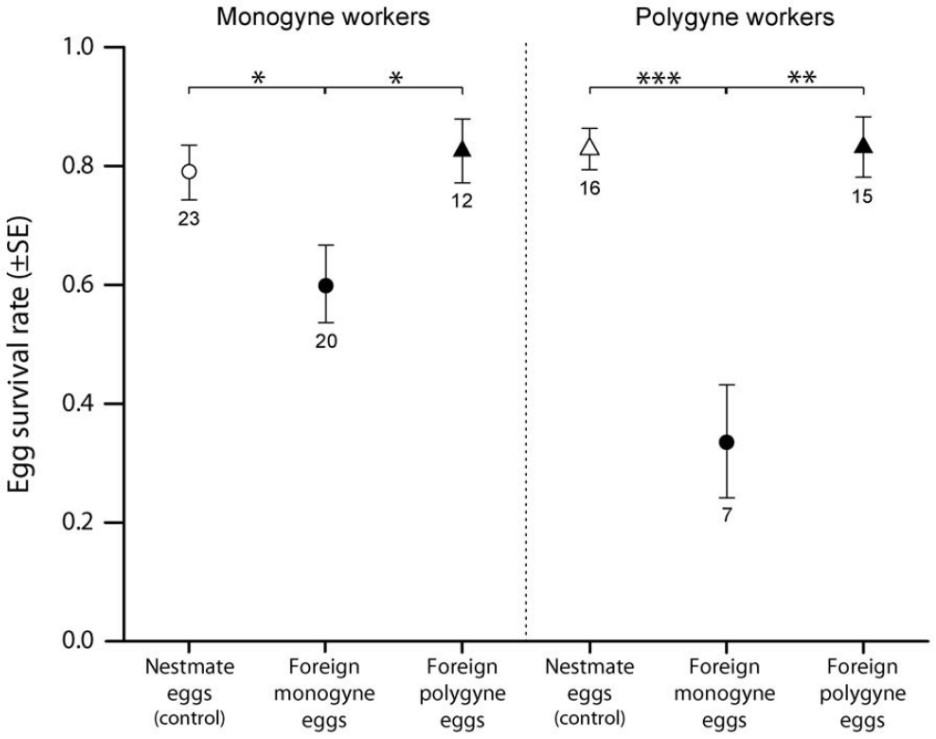


**Figure 12.** Survival rate of eggs introduced in groups of monogyne and polygyne workers. In both groups of workers, foreign monogyne eggs (black circle) had a significantly lower survival rate than both foreign polygyne eggs (black triangle) and nestmate eggs (white circle and white triangle), whereas there was no significant difference between the survival rates of foreign polygyne eggs and nestmate ones. The number of recipient groups is indicated below the SE bars. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Meunier *et al.*, 2011).

Ces résultats suggèrent que les ouvrières sont capables de détecter des signaux informant sur la structure sociale à partir d'informations provenant des œufs. Tout laisse à penser que cette information serait codée par les HCs situés à la surface des œufs. Même s'il est difficile d'écarter l'hypothèse que cette information puisse résider dans la structure même des œufs, notamment la taille qui est différente en fonction de la structure sociale d'origine (Meunier & Chapuisat, 2009). Nous avons donc procédé à l'analyse des HCs et comparé les extraits chimiques de 10 ouvrières ou 30 œufs, échantillonnés directement sur le terrain à partir de 23 colonies monogynes et 23 polygynes. L'analyse comparée des profils par GC-MS des deux formes sociales, a montré des différences significatives de quantité relative entre monogynes et polygynes à la fois chez les œufs et chez les ouvrières mais aucune différence qualitative.

Dans l'ensemble, ces travaux montrent que l'origine sociale des œufs et des ouvrières est encodée par les HCs et qu'ils interfèrent avec la discrimination des apparentés. Des expériences supplémentaires en manipulant le nombre de reines dans les colonies pourraient permettre de mieux comprendre si les quantités relatives des HCs dépendent de l'environnement social (nombre de reines dans la colonie) ou d'une base génétique (origine sociale des reines résidentes). La découverte récente de l'existence d'un "chromosome social" regroupant plusieurs centaines de gènes sur deux chromosomes différents non-recombinants, l'un lié à la monogynie et l'autre lié à la polygynie oriente davantage sur la piste de différence de la base génétique des signatures coloniales (Wang *et al.*, 2013; Purcell *et al.*, 2014).

### 1.3.2 Influence parentale

Comme nous venons de le voir, les hydrocarbures cuticulaires renferment des informations sur l'origine sociale des individus qui sont possiblement issus d'un héritage génétique. Chez les insectes sociaux, il est prédit que les potentiels conflits entre groupes appartenant à différentes lignées paternelles devraient aboutir à une diminution de l'expression de médiateurs chimiques spécifiques à la lignée paternelle (Keller, 1997; Boomsma *et al.*, 2003). Cette prédiction clé dans l'évolution des systèmes de communication n'a été étudiée que chez les insectes eusociaux (Page *et al.*, 1991; Breed *et al.*, 1994; Arnold *et al.*, 2000; Nehring *et al.*, 2010). La question se posait donc de savoir si cela pouvait être généralisé à d'autres modèles d'insectes, ce que nous avons fait avec le perce-oreille européen *Forficula auricularia*.

*Forficula auricularia* présente une vie de famille où les raisons de la présence des parents au sein de la cellule familiale restent encore peu comprises. Classiquement, il est considéré que les parents bénéficient directement de l'expression des soins parentaux en augmentant le développement et la survie de leur descendance. La vie familiale exprimée chez le forficule européen est souvent décrite comme une forme moins diversifiée de la socialité dite "vraie" des insectes eusociaux et est à ce titre moins considérée dans les études actuelles. Pourtant, la compréhension des mécanismes de transition évolutive de la vie sub-sociale à la vie eusociale peut permettre de mieux comprendre les facteurs à l'origine de l'évolution de la vie sociale chez les insectes en général.

Nous avons donc testé l'hypothèse de conflits entres lignées paternelles par des manipulations expérimentales impliquant plusieurs séries de mâles lors d'expériences de reproductions croisées (Wong *et al.*, 2014b). Pour ce faire, nous avons analysé les profils chimiques extraits des nymphes et des adultes issus de croisements contrôlés avec 1 ou 4 mâles. La descendance a ensuite été élevée soit en groupes d'individus apparentés ou étrangers (Fig. 13).

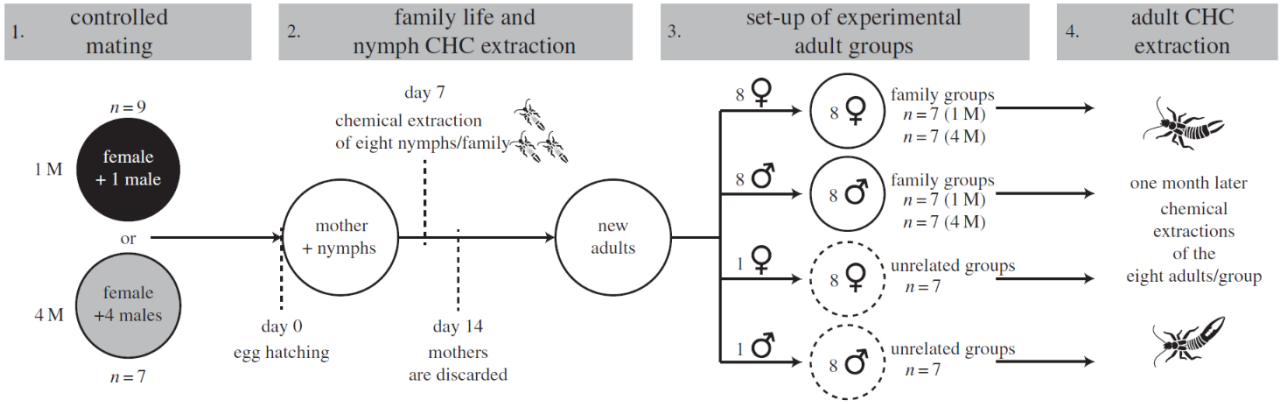


**Figure 13.** Experimental design used to extract the CHC profiles from nymph and adult earwigs (Wong *et al.*, 2014b).

Une série d'analyses discriminantes a montré que non seulement le profil chimique possède une composante héritable mais qu'il reflète aussi les conditions sociales et environnementales dans lesquelles les individus ont grandi. Ceci a été testé à l'aide d'analyses discriminantes afin de déterminer si la signature chimique reflète la famille d'origine. La significativité des analyses discriminantes étant faites entre autres en estimant le succès prédictif (Fig. 14). Ainsi, la signature chimique des nymphes et des adultes reflète leurs familles d'origine et les variations des profils chimiques entre membres de la famille sont inférieures à celles observées entre différentes familles. Enfin, lorsque plusieurs mâles engendrent la descendance, les variations chimiques familiales sont plus grandes que lorsqu'un seul mâle est l'unique parent. Un point particulièrement intéressant aussi présent dans la figure 14 est que les variations des signatures chimiques sont absentes lorsque la descendance est jeune (nymphes) mais se révèlent à l'état d'adulte (mâles/femelles). Ce qui démontre une expression âge-dépendante des médiateurs chimiques spécifiques du père. Cette expression différentielle pourrait permettre une limitation des risques de népotisme durant les phases de vie familiale des juvéniles, favorisant par la même l'évitement d'actes de reproduction consanguine pendant la phase adulte (Wong *et al.*, 2014b).

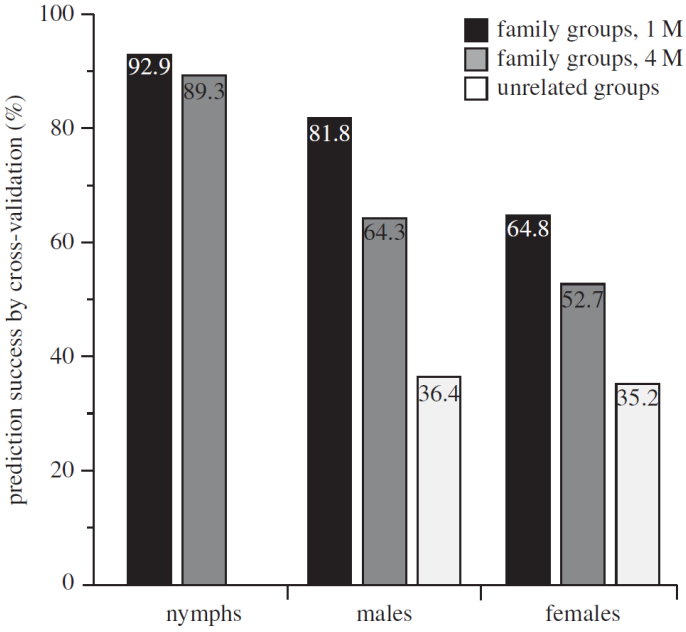


**Figure 14.** Prediction success (estimating the percentage of correct assignment of individuals to their family of origin) by jack-knife cross validation of earwig individuals according to their life stage, their sexes (only for adults), the type of rearing groups (family or unrelated) and the mating treatments (1 M or 4 M). The corresponding values are given at the top of each bar; M: male (Wong *et al.*, 2014b).

Il existe donc une base génétique transmise par le père à sa descendance. Mais les signaux chimiques peuvent-ils refléter l'état physiologique des parents ? Afin de maximiser la fitness des parents et minimiser les coûts associés aux soins parentaux, il est attendu que les parents ajustent leurs soins envers leur descendance en fonction de l'état de celle-ci (Godfray & Johnstone, 2000; Mock *et al.*, 2011; Kilner & Hinde, 2012). Inversement, la descendance doit aussi ajuster son taux de demande de nourriture en fonction de l'état général de leurs parents afin de modérer le coût d'investissement des parents (Bateson, 1994). En conséquence, c'est dans l'intérêt des parents et de la descendance d'être sensible à l'état physiologique de chacun, si bien que l'échange de signaux d'information devrait être sélectionné au cours de l'évolution. Les signaux impliqués dans l'état physiologique de la descendance ont été étudiés (Grafen, 1990; Godfray, 1991; 1995; Johnstone & Godfray, 2002; Mock *et al.*, 2011), alors que peu de choses avait été faite sur les signaux issus des parents (Bateson, 1994).

Avec les mêmes procédés que ceux décrits précédemment (Wong *et al.*, 2014b), nous avons donc testé chez *Forficula auricularia* l'hypothèse selon laquelle les conditions physiques de la mère influenceraient la demande de nourriture formulée par la descendance lors des soins parentaux (seule la femelle élève la descendance). Le but de l'expérience était de permettre à la mère de relâcher les signaux chimiques dans le substrat et d'exposer les nymphes tout en empêchant les contacts physiques. Les mères ayant accès à des grandes ou des petites quantités de nourriture. A la fin des 40 jours de l'expérience les mères ont été extraites et les profils chimiques ont été analysés par GC-MS.

Nos résultats montrent que non seulement, l'état physique général de la mère est corrélé à son profil chimique cuticulaire, mais que de surcroit, les juvéniles modifient leurs interactions sociales en fonction de l'état de leur mère. Il existe donc un échange d'informations entre la mère et ses juvéniles

qui permet à l'ensemble de la famille d'adapter son comportement nourricier en fonction de la disponibilité en nourriture, le tout apparemment encodé par des médiateurs chimiques déposés dans leur environnement (Wong *et al.*, 2014a).

## 1.4 INFLUENCE DU PARASITISME SUR LA NATURE D'UN SIGNAL

Dans l'environnement de nombreux facteurs abiotiques ou biotiques externes sont présents et peuvent altérer les signaux de communication. Que ce soient des contaminants (pesticides, polluants, perturbateurs endocriniens), des variations de température, d'humidité ou encore la présence de parasites (Vander Meer *et al.*, 1998; Chirault *et al.*, 2015). Ces facteurs peuvent soit altérer la nature des signaux produits par les insectes, soit brouiller la perception des signaux au niveau des récepteurs dédiés. De ce fait la capacité des individus à fournir une réponse adaptée aux situations qu'ils rencontrent s'en trouve perturbée.

La présence de parasites est connue pour modifier plusieurs fonctions physiologiques des insectes et leur vulnérabilité à d'autres maladies, ainsi que leurs capacités à communiquer (Vale *et al.*, 2018). Mais l'impact de leur présence dans les interactions multi espèces présentant plusieurs niveaux d'action a été peu étudié (Strauss & Irwin, 2004). Dans les sociétés animales, les changements induits par les parasites au niveau individuel peuvent aussi entrainer des conséquences multiples au niveau de la société, rendant d'autant plus complexe leur analyse. Certains champignons ectoparasites comme *Rickia wasmannii* sont communs dans certaines populations de fourmis de l'espèce *Myrmica scabrinodis*, rendant ainsi plus facile l'étude de leur présence chez une fourmi qui elle-même est la cible de parasites sociaux, les papillons du genre *Maculinea*. L'ensemble représente un système biologique multipartite impliquant des insectes sociaux et deux espèces parasitaires, rendant possible l'analyse de l'influence de la présence d'un parasite sur les chances d'infestation d'un autre parasite.

Nous avons donc regardé les potentielles conséquences au niveau des interactions sociales des fourmis, ainsi que l'effet du champignon parasite sur la capacité de discrimination des apparentés chez la fourmi *Myrmica scabrinodis*, en regardant les médiateurs chimiques. Puis, nous avons regardé les changements qui s'en suivent dans les succès d'infiltration de deux espèces de papillons (*M. alcon* et *M. teleius*), eux-mêmes parasites de cette espèce de fourmis (Csata *et al.*, 2017).

Les analyses des hydrocarbures cuticulaires, connus pour être impliqués dans les processus de discrimination des insectes, ont révélé des variations au niveau des profils chimiques en corrélation avec le degré d'infection des fourmis, qui ne sont pas explicables par des variations génétiques (testées par marqueurs microsatellites ; Fig. 15).

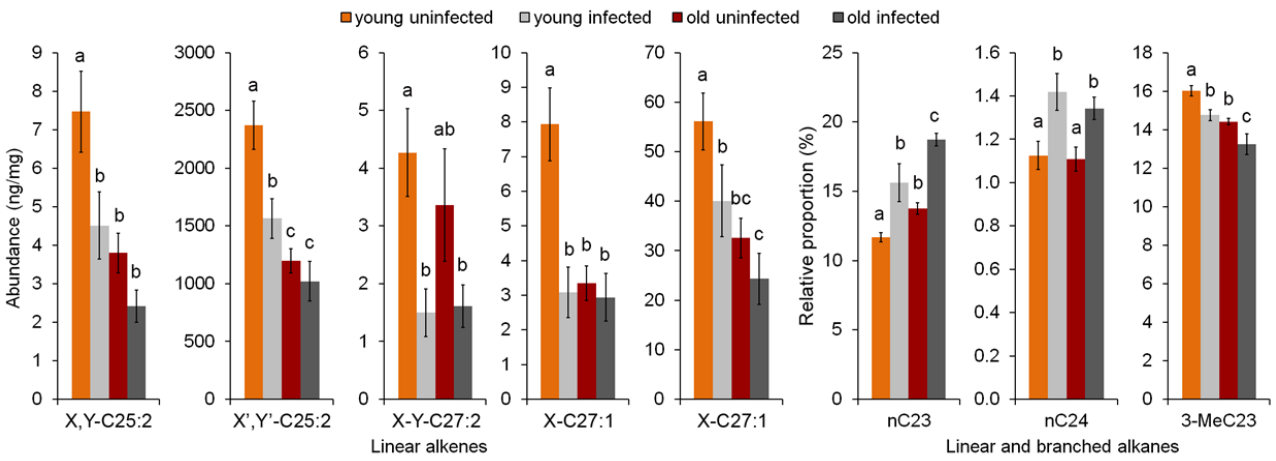


**Figure 15.** Mean relative proportions (±SE) of specific cuticular HCs of young and old *M. scabrinodis* workers from infected and uninfected colonies. These specific cuticular HCs were pointed out by the principal component analyses for which a fungal effect was detected. Bars with different letters are statistically different according to Linear Mixed Model pairwise comparisons (Csata *et al.*, 2017).

Dans les tests comportementaux, les fourmis infectées sont moins agressives envers les individus n'appartenant pas à leur colonie, alors qu'en condition saine les intrus sont systématiquement éliminés. De plus, les reproducteurs non apparentés sont plus acceptés, ce qui augmente d'autant plus la probabilité que les colonies passent du statut de monogyne (une seule reine) à celui de polygyne (plusieurs reines). Il en résulte des modifications importantes de l'organisation sociale de la colonie de pair avec l'apparition de conflits sociaux. Les larves du papillon parasite *Maculinea* ont aussi une plus grande chance d'être adoptées par les colonies de fourmis infectées par le champignon que par les colonies saines (Fig. 16).

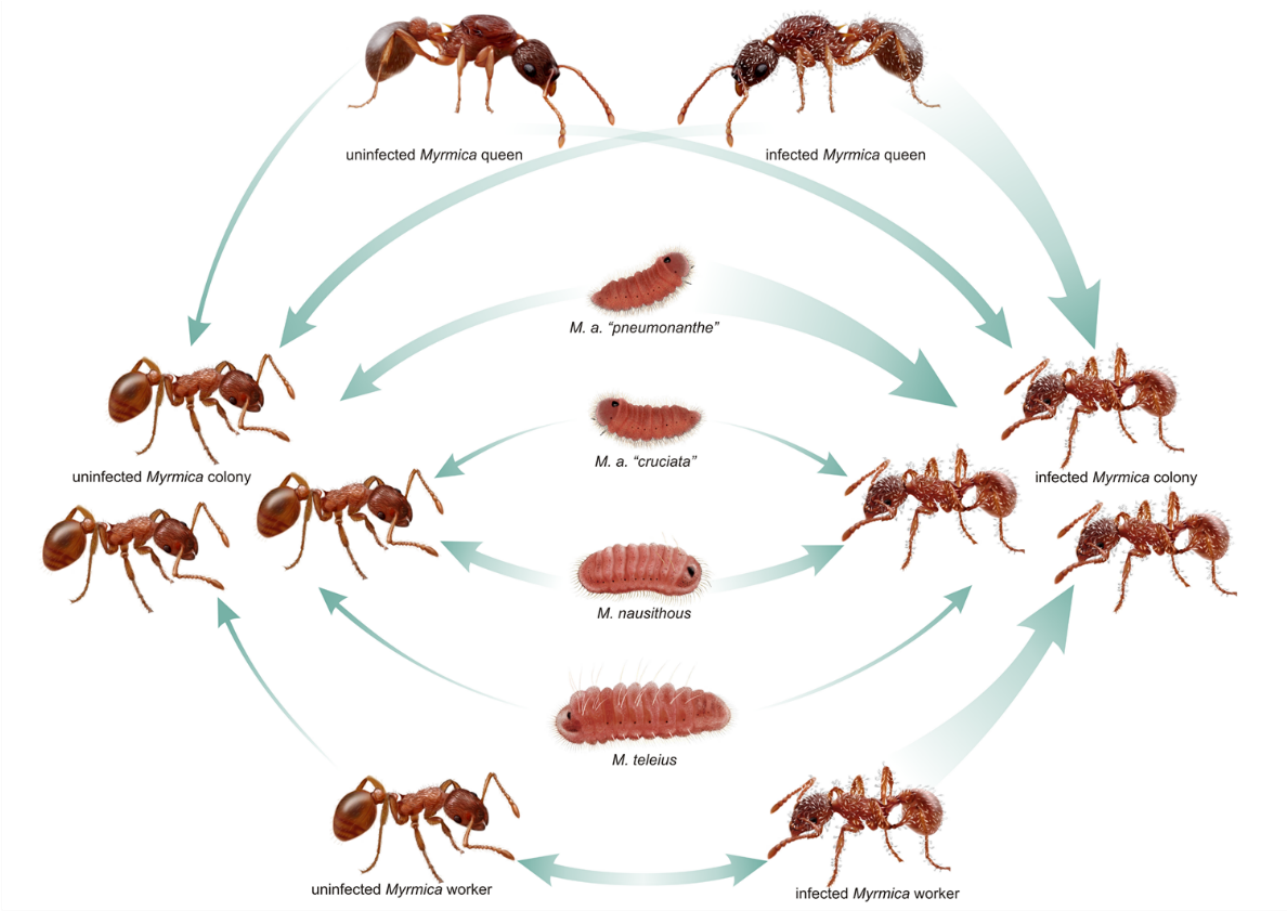


**Figure 16.** Summary of the experimental setup and the findings of the study. The thickness of arrows correlates with the degree of acceptance by ant colonies of different infection status (Csata *et al.*, 2017).

Notre étude démontre donc que le pathogène modifie drastiquement les capacités de reconnaissance de la fourmi hôte rendant leurs sociétés particulièrement vulnérables et plus à même d'accepter d'autres organismes conspécifiques et allospécifiques (Csata *et al.*, 2017).

Le parasite a donc une action directe sur la composition de la signature coloniale, mais quelle est sa dynamique au fur et à mesure de l'infestation ? Nos études, sur un autre modèle de parasite social, montrent que cette signature chimique est modifiée dès l'arrivée du parasite et que celle-ci va finir par diverger au bout de plusieurs mois. Chez les guêpes sociales comme *Polistes biglumis*, la signature chimique du nid est utilisée par les individus émergeant pour apprendre l'odeur de leurs colonies, formant ainsi leur "visa" colonial. Or durant cette période, certains de ces nids sont la cible du parasite social *Polistes atrimandibularis*, ce qui offre l'opportunité d'étudier l'évolution de la signature coloniale de la fondation du nid jusqu'à son déclin.

Dans cette étude, nous avons déterminer les variations temporelles de la signature chimique qui a été déposée sur le nid cartonné des guêpes *Polistes biglumis* au cours du temps. Toutes les semaines, les nids étaient vérifiés pour déterminer la présence ou non de parasites, puis un fragment du nid de 1 $cm^2$ était prélevé pour les extractions chimiques. L'étude a porté sur 129 nids de début juin (période de fondation) à fin aout (déclin des colonies) directement sur le terrain. Nos résultats montrent qu'au cours de la saison, l'odeur du nid change avec une augmentation globale des quantités d'hydrocarbures et une augmentation de la taille des molécules, ainsi qu'une augmentation du nombre d'hydrocarbures branchés (Elia *et al.*, 2017). Lorsque le nid a été parasité, on constate une altération chimique qualitative (présence d'alcènes originaires du parasite) et quantitative (interférence de l'abondance totale d'hydrocarbures) (Fig. 17).

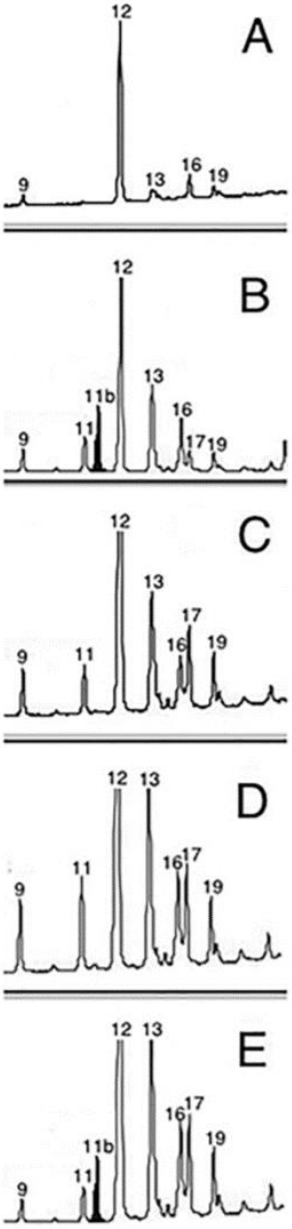


**Figure 17.** Representative gas chromatograms (GC) of a parasitized nest of *P. biglumis* at five different time points during the colony cycle. (**A**) GC before parasite invasion; (**B**) one week after parasite invasion; (**C**) two weeks after parasite invasion (**D**) three weeks after parasite invasion; (**E**) six weeks after parasite invasion. Only hydrocarbons between peak 9 and peak 19 are shown. The picture shows the presence/absence of (Z)-9-heptacosene (the black peak), the most abundant alkene on parasitized nests. Peak 9: hexacosane; Peak 11: 2-methylhexacosane; Peak 11b: (Z)-9-heptacosene; Peak 12: n-heptacosane; Peak 13: 9-+11-+13-methylheptacosane; Peak 16: 11,15-dimethylheptacosane; Peak 17: 3-methylheptacosane; Peak 19: n-octacosane (Elia *et al.*, 2017).

L'odeur coloniale se révèle donc comme étant hautement dynamique, que les nids soient parasités ou non, et s'avère majoritairement produite par la reine fondatrice avec très peu d'influence des nouveaux individus émergents. De plus, le parasite social qui va par la suite mimer l'odeur de la fondatrice hôte, présente un comportement de marquage actif avec ces propres hydrocarbures spécifiques.

Dans l'ensemble, nos travaux montrent que les parasites, en perturbant le système de communication des insectes, peuvent être considérés comme une force évolutive qui façonne les sociétés, avec une empreinte neuronale de l'odeur référente ("visa") en évolution constante au cours du temps.

# 2. Adaptation des espèces à leurs environnements

Face à leurs environnements, les individus développent une réponse comportementale spécifique adaptée aux différents signaux perçus. Evidement ces signaux changent au cours du temps et dans l'espace telle que la présence de prédateurs ou de compétiteurs. Cette modulation comportementale autrement appelée "plasticité comportementale", permet aux individus d'émettre la réponse la plus adaptée possible face aux contraintes environnementales (Córdoba-Aguilar *et al.*, 2018). Le comportement devient ainsi un acteur central dans l'évolution des espèces (West-Eberhard, 1989; West-Eberhard, 2005), ce qui amène à distinguer la plasticité comportementale de la plasticité phénotypique, dès lors que l'on considère cette dernière comme un équivalent généralisé du comportement (Mayley, 1996; Novoplansky, 2002; Paenke *et al.*, 2007; Nicoglou, 2011). Dans l'étude des insectes sociaux, ces deux notions ont tendance à se confondre, bien qu'intuitivement distinctes, avec un polyphénisme morphologique qui s'accompagne d'un polymorphisme comportemental (voir chapitre 3).

Etudier les bases de la plasticité comportementale est donc un point crucial pour comprendre les mécanismes d'adaptation des espèces à leurs environnements. Nous allons voir des exemples, issus de mes travaux de recherche, de situations environnementales qui amènent à une modulation comportementale, avant de regarder certains gènes impliqués dans la plasticité comportementale.

## 2.1 HETEROGENEITE DU GROUPE & COMPORTEMENT SEXUEL

La vie sociale d'un animal dépend de sa capacité de communication avec les autres. Chez la drosophile, le contexte social (défini ici comme la taille et la composition génotypique du groupe) agit sur les comportements de locomotion (Levine *et al.*, 2002), d'agrégation (Wertheim, 2001), d'agressivité (Wang *et al.*, 2008), d'évitement (Suh *et al.*, 2004), d'alimentation (Tinette *et al.*, 2004), de reproduction (Ferveur, 1997; 2005; Svetec *et al.*, 2005; Fujii *et al.*, 2007) et de sommeil (Ganguly-Fitzgerald *et al.*, 2006). De plus, ces études soulignent le rôle important des signaux chimiques comme médiateurs de ces effets sociaux. Dans le chapitre précédent, nous avons vu que l'expression des gènes impliqués dans la production des phéromones sexuelles est rythmique, permettant aux individus d'interagir avec le plus grand nombre et au meilleur moment lors d'interactions sociales (Dunlap *et al.*, 2004). Si tel est le cas, quel est l'impact d'un individu n'ayant pas le même rythme circadien sur les signaux de communication et qu'est-ce que cela change pour le groupe avec lequel il interagit ? C'est ce que nous avons étudié chez la drosophile avec des groupes hétérogènes d'individus contenant des mutants de l'horloge circadienne.

Les expériences ont utilisé deux groupes de mouches composés soit uniquement de type sauvage (groupe homogène) soit d'un mélange de type sauvage ou mutant *per*$^{0}$ (groupe hétérogène). Les mutants *per*$^{0}$ présentent un déficit de l'horloge circadienne. Les mouches ont interagi librement pendant 24h, période durant laquelle nous avons mesuré les comportements d'accouplement (Fig. 18). De façon surprenante, nos résultats montrent que les mouches en groupe social mixte, copulent davantage que ceux en groupe uniforme avec une augmentation de 22% du nombre total d'accouplements (Krupp *et al.*, 2008).

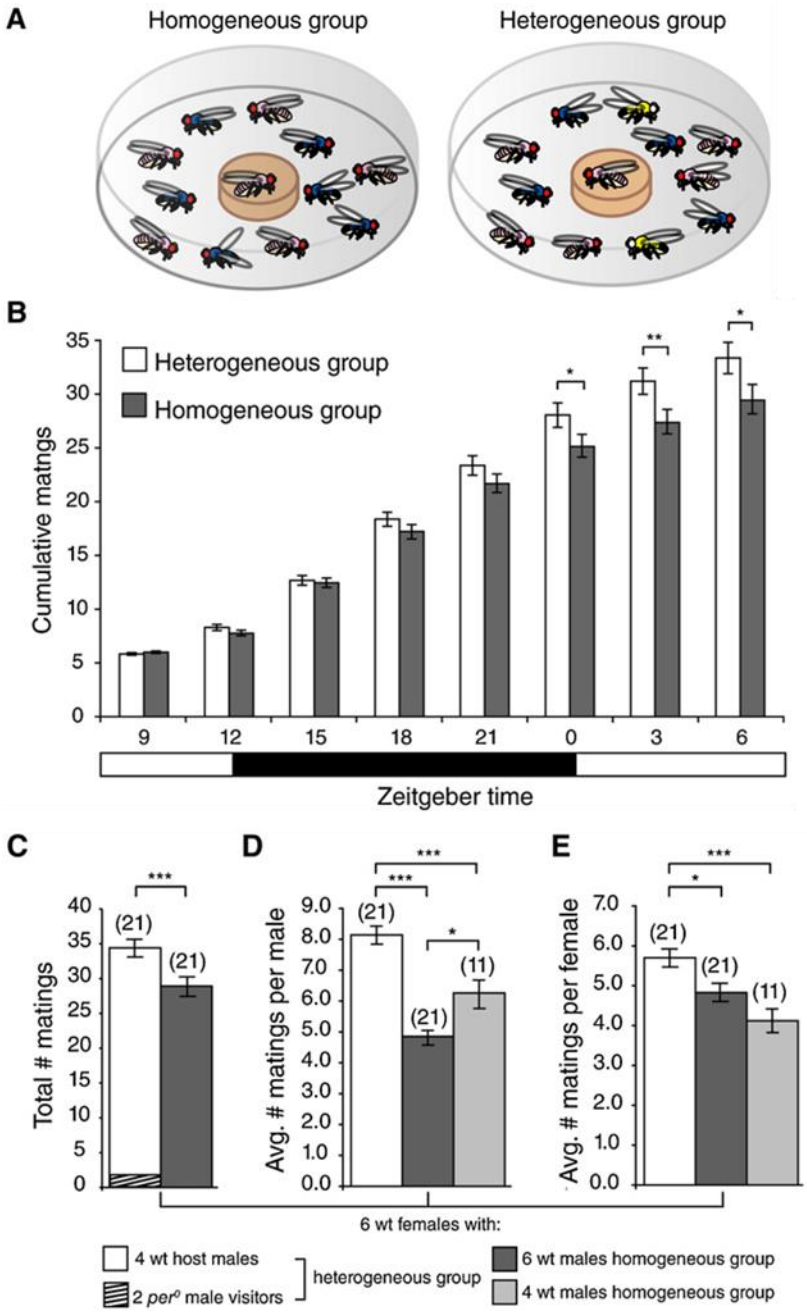


**Figure 18.** Social context changes the amount and temporal distribution of mating.

(**A**) Schematic representation of the social-mating assay. The homogeneous group consists of six wild-type (wt) females (pink) and six wt males (blue). The heterogeneous group consists of six wt females, four wt host males, and two *per*$^{0}$ mutant visitor males (yellow).

(**B**) Cumulated number of matings over a 24h observation period shown at 3h interval time points. n=13 for both heterogeneous (white) and homogeneous (gray) groups. (ANOVA: *p < 0.05, **p < 0.01; after False Discovery Rate correction at q = 0.1).

(**C**) Total number of matings in a heterogeneous genotype (white) or homogeneous (gray) group. ***p < 0.001, paired t-test. Number of repeats (n) is between parentheses.

(**D**) Mean number of matings per wild-type male in the indicated social context. Values represent the number of wt male matings over 24h divided by the number of wt males present (either 4 or 6) averaged across independent experiments. ANOVA: *p < 0.05; ***p % 0.001.

(**E**) Mean number of matings per wt female in the indicated social context. ANOVA: **p < 0.01; ***p < 0.001. Error bars indicate ± SEM in all panels (Krupp *et al.*, 2008).

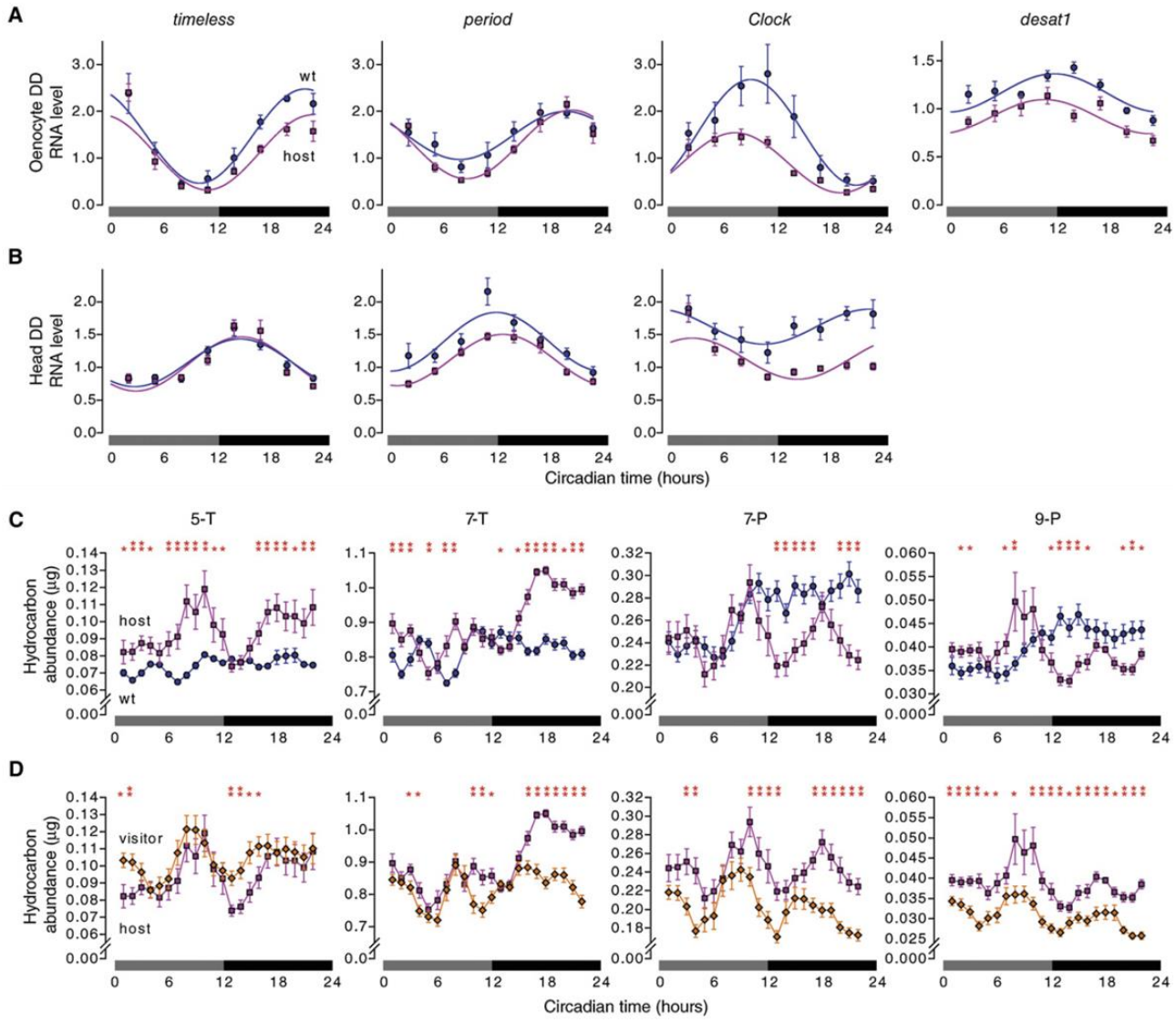


**Figure 19.** Social interactions affect the temporal profile of gene expression and cuticular hydrocarbon accumulation.

(**A** and **B**) The temporal patterns of *tim*, *per*, *Clk*, and *desat1* expression in wild-type control and host œnocyte preparations (**A**) and whole-head extracts (**B**) as determined by quantitative RT-PCR analysis. Œnocytes and heads were isolated from the same flies. Best-fit cosine curves (wt: wild-type, wt: blue line, host: magenta line) are fitted to RNA expression values ± SEM (wt DD: blue circles, host DD: magenta squares). Expression was assayed on the second day of constant darkness. Each time point represents the average of three replicate qPCR reactions from three separate experiments. (**C** and **D**) Comparison of the temporal profiles of 5-T, 7-T, 7-P, and 9-P between wt control and host males in DD (**C**) and between host and visitor males in DD (**D**). Shown are values calculated from a 3h moving average ± SEM (n = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two-sided Student's t-test (**p < 0.01, *p < 0.05). wt: wild-type, wt DD: filled blue circles, host DD: filled magenta squares, visitor DD: filled orange diamonds, DD: Dark/Dark conditions following the regular 12/12 clock period (Krupp *et al.*, 2008).

Ces groupes sociaux mixtes ont aussi un impact sur l'expression des gènes *tim*, *per*, *Clk*, et *desat1*. Trois de ces gènes sont impliqués dans l'activité circadienne des mouches, tandis que le gène *desat1* est impliqué dans la synthèse des principales phéromones sexuelles de la drosophile. Le contexte social induit des différences prononcées dans le niveau et l'amplitude de l'expression de ces gènes aussi bien dans la tête que dans les œnocytes (cellules spécialisées dans la production des phéromones). L'expression des gènes est inférieure chez les "host" (mouches wild-type "wt" issues des groupes hétérogènes) que chez les "wt" (mouches "wt" issus des groupes homogènes) (Fig. 19A, B). De plus, le pattern général d'accumulation des HCs est globalement différent entre les individus "host" et les "wt", ce qui préfigure là aussi de modifications du profil chimique chez les "host" des groupes hétérogènes par rapport aux mouches "wt" des groupes homogènes (Fig. 19C). La comparaison des profils temporels d'accumulation des HCs cuticulaires des "host" et des "visitor" (les "visitor" sont les mutants $per^0$ présents dans les groupes hétérogènes), tous les deux issus des groupes hétérogènes, montre des similitudes plus marquées entre eux que lorsqu'ils sont comparés avec leurs contrôles génétiques (Fig. 19D ; voir les détails des résultats en annexe).

Le contexte social présente donc une influence environnementale régulatrice de l'expression de signaux chimiques qui module le comportement sexuel de la drosophile.

## 2.2 DENSITE & GREGARISATION

Un exemple spectaculaire connu de polymorphisme comportemental est le criquet du désert *Schistocerca gregaria* (Simões *et al.*, 2016; Song *et al.*, 2017). Cette espèce peste, de l'Afrique du nord, du Moyen Orient et de l'Inde, présente deux formes d'individus associés à des différences de comportement de grégarisation et de fourragement. Au point que les deux formes étaient décrites comme deux espèces différentes pendant de longues années. Sous la forme solitaire, les criquets sont cryptiques, de couleur verte et se déplacent sur de courtes distances. Tandis que sous la forme grégaire, les mêmes individus sont de couleur sombre et se déplacent sur de longues distances avec une haute fréquence de prise alimentaire.

Tom Fayle

Lorsque la quantité de nourriture diminue, la densité d'individus autour des points de fourragement augmente. Cela induit des changements des conditions environnementales, telles que les odeurs et les contacts physiques entre congénères, ce qui entraine des modifications d'expression génique, amenant les individus à passer de la forme solitaire à la forme grégaire (Simpson *et al.*, 2011). L'augmentation de la densité d'individus entraine à son tour une diminution de la quantité totale de nourriture, ce qui amplifie encore plus la grégarisation de la population et provoque des essaimages allant jusqu'à plusieurs centaines de millions d'individus, dévastant les zones rencontrées

et posant de vastes problèmes écologiques et économiques. La grégarisation s'effectue en quelques heures, même si l'ensemble des caractéristiques de la forme grégaire se développent en totalité en quelques générations, de la même manière que le passage retour à la forme solitaire. Les individus grégaires ont une durée de vie plus courte, sont moins féconds et transmettent les caractéristiques de leur forme à leur descendance.

La quantité de contacts entre criquets, est considérée comme une information issue de l'environnement social. Les criquets sous forme solitaire qui reçoivent expérimentalement un grand nombre de contacts au niveau de leurs pattes arrière, voient leurs taux de sérotonine augmenter (Anstey *et al.*, 2009). Si on manipule la concentration de ce neuromodulateur, les criquets passent en forme grégaire et passent d'un régime alimentaire spécifique à un régime généraliste (Simões *et al.*, 2016).

La régulation implique des hormones, des neuropeptides, des neurotransmetteurs et certainement des facteurs épigénétiques (Ernst *et al.*, 2015). A ceci on peut ajouter une enzyme pléiotropique, la protéine kinase cGMP dépendante, comme le suggère nos travaux. En effet, les mesures d'activité enzymatique montrent des différences entre les formes solitaire et grégaire. Cette enzyme est particulièrement présente au niveau du cerveau, où seule la *pars intercerebralis* est immunoréactive aux anticorps spécifiques de l'enzyme. Cette partie du cerveau contient la plus grande collection de cellules neurosecrétrices et la plupart de ces cellules ont des fibres en connexion directe avec le *corpora cardiaca* via le *nervus corporis cardiaci I* (Lucas *et al.*, 2010b). D'autres analyses semblent indiquer que l'injection d'un activateur de cette enzyme peut déclencher le passage à la forme grégaire mais aussi augmenter la durée de vol et son temps d'initialisation (Ayali, Pers. Com.).

## 2.3 PRESENCE DE PREDATEURS & ORGANISATION SOCIALE

La capacité à détecter un prédateur, est cruciale pour la survie de l'individu et à plus haute échelle pour la survie de l'espèce. Tout indice informant de la présence d'un prédateur qui a été laissé, activement ou involontairement dans l'environnement, correspond donc à une information vitale utilisable par la proie (notion de kairomone, voir l'introduction) (Davies *et al.*, 2012). Parmi les médiateurs possibles, les hydrocarbures présents sur la cuticule et produits en permanence par les insectes peuvent représenter une source d'information fiable (Blomquist & Bagnères, 2010). C'est la question que nous nous sommes posée en nous intéressant de surcroit à une relation proie-prédateur particulière puisqu'elle implique deux espèces d'insectes sociaux à savoir la relation fourmi-termite. En effet, les fourmis et les termites utilisent les hydrocarbures cuticulaires dans leur système de communication et notamment dans la reconnaissance coloniale (voir chapitre précédent). Chez la fourmi *Pheidole pallidula*, la présence d'un compétiteur (une colonie étrangère) dans l'environnement proche, sans contact physique, entraine une augmentation du nombre de soldats produits. Ici, l'information du compétiteur, sous la forme d'un médiateur chimique, suffit à déclencher une réorganisation de la colonie (Passera *et al.*, 1996). Nous avons émis l'hypothèse que cet effet de la

présence d'un compétiteur pouvait aussi se faire en présence d'un prédateur dans le cadre d'une interaction proie-prédateur de deux espèces eusociales.

Nous avons donc testé l'influence de la présence d'hydrocarbures d'une fourmi prédatrice (*Lasius niger*) dans l'environnement proche de deux termites *Reticulitermes grassei* et *R. flavipes*. Pour cela nous avons extrait les hydrocarbures du prédateur que nous avons déposés au cœur de plusieurs micro-nids issus de plusieurs colonies de termites des deux espèces (le solvant a été utilisé comme contrôle). Les micro-nids étaient constitués de 3 feuilles de papier de pure cellulose, servant à la fois de nourriture, d'élément de construction, de zone de dépôt des extraits et permettant un accès direct facile à l'ensemble des membres de la colonie (Fig. 20). À la suite de deux mois d'exposition nous avons suivi le ratio de caste (soldats et reproducteurs néoténiques) mais aussi la dérive du profil chimique pour déterminer si l'odeur étrangère influençait l'évolution de l'odeur coloniale. Enfin, nous avons testé le comportement des termites lors de tests de compétition soit à l'encontre de termites naïfs, n'ayant subi aucun traitement, soit à l'encontre de termites ayant été mis en présence de l'odeur du prédateur pendant les 2 mois. Les résultats ne montrent aucune influence de la présence de l'odeur du prédateur sur le ratio de caste ou sur la mortalité, en revanche des différences au niveau des profils chimiques et du comportement ont été trouvées (Lucas *et al.*, 2018).

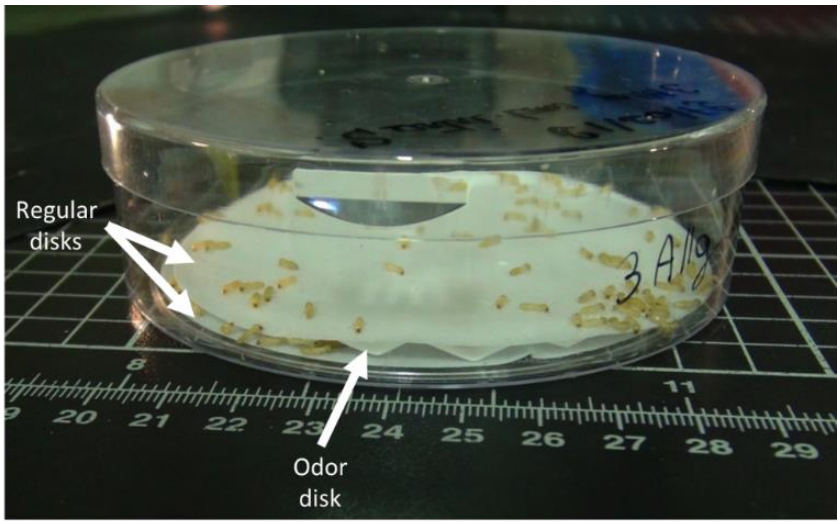


**Figure 20.** Profile view of the experimental nest setup used for odor tests (Lucas *et al.*, 2018).

Les analyses chimiques ont permis d'identifier 49 pics chez *R. grassei* et 31 pics chez *R. flavipes*, ce qui a enrichit la liste des composés connus par rapport à la littérature. Les résultats montrent que les profils des termites présentent des différences quantitatives mais pas qualitatives. Il est intéressant de noter que l'absence de différence qualitative entre les groupes mis en présence de l'odeur du prédateur et les contrôles, signifie qu'aucun hydrocarbure du prédateur n'a été transféré à

la proie, que ce soit par contact physique ou bien par internalisation alimentaire. Tout au moins, si cela s'est produit, les hydrocarbures du prédateur ont été filtrés du profil chimique des termites. De plus, pour l'espèce *R. grassei*, l'analyse des différences quantitatives des profils chimiques par l'analyse des distances de Nei (indice d'hétérogénéité, Nei, 1972) montre que les termites assujettis à la présence de l'odeur du prédateur présentent un profil chimique plus homogène (Fig. 21).

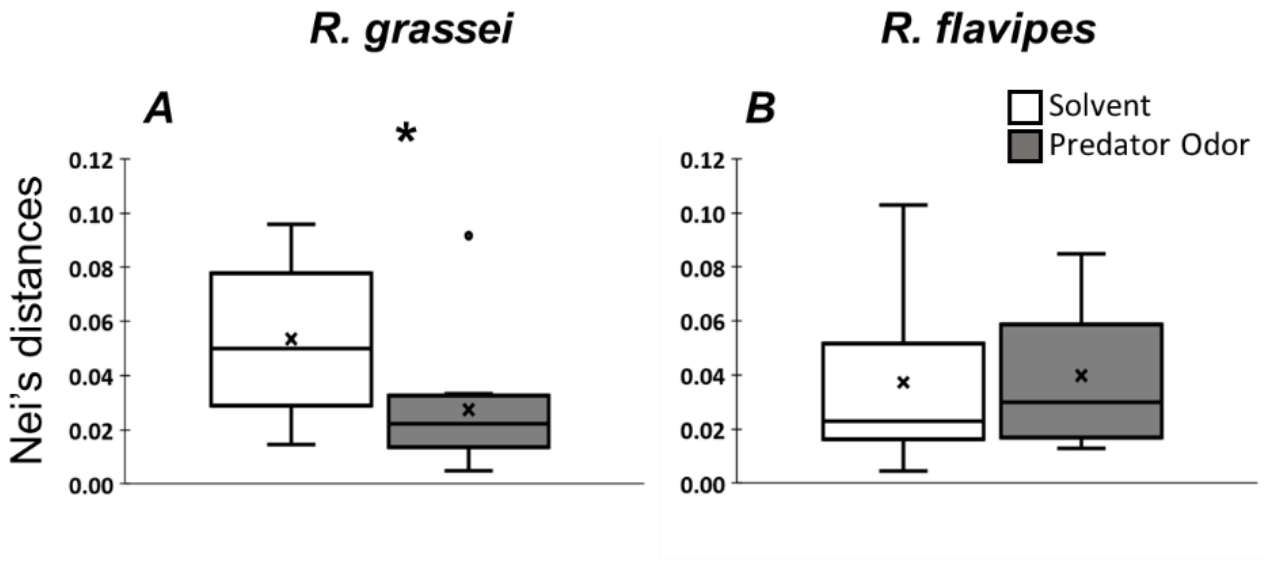


**Figure 21.** Chemical heterogeneity indexes (Nei's distances, high values mean that profiles are more heterogeneous) for *R. grassei* (**A**) and *R. flavipes* (**B**). They were calculated using the relative areas of the chemical compounds present on termite cuticles after 2 months of treatments (solvent and predator-odor treatments), * $p<0.05$. Ten pairwise comparisons per treatment and per species were analyzed. Box plots show the median, upper and lower quartiles and range; dot indicates outliers; x indicates the mean (Lucas *et al.*, 2018).

Du point de vue comportemental, les deux espèces présentent des différences dans les tests de compétition. En effet, l'espèce *R. grassei* exposée à l'odeur de prédateur présente un taux de survie supérieur comparé aux individus exposés à l'odeur contrôle, ce qui n'est pas le cas de *R. flavipes*. Deux explications sont possibles, soit ces termites sont devenus plus agressifs, soit leur capacité de défense a augmenté, les deux ayant comme résultante une augmentation de la survie des individus. Nos expériences complémentaires semblent indiquer que l'hypothèse privilégiée est l'augmentation de la défense, ce qui semble être corroboré par l'homogénéisation des profils chimiques observée pour les individus exposés à l'odeur du prédateur (Fig. 22). L'absence de réponse à l'odeur pour *R. flavipes* peut trouver des explications dans son statut d'espèce invasive allant souvent de pair avec une réduction de l'agressivité intraspécifique, une augmentation de l'homogénéité chimique au niveau spécifique, permettant des événements de fusion coloniale (Brandt *et al.*, 2009; Vásquez *et al.*, 2009; Perdereau *et al.*, 2010; Perdereau *et al.*, 2011).

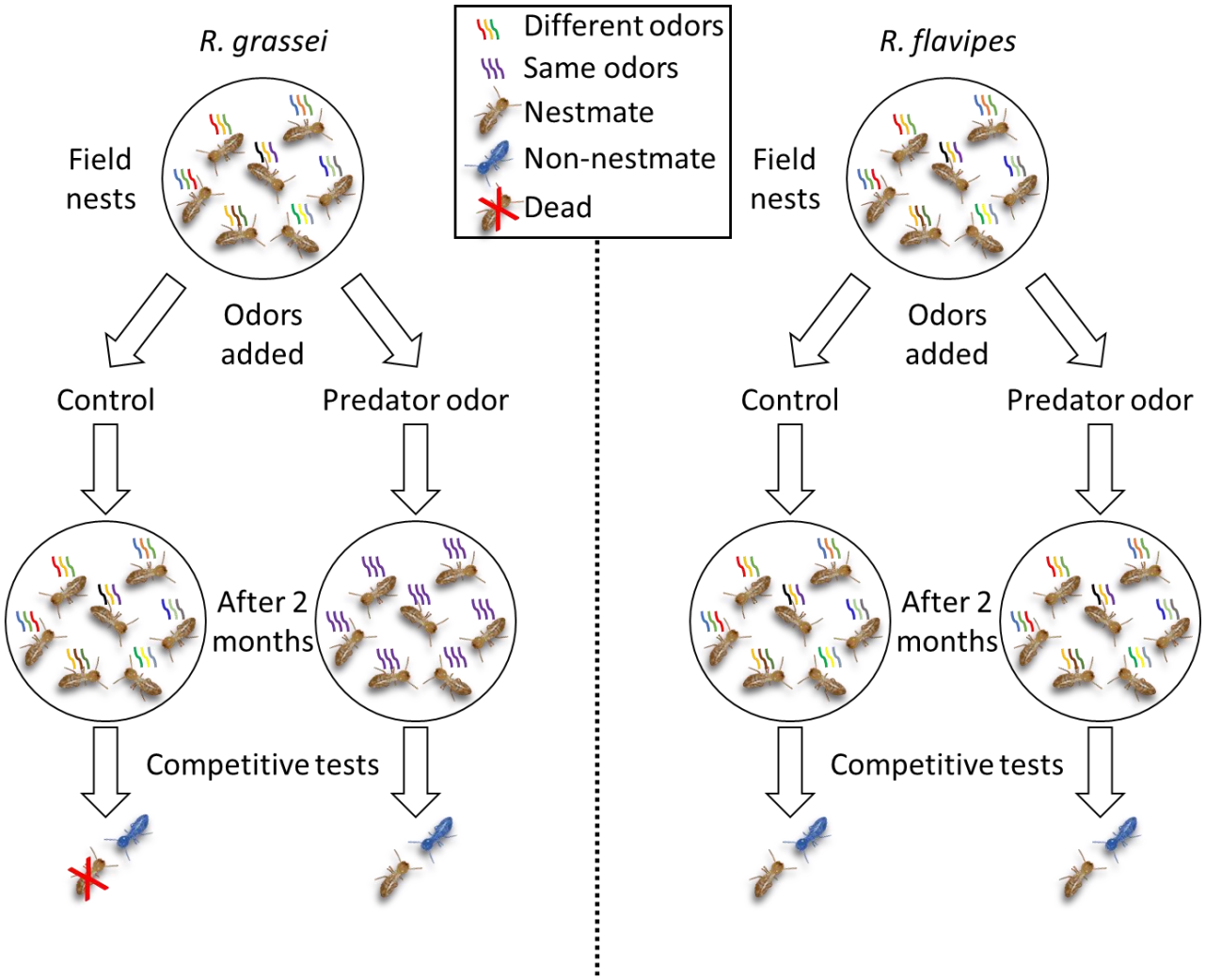


**Figure 22.** Résumé des résultats et du système expérimental. L'hétérogénéité des odeurs est représentée par des couleurs plus ou moins variées (Lucas *et al.*, 2018).

Ainsi, la présence de l'odeur du prédateur dans l'environnement influence les colonies de termites en (1) induisant une homogénéisation de l'odeur coloniale et (2) en augmentant la survie lors de tests de compétition. Ce qui montre que non seulement l'odeur du prédateur a été détectée par les termites mais qu'en plus l'odeur a modifié la cohésion sociale et l'organisation sociale de la colonie (Lucas *et al.*, 2018).

## 2.4 GENETIQUE DE LA PLASTICITE COMPORTEMENTALE

Un exemple de plasticité comportementale est le polyéthisme d'âge des insectes sociaux, qui amène un individu à changer de tâche comportementale au cours de sa vie. Dans certains cas, des individus ont des différences morphologiques et physiologiques importantes et sont spécialisés dans des tâches comportementales, comme c'est le cas de la caste des soldats ou celle des reproducteurs,

on parle alors de polyéthisme de castes. Sous certaines conditions, cette division du travail peut être réorganisée et les tâches comportementales normalement inféodées à un type d'individu peuvent être modifiées en fonction des besoins de la colonie (Villalta *et al.*, 2016). Ainsi des individus spécialisés dans la défense ou le fourragement peuvent s'impliquer dans le soin au couvain ou la reproduction par l'action d'une plasticité comportementale.

Dans les parties précédentes, nous avons vu que la plasticité comportementale est le fruit de signaux de l'environnement au sens large. Cette plasticité s'accompagne aussi de changements dans l'expression de certains gènes. En effet, il existe un jeu complexe entre expression génique, facteurs environnementaux et comportements, qui doit être appréhendé comme un ensemble et qui peut être élargi avec le concept du phénotype étendu (Dawkins, 2016). Les différences génotypiques impliquent que les différents allèles sont sélectionnés sous les pressions de l'environnement au cours de l'évolution. Les changements de l'expression génique sont, quant à eux, le reflet de l'existence de différents types de réponses aux pressions environnementales (Córdoba-Aguilar *et al.*, 2018). Les informations perçues par le système nerveux peuvent entraîner des changements dans l'expression génique qui, en retour, modifie la structure et le fonctionnement de celui-ci durant la vie de l'individu. Sans connaissance sur ces gènes spécifiques, il est très difficile de prédire le résultat de leurs influences mutuelles. Identifier les gènes responsables de changements comportementaux, c'est-à-dire les bases moléculaires de la plasticité comportementale, est donc primordial pour la compréhension des mécanismes contrôlant le polyphénisme (Simpson *et al.*, 2011). Pour comprendre quels sont les mécanismes moléculaires responsables d'une telle flexibilité comportementale, j'ai étudié le comportement alimentaire et de défense de la fourmi *Pheidole pallidula* et le polymorphisme social de *Solenopsis invicta*.

### 2.4.1 D'un rôle à l'autre : l'implication d'un gène

Très peu de gènes ayant une influence claire sur le comportement ont été identifiés et étudiés en détails à ce jour. L'exemple le plus marquant de tels gènes est le gène *foraging* (*for*) découvert chez la drosophile (Sokolowski, 2001). Chez *Drosophila melanogaster*, il existe deux variants sauvages alléliques qui affectent le comportement de fourragement des larves et des adultes. Les individus "rover" couvrent de grandes distances et mangent moins comparés aux individus "sitter". Dans ce système biologique, les variations alléliques de *for* sont importantes pour l'expression comportementale, néanmoins il existe aussi une plasticité comportementale exprimée qui module les comportements en fonction des informations environnementales (Kaun *et al.*, 2007a; Kaun *et al.*, 2007b; Mery *et al.*, 2007). Chez la drosophile, le gène *for* encode pour une protéine kinase dépendante du GMPc (PKG) qui possède une action cellulaire pléiotropique de phosphorylation d'une multitude de protéines cibles (Wang & Robinson, 1997; Douglas *et al.*, 2005). Il est possible de transformer le comportement d'un individu "sitter" en comportement "rover" en lui faisant exprimer un ADN complémentaire provenant d'un individu "rover" (Osborne *et al.*, 1997). Ainsi, le gène *for* montrerait à la fois un polymorphisme allélique naturel qui influence les différences de comportements entre individus et des variations d'expression génique qui influencent la plasticité comportementale chez le même individu (Fitzpatrick *et al.*, 2005; Reaume & Sokolowski, 2006).

Chez les insectes sociaux, la plasticité comportementale peut se montrer particulièrement impressionnante, comme nous venons de le voir (chapitre 2), avec un polyéthisme d'âge et de caste, ainsi qu'une organisation sociale du travail alors que tous les individus de la colonie possèdent le même génome. Les insectes sociaux proposent donc un modèle d'étude propice pour comprendre les mécanismes moléculaires de la plasticité comportementale. Il a été montré que la maturation comportementale de l'état de nourrice à l'état de fourrageuse, chez l'abeille *Apis mellifera* ou la fourmi *Pogonomyrmex barbatus* est associée à une augmentation à la fois de l'expression du gène *for* mais aussi à celle de l'activité de la PKG (Ben-Shahar *et al.*, 2002; Ingram *et al.*, 2005). Cependant, les nourrices et les fourrageuses diffèrent en âge et sont confrontées à des environnements différents.


La fourmi *Pheidole pallidula*, possède deux groupes d'ouvrières ultra spécialisées dans des tâches comportementales différentes : les majors (soldats) et les minors (fourrageuses). Ces deux types d'ouvrières présentent un dimorphisme discontinu et sont confrontés aux mêmes environnements. Les majors ont une tête large avec de grosses mandibules et sont principalement impliqués dans la défense de la colonie. Cette caste de majors, autrement appelée "soldat", patrouille à l'entrée du nid et attaque les intrus. Les minors sont plus petites et sont principalement impliquées dans des tâches de recherche de nourriture et de recrutement. Chaque caste d'ouvrières est prédisposée à des tâches comportementales propres et leurs répertoires comportementaux sont généralement limités à ces rôles. Néanmoins, les majors sont capables d'aider les minors dans les activités de fourragement en fonction des besoins de la colonie et vice versa. Ainsi, il existe une plasticité comportementale dans leurs répertoires spécialisés (Aarab, 1991). C'est dans ce cadre que nous avons entrepris l'étude de l'influence du gène *foraging* sur la plasticité des comportements de fourragement et de défense chez *P. pallidula* (Lucas & Sokolowski, 2009).

Nos travaux ont démontré un lien causal entre le gène *foraging* et la plasticité comportementale à la fois sur le comportement de recherche de nourriture et de façon plus surprenante (car nouveau) sur le comportement de défense (Lucas & Sokolowski, 2009). Pour ce faire, nous avons réalisé des manipulations environnementales en ajoutant un vers de farine pour induire un comportement de recherche de nourriture. Les analyses montrent que les minors fourrageuses présentent une activité de la PKG inférieure aux majors soldats et en plus que cette activité est modulée à la baisse en présence d'un vers de farine comparé aux individus mis en présence d'un leurre en plastique (Fig. 23A). Dans une autre série d'expériences, nous avons stimulé l'activité de défense en utilisant le même dispositif expérimental mais cette fois en introduisant des fourmis de la même espèce provenant soit de colonies étrangères soit de la même colonie que les individus testés. Chez *P. pallidula*, les colonies sont dites fermées si bien que la présence de fourmis étrangères déclenche des comportements agressifs. Une fois de plus, cette stimulation entraine des modulations de l'activité de la PKG mais cette fois-ci à la hausse c'est-à-dire à l'opposé de la réponse à la stimulation de fourragement (Fig. 23B).

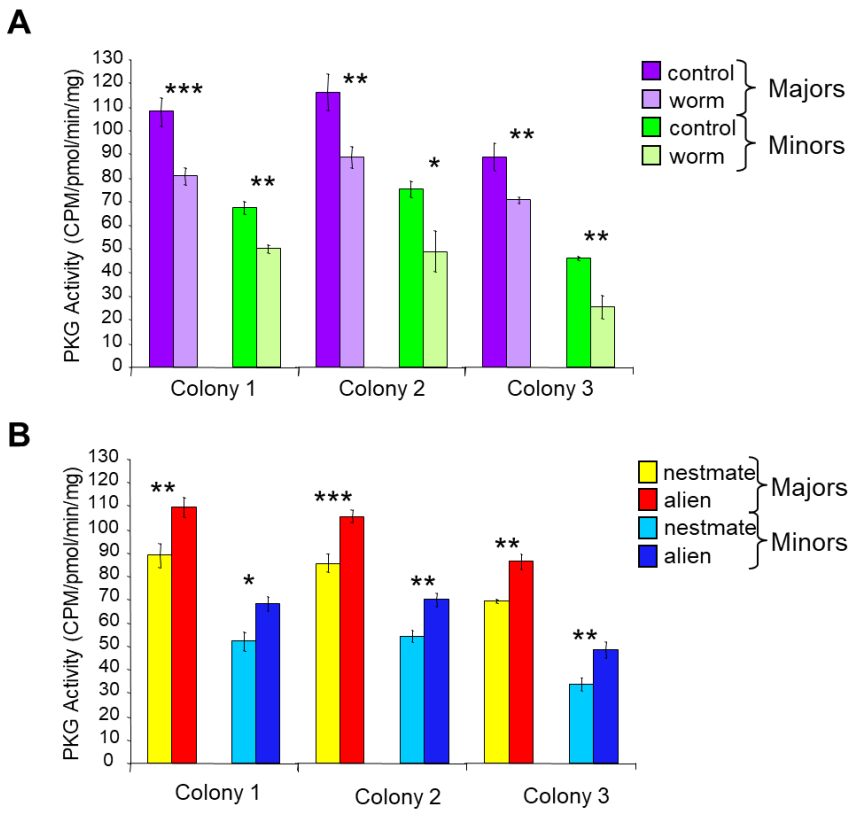


**Figure 23.** PKG activity of major and minor *P. pallidula* worker ants (mean ± S.E). (**A**) PKG activity of majors and minors was lower in the presence of the mealworm (foraging stimulus) as compared to the control ($F_{(3,45)}$=24.13, p<0.001); majors had higher PKG activity than minors ($F_{(3,45)}$=73.41, p<0.001). (**B**) Majors and minors had higher PKG activity in the presence of aliens (defense stimulus) compared to nestmate intruders ($F_{(3,41)}$=23.07, p<0.001); majors had higher PKG activity than minors ($F_{(3,41)}$=103.19, p<0.001). For **A** and **B**, the significance of all within colony comparisons not indicated on the figure was p< 0.001. *P* levels of *P*<0.001, P < 0.01, and P < 0.05 are represented as ***, **, and *, respectively (Lucas & Sokolowski, 2009).

Il existe donc une activité de la PKG spécifique à chaque caste en plus de l'existence d'une modulation d'activité en lien avec les signaux environnementaux. On peut donc émettre l'hypothèse que si la colonie a besoin de plus de nourriture les majors vont aider les minors à fourrager et si la colonie a besoin de plus de défense les minors vont aider les majors à défendre et tout ceci sous l'influence de l'expression du gène *foraging*. Pour tester cette hypothèse, afin de passer de corrélation à causalité, nous avons manipulé pharmacologiquement l'activité de la PKG et avons mesuré les comportements qui en résultent. Le 8-Br-GMPc est un activateur de la PKG qui, ajouté à la nourriture, induit une augmentation de l'activité de l'enzyme (Ben-Shahar *et al.*, 2002). Après quelques jours, le traitement pharmacologique a en effet modifié le comportement des ouvrières en induisant plus de défense et moins de recherche de nourriture.

Afin d'élucider les bases neuronales associées à cette plasticité, nous avons aussi comparé l'expression de *for* dans les cerveaux des minors et des majors en utilisant un anticorps spécifique de la PKG. L'analyse de la distribution spatiale dans les cerveaux par microscopie confocale montre l'existence de 3 régions immunoréactives communes aux minors et aux majors : (i) la face interne des lobula sur la partie dorsale du cerveau, (ii) les pédoncules des "mushroom bodies" en position postérieure et ventrale, (iii) et la face antérieure du ganglion sous-œsophagien. Enfin, une 4$^{ème}$ région, exclusivement présente chez les majors, est constituée de 5 groupes de cellules qui se trouvent en position antérieure sur la face externe des pédoncules des "mushroom bodies". Ce dernier groupe de

cellules, spécifiques des majors, pourrait être en partie responsable des différences comportementales entre minors et majors (Fig. 24).

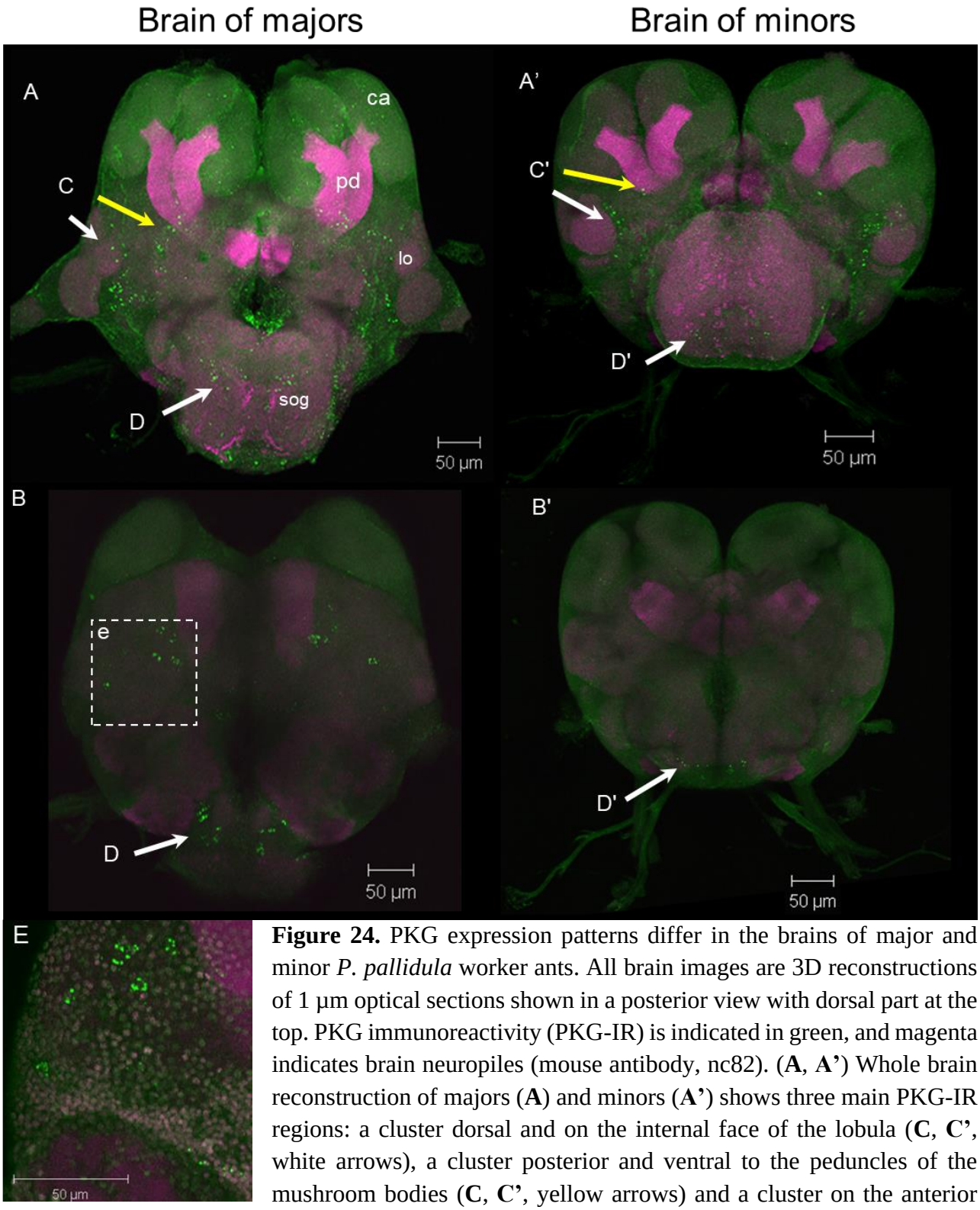


**Figure 24.** PKG expression patterns differ in the brains of major and minor *P. pallidula* worker ants. All brain images are 3D reconstructions of 1 µm optical sections shown in a posterior view with dorsal part at the top. PKG immunoreactivity (PKG-IR) is indicated in green, and magenta indicates brain neuropiles (mouse antibody, nc82). (**A**, **A'**) Whole brain reconstruction of majors (**A**) and minors (**A'**) shows three main PKG-IR regions: a cluster dorsal and on the internal face of the lobula (**C**, **C'**, white arrows), a cluster posterior and ventral to the peduncles of the mushroom bodies (**C**, **C'**, yellow arrows) and a cluster on the anterior face of the sub-esophageal ganglion (**D**, **D'**) (zoom details in, Lucas & Sokolowski, 2009). (**B**, **B'**) 3D reconstruction of subsections (40 sections) of the whole brains **A** and **A'**, spanning the posterior part of the brain of majors (**B**) and minors (**B'**) respectively shows a cluster of PKG-IR cells found in

the brain of majors (**E**, 30 sections) but not in minors. This specific cluster is localized on the anterior face external to the peduncles of the mushroom bodies. Negative controls using a pre-absorption step to abolish PKG immunoreactivity and blank controls using only the secondary antibody was also done (Lucas & Sokolowski, 2009). ca: calyx, pd: peduncles, lo: lobula, sog: sub-esophageal ganglion.

La plasticité comportementale des ouvrières est donc sous l'influence du gène *foraging*, non seulement pour la recherche de nourriture comme c'est le cas chez d'autres espèces mais aussi dans le comportement de défense. Nous voyons donc que le centre d'action du gène *foraging* ne semble pas uniquement limité au comportement alimentaire, ce qui semble être corroboré par des études plus récentes (Kohn *et al.*, 2013; Wang & Sokolowski, 2017; Anreiter & Sokolowski, 2019; Kohlmeier *et al.*, 2019). La fonction de ce gène a été conservée chez de très nombreuses espèces, de l'insecte à l'homme (Lucas *et al.*, 2010b; Tarès *et al.*, 2013; Chardonnet *et al.*, 2014; Malé *et al.*, 2017; Struk *et al.*, 2019). Tout ceci nous amène à penser que le gène *foraging* pourrait être un acteur clé qui régule les réponses comportementales au sens large, en fonction des besoins des individus et des changements de l'environnement (Lucas *et al.*, 2010a). Même s'il n'est pas exclu que d'autres gènes puissent agir sur ces comportements, nos expériences montrent que l'expression différentielle d'un seul gène suffit pour les modifier. De futures études sur le fonctionnement cellulaire de cette enzyme, pourraient nous aider à mieux comprendre la modularité de ces effets pléiotropiques sur le comportement.

### 2.4.2 Polymorphisme social

Les stratégies de reproduction des insectes sociaux présentent différentes structures sociales, passant de colonies monogynes/monoandres à des colonies polygynes/polyandres via l'ensemble des intermédiaires possibles. Ce polymorphisme social se traduit par une grande variabilité du nombre de reproducteurs et une diversité de leur mode de reproduction, sans oublier la diversité comportementale associée. Il s'observe entre différentes espèces mais aussi entre populations d'une même espèce ou entre colonies issues d'une même population (Pearcy *et al.*, 2004; Timmermans *et al.*, 2008; Cronin *et al.*, 2013; Leniaud *et al.*, 2013). Ce qui donne une grande capacité adaptative à l'espèce face à différentes conditions environnementales plus ou moins propices à chaque structure sociale (Ross & Keller, 1995b; Ingram, 2002). Chez la fourmi *Solenopsis invicta*, il existe deux structures d'organisation sociale des colonies : les monogynes et les polygynes (monoandre facultatif, voir Lawson *et al.* (2012)). Les colonies monogynes possèdent une seule reine fertile de grande taille. Les ouvrières stériles monogynes, constituant le reste de la colonie, sont très territoriales et agressives. Tandis que les colonies polygynes possèdent de multiples reines fertiles de petite taille avec des ouvrières moins agressives que les monogynes (Ross & Keller, 1995a; Tschinkel, 2006). De


Photo © Wild

plus, les futures reines (gynes) issues des colonies monogynes dispersent loin de la colonie mère pour fonder une nouvelle colonie de façon indépendante. Tandis que les gynes issues de colonies polygynes ne dispersent que très peu et infiltrent une colonie déjà existante, voire la colonie mère elle-même (DeHeer, 2002).

Ce polymorphisme social est connu pour être sous l'influence d'un supergène (Wang *et al.*, 2013). Un supergène est un cluster de loci fermement liés ensemble qui est maintenu grâce à des combinaisons alleliques spécifiques qui facilitent la co-ségrégation des gènes impliqués dans l'adaptation phénotypique (Mather, 1950). Le gène *GP-9* (*General Protein-9*) est classé parmi les "odorant-binding protein" et fait partie d'une région non recombinante de ce supergène. Il est associé au polymorphisme social ainsi qu'à différents traits affectant la physiologie, la fécondité et le comportement (Thompson & Jiggins, 2014). Ce gène est aussi appelé "barbe verte" car il est associé à la tolérance de la présence de plusieurs reines au sein d'une même colonie (Keller & Ross, 1998). En effet, toutes les reines d'une colonie polygyne sont hétérozygotes *Bb* pour le gène *GP-9*, les reines $GP\text{-}9^{BB}$ sont systématiquement tuées de façon prépondérante par les ouvrières $GP\text{-}9^{Bb}$. Le bénéfice apporté par *b* dans le succès reproducteur est contrebalancé par une fitness plus faible des mâles *b* et la léthalité des femelles homozygotes *bb* (Lawson *et al.*, 2012) (*bb* est léthal, mais voir Fritz *et al.* (2006)). Le polymorphisme social de *S. invicta* est donc directement en lien avec la composition allélique de *GP*-9, lui-même associé à de grandes différences comportementales. Mais chez les individus ayant des génotypes différents, existe-t-il une variabilité d'expression génique de *GP*-9 pouvant être associée à une plasticité comportementale ? Le gène *foraging* est aussi un autre candidat possible, connaissant son rôle central dans la régulation des comportements sociaux et en se basant sur nos recherches précédentes reliant fourragement, défense et déplacement (Lucas & Sokolowski, 2009; Lucas *et al.*, 2010a; Lucas *et al.*, 2010b). Nous nous sommes donc posé la question si le polymorphisme social pouvait présenter des différences d'expression du gène *for* et du gène *GP-9*.

Nous avons donc étudié l'expression de ces 2 gènes clés par analyse en RT-qPCR sur des futures reines (gynes) et des ouvrières provenant soit du nid soit de l'aire de fourragement (Lucas *et al.*, 2015). Les ouvrières appartenant à ces deux zones sont impliquées dans des tâches comportementales différentes comme le soin au couvain ou la recherche de nourriture. En ce qui concerne les gynes, elles sortent de la colonie au moment de la recherche de partenaire avant de disperser soit en établissant une nouvelle colonie indépendamment, soit en colonisant un nid déjà établi, en fonction de leur origine sociale (Porter *et al.*, 1991; Gotzek & Ross, 2007).

L'expression du gène *GP-9* se révèle significativement différent, uniquement pour les ouvrières, à la fois entre formes sociales et aussi en fonction de la localisation des individus. De plus, l'analyse par pyroséquençage révèle une expression supérieure de l'allèle *b* comparé à l'allèle *B* et ceux chez les ouvrières et les gynes hétérozygotes (Fig. 25). Cette variabilité inattendue d'expression de *Gp-9* pourrait être associée au comportement d'élimination sélective des gynes $GP\text{-}9^{BB}$ observé dans les colonies polygynes qui est principalement perpétué par les ouvrières de génotype $GP\text{-}9^{Bb}$ (Keller & Ross, 1998).

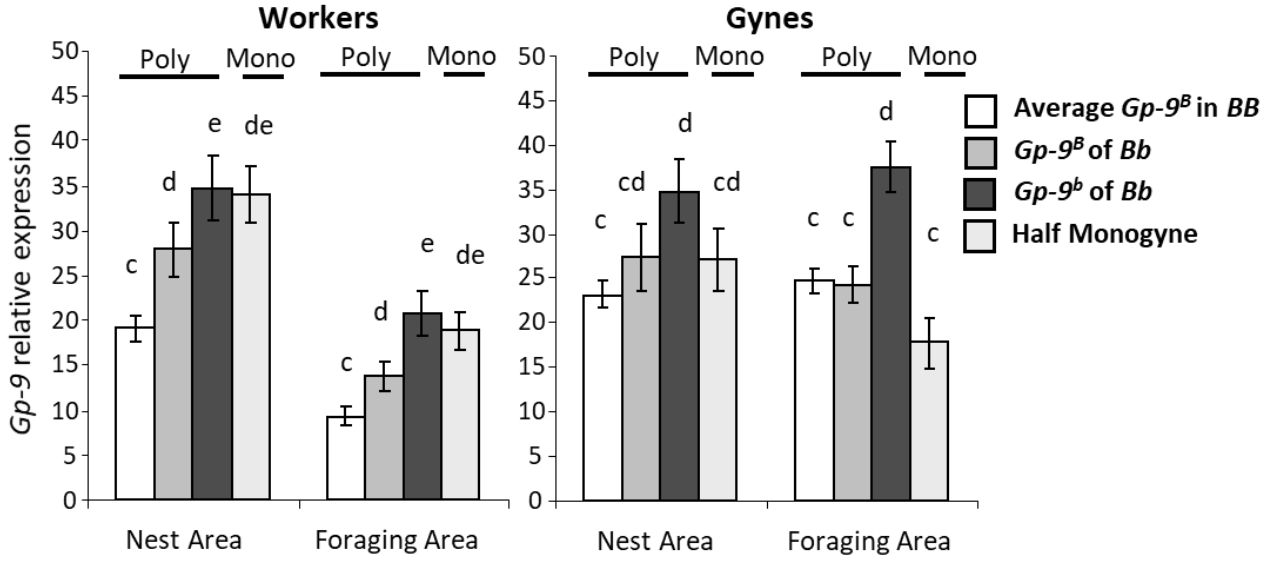


**Figure 25.** Relative expression of *Gp-9* per allele [significant differences between pairwise tests ($p<0.05$) within same location are represented with different letters]. Statistical comparisons between locations are not shown but within each genotype, comparisons were all significant for workers ($p<0.05$) and only one was significant for gynes: half monogyne versus $Gp\text{-}9^{B}$ allele ($p<0.01$). *BB*: individuals with $Gp\text{-}9^{BB}$ genotype, *Bb*: individuals with $Gp\text{-}9^{Bb}$ genotype (Lucas *et al.*, 2015).

Concernant le gène *foraging*, nos résultats montrent que non seulement il existe des différences d'expression en fonction de la forme sociale des colonies mais aussi de la localisation des individus (nid/aire de fourragement) (Fig. 26A). Ces résultats sont en correspondance avec nos travaux précèdents sur *for* chez la fourmi *Pheidole pallidula*, indiquant un lien entre fourragement et agressivité. En effet, on retrouve un niveau d'expression de *for* plus bas pour les individus situés dans l'aire de fourragement comparé à ceux situés dans le nid. De plus, on constate que le niveau d'expression est plus grand chez les ouvrières monogynes que chez les polygynes. Là encore cela reste cohérent avec ce que l'on connait sur la relation entre le comportement de défense et le gène *for* (Lucas & Sokolowski, 2009; Lucas *et al.*, 2010a), sachant que les ouvrières monogynes sont réputées pour être plus agressives que les polygynes (Tschinkel, 2006; Chirino *et al.*, 2012). Nos résultats montrent aussi des différences entre ouvrières et gynes avec le rapport d'expression qui s'inverse (Fig. 26A). Les gynes issues de colonies monogynes possèdent une expression de *for* inférieure aux gynes polygynes. Notre hypothèse est que cela pourrait être mis en correspondance avec la capacité de dispersion plus importante des gynes monogynes comparé aux polygynes, à l'instar de nos résultats sur le comportement alimentaire et de grégarisation du criquet du désert dont nous avons parlé précédemment (Lucas *et al.*, 2010b). Ceci pourrait ouvrir des perspectives de recherche sur les mécanismes de dispersion des insectes, dont l'étude représente un enjeu économique et écologique important. Le rôle du gène *for* dans l'adaptation écologique aux changements environnementaux reste encore obscure chez *S. invicta* bien que des expériences récentes montrent que l'expression du gène semble expliquer les capacités d'acclimatation des individus à des variations de nourriture et de température (Zhou *et al.*, 2019).

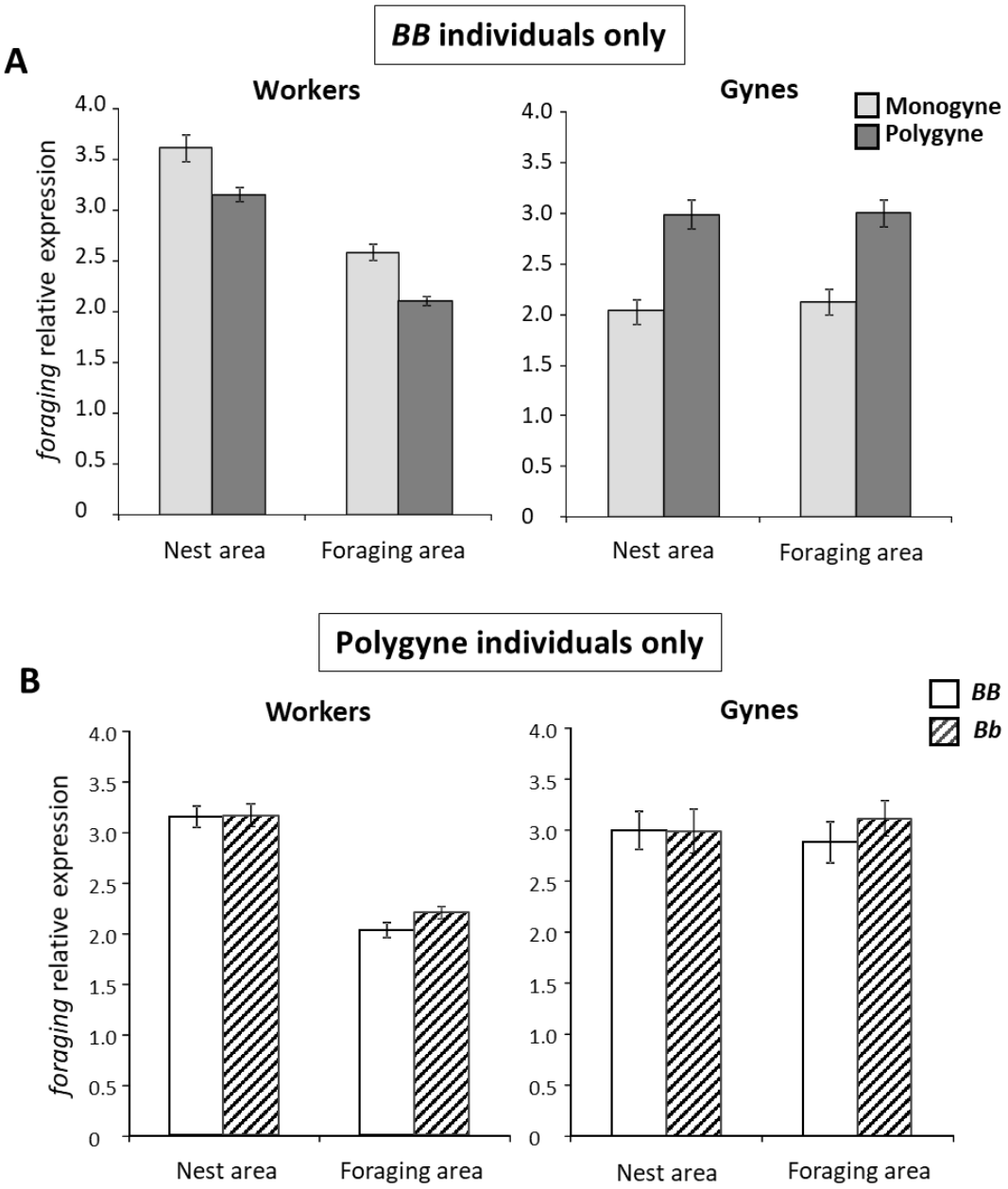


**Figure 26.** Relative expression of the *foraging* gene with (**A**) *BB* individuals only [statistics for workers (social form: $p<0.05$, location: $p<0.001$, social form x location: NS); statistics for gynes (social form: $p<0.05$, location: NS, social form x location: NS)]. (**B**) Polygyne individuals only [statistics for workers (social form: NS, location: $p<0.001$, social form x location: NS); statistics for gynes (social form: NS, location: NS, social form x location: NS)]. *BB*: individuals with *Gp-9*$^{BB}$ genotype, *Bb*: individuals with *Gp-9*$^{Bb}$ genotype (Lucas *et al.*, 2015).

Le niveau d'expression de *for* n'est pas significativement associé aux génotypes des ouvrières et des gynes chez les colonies polygynes (Fig. 26B). Ce qui suggère que les gènes *for* et *GP-9* ont des effets indépendants non-épistatiques sur le comportement de *S. invicta*. Ces deux gènes agissant sur

la division du travail et sur le polymorphisme social, amènent une répartition des individus en fonction de leurs rôles au sein de la société. Ceci se traduit entre autres par une répartition spatiale à la fois dépendante de l'expression de ces deux gènes mais aussi du génotype des individus, comme le démontre, au moins en partie, nos résultats indiquant une répartition des ouvrières dans les colonies polygynes en fonction de leurs génotypes, résumé dans la figure 27 (voir les détails en annexe). Il faut garder à l'esprit que *Gp-9* est lié à plus de 600 gènes dans une région non recombinante (Wang *et al.*, 2013), il est donc actuellement impossible de déterminer précisément l'association observée entre génotype et répartition spatiale (Lucas *et al.*, 2015).

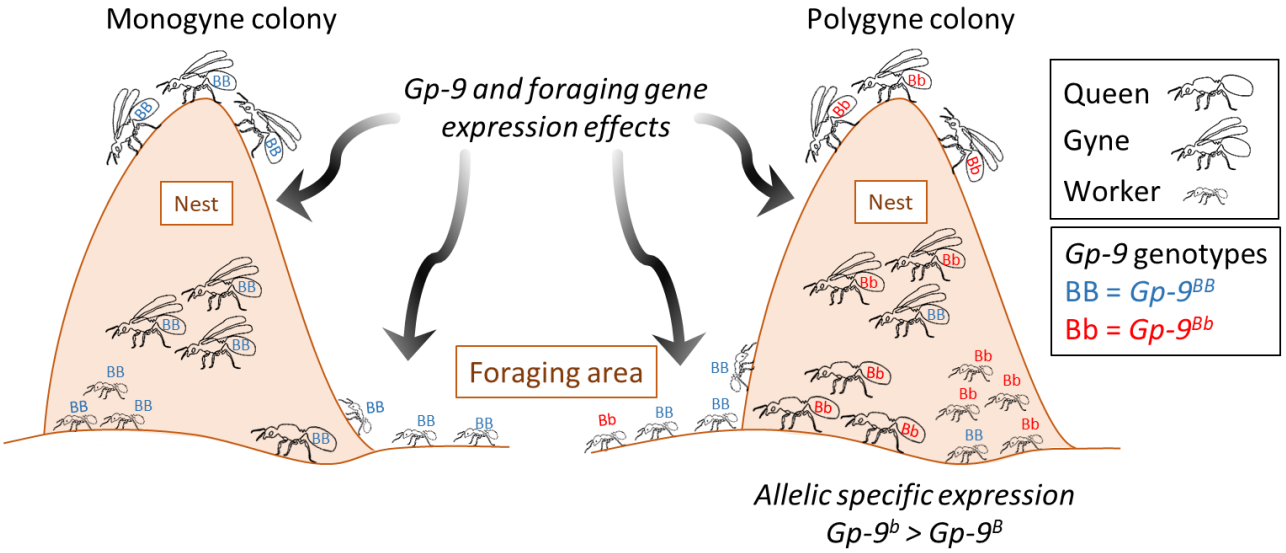


**Figure 27.** Fréquences et répartition observées du nombre d'ouvrières et de gynes, entre nid et aire de fourragement, en fonction de leurs génotypes et de la forme sociale (Lucas *et al.*, 2015).

L'inversion d'expression de *for* entre ouvrières et gynes montre qu'un même gène peut s'exprimer différemment au sein d'individus présentant le même génome et que cela induit des différences comportementales marquantes. Evidemment, ce qui est observable ici à l'échelle d'un seul gène peut se retrouver d'autant plus amplifié lorsque l'on prend en considération l'ensemble du génome dont les gènes interagissent et s'expriment différemment entre individus de castes différentes, amenant à des spécialisations comportementales dont nous allons parler dans le chapitre suivant.

# 3. Spécialisations comportementales : système de castes

L'une des transitions majeures dans l'évolution du vivant est l'apparition de la socialité. En commençant avec la réplication des molécules, la vie sur terre est devenue organisée selon un modèle de plus en plus complexe, à l'image des sociétés (Maynard Smith & Szathmáry, 1995). Chez les insectes sociaux (termites, fourmis, abeilles et guêpes), la socialité est caractérisée par une division du travail où chaque individu est spécialisé dans certaines tâches comportementales. Cette division s'accompagne souvent de transformations morphologiques et physiologiques de groupes d'individus, en relation avec leur rôle (leur caste) au sein de la colonie. Ainsi, certains individus sont spécialisés dans la reproduction tandis que d'autres le sont dans la défense, le soin au couvain ou encore la recherche de nourriture. Les reproducteurs sont des individus hautement féconds qui peuvent vivre jusqu'à 30 ans chez certaines espèces (Keller & Genoud, 1997; Rueppell *et al.*, 2004; Bignell *et al.*, 2011), tandis que les ouvriers possèdent des gonades peu ou pas développées (Roisin, 2000; Dijkstra *et al.*, 2005; Dijkstra & Boomsma, 2006) et vivent en moyenne moins d'un an. Les ouvriers participent à des tâches coopératives telles que la construction du nid, la collecte de nourriture, l'élevage des jeunes et la défense de la colonie. Certains ouvriers peuvent posséder des mandibules très développées augmentant leur efficacité dans la défense tandis que les fourrageuses sont plus petites et recherchent la nourriture le plus souvent à l'extérieur du nid. Les castes non reproductives ont évolué à travers la sélection de parentèles (Hamilton, 1964) : elles gagnent une fitness indirecte en favorisant la reproduction d'autres individus génétiquement apparentés (inclusive fitness, avec des particularités pour le système termite décrit plus bas). Cette organisation sociale avec son système de castes apporte de nombreux avantages et forme la base de l'énorme succès évolutif des insectes sociaux, comme le démontrent leur dominance écologique et leur haute diversité d'espèces (Wilson, 1971; Hölldobler & Wilson, 1990).

Ce système de castes est le fruit de millions d'années d'évolution des sociétés d'insectes et est l'une des caractéristiques marquantes de l'eusocialité (socialité véritable). C'est un exemple marquant de ce que l'on appelle la plasticité phénotypique, c'est-à-dire plusieurs morphotypes avec des physiologies et des variants comportementaux produits par le même génotype. Le fonctionnement et le maintien de ce système de castes met en jeu une communication spécialisée intense qui régit les comportements des différents membres de la société et qui peut avoir un rôle prépondérant dans le devenir des individus au sein de la colonie. Chez les termites souterrains, le devenir de chaque individu est déterminé par la présence ou l'absence de chaque caste. Ainsi, une colonie privée de reproducteurs ou de soldats se met à produire des individus de la caste manquante. Si la colonie se trouve en situation de compétition avec d'autres colonies agressives, alors il y aura une augmentation de la production du nombre de soldats. Mais une fois les soldats présents au sein de la colonie, leur production va être réduite par un système complexe de rétrocontrôle chimique via des phéromones modificatrices (Zhou *et al.*, 2007; Le Conte & Hefetz, 2008; Tarver *et al.*, 2009). Chez les fourmis du genre *Pheidole*, la présence physique des soldats n'est pas requise. En effet, le contact du couvain avec des ouvrières, elles-mêmes en contact avec des soldats, suffit à inhiber le développement du

couvain en soldats (Passera *et al.*, 1996). Ainsi, à la suite de certains signaux dans l'environnement, un individu peut s'orienter vers différents rôles dans la colonie en fonction des besoins de celle-ci. Les systèmes de communication ont donc un rôle dans le déterminisme des castes au même titre que certains facteurs purement génétiques. Une fois de plus, les signaux dans l'environnement et notamment les interactions sociales jouent un rôle prépondérant dans la régulation des castes. Malgré la littérature importante sur ce sujet, des zones d'ombre persistent toujours concernant les conditions environnementales et/ou génétiques amenant un membre de la colonie à s'orienter dans une caste plutôt qu'une autre.

## 3.1 BASE MOLECULAIRE DE LA DIVISION DU TRAVAIL

Chez la plupart des espèces d'insectes sociaux, le système de castes ne dépend pas du génome de l'individu mais il est déterminé par l'environnement qui modifie l'expression de ce génome durant plusieurs périodes critiques du développement larvaire (Wheeler, 1986; 1991). Comme par exemple, l'effet de la gelée royale sur le développement des reines décrit chez l'abeille (Kamakura, 2011) ou l'effet des facteurs sociaux avec la présence de la reine qui change directement le nombre de gynes (futures reines) produites chez la fourmi *Linepithema humile* (Ingram, 2002). Ces mécanismes longtemps associés à des facteurs purement environnementaux peuvent en réalité être beaucoup plus complexes (Buttstedt *et al.*, 2016). Par conséquent, les castes sont un exemple de polyphénisme où les individus appartenant à des castes différentes possèdent les mêmes gènes mais ces gènes s'expriment différemment (Hamilton, 1964; Keller & Genoud, 1997; Evans & Wheeler, 1999; 2000; 2001; Robinson, 2002; Nijhout, 2003; Rueppell *et al.*, 2004; Dijkstra *et al.*, 2005; Pereboom *et al.*, 2005; Dijkstra & Boomsma, 2006; Sumner *et al.*, 2006). Il existe aussi des systèmes de castes dont le déterminisme est purement de base génétique aboutissant parfois à des lignées génétiques indépendantes au sein de la même espèce avec des effets maternels ou paternels sur le déterminisme des castes comme chez les fourmis (Schwander & Keller, 2008; Nygaard *et al.*, 2011; Libbrecht & Keller, 2013) ; ou enfin à des systèmes mixtes déterminés à la fois par des facteurs génétiques et environnementaux (Schwander *et al.*, 2010). L'étude approfondie des mécanismes de ce polyphénisme permet de comprendre comment l'expression différentielle de gènes peut générer des variations biologiques complexes (West-Eberhard, 2003). C'est aussi la clé pour comprendre la base génétique de la division du travail et ses implications comportementales.

Chez la fourmi de feu *Solenopsis invicta*, les tâches comportementales des ouvrières sont sous l'influence de facteurs internes (âge et taille), de facteurs externes environnementaux (interactions sociales), et de facteurs phéromonaux issus de la reine (Mirenda & Vinson, 1981; Vargo, 1998; Vander Meer & Alonso, 2002; Wyatt, 2003; Duarte *et al.*, 2011). Nous avons utilisé ce modèle biologique pour étudier les gènes impliqués dans la régulation moléculaire et sociale de la division du travail (Manfredini *et al.*, 2014). Nous avons ainsi identifié plusieurs gènes dont l'expression varie en fonction du comportement de fourragement des ouvrières. La plupart de ces gènes s'avère différent de ceux impliqués dans la division du travail découverts chez l'abeille ou la guêpe (Fig. 28). Parmi l'ensemble de ces gènes, 16 sont communs aux 3 espèces eusociales.

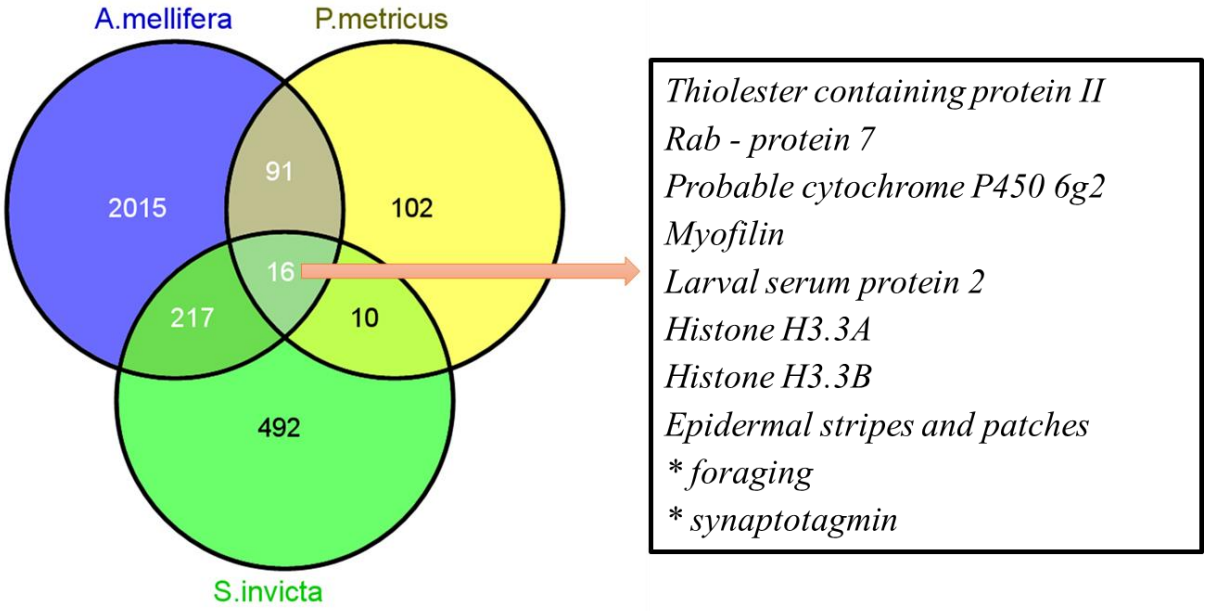


**Figure 28.** Comparative analysis of transcripts associated with worker division of labor across social insect species. Overlap analysis of FlyBase numbers corresponding to transcripts that were differentially expressed between foraging and nonforaging workers in *Apis mellifera* (brain tissue: Alaux *et al.* (2009) and fat bodies: Ament *et al.* (2011), *Polistes metric*us (brain tissue: Toth *et al.* (2010)) and *Solenopsis invicta* (whole-body: Manfredini *et al.* (2014)). Statistical analysis revealed that in both cases there was less overlap than expected by chance. The list includes the 11 genes annotated. Note that the patterns of expression of these 16 genes in foraging and nonforaging workers were not consistent across the three social insects.
*These genes were not listed in Alaux *et al.* (2009) and Ament *et al.* (2011), but were differentially expressed in *Apis mellifera* based on different studies (Ben-Shahar *et al.*, 2002; Whitfield *et al.*, 2003).

De plus, nos analyses montrent que si l'on retire la reine de la colonie, le niveau de transcription n'est plus différent entre ouvrières fourrageuses et non-fourrageuses. Enfin, toutes tâches comportementales confondues, nous avons détecté des différences d'expression en fonction de la présence ou non de la reine, démontrant l'importance des interactions sociales dans les mécanismes moléculaires régulant l'activité des ouvrières. Tout ceci indique qu'il existe des différences consistantes dans l'expression génique associée à la division du travail avec l'existence d'une régulation sociale exercée par la reine (Manfredini *et al.*, 2014).

## 3.2 PLASTICITE PHENOTYPIQUE DES TERMITES

Parmi les insectes eusociaux, les termites apparaissent comme complètement différents des fourmis, abeilles et guêpes dans leur développement et leur système de reproduction tout en gardant des traits caractéristiques des sociétés d'insectes (Wilson, 1971; Roisin, 2000; Zhou *et al.*, 2006; Korb *et al.*, 2009; Nalepa, 2010; Bignell *et al.*, 2011; Brian, 2012; Watson *et al.*, 2014). Ces spécificités du modèle termite ouvrent des opportunités d'études uniques comparées aux autres insectes eusociaux. Le premier point est la présence de mâles et de femelles dans toutes les castes, selon des ratios dépendant des espèces. Il y a donc présence de mâles et de femelles chez les ouvriers et les soldats mais aussi chez les reproducteurs avec au moins un couple royal par colonie qui s'accouple régulièrement. Les autres particularités majeures sont la diploïdie et le système de développement hémimétabole, là où tous les autres insectes eusociaux sont haplodiploïdes et holométaboles. L'hémimétabolie apporte un déterminisme de castes post-embryonnaire qui donne une très haute plasticité phénotypique. Ainsi, la capacité d'adaptation aux contraintes environnementales est augmentée car la plupart des membres de la colonie peut sous certaines conditions passer d'une caste à l'autre avec, chez certaines espèces, des mues régressives vers la caste d'origine.

Chez certains termites, les processus de différenciation des castes apportent une plasticité phénotypique encore plus grande (Nalepa, 2010), puisque tous les individus de la colonie peuvent sous certaines conditions accéder à la reproduction en se développant en reproducteurs sauf les soldats. Bien qu'il existe des soldats reproducteurs (Myles, 1986; Johnson *et al.*, 2011) considérés comme un reliquat évolutif (Thorne *et al.*, 2003), hypothèse néanmoins nuancée par Saiki *et al.* (2014). Cette "totipotence" reproductive est soumise à des conditions environnementales et sociales qui ne sont pas entièrement élucidées mais qui implique des facteurs biotiques, abiotiques, phéromonaux et hormonaux (Zhou *et al.*, 2006; Matsuura *et al.*, 2009; Matsuura *et al.*, 2010; Miura & Scharf, 2010; Simpson *et al.*, 2011; Tarver *et al.*, 2012; Sun *et al.*, 2017) et potentiellement génétique (car cela manque de démonstration expérimentale) (Hayashi *et al.*, 2007). Ainsi, chez ces termites, les ouvriers stériles peuvent muer en reproducteurs fertiles, permettant le passage direct de leur patrimoine génétique. Néanmoins, la stabilité sociale au sein des colonies est garantie par un équilibre entre les avantages de la reproduction directe et les coûts associés. Comme par exemple l'investissement dans la voie de développement en reproducteur et la fondation de nouvelles colonies avec leur faible taux de survie lié à la niche écologique qu'occupent les termites (Vargo, 2019). En effet, les termites font face à une pression écologique unique à cause de leurs ressources alimentaires (le bois) qui sont dispersées, limitées et de faible qualité nutritive, sans compter les coûts élevés associés à la recherche de partenaires sexuels (Nalepa & Jones, 1991).

Ainsi, issus de ces différentes voies de développement possibles, chez les termites souterrains, trois types de reproducteurs existent en ayant chacun ses propres stratégies de reproduction, ses avantages et ses coûts (Fig. 29) : (i) Les reproducteurs adultoïdes (reproducteurs primaires), issus de la lignée imaginale, se développent en passant par une phase ailée appelée stade alate. Ils présentent un comportement de dispersion (essaimage par vol) et de colonisation en fondant de nouvelles

colonies sans aide extérieure ; (ii) les reproducteurs néoténiques nymphoïdes brachyptères (reproducteurs secondaires), sont aussi issus de la lignée imaginale. Ils restent principalement à l'intérieur de la colonie mère, tout comme (iii) les reproducteurs néoténiques ergatoïdes aptères qui eux sont issus d'ouvriers et sont donc dépourvus de bourgeons alaires (nommés reproducteurs secondaires, parfois nommés tertiaires, voir tableau 1 en annexe ; Fig. 30).

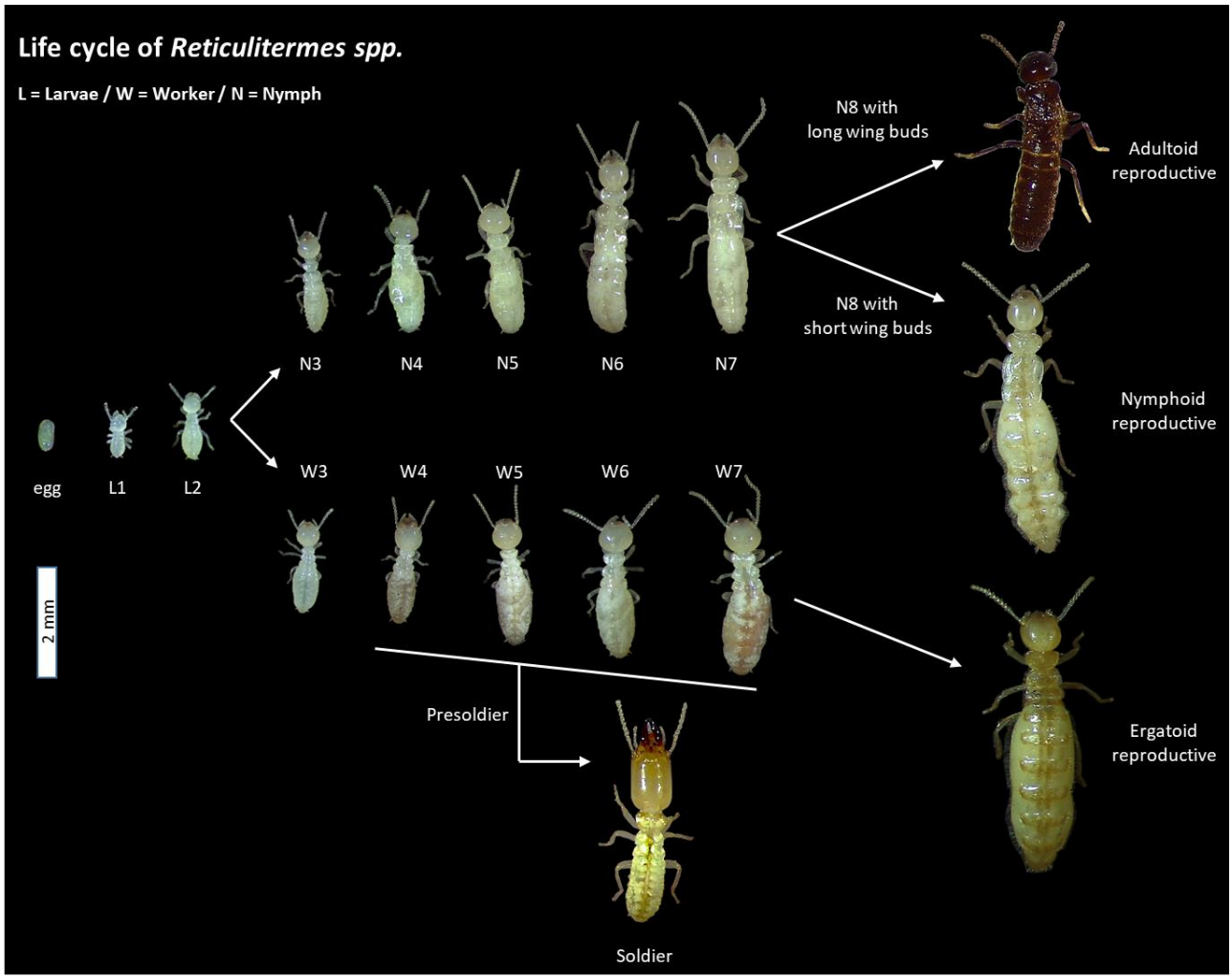


**Figure 29.** Life cycle of *Reticulitermes spp*. Only female reproductives are represented (for more details on reproductives see figure 30). Adultoid reproductives are also called, primary reproductives. They developed through a winged alate form. Soldier develop through a presoldier step also called white soldiers. Nymphoid and ergatoid reproductives are also called respectively brachypterous and apterous neotenics (see table 1 in annexes for details). L: larvae, W: worker, N: Nymph, (picture credits: Dupont S. / CNRS).

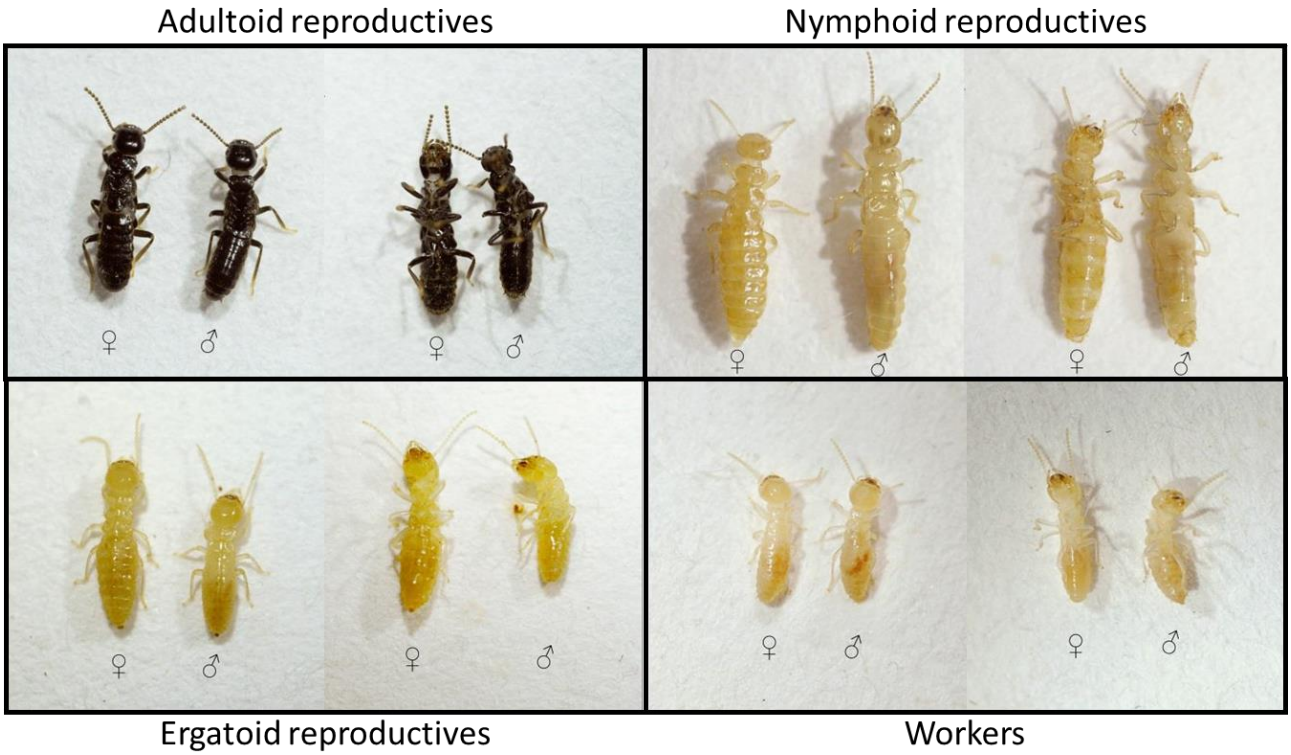


**Figure 30.** Pictures from top and bottom sides of male and female reproductives and workers of *Reticulitermes flavipes*. Scales were conserved across pictures (picture credits: Bredon M. / CNRS).

Notez que la nomenclature est parfois complexe. Par exemple, les notions de primaire et secondaire se réfèrent à la capacité des reproducteurs à fonder une colonie indépendamment (reproducteurs primaires) ou bien de renforcer le pouvoir reproductif de la colonie mère (reproducteurs secondaires) regroupant ainsi les reproducteurs nymphoïdes et ergatoïdes. Néanmoins, dans la littérature la notion de primaire / secondaire / tertiaire peut supplanter les termes adultoïde / nymphoïde / ergatoïde (Thompson & Snyder, 1920; Noirot, 1956; Nutting, 1956; Thorne *et al.*, 1999; Lainé & Wright, 2003; Dean & Gold, 2004; Zhou *et al.*, 2006; Maekawa *et al.*, 2008; Vargo & Husseneder, 2009; Steller *et al.*, 2010; Tarver *et al.*, 2012). On peut aussi parler de "alate-derived / nymph-derived / worker-derived" (Vargo, 2019), ou encore respectivement d'imago, de néoténique brachyptère et de néoténique aptère (Tab. 1 en annexe). La notion de néoténique se référant aux individus présentant la capacité à se reproduire alors qu'ils sont à un stade larvaire, ainsi seuls les reproducteurs primaires et les soldats sont considérés comme de véritables adultes (Bignell *et al.*, 2011).

Cette formidable plasticité phénotypique et comportementale est sous l'influence de facteurs environnementaux et sociaux qui modifient l'expression de certains gènes (Evans & Wheeler, 1999; 2001; Weil *et al.*, 2009b), dont une partie est encodée par des signaux chimiques (Weil *et al.*, 2009a) et ceci via des régulations endocriniennes telle que l'hormone juvénile III (Scharf *et al.*, 2003; Mao *et al.*, 2005; Park & Raina, 2005; Scharf *et al.*, 2005b). Chaque caste partage le même génome et pourtant chaque caste possède un comportement spécifique et un phénotype qui lui est propre, le tout influencé par l'environnement au sens large et les interactions sociales. C'est dans ce cadre que nous avons abordé l'étude de la plasticité phénotypique des termites du genre *Reticulitermes*.

### 3.2.1 Caste des reproducteurs et stratégies de fondation


Une colonie de termites souterrains est donc en constante évolution avec des individus changeant de caste en fonction des besoins de la colonie en suivant certaines contraintes. Les différents types de reproducteurs présentent un polymorphisme comportemental et morphologique, associé à des stratégies de reproduction différentes. Leur nombre au sein des colonies est donc variable et est considéré comme une des raisons du succès évolutif de ces termites. Pour comprendre comment de nouveaux reproducteurs émergent au sein d'une colonie de termites, il faut dans un premier temps connaitre le rôle des reproducteurs au cours de la genèse de la colonie, ainsi que les différentes étapes de développement de la colonie, avant l'apparition des autres castes. De plus, si la morphologie des différents reproducteurs est connue, peu d'études avaient décrit le comportement de ces reproducteurs lors de la phase de fondation. C'est ce que nous avons abordé au cours d'une thèse que j'ai co-encadrée.

Durant cette thèse, nous avons créé un dispositif expérimental optimisé pour observer finement les comportements et le développement de chaque individu, dès les premiers jours de la fondation. Il est constitué de deux plaques de verre renfermant du sable humidifié ainsi qu'un disque de papier en pure cellulose appliqué sur une des deux faces. Des lanières de plastique assurent l'étanchéité relative du système, pour éviter que les individus ne s'échappent, tout en permettant l'humidification par capillarité en trempant le dispositif dans un bac d'eau. Chaque unité peut facilement être manipulée permettant à la fois de visualiser les individus, de les dénombrer et d'observer finement leur comportement, le tout en lumière infrarouge afin de respecter le mode de vie cryptique de ces termites. L'ensemble des matériaux est stérilisé sous UV participant ainsi au maintien des fondations sur plus de 6 mois (Fig. 31).

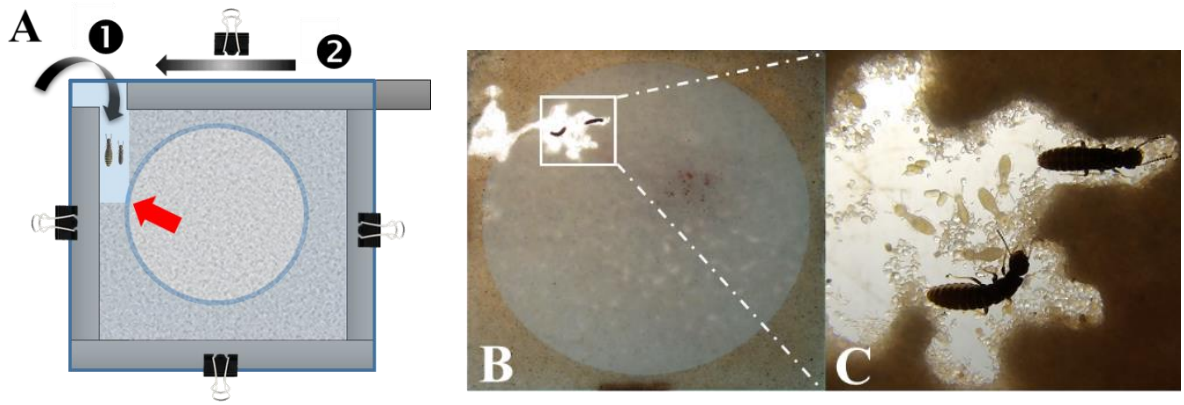


**Figure 31.** Overview of the experimental setup. (**A**) Schematic representation of the glass case and colony initiation including the two methodological steps. The colony was founded by placing the royal pair in a spot where no sand was present (Step 1). Then, the glass case was closed by sliding the upper plastic spacer in place (Step 2). The pair had access to the cellulose disk so that the termites did not have to excavate to obtain food (indicated by the red arrow). (**B**) A picture of a colony 63 days post establishment. (**C**) Magnified view of the pair and its offspring within the glass case.

Ce dispositif a permis une approche comparative entre deux espèces sympatriques, l'invasive *Reticulitermes flavipes* et la native *R. grassei*, montrant l'existence de différences de stratégies de fondation, de succès reproducteurs ainsi que des différences comportementales (Brossette *et al.*, 2017; Brossette *et al.*, 2019). Notamment, on constate que lors de la fondation de la colonie par un couple royal de reproducteurs primaires, l'espèce native *R. grassei* produit moins d'œufs, survit moins longtemps et le nombre d'individus total est moindre, à échelle de temps constant, comparé à l'espèce invasive. Pourtant les temps d'apparition et de génération sont identiques (Fig. 32). Dans l'ensemble cela indique que *R. flavipes* est plus apte à faire des fondations indépendantes, ce qui pourrait être un facteur explicatif de son succès d'invasion.

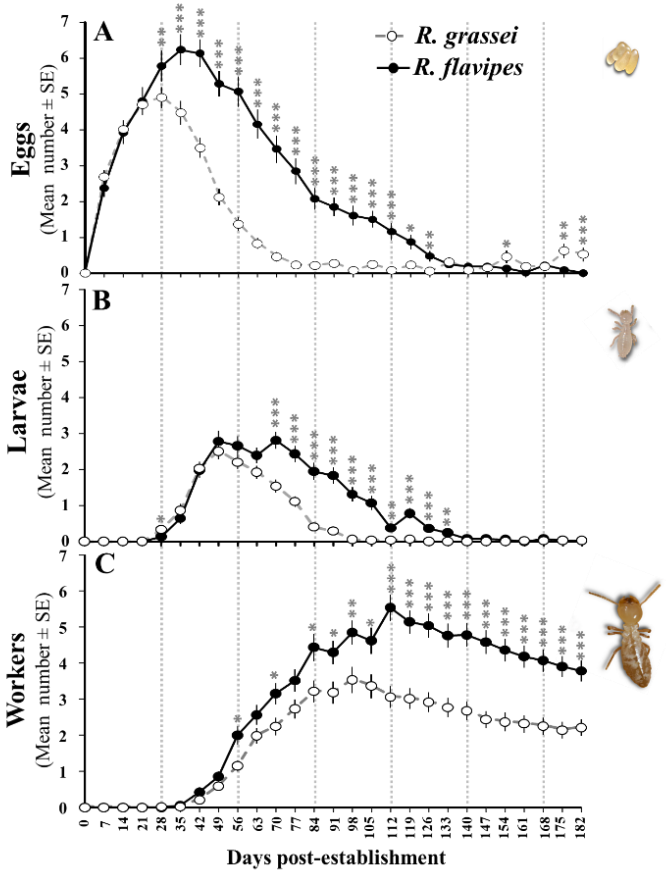


**Figure 32.** Mean number of eggs (**A**), larvae (**B**), and workers (**C**) produced per royal pair over the six months of the experiment for *R. grassei* (dashed gray lines, N = 87) and *R. flavipes* (solid black lines, N = 78) (mean ± S.E.; ***P-values < 0.001, ** P-values < 0.01, * P-values < 0.05). See annexes for the detailed statistics. The vertical dotted lines indicate a month period (Brossette *et al.*, 2017).

L'un des points les plus marquants de cette thèse porte sur le déséquilibre d'investissement dans les soins parentaux au sein du couple royal. En effet, l'investissement des parents dans les soins est généralement couteux, si bien qu'il est prédit que chaque parent va tenter de maximiser sa propre fitness en prodiguant moins de soins que son partenaire (Lessells, 2012). Il est attendu que ces conflits sociaux soient moindres chez les espèces à monogamie vraie (fidèles à vie), puisque la fitness de chaque parent est liée à l'investissement de l'autre (Boomsma, 2009). Les analyses comportementales des fondations de reproducteurs dans nos dispositifs ont permis d'aborder cette question qui a été finalement peu étudiée chez les invertébrés. Les comportements de toilettage, d'antennation, de trophallaxies stomodéale et proctodéale ont été étudiés à plusieurs niveaux entre les parents (reines/rois), les œufs et les descendants (larves/ouvriers) en tant que donneurs ou receveurs et ce sur plus de 6 mois (Brossette *et al.*, 2019).

Nos travaux montrent que, contrairement à ce qui était attendu (Shellman-Reeve, 1990; Rosengaus & Traniello, 1991), l'investissement des membres du couple n'est pas équivalent. Dans l'ensemble, les rois prodiguent moins de soins que les reines lorsque la descendance est présente. Cela inclut les toilettages, les antennations et les trophallaxies quelle que soit l'espèce ou la nature du receveur (descendants ou partenaires) (Fig. 33). Il est important de noter que le poids des reines et rois ne présentent pas de différence marquée durant la phase de fondation (Bignell *et al.*, 2011). Les deux parents s'investissent davantage vers la descendance que vers leurs partenaires (Fig. 34). Enfin, l'espèce invasive *R. flavipes* présente une durée totale de toilettage et d'antennation plus courte envers leurs partenaires et les œufs mais une durée de trophallaxies stomodéales plus longue, ce qui pourrait expliquer l'existence de différence dans la survie des fondations qui a été observée précédemment (Leniaud *et al.*, 2011; Brossette *et al.*, 2017). Un dernier résultat de ces travaux à pointé du doigt des comportements vibratoires que nous avons étudiés plus en détail dans d'autres expériences décrit plus bas.

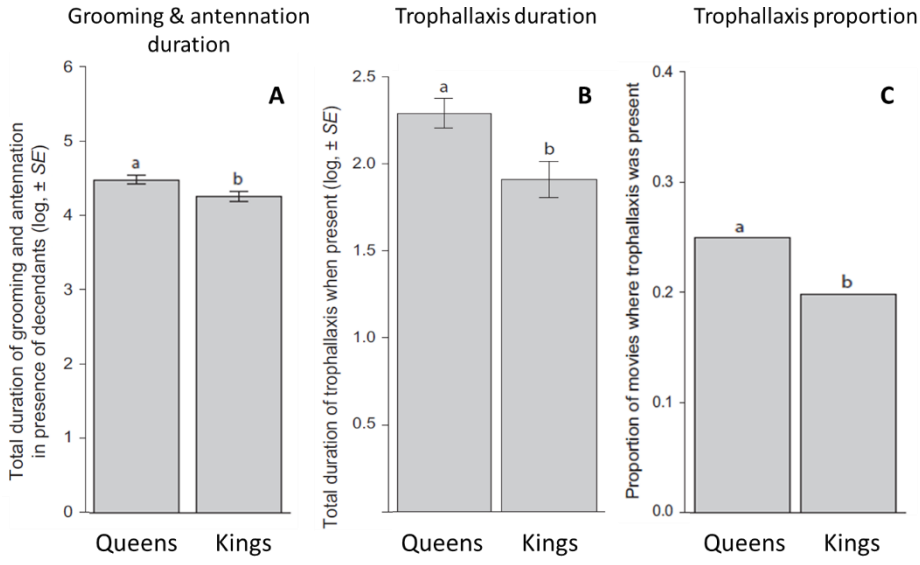


**Figure 33.** Behaviors with Queens or Kings as donors. (**A**) total duration of allogrooming and antennation in presence of descendants, (**B**) total duration of trophallaxis and (**C**) proportion of trophallaxis. Note that species, recipients and types of trophallaxis (proctodeal/stomodeal) are presently pooled, because of no significant interactions (see detailed statistics in the annexes). Bars represent (**A**) mean values of the log(+1)-transformed total duration ±SEM, (**B**) mean values of the log-transformed total duration ±SEM and (**C**) proportion of movies. Different letters refer to $p < 0.05$.

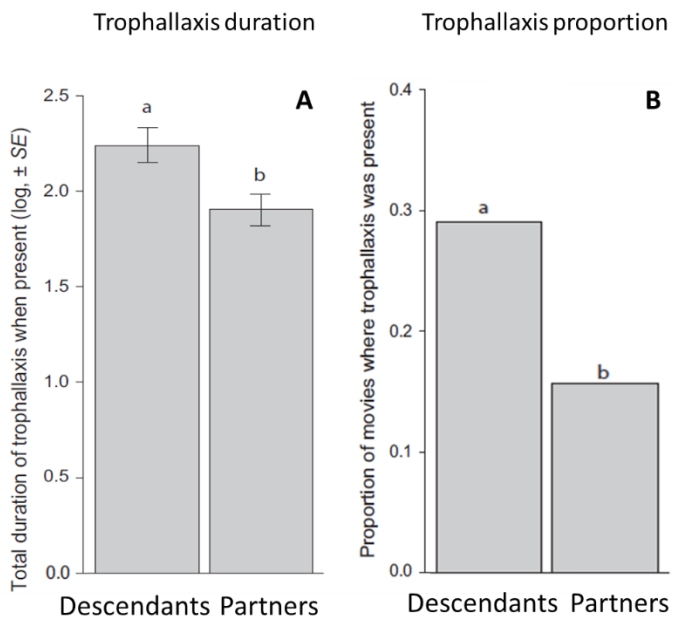


**Figure 34.** Trophallaxis with Descendants or Partners as recipients. (**A**) total duration and (**B**) proportions. Note that species, recipients and types of trophallaxis (proctodeal/stomodeal) are presently pooled, because of no significant interactions (see detailed statistics in annexes). Moreover, these models were restricted to the data set where descendants were present, because trophallaxis is not possible towards eggs. Bars represent (**A**) mean values of the log-transformed total duration ±SEM and (**B**) proportion of movies. Different letters refer to $p < 0.05$.

Il existe donc un déséquilibre d'investissement dans les soins parentaux dans ces deux espèces à monogamie vraie contrairement à ce qui était connu chez d'autres termites (Shellman-Reeve, 1990; Rosengaus & Traniello, 1991), et à ce qui est théoriquement attendu (Boomsma, 2009). De plus, l'investissement plus grand des reines par rapport aux rois suggère que ceux-ci sont soit spécialisés dans d'autres tâches comportementales, soit sont moins actifs durant les 6 premiers mois de la fondation de la colonie. De façon plus générale, ces travaux pointent du doigt le rôle central, jusqu'alors négligé, du roi dans l'évolution et le maintien de l'organisation sociale des termites (Brossette *et al.*, 2019).

### 3.2.2 Signal spécialisé dans la régulation sociale ?


La particularité de l'existence d'un couple royal chez les termites comparés aux autres insectes sociaux, permet de se pencher plus finement sur les mécanismes de la régulation sociale liée au sexe. Cette spécificité des termites, amène à l'existence de parents des deux sexes en permanence au sein de la colonie avec présence d'accouplements réguliers. Mais quels sont les mécanismes de cette régulation ? L'émergence de nouveaux reproducteurs est sous l'influence de la présence des autres reproducteurs (Sun *et al.*, 2017).

Les reines stimuleraient l'émergence de nouveaux rois tout en inhibant l'émergence de nouvelles reines et inversement pour les rois. Ainsi, un jeu subtil entre stimulation et inhibition amènerait à la régulation du nombre de reproducteurs de chaque sexe. Les signaux et les comportements de régulation en cause semble pointer vers une hiérarchie chimique complexe qui est admise depuis les années 70 dans la littérature mais qui manque de démonstrations expérimentales (Wilson, 1971). Afin d'identifier les facteurs amenant à réguler l'accès à la reproduction chez les termites, une première approche consiste à identifier les comportements associés à la présence de reproducteurs ou de leurs œufs. L'étude de tels comportements permet, par la suite, de disséquer plus en détail les jeux de stimulation/inhibition phéromonale existants au sein de la colonie par une approche expérimentale d'observation comportementale ; ces comportements étant potentiellement impliqués dans la régulation de l'accès à la reproduction.

Chez les insectes sociaux, il est montré que la présence de reproducteurs influence plusieurs traits biologiques des autres membres de la colonie. Par exemple, la présence de reines chez l'abeille attire les ouvrières et entraine une augmentation des comportements de toilettage et d'antennation envers elles (Slessor *et al.*, 1988; Liebig *et al.*, 2009). Chez les autres insectes sociaux, le retrait de la reine bouleverse l'expression génique des ouvrières, modifiant la division du travail (voir chaptre 3, Manfredini *et al.* (2014)) et pouvant entrainer une augmentation de comportement agressif entre ouvrières (Monnin & Ratnieks, 2001; Korb *et al.*, 2009) ou encore inhiber le développement des ovaires des ouvrières (Stroeymeyt *et al.*, 2007; Matsuura, 2012). La présence de la reine peut aussi avoir un effet par l'intermédiaire de la présence des œufs (Endler *et al.*, 2004; Matsuura *et al.*, 2010). Ces effets de la présence de reproducteurs sur l'organisation sociale de la colonie, sont souvent étudiés à long terme. Pourtant, étudier leur influence à court terme permettrait de mieux comprendre les mécanismes qui peuvent tamponner l'impact des perturbations sociales sur l'organisation de la société.

Nous avons donc regardé l'effet du retrait des reproducteurs sur les ouvriers, leurs poids, leur consommation de nourriture, leur mortalité et le comportement d'alarme chez le termite *R. flavipes*. Puisque la présence de reproducteurs peut aussi se faire par l'intermédiaire des œufs, nous avons aussi testé leurs présence/absence. Pour ce faire, 15 colonies de *R. flavipes* ont été réparties dans 4 types de micro-nids (Fig. 20) avec 30 ouvriers et présence/absence de reproducteurs/œufs (Fig. 35). Le poids des individus, la prise alimentaire, la mortalité et le nombre d'œufs ont été mesurés avant et après 24h. Enfin, chaque micro-nid a été filmé pendant 5 min pour permettre de comptabiliser le comportement vibratoire (nommé "body-shaking"). Ce comportement est omniprésent chez la majorité des termites et est décrit comme un comportement d'alarme (Howse, 1965; Rosengaus *et al.*, 1999; Reinhard & Clément, 2002; Hertel *et al.*, 2011; Cristaldo *et al.*, 2015; Yamanaka *et al.*, 2019).

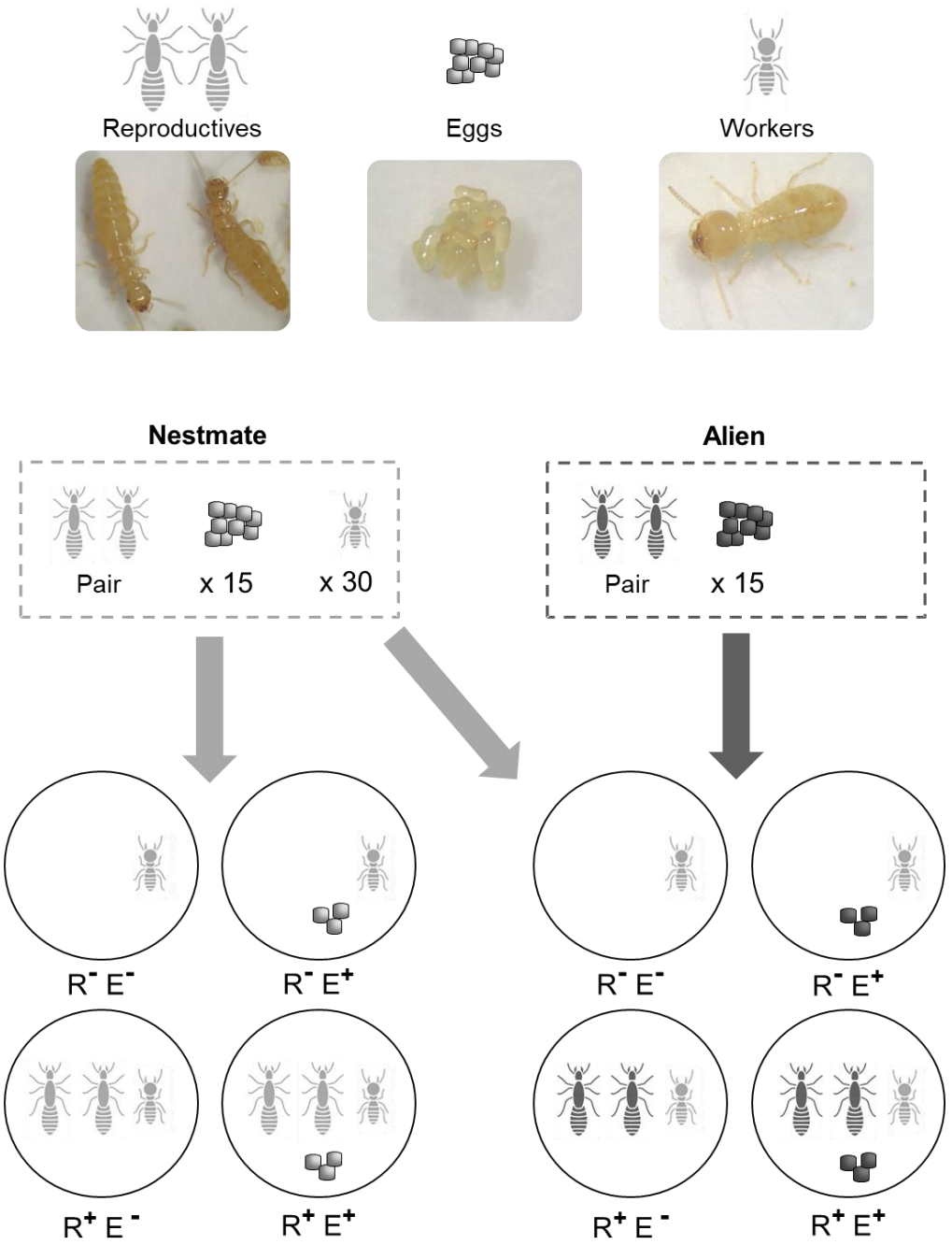


**Figure 35.** Experimental micro-nest with treatments: workers in absence of reproductives and eggs ($R^-E^-$), in presence of eggs ($R^-E^+$), in presence of reproductives ($R^+E^-$) and in presence of reproductives and eggs ($R^+E^+$). Nestmate and Alien conditions are represented with different colors (Ruhland *et al.*, Submitted).

Nos résultats démontrent, qu'après 24h, aucun changement significatif n'est perçu sur le poids, la nourriture consommée, ou la mortalité des ouvriers. En revanche, la présence/absence des reproducteurs/œufs agit sur le body-shaking (Fig. 36). Dans ces expériences, nous avons utilisé soit des reproducteurs/œufs provenant de la colonie d'origine des ouvriers soit provenant de colonies

différentes. L'ajout de reproducteurs/œufs provenant d'une colonie étrangère n'influence pas le body-shaking alors que leur présence pourrait être considérée comme un stress pour la colonie, ce qui par voie de conséquence devrait induire un comportement d'alarme. A ce stade, nous ne pouvons pas exclure qu'un signal de fertilité issu des reproducteurs puisse interférer avec le signal de reconnaissance du "visa" colonial, empêchant ainsi l'émergence d'un signal d'alarme. Une hypothèse alternative est que les ouvriers auraient perdus la capacité à distinguer les intrus. Néanmoins, nos expériences complémentaires suggèrent que le système de reconnaissance ouvert de *R. flavipes* n'affecte pas la capacité des individus à discriminer les aliens. Nos résultats sont donc en accord avec l'idée que des différences au niveau des signaux de reconnaissance entre deux colonies n'entrainent pas de manière systématique un comportement d'agressivité (Su & Haverty, 1991; Grace, 1996). Ces résultats font l'objet d'une publication actuellement soumise (Ruhland *et al.*, Submitted).

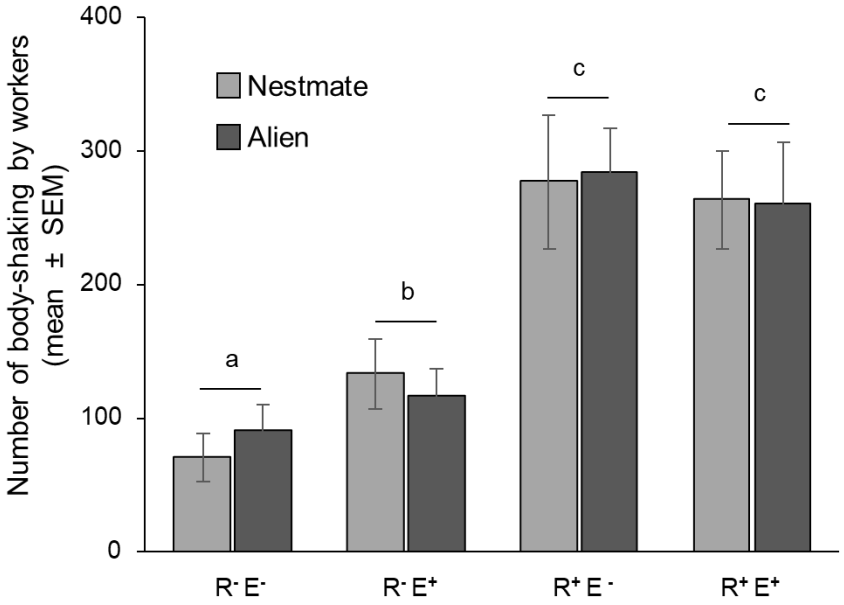


**Figure 36.** Number of body-shaking from workers in presence of related (Nestmate) or unrelated (Alien) reproductives and eggs. Letters indicate significant differences between treatments (LMM, $\alpha = 0.05$). $R^-E^-$: absence of reproductives and eggs, $R^-E^+$: presence of eggs, $R^+E^-$: presence of reproductives, $R^+E^+$: presence of reproductives and eggs.

La modulation du body-shaking en fonction de la présence de reproducteurs/œufs semble indiquer que ce comportement a un rôle plus complexe que décrit précédemment, puisqu'il était principalement inféodé au comportement d'alarme. Ce potentiel rôle spécialisé en lien avec la reproduction est corroboré par d'autres études récentes (Funaro *et al.*, 2018; Brossette *et al.*, 2019; Yamanaka *et al.*, 2019). Les démonstrations expérimentales directes sont toujours manquantes, si bien que l'étape suivante naturelle est la manipulation de ce comportement afin d'observer son influence sur la production des reproducteurs, à l'aide, par exemple, d'expériences play-back.

Dans l'ensemble, nos résultats apportent de nouvelles informations sur la capacité des sociétés d'insectes à s'adapter à une perturbation de la composition sociale, surlignant le rôle potentiellement sous-estimé des comportements vibratoires dans ces processus. Les comportements vibratoires étant pourtant de très bons candidats pour appuyer ou complémenter un signal chimique (Delattre *et al.*, 2019). Nous avons donc décidé de pousser leurs études dans une thèse financée par l'école doctorale SSBCV, qui a débuté en octobre intitulée : "Reproductive differentiation in termites: study of the signals and the behaviors involved".

### 3.2.3 Spécificités du modèle termite en lutte biologique

Bien qu'essentiellement fondamentaux, mes travaux ont aussi un aspect plus appliqué, avec des enjeux économique, écologique et sociétal importants, étant donné le statut de ravageurs urbains que portent les termites souterrains en France. C'est dans ce double cadre d'espèces invasives et d'insectes xylophages que j'ai développé le projet de recherche BioControleTermite actuellement financé par la région Centre-Val de Loire. L'objectif principal est d'identifier et d'étudier le rôle des phéromones dans l'organisation sociale des termites afin de pouvoir les utiliser dans la détection et le contrôle écoresponsable des populations d'espèces invasives en milieu urbain. L'idée étant que toute perturbation du système de communication pourrait amener à des bouleversements de la plasticité phénotypique des termites entrainant un potentiel déclin des colonies.

Les termites souterrains, présents en région Centre-Val de Loire, sont parmi les plus nuisibles au monde. Ce sont des insectes xylophages indispensables en forêt qui participent de manière prépondérante au recyclage de la biomasse végétale. Malheureusement en zone urbanisée, les termites s'avèrent être des ravageurs importants qui causent de lourds dommages aux monuments historiques, aux bâtiments municipaux, aux maisons individuelles, aux immeubles et aux archives. Avec 280 km de rives inscrites au patrimoine mondial de l'Unesco, la région Centre-Val de Loire attire chaque année plus de 9 millions de touristes grâce aux nombreux châteaux de la Loire et monuments historiques, joyaux de l'Histoire de France. Le projet régional TermiCentre (2011-15) a démontré l'étendu de l'infestation dans notre région. L'une de ces principales conclusions est que les termites vont poursuivre leur progression si rien n'est fait. Les termites représentent donc une menace importante pour notre patrimoine et pour l'économie régionale qui en découle. A ce jour, la lutte contre ces ravageurs est principalement d'ordre chimique et se fait soit par l'emploi de pesticides toxiques avec des effets secondaires lourds sur la santé publique, soit par des pièges passifs imprégnés de pesticides à effet retard. Mais ces méthodes chimiques sont contraires aux recommandations de diminution de l'emploi de pesticides préconisée par le plan national ECOPHYTO 2025. Trouver de nouveaux systèmes de lutte contre les termites est donc aujourd'hui une nécessité afin de mieux protéger le patrimoine régional, tout en répondant aux directives législatives.

Le projet BioControleTermite intervient sur 3 points clés pour enrayer la progression annoncée du termite en région Centre-Val de Loire : la détection, le biocontrôle écoresponsable et la mise en place d'outils d'information. (1) La détection des termites se fait actuellement par constats ponctuels (souvent annuels) des dégâts faits aux bâtiments en vérifiant de manière destructive les zones suspectées. Cette méthode ne permet donc pas de prévenir les dégâts, car la colonie de termites doit être très développée pour être détectable. Nous proposons une approche innovante visant à détecter la présence des termites grâce aux odeurs qu'ils dégagent. A l'instar des détecteurs de fumée, ce système offrira l'unique avantage de pouvoir contrôler de façon continue la présence de ce nuisible dans une zone protégée. (2) Le biocontrôle écoresponsable des termites se fera par l'utilisation de leurs propres phéromones sexuelles. Cela permettra de contrôler le développement des populations en agissant par confusion sexuelle et castration chimique. Ces effets s'accompagneront d'un effet répulsif des zones traitées, minimisant ainsi les risques de re-contamination des zones déjà traitées. Les phéromones étant des substances naturelles, elles n'induisent pas de danger pour la population et

représentent un système alternatif de biocontrôle écoresponsable car elles se substituent à l'emploi des pesticides. Outre leurs sélectivités remarquables envers leurs cibles, ils présentent l'avantage d'être biodégradables. Une lutte identique a déjà été réalisée avec succès dans le cas des mites, où 100% des composés chimiques de synthèse ont été remplacés par des phéromones (Arnault *et al.*, 2012). (3) Parce que le manque d'information est un point crucial qui favorise la propagation des termites, la mise en place d'outils de communication est primordiale pour assurer le succès des méthodes de lutte. Le projet comprend donc une forte composante de diffusion de la culture scientifique vers le grand public accompagnée d'un programme de formation sur la gestion de la problématique termite (public/privé).

En France, une loi portant sur les termites existe depuis 1999 (n°99-741) afin de déclarer les zones termitées et protéger les biens. Même si les grandes agglomérations sont généralement bien informées, il s'avère nécessaire de diffuser plus largement les caractéristiques du système termite et de ses modes de propagation (Fig. 37). En effet, les termites portent en eux un paradoxe en termes de communication. Connus de tous, avec une appréhension négative, leur véritable nature est pourtant bien souvent largement méconnue. Leur rôle écologique indispensable dans le renouvellement des forêts est totalement occulté par leurs impacts négatifs sur le patrimoine. Une des raisons principales de cette méconnaissance tient à leur vie cryptique et un cycle de vie très complexe. Ainsi, le publique n'entre jamais en contact direct avec le termite et n'en retient qu'une image générale. Cette connaissance biaisée de la biologie des termites accroît les mauvaises pratiques de gestion du patrimoine, ce qui évidement accélère la propagation de cette espèce invasive en zone urbanisée. C'est pourquoi il est primordial de diffuser le plus largement possible des informations précises validées scientifiquement pour pouvoir enrayer la dispersion du termite.

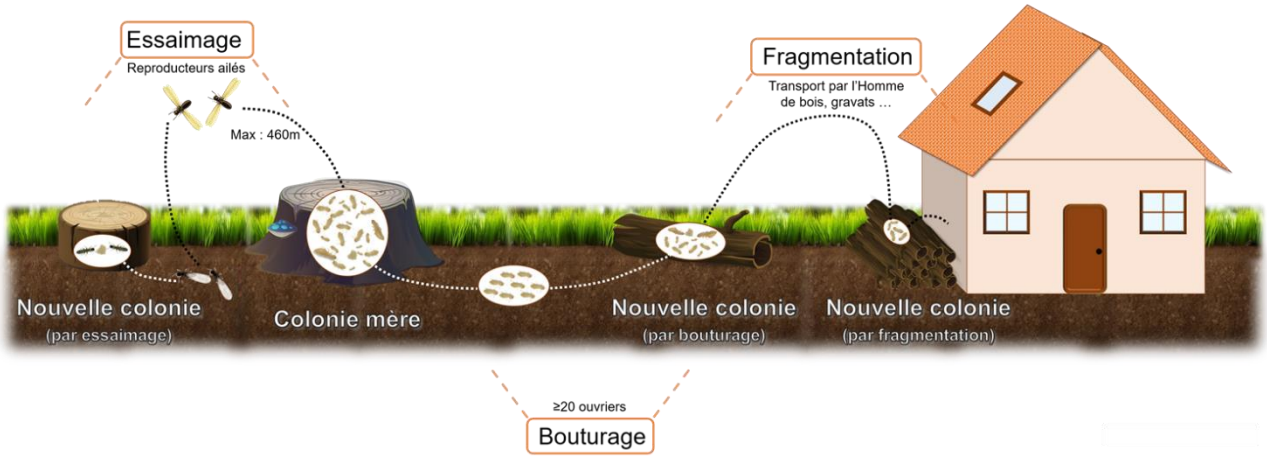


**Figure 37.** Représentation des différents modes de dispersion des termites souterrains d'après Büchli (1958) et Nutting (1969) (Lucas & Ruhland, 2018).

Une chance unique de trouver des moyens de contrôler ces sociétés d'insectes est d'utiliser nos connaissances fondamentales pour contrôler les reproducteurs. Nous l'avons vu la communication chimique est la clé de voute des sociétés d'insectes puisqu'elle est à la base des relations intra- et interspécifiques (Vander Meer *et al.*, 1998; Blomquist & Bagnères, 2010), si bien qu'agir sur cette communication chimique c'est agir directement au cœur du système termite. Il apparait donc évident

que l'un des meilleurs moyens de lutte contre les invasions biologiques est l'utilisation des phéromones (Minks & Kirsch, 1998). Dans de nombreux exemples, les phéromones ont remplacé l'utilisation conventionnelle des pesticides (Carde & Minks, 1995). L'utilisation des mécanismes de communication chimique pour le contrôle des populations d'insecte est une thématique qui a plus de 30 ans et qui a démontré son efficacité. Néanmoins, dans le cadre du contrôle des termites cette problématique reste peu étudiée. D'ailleurs, les phéromones ont été envisagées dans la lutte contre les termites depuis une vingtaine d'années mais cela n'a jamais été mis en place à cause du manque de connaissance sur leurs systèmes de reproduction et sur les phéromones impliquées (Su & Scheffrahn, 1998).

C'est dans ce cadre que s'inscrit notre projet de recherche en proposant de nouveaux systèmes de contrôle écoresponsable des termites sans utilisation de pesticides, en s'appuyant sur des études plus approfondies du système social des termites et de leurs spécificités. Le but ultime de ce projet, actuellement en cours, est à la fois de réduire l'emploi de phytosanitaire et de permettre la protection des biens. Notre projet apporte donc des alternatives crédibles aux attentes sociétales actuelles tout en renforçant nos connaissances fondamentales de ces sociétés d'insectes.

# Discussion & Perspectives

L'ensemble de mes recherches, que ce soit sur les insectes sociaux ou solitaires, s'est penché sur la production, la circulation et le rôle des signaux de communication dans les interactions sociales au sens large, ainsi que sur les bases moléculaires à l'origine de la plasticité comportementale et du polyphénisme, sans oublier les influences des conditions environnementales. Caractériser les signaux de communication et les interactions comportementales qui lui sont liés, et mettre en évidence les mécanismes moléculaires impliqués, permettent de mieux comprendre l'évolution des comportements complexes et le fonctionnement des interactions écologiques dans la dynamique "environnement – gène – comportement". Mes recherches se situent donc à l'interface de l'écologie chimique, la génétique moléculaire, l'écologie comportementale, l'éthologie et la sociogénomique. L'ensemble de mes travaux étant approvisionné par des collaborations scientifiques qui enrichissent mes recherches. Evidemment, les opportunités de recherche restantes sont innombrables et particulièrement stimulantes. De l'ensemble des objectifs à court, moyen et long terme qui se dessinent, mes projets de recherche futures vont s'orienter vers les mécanismes de la plasticité comportementale et phénotypique des termites. Notamment sur les signaux de communication induits par la présence des reproducteurs et leur impact sur l'organisation sociale des termites, mais aussi sur la sociogénomique du polymorphisme des termites et son évolution.

Une des conclusions directes de nos projets de recherche actuels sur la plasticité phénotypique des termites est le rôle centrale joué par les signaux de communication. En effet, nous avons vu que chez les termites souterrains, l'émergence de nouveaux reproducteurs est sous l'influence de médiateurs chimiques produits par les reproducteurs (Matsuura *et al.*, 2010), même si cette relation directe manque encore de démonstration expérimentale (Sun *et al.*, 2017). Ce système est d'autant plus complexe chez les termites, par rapport aux autres insectes sociaux, puisqu'ils ont la particularité de présenter un couple royal en relation continue par le biais notamment d'accouplements réguliers (Bignell *et al.*, 2011). Ainsi, un jeu subtil entre stimulation et inhibition amènerait à la régulation du nombre de reproducteurs de chaque sexe et donc à l'émergence du couple royal ou de son remplacement (Wilson, 1971). La principale avancée dans ce domaine, ces dernières années, porte sur deux phéromones produites par les reines de *Reticulitermes speratus* qui ont un effet inhibiteur sur l'émergence de nouvelles reines (Matsuura *et al.*, 2010). Mais comme nous l'avons déjà expliqué, cette avancée reste incomplète puisque nous ne connaissons toujours rien sur la régulation de la production des reproducteurs mâles. Trouver de telles phéromones spécifiques au roi liées soit à la persistance du couple royal (communication intra-couple), soit liées à une stimulation de leur émergence, représenterait une avancée significative pour la compréhension des sociétés d'insectes.

La recherche de ces 20 dernières années s'est concentrée sur les composés organiques volatiles, laissant de côté la possibilité de phéromones de contact. Chez les vertébrés, ainsi que chez certains invertébrés aquatiques, il est intéressant de noter que la communication chimique est assurée par des protéines et des peptides (Muller-Schwarze, 1979; Johnston *et al.*, 1999; Brönmark & Hansson, 2000; Marchlewska-Koj *et al.*, 2000; Wyatt, 2003; Muller-Schwarze, 2006; Chamero *et al.*, 2007). Par leur nature, ces composés peuvent transporter et coder l'information différemment comparé aux composés organiques volatiles ou aux hydrocarbures cuticulaires. Malgré un nombre croissant de rapports impliquant le rôle des peptides dans la communication chez les insectes, leur étude reste rare (Bagnères & Hanus, 2015), alors qu'ils peuvent représenter une transition majeure dans notre compréhension de ces systèmes. Cette possibilité d'une nature non volatile du signal chez les termites est pour le moment théorique car même si une protéine issue des reproducteurs mâles a été trouvée (Hanus *et al.*, 2010), aucune démonstration comportementale n'a encore été effectuée. Pourtant chez les blattes, qui sont phylogénétiquement proches des termites, il a été montré que la communication sexuelle peut passer par ces protéines (Cornette *et al.*, 2001; Cornette *et al.*, 2002; Cornette *et al.*). En effet, lors de l'accouplement, le mâle récupère des protéines sur le dos de la femelle, dont les effets sont aphrodisiaques. Des études du métabolome externe de la cuticule des termites croisées avec des expériences comportementales pourront nous en apprendre davantage sur l'existence et le fonctionnement de ces phéromones de contact.

Pour aller encore plus loin et sortir de l'aspect purement chimique, nos recherches actuelles sur les comportements vibratoires des termites montrent qu'il est probable que des signaux non chimiques pourraient être employés par les termites. En effet, dans un environnement souterrain, confiné et solide à l'intérieur du sol ou du bois, comme c'est le cas pour les nids de termite, les vibrations peuvent permettre la transmission de messages de façon plus efficace que des composés volatiles (Hill, 2009). En fait chez les insectes, les comportements vibratoires sont omniprésents alors qu'ils sont très peu étudiés et en particulier chez les insectes eusociaux (Hill, 2008). Cocroft et Rodríguez (2005) certifient que plus de 80% des espèces d'insectes décrits utilisent des vibrations. Les signaux vibratoires sont très diversifiés au regard de leurs origines et de leurs modulations entre les espèces : (i) frottement sur le substrat avec les mandibules ou les pattes avant (Hunt & Richard, 2013), (ii) organes spécialisés comme la stridulation des fourmis (Roces *et al.*, 1993) ou les muscles alaires chez l'abeille (Kirchner, 1997) ou encore (iii) les mouvements oscillatoires distingués en 4 grands types : "drumming", "abdominal wagging", "butting" et "body-shaking" (ou "longitudinal oscillatory movements"). La communication vibratoire est liée à différentes fonctions comme le recrutement et l'augmentation générale d'activité (Cocroft & Rodríguez, 2005). Chez les termites, les comportements vibratoires sont omniprésents puisque la grande majorité des espèces le fait, toutes castes confondues (Sieber & Leuthold, 1981; Maistrello & Sbrenna, 1996; Rosengaus *et al.*, 1999; Reinhard & Clément, 2002; Whitman & Forschler, 2007; Šobotník *et al.*, 2008; Hertel *et al.*, 2011; Cristaldo *et al.*, 2015). Ce comportement est principalement décrit comme un signal d'alarme en réponse à une phéromone, à des stimuli abiotiques ou encore peut être spontanément émis (Yamanaka *et al.*, 2019). La nature physique même de ces vibrations étant décrite très sporadiquement (Delattre *et al.*, 2015). A noter que chez *Cryptotermes secundus* les ouvriers utilisent les vibrations pour évaluer la taille du bois dont ils se nourrissent (Evans *et al.*, 2007).

Ainsi, curieusement, malgré l'omniprésence des comportements vibratoires chez les termites, leur potentiel rôle dans l'organisation sociale ou le déterminisme des castes n'a jamais été étudié. Seuls, dans une expérience qui n'était pas dédiée à cette question, Evans *et al.* (2005) concluent que chez *Cryptotermes secundus*, le play-back de vibrations artificielles issues de l'activité de fourragement des ouvriers entraine une diminution de la différenciation en reproducteur. Tout ceci démontre bien de l'urgence d'approfondir nos connaissances à ce sujet chez les termites, au regard de la riche littérature chez les oiseaux et les mammifères à ce sujet (Cocroft & Rodríguez, 2005; Hill, 2008) et particulièrement en comparaison avec les décennies d'études du rôle des signaux chimiques dans le déterminisme et le polyphénisme des insectes sociaux (Blomquist & Bagnères, 2010).

Plusieurs perspectives d'étude s'ouvrent donc à nous que ce soit sur la phéromone du roi ou encore sur la nature non volatile d'un messager impliqué dans la régulation des reproducteurs, qu'il soit peptidique ou vibratoire, ces découvertes sont pleines de promesses et font l'objet de notre activité de recherche actuelle. Mais il ne faut pas négliger l'influence des facteurs génétiques associés à l'action de ces signaux sur l'organisation sociale et sa composante comportementale. C'est pourquoi, afin de mieux appréhender les mécanismes génétiques impliqués dans la différenciation des castes, il est important aussi d'accentuer les efforts de recherche sur la sociogénomique du polymorphisme des termites et son évolution. Notamment, les spécificités du modèle termite, croisées avec les opportunités techniques qu'offrent les séquençages à haut débit, ouvrent des opportunités d'études très prometteuses (Van Dijk *et al.*, 2014; Baudino *et al.*, 2016; 2017). En effet, l'hémimétabolie des termites ouvre des possibilités de réponses à certaines questions sur le déterminisme des castes qui ne peuvent trouver aisément de solution chez les hyménoptères sociaux, puisque chez les termites, les individus ne sont pas arrêtés dans leurs développements (état larvaire permanent). D'un point de vue pratique deux options s'ouvrent à nous : la stratégie du gène candidat et/ou la transcriptomique, sachant que les études transcriptomiques peuvent déboucher sur l'identification de nouveaux gènes candidats qui pourront être validés par des études fonctionnelles.

L'approche gène candidat ne fonctionne que si le gène identifié comme "d'intérêt" est fortement marqué de potentialités pour répondre à la question posée. Etant donné que mes études précédentes amènent à redéfinir la fonction du gène *foraging* au-delà de son implication dans le comportement de recherche de nourriture, *foraging* se présente donc comme un bon candidat. En effet, présent chez de nombreuses espèces différentes, il a été conservé au cours de l'évolution et apparaît de plus en plus comme un régulateur général de la plasticité comportementale (Lucas *et al.*, IMB 2015 ; Lucas *et al.*, CIB 2010 ; Lucas *et al.*, AIBP 2010 ; Lucas & Sokolowski, PNAS 2009)(Malé *et al.*, 2017; Anreiter & Sokolowski, 2019). Le rôle supposé de *foraging* sur la colonisation et la dispersion des reproducteurs est d'autant plus intéressant lorsque l'on étudie un modèle biologique invasif comme les termites. Ainsi, une étude comparative permettant de mieux comprendre les stratégies de colonisation en milieu naturel et urbanisé des termites au niveau moléculaire, revêt des aspects à la fois scientifiques et économiques d'actualité. C'est pourquoi nous envisageons d'entreprendre des expériences chez le termite invasif *R. flavipes* afin de corréler l'expression du gène *foraging* avec les comportements (1) de colonisation par creusage de milieux non nutritifs, (2) de fourragement par creusage de milieux nutritifs et (3) de dispersion des reproducteurs.

De plus, les différentes populations de *R. flavipes*, qui ont la particularité d'être une espèce invasive en France en provenance des Etats-Unis, présentent des changements majeurs de structure sociale, passant de colonies avec peu de reproducteurs à des colonies possédant plusieurs centaines de reproducteurs (Vargo & Husseneder, 2009; Perdereau *et al.*, 2013; Vargo, 2019). Mes travaux précédents ont déjà montré un lien entre structure sociale et expression du gène *foraging* chez la fourmi *Solenopsis invicta* (Lucas *et al.*, IMB 2015), mais c'est l'opportunité d'approfondir le rôle de *foraging* dans la biologie de l'invasion et de mieux appréhender les liens supposés entre l'expression de *foraging* et la voix insuline (hormone juvénile, vitellogénine) connue pour être impliquée dans la reproduction (Chandra *et al.*, 2018). En effet, l'action de l'expression du gène *foraging* sur le comportement des individus est désormais avérée mais la cascade moléculaire impliquée est beaucoup plus hypothétique. L'étude de ce modèle biologique apporterait donc des avancées scientifiques novatrices sur les liens supposés entre structure sociale, équilibre hormonale et expression génique tout en élargissant nos connaissances sur l'effet comportemental pléiotropique du gène *foraging*.

Evidemment, d'autres gènes pourraient avoir un rôle décisif, c'est pourquoi il apparait nécessaire de faire des études plus larges de transcriptomique pour les identifier, en s'intéressant dans un premier temps, à la caste des reproducteurs. En effet, comme nous l'avons déjà expliqué, la caste des reproducteurs, qui représente une caste clé dans la pérennité des colonies d'insectes sociaux, présente une composition particulièrement complexe chez les termites souterrains. Les conditions qui amènent les individus à se différentier en reproducteurs sont encore mal définies (médiateurs chimiques, interactions sociales, environnements favorables) et pourraient être croisées avec certains facteurs génétiques (reconnus mais non clairement identifiés) (Scharf *et al.*, 2003; Scharf *et al.*, 2005b). J'envisage donc d'étudier le transcriptome de *R. flavipes* par séquençage haut débit (RNAseq) couplé avec des expériences d'écologie comportementale pour étudier les stratégies de reproduction, de colonisation et de dispersion. Les reproducteurs seront comparés selon 4 critères : (i) leurs morphotypes ; (ii) leurs sexes ; (iii) leurs types native-invasive ; (iv) les conditions expérimentales (stress alimentaire, compétition spécifique, présence/absence d'autres reproducteurs). En plus des aspects fondamentaux pour comprendre les gènes impliqués dans l'organisation sociale des termites, nous espérons apporter de nouvelles opportunités appliquées aux besoins des collectivités territoriales et des industriels pour le contrôle des populations, dont l'impact économique et écologique est important dans nos sociétés. Ceci permettra peut-être d'apporter, entre autres, plus d'éléments concernant le rôle supputé du gène *dubbed worker* considéré comme impliqué dans le déterminisme génétique des castes des termites mais qui nécessite une démonstration expérimentale (Hayashi *et al.*, 2007).

Les potentiels gènes candidats issus des différentes analyses transcriptomiques pourront être validés par des études fonctionnelles avec la technique des ARN interférences qui fonctionnent déjà chez *Reticulitermes* (Zhou *et al.*, 2006), pour inhiber l'expression de gènes cibles. Néanmoins, cette technique n'entraine pas toujours la suppression totale de l'expression génique. Pour cela l'utilisation du système CRISPR/Cas9 permet lui de générer des lignées mutantes avec absence du gène qui a été excisé du génome (Cong *et al.*, 2013; Sampson & Weiss, 2014). C'est à l'aide de cet outil que le rôle

du gène *orco* a été identifié chez la fourmi (Trible *et al.*, 2017; Yan *et al.*, 2017). Ce gène est impliqué dans le fonctionnement des récepteurs olfactifs affectant l'organisation sociale, la reproduction et la plasticité comportementale. Cet outil ouvre donc la porte sur le fonctionnement de l'interaction entre gènes et environnement social et pourrait permettre de comprendre comment cela impacte les traits comportementaux des individus et des colonies dans les sociétés d'insectes.

L'analyse transcriptomique de la caste des reproducteurs des termites ouvrira d'autres pistes de recherche en apportant des gènes candidats marqueurs de la fonction de reproduction. Notamment peu d'études chez les termites se sont intéressées à la caractérisation de la fonction de reproduction aux niveaux moléculaire, cellulaire et physiologique (Scharf *et al.*, 2003; Raina *et al.*, 2004; Scharf *et al.*, 2005a; Brent *et al.*, 2007; Elliott & Stay, 2007; Raina *et al.*, 2007; Weil *et al.*, 2007; Maekawa *et al.*, 2010; Husseneder *et al.*, 2012). Scharf *et al.* (2005a) ont pour la première fois identifié des gènes différemment exprimés chez les nymphes, les reproducteurs primaires et les reproducteurs néoténiques. Weil *et al.* (2007; 2009b) ont par la suite caractérisé des gènes surexprimés chez les femelles néoténiques et les reproducteurs primaires. Enfin Husseneder *et al.* (2012) ont mis en évidence huit gènes particulièrement exprimés chez les reproducteurs primaires femelles. La caractérisation moléculaire des différents types de reproducteurs demeure néanmoins encore assez floue. Qui plus est, la fonction de reproduction chez les mâles est encore moins caractérisée que chez les femelles. Seuls les séquençages récents des génomes de *Zootermopsis nevandensis* (Terrapon *et al.*, 2014) et de *Macrotermes natalensis* (Korb *et al.*, 2015) ont permis d'identifier des familles géniques surexprimées chez les mâles reproducteurs par rapport aux autres castes. Il est intéressant de noter que des études ont montré que les ouvriers mâles ont accès à la spermatogenèse très tôt dans leur développement (Grandi, 1992; Dean & Gold, 2004). Plusieurs études ont également signalé la présence de mâles cachés chez les termites du genre *Reticulitermes* au sein des colonies (Büchli, 1961; Pichon *et al.*, 2007; Fujita & Watanabe, 2010; Wu *et al.*, 2013). Ces mâles matures sexuellement ne se différencieraient pas ou peu des ouvriers morphologiquement et pourtant ils participeraient à la reproduction.

Ce statut de reproducteur est souvent apprécié en présence/absence des reproducteurs mais est-il possible de quantifier plus finement ce statut de reproducteur ? Et les mâles cachés participent-il réellement à la reproduction ? Pour répondre à ces questions, il serait intéressant d'identifier des marqueurs génétiques de la fonction de reproduction et de les mettre en lien avec des critères comportementaux caractéristiques de l'état de "reproducteur actif". Ces marqueurs, qui auraient une expression dépendante de la caste et de l'âge, nous permettraient d'avoir une image fixe de la reproduction au sein de la colonie à un temps donné. Ainsi, grâce à ces marqueurs moléculaires, il serait envisageable d'identifier des reproducteurs "génétiquement actifs" dans des colonies pourtant dénuées de mâles morphologiquement différenciés en reproducteurs. En caractérisant au niveau moléculaire la fonction de reproduction du termite souterrain *R. flavipes*, nous pourrons évaluer le rôle joué par ces reproducteurs cachés sur les facteurs de colonisation et de dispersion de cette espèce invasive. D'ailleurs l'existence de ces mâles cachés pourrait expliquer pourquoi certaines études mettent en évidence des sex-ratios en faveur des femelles chez les néoténiques (Myles, 1999), ce qui pourrait changer notre vision actuelle du fonctionnement du système de reproduction des termites basaux.

Par la suite, les études transcriptomiques pourraient être élargies aux autres castes (ouvriers, soldats, nymphes). Ainsi cela permettrait de mieux comprendre les mécanismes de convergence évolutive ayant conduit à l'émergence de l'eusocialité chez les isoptères (Korb & Hartfelder, 2008). En effet, parmi les insectes sociaux, les termites possèdent l'une des plus anciennes formes d'eusocialité qui est apparue indépendamment de celles des hyménoptères, avec leur mode de développement très spécifique (diploïde, hémimétabole, couple royal) (Wilson, 1971; Bignell *et al.*, 2011). L'existence d'un "chromosome social" décrit chez la fourmi *Solenopsis invicta* (Wang *et al.*, 2013) pose des questions sur l'apparition d'un tel système dans le cadre de l'évolution sociale. On peut notamment se poser la question de savoir si un tel chromosome existe chez les isoptères. En d'autres termes, la convergence évolutive observée entre hyménoptères sociaux et isoptères repose-t-elle sur une base génomique commune ? Un moyen de commencer à répondre à cette problématique serait d'étudier si les 600 gènes liés par une inversion chromosomique dans une zone non-recombinante chez *S. invicta* sont présents et regroupés à plus ou moins grande distance chez les termites. Une première comparaison chez une autre espèce de fourmis, *Formica selysi*, semble indiquer que ce n'est pas un phénomène tranché, car même si une architecture génomique identique existe entre les deux espèces de fourmis, aucune similarité n'a été détectée dans le contenu en gènes (Purcell *et al.*, 2014). Une comparaison à plus grande échelle entre hyménoptères et isoptères (en y ajoutant d'autres espèces non sociales) pourrait permettre de déterminer si une zone chromosomique non recombinante est indispensable pour l'évolution de la vie sociale et si certains gènes identifiés chez les fourmis sont obligatoires pour la mise en place de la socialité vraie.

Une autre orientation de recherche serait l'étude du méthylome qui revêt une dimension très particulière chez les insectes sociaux, due à leur unique polyphénisme et leur plasticité comportementale comme nous avons pu le voir au cours de ce mémoire. Ainsi, les insectes sociaux sont sur le point de devenir le modèle phare d'investigation du rôle de la génétique et l'épigénétique dans les sociétés animales (Yan *et al.*, 2014; Yan *et al.*, 2015). L'étude de la transmission intergénérationnelle et transgénérationnelle permettra de mieux comprendre les mécanismes par lesquels les marqueurs épigénétiques peuvent affecter l'évolution des comportements (Córdoba-Aguilar *et al.*, 2018). Les termites présentent d'ailleurs des avantages par rapport aux autres insectes sociaux, en plus du développement hémimétabole, à savoir la présence des deux sexes au sein de chaque caste, la structure sociale spéciale représentée par les différents types de reproducteurs, ainsi que l'existence de reproduction clonale (Asexual Queen Succession) présentes chez certaines espèces de *Reticulitermes*. A l'heure actuelle les études sur le méthylome aboutissent à des résultats contradictoires avec des preuves et des contre-exemples de méthylation orientée ayant une influence sur le déterminisme des castes (Libbrecht *et al.*, 2016). Ces études contradictoires ont toutes été faites chez les hyménoptères sociaux, l'analyse d'un autre modèle biologique eusociale pourrait apporter un éclairage nouveau sur cette problématique d'actualité.

Cette discussion tente d'anticiper les prochaines années pour faire suite à 15 ans de recherche réduits à quelques pages qui ont tenté d'ajouter des connaissances sur la communication chez les insectes. Il est difficile de savoir avec certitude si mes futures recherches vont prendre le chemin que nous venons de tracer, car la science se doit d'évoluer et de s'adapter en fonction des découvertes. Elles seront probablement bousculées par le développement de futures méthodes grâce aux évolutions techniques, car science et technologie vont de pair, comme, pourquoi pas, le tracking automatisé et immédiat des comportements ou encore la "transcriptomique de paillasse" directement réalisée sur des individus en pleine expression comportementale. Bref, la révolution technologique va surement bouleverser notre façon de faire et de penser la science en ce siècle de la communication (Bockaert, 2017). Tandis que les insectes sociaux sont loin d'avoir livrés tous leurs secrets, pleins de promesses, riches de leur spécificité exceptionnelle, comme le montre la déjà longue liste des transformations de nos sociétés, directement issue de leurs études : communication, interactions sociales, polymorphisme, optimisation des ressources, évolution de la vie, rythme de travail, vieillissement, adaptation, construction, transport, réseautage, coordination, exosquelettes mécanisés, robotique bio-inspirée, exploration en milieux hostiles…

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# *III – Annexes*

**Tableau 1.** Correspondances des terminologies utilisées pour les différents types de reproducteurs.

| Adultoïde | Nymphoïde | Ergatoïde |
|---|---|---|
| Imago | Brachyptère | Aptère |
| Primaire | Secondaire | Tertiaire |
| Alate-derived | Nymph-derived | Worker-derived |

# Publications en annexe


Brossette L, Bagneres A-G, Millot A, Blanchard S, Dupont S, Lucas C. **2017** Termite's royal cradle: does colony foundation success differ between two subterranean species? ***Insectes sociaux*** 64 (4): 515-523.

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Insectes Sociaux

RESEARCH ARTICLE

# Termite's royal cradle: does colony foundation success differ between two subterranean species?

**L. Brossette[1] · A.-G. Bagnères[1] · A. Millot[1] · S. Blanchard[1] · S. Dupont[1] · C. Lucas[1]**

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**Abstract** Colony foundation modes play a key role in social insect societies as they strongly impact colony survival. In subterranean termites, we do not yet know which factors most influence the early stages of colony foundation since studies on the topic are scarce. In the present study, we examined how variation in life-history traits can influence colony foundation success in two European termite species: *Reticulitermes grassei*, an indigenous, and *R. flavipes*, an invasive. We conducted an experiment in which we observed the development of colonies founded by 165 royal pairs on a weekly basis over a 6-month period. Several key results emerged: (1) survival rates of royal pairs were lower in *R. grassei*; (2) *R. grassei* pairs laid fewer eggs; and (3) all castes contained fewer individuals in *R. grassei* colonies, but (4) their timing of appearance are similar for both species. Overall, *R. grassei* pairs seemed less robust and produced fewer offspring. To our knowledge, our study is the first to describe the foundation of *R. grassei* incipient colonies in detail over a 6-month period. We also discovered new information about *R. flavipes'* colony foundation. The data suggest that the two species have different levels of colony foundation success, resulting in differences in colony development. This finding could help explain the invasiveness of *R. flavipes*. Our study contribute to our understanding of the spread of two termite pest species and shed light on critical steps in the colony foundation process.

**Keywords** Termite · Royal pair · Colony foundation · Incipient colonies · *Reticulitermes grassei* · *Reticulitermes flavipes*

## Introduction

In social insects, the production and development of offspring rely on the colony, which contains several castes specialized in different tasks. Altruistic behaviors are an essential part of this organization as colony members forego their own reproduction to help other members reproduce, thus ultimately increasing colony fitness (Hamilton 1964). The reproductive caste is the only one to directly transmit its genes via offspring production. Workers, in contrast, are generally sterile and carry out the tasks of nest building, foraging and offspring care; soldiers mainly defend the nest. Reproductives may exploit specialized dispersal strategies, which are reflected by their morphological features (Grassé 1982). Winged individuals can disperse towards new habitats located further away, while wingless individuals disperse on foot in the local environment around the nest. This caste polyethism impacts colony foundation because it is associated with different colony foundation modes. Social insects have developed dependent (DCF) and independent colony foundation (ICF) (Cronin et al. 2013). In DCF, workers help the reproductives to establish a new colony, while in ICF, reproductives (a single queen or queen-king pair) found new colonies without any worker assistance (Peeters and Molet 2010). Consequently, this mode of colony foundation is associated with much greater risks of mortality and colony failure (Ross and Matthews 1991;

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✉ L. Brossette
brossette.irbi@gmail.com

✉ C. Lucas
christophe.lucas@univ-tours.fr

[1] Institut de Recherche sur la Biologie de l'Insecte (UMR7261) CNRS, University of Tours, Tours, France

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Korb and Schneider 2007; Hölldobler and Wilson 2009). At the same time, ICF can be advantageous in that it is the only foundation mode allowing the colonization of new habitats with new resources.

Although Hymenoptera (ants, bees, and wasps) and Isoptera (termites) share a similar system of social organization, they differ in their development (holometabolous vs. heterometabolous), nesting patterns, and reproductive styles. Hymenopteran queens copulate during their nuptial flights and then store male sperm for years (Hölldobler and Bartz 1985; Pamilo 1991; Keller and Genoud 1997). In contrast, the termite king and queen form a monogamous pair in incipient colonies (Myles 1999), where the king regularly inseminates the queen (Raina et al. 2007; Ye et al. 2009). Several colony foundation forms have been observed in termites: colony foundation by paired alates (Nutting 1969; Matsuura 2010), pleometrosis (Thorne 1984; Darlington 1988; Hacker et al. 2005), budding (Husseneder et al. 1998), and even parthenogenesis (Howard et al. 1981; Matsuura et al. 2004; Kawatsu and Matsuura 2013). For higher termites (i.e., Termitidae), the factors influencing incipient colony foundation and development have been described in many species from different angles (Sands 1965; Garcia and Becker 1975; Sieber and Leuthold 1981; Rosengaus and Traniello 1993b; Thorne and Haverty 2000; Calleri et al. 2005; Adam and Mitchell 2009; Hartke and Baer 2011; Okot-Kotber 2011; Hartke and Rosengaus 2013). For subterranean termites (i.e., Rhinotermitidae), these processes are far less characterized, especially those concerning independent colony foundation (Thorne et al. 1997; Matsuura et al. 2002; Fei and Henderson 2003; Grube and Forschler 2004; Ghesini and Marini 2009; Janowiecki et al. 2013; Chouvenc et al. 2015; Roisin and Parmentier 2016). However, it is known that in subterranean termites of the genus *Reticulitermes* colony foundation is not simple, due to the complexity of their reproductive caste system. *Reticulitermes* termites produce three types of reproductives that display different modes of dispersal resulting in flexibility in colony foundation modes: alates (ICF by swarming); brachypterous neotenics that develop from nymphs (DCF by budding); and apterous neotenics that develop from workers (DCF by budding). Only alates can fly and thus disperse to new habitats (Nutting 1969). After swarming, alates run along the ground, employing pheromones to encounter an appropriate mate (Clément 1982a). Once a pair is formed, the partners run in tandem until a suitable nest site is found. Afterwards, they shed their wings and excavate a nest (copularium), in which they will establish a new colony (Nutting 1969). Once the first eggs have been laid, the two assume their roles as king and queen of the incipient colony (Nutting 1969).

Because of predation, competition, and environmental conditions during swarming and the nuptial flight, as well as the occasional inability of the royal pair to raise their first brood (Nutting 1969; Oster and Wilson 1978), colony foundation by ICF has a very low success rate and constitutes a bottleneck event (Chouvenc et al. 2014, 2015). To better understand these first steps of colony foundation, we carried out laboratory experiments in which we triggered ICF events in several colonies of two subterranean termites present in Europe: the indigenous *Reticulitermes grassei* and the invasive *R. flavipes*. The biology of European *Reticulitermes* termites is relatively well known (Büchli 1958; Nutting 1969; Lainé and Wright 2003; Field and Brace 2004; Pichon et al. 2007; Perdereau et al. 2010, 2015; Leniaud et al. 2011), even if the subterranean lifestyle of these insects makes them difficult to observe in nature. As part of the soil fauna, they are essential in forest ecosystems (La Fage and Nutting 1978; Gentry and Whitford 1982; Ulyshen et al. 2014). However, they are also urban pests that cause extensive and costly damage to anthropogenic structures (Su 2002). *R. grassei* Clément is an indigenous species that naturally occurs along the Atlantic coast of France (Clément et al. 2001). Populations migrated from Spain via the Pyrenees Mountains following the last glaciation period (Kutnik et al. 2004; Lefebvre et al. 2016). In contrast, *R. flavipes* (Kollar) was introduced into Europe almost two centuries ago, around the 1800 s. It arrived in France on the Atlantic coast, possibly carried on commercial ships coming from United States. An invasive species is defined as an introduced species whose presence is attributable to human actions that enable them to overcome their natural dispersal range and who has overall negative effects on ecosystems and economy (Early et al. 2016; Russell and Blackburn 2017). *R. flavipes* is, therefore, considered to be an invasive species (Bagnères et al. 1990; Clément et al. 2001; Austin et al. 2005; Dronnet et al. 2005; Perdereau et al. 2013). The two species live in sympatry in different parts of southwestern France and in Oléron Island forests. Studies of population genetics have revealed major differences in social structure between the two species, underscoring the important role played by reproductives in colony and species success (Vargo 2003; DeHeer et al. 2005; Perdereau et al. 2011, 2015). However, the early, integral steps of colony foundation following swarming by alate reproductives remain uncharacterized in the invasive *R. flavipes* and indigenous *R. grassei* although it represents a major step of colonization. Thus, a comparative evaluation of the foundation success between invasive and indigenous species by the alate reproductives might bring complementary results to explain the invasive success of *R. flavipes*. In this study, the development of incipient colonies of both species was followed weekly over the period of 6 months following the royal pair's establishment in the invasive termite *R. flavipes*

and the indigenous termite *R. grassei*. To do so, specially designed experimental glass enclosures were used. Several life-history traits were examined such as egg laying, caste development, and survivorship, which could be associated with colony foundation success and status (i.e., indigenous vs. invasive). Because of the high invasive success of *R. flavipes* worldwide, we expected higher survivorship and fertility for this invasive species compared to the indigenous species *R. grassei*.

## Materials and methods

A total of 13 colonies of *R. grassei* and 13 colonies of *R. flavipes* were collected in March 2013 in forests on Oléron Island (France, 45° 55' 0" N 1° 18' 0" W). Species were identified based on worker post-clypeus shape. *R. grassei* has a curved post-clypeus, while *R. flavipes* has a rectilinear post-clypeus (Clément 1982b). To ensure colony independence, we sampled colonies that were separated from each other by at least 100 m for *R. grassei* and by 300 m for *R. flavipes* (Perdereau et al. 2010). Upon collection, colonies were immediately placed in plastic boxes (18 × 24 × 9.5 cm) along with some of their original nest material. In the laboratory, they were maintained under standard conditions (80% relative humidity, 26 °C, 13.5L/10.5D cycle) until the long-winged nymphs became alates (1–2 weeks). At that time, to prevent mating, all the newly emerging adults were separated by sex and transferred into different plastic boxes (50 mm in diameter; Starpack) containing moistened paper made of cellulose (47 mm in diameter; Whatman, GE Healthcare). Termite sex was determined by examining the size of the seventh posterior sternite, which is more elongated in females than in males (Büchli 1958; Grassé 1982; Zimet and Stuart 1982).

Experimental incipient colonies were produced by pairing adult males and adult females from different colonies of origin. For each species, four colonies of origin were used and, thus, a total of 12 pairing combinations were obtained (Sup. Table 1). Each royal pair was placed in a plastic box (50 mm in diameter; Starpack) containing cellulose paper (47 mm in diameter; Whatman, GE Healthcare) that had been supplemented with a nutritive solution (Argoud et al. 1982). Seven days later, the different termite pairs (i.e., for each species: 12 pair combinations with 3–9 biological replicates each) were used in experiments. Each cross was assigned an ID code (cross ID) composed of the female's colony of origin and the male's colony of origin. To the cross ID, we added a unique code for each replicate and thus formed the pair ID. Each pair was, therefore, defined by a unique pair ID. The cross ID and pair ID were included in the statistical analyses (see the description of the statistical models below for more details).

A total of 87 and 78 pairs of *R. grassei* and *R. flavipes*, respectively, were transferred to the experimental glass cases in which the incipient colonies were housed. The cases comprised two 12 × 12 cm sheets of glass that were separated by 1.8-mm-thick plastic spacers (Kunststofshop). They were filled with Fontainebleau Sand (Carlo Erba Reagents) and contained a centrally located cellulose disk (90 mm in diameter; Whatman, GE Healthcare) that had been supplemented with a nutritive solution (Argoud et al. 1982) (Sup. Figure 1). The colonies were maintained under standard conditions: 26 °C and 80% RH in complete darkness in tanks where temperature and humidity were verified using thermometers (KHT-1, Basetech) and humidity levels were kept consistent using potassium nitrate wells (35 ml $KNO_3$/100 ml $H_2O$) (Thermo Fisher Scientific).

On a weekly basis, we recorded events, such as the death of one or both reproductives, the date on which the first egg was laid, and the emergence dates for the first larvae, workers, soldiers, and nymphs. If one member of a royal pair died, their colony was excluded from further analysis and the pair was treated as dead. On the same day, to follow colony development, pictures were taken of each colony (HDR CX700V, Sony), meaning we had weekly pictures for the entire period of the experiment. The pictures were used to estimate the number of eggs, undifferentiated larvae (first and second larval stages), workers (third stages and up), soldiers, and nymphs present each week. All measurements and analyses were double blind with regard to species and the time at which the picture had been taken (Gamboa et al. 1991).

Data on the numbers of eggs, larvae, and workers were analyzed using three different general linear mixed effects models (GLMMs), where the explanatory variables were species, Day post-establishment (a continuous variable), the quadratic of Day post-establishment, and the two interactions (Species:Day post-establishment and Species:Day post-establishment$^2$). We included cross ID and pair ID as random factors in the GLMMs. We did not perform GLMMs on soldier and nymph numbers because no soldiers and very few nymphs were produced (Sup. Figure 2). Multiple pairwise comparisons were used to assess the effect of species on each observation date (Sup. Table 2). The survivorship of the royal pairs and the timing of appearance of each caste were tested using several Cox proportional hazards regression models, which allow for censored data. In these models, the explanatory factor was species and the random factor was cross ID. All models were checked using Akaike's Information Criterion. Statistics were performed using R (v. 3.1.1, R Development Core Team).

## Results

Overall, *R. grassei* pairs had lower survivorship than *R. flavipes* pairs over the 6 months of the experiment (Fig. 1, $Z = 5.49$, $P = 0.019$).

The timing of caste appearance was similar for both species (i.e., for pairs still alive at the end of the experiment; $P > 0.05$, Fig. 2). Of the 42 *R. grassei* pairs, 1 pair never laid eggs; all 56 *R. flavipes* pairs laid at least one egg (Fig. 2a). Overall, 92.9% ($N = 39$) of the *R. grassei* pairs produced at least one larvae; this percentage was 94.6% ($N = 53$) for *R. flavipes* (Fig. 2b). In *R. grassei*, 95.2% ($N = 40$) of pairs generated at least one worker vs. 91.0% ($N = 51$) in *R. flavipes* (Fig. 2c). At least one nymph was produced by 59.5% ($N = 25$) of *R. grassei* pairs and 50% ($N = 28$) of *R. flavipes* pairs (Fig. 2d); this difference was not statistically significant ($P > 0.05$).

The numbers of eggs, undifferentiated larvae and workers over time differed between species (Table 1). Indeed, colony development was influenced by the species-by-day post-establishment interaction (Species:Day post-establishment). More detailed analyses revealed that, compared to *R. flavipes* pairs, *R. grassei* pairs had fewer eggs from the second through the fifth month included (Fig. 3a), fewer undifferentiated larvae from the third through the fifth month included (Fig. 3b), and fewer workers from the third month through the end of the experiment (Fig. 3c). It is important to note the following: (1) mean nymph number was less than one (Sup. Figure 2) and (2) no soldiers were produced by either species.

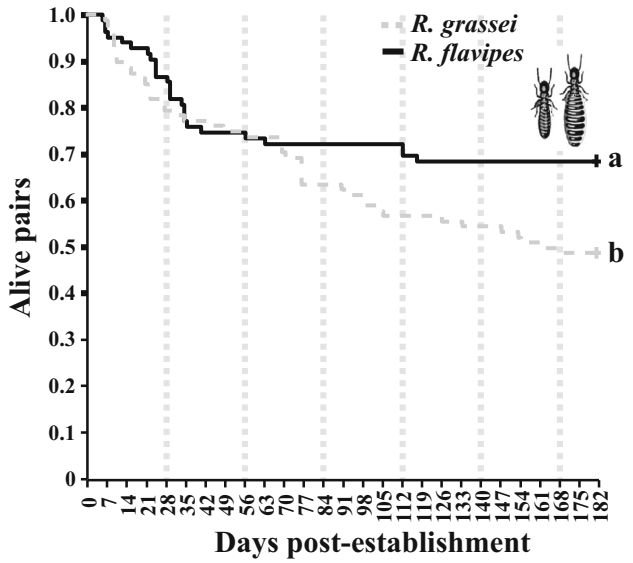


**Fig. 1** Proportion of pairs still alive on each observation date over the 6 months of the experiment for *R. grassei* (*dashed gray lines*) ($N = 87$) and *R. flavipes* (*solid black lines*) ($N = 78$) (mean ± SE). *Different letters* (*a*, *b*) indicate statistically significant differences ($P < 0.05$). The *dotted lines* indicate the different months

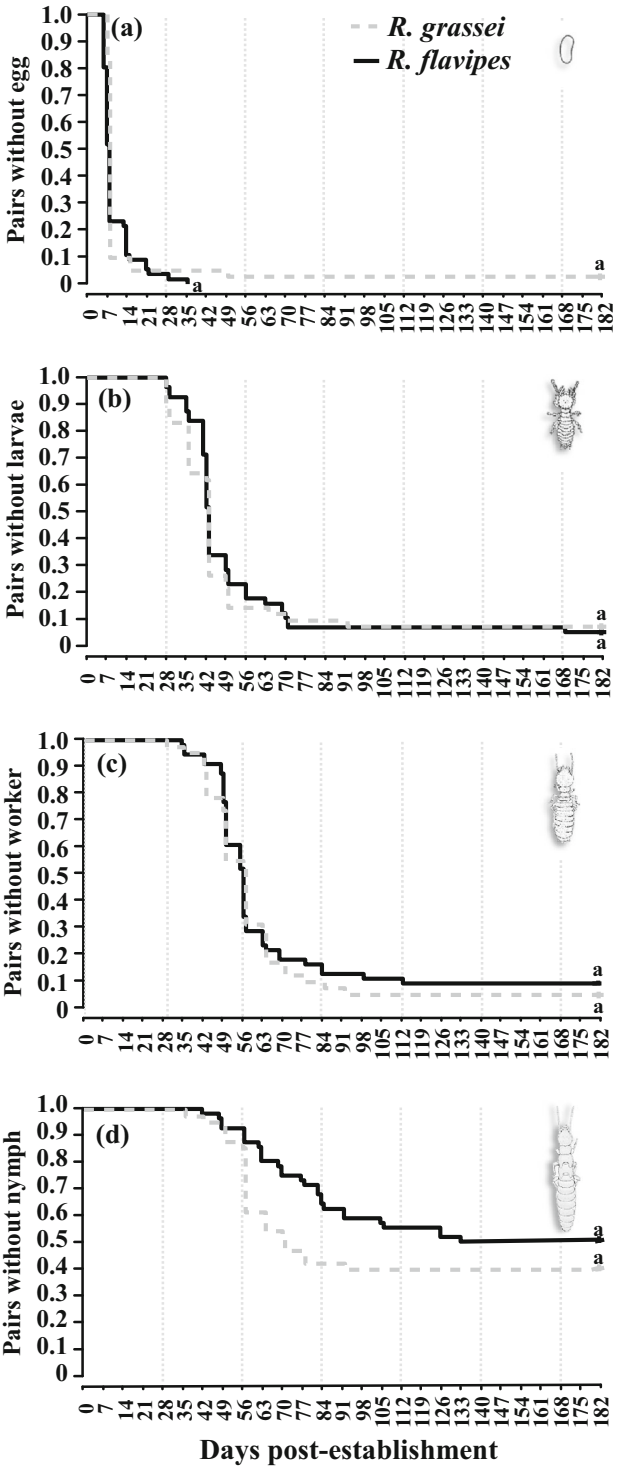


**Fig. 2** Time to the first appearance of eggs (**a**), larvae (**b**), workers (**c**), and nymphs (**d**) in royal pairs that survived the 6-month experiment for *R. grassei* (*dashed gray lines*, $N = 42$) and *R. flavipes* (*solid black lines*, $N = 56$). The data points represent the total proportion of pairs without any eggs, larvae, workers, or nymphs at a given point in time. No statistical differences were found ($P > 0.05$). The *dotted lines* indicate the different months

**Table 1** Results of the general linear models used to test the effects of species and day post-establishment on the numbers of eggs, larvae, and workers

| | Number of eggs | | Number of larvae | | Number of workers | |
|---|---|---|---|---|---|---|
| | LR $\chi^2_1$ | *P* value | LR $\chi^2_1$ | *P* value | LR $\chi^2_1$ | *P* value |
| Species | 16.04 | **<0.0001** | 31.34 | **<0.0001** | 11.59 | **<0.0001** |
| Day post-establishment | 26.02 | **<0.0001** | 503.44 | **<0.0001** | 1830.01 | **<0.0001** |
| Day post-establishment$^2$ | 30.21 | **<0.0001** | 694.115 | **<0.0001** | 922.31 | **<0.0001** |
| Species:Day post-establishment | 136.26 | **<0.0001** | 62.24 | **<0.0001** | 61.39 | **<0.0001** |
| Species:Day post-establishment$^2$ | 167.74 | **<0.0001** | 58.85 | **<0.0001** | 12.96 | **<0.0001** |

Bold values are highly significant. "Day post-establishment$^2$" and "Species:Day post-establishment$^2$" represent the quadratic effects of the variables

## Discussion

This study has revealed details about early colony foundation in two sympatric termite species, the indigenous *R. grassei* and the invasive *R. flavipes*. First, *R. grassei* royal pairs had lower survivorship than *R. flavipes* royal pairs. Second, the first eggs, larvae, workers, and nymphs appeared at the same time in both species. Third, no soldiers and almost no nymphs were observed during the experiment. Overall, in our conditions *R. grassei* appeared to be less robust and produce fewer offspring than *R. flavipes* and the two species seem to display a caste-specific developmental pathway over time. In general, *R. flavipes* seemed to be better at ICF than *R. grassei*, which could be a factor explaining *R. flavipes*' success as an invader.

*R. grassei* royal pairs had lower survivorship than *R. flavipes* royal pairs. Early colony foundation is a period of extreme stress for incipient colonies of social insects, especially those employing solitary ICF (Cronin et al. 2013). Foundation success may be drastically reduced and risks of colony failure are at their peak. This reduction might be especially strong during the first year, after the king and queen lay their first clutch (Nutting 1969; Chouvenc et al. 2015). There are a few studies on colony development in *Reticulitermes* (Büchli 1958; Thorne et al. 1997; Pichon et al. 2007; Leniaud et al. 2011). However, those examining the colony foundation success of royal pairs are scarce (Thorne et al. 1997; Grube and Forschler 2004; Janowiecki et al. 2013; Kawatsu and Matsuura 2013). Indeed, termite reproductives are cryptic and quite difficult to observe in the field, except during the swarming period (which is when we collected our study species). Ghesini and Marini (2009) showed that the survival of incipient colonies varied across seasons for *R. urbis*. Vargo et al. (2013) showed also that bioclimatic factors could influence colony breeding structure in the two studied species. In our study, we controlled for temperature, humidity, food availability, and soil composition but not for atmospheric pressure. Consequently, survivorship might have been influenced by seasonal variation in atmospheric pressure. The survival of primary reproductives and the success of colony establishment can also be strongly impacted by socially transmitted diseases, which are often linked to entomopathogenic fungi (Rosengaus et al. 2000; Calleri et al. 2005). Indeed, in *Zootermopsis angusticollis*, sibling reproductives are immunized against the same pathogens because they are similarly exposed in their colony of origin (Rosengaus and Traniello 1993a). In contrast, non-sibling pairs might transmit new pathogens to each other via grooming and trophallaxis, leading to mortality and colony failure (Rosengaus and Traniello 1993a). These results suggest that these two behaviors (i.e., grooming and trophallaxis) might strongly participate to offspring survivorship during the establishment of new nests and by extent to the foundation success of colonies (Rosengaus et al. 1998; Matsuura et al. 2004). To go further, it would be interesting to investigate in more details the parental care in incipient colonies linked to their survivorship and their potential connection with genotypic variations. Indeed, genotypic variations among colony members can also influence colony productivity and colony foundation success as shown in *R. speratus* (Miyazaki et al. 2014) and *R. flavipes* (Thorne et al. 1997).

*R. grassei* pairs produced fewer eggs than did *R. flavipes* pairs. A differential lipid storage ability between reproductives of the two species might explain the higher production of eggs observed in *R. flavipes* incipient colonies. Indeed, body mass and composition (like lipid storage) are factors which impact the colony foundation success. Fat reserves are important for early offspring production (Lenz 1987; Nalepa and Jones 1991; Lenz 1994; Shellman-Reeve 1997) and especially for egg production (Kaib et al. 2001). Therefore, nutrition plays a role in colony development, as shown in *Z. angusticollis* (e.g., Nalepa and Jones 1991). Such variations in egg number could have a direct impact on the colony development like it has been shown in a close relative species *R. speratus* where a pheromone has been

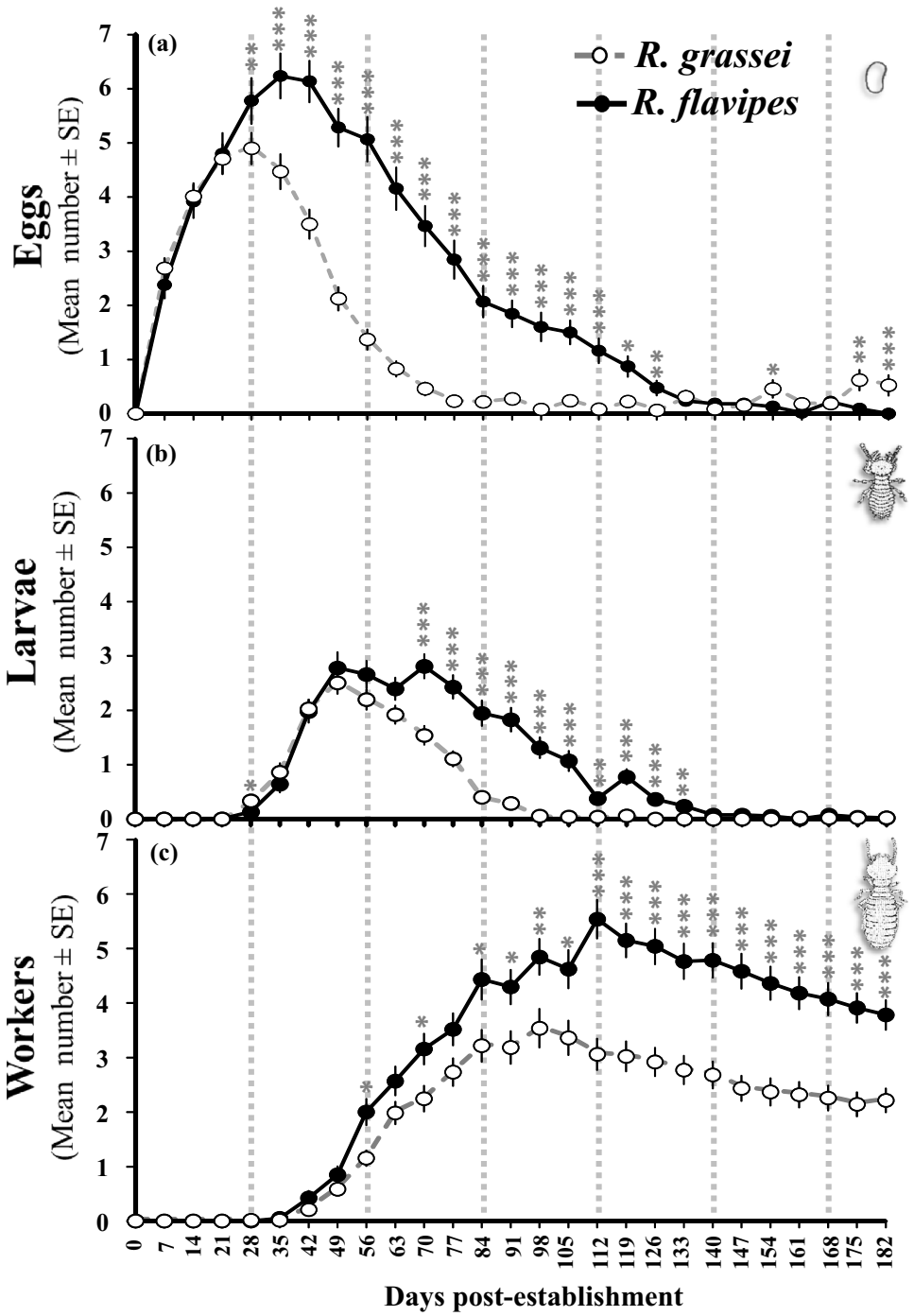


**Fig. 3** Mean number of eggs (**a**), larvae (**b**), and workers (**c**) produced per royal pair over the 6 months of the experiment for *R. grassei* (*dashed gray lines*, $N = 87$) and *R. flavipes* (*solid black lines*, $N = 78$) (mean ± SE; $***P < 0.001$, $**P < 0.01$, $*P < 0.05$). See Table 1 and Supplementary Table 2 for the detailed statistics. The *dotted lines* indicate the different months

identified on the egg surface (Matsuura et al. 2007) that suppresses the emergence of female neotenics (Matsuura et al. 2010; Matsuura 2012). These findings underscore the need for further experiments examining the impact of egg number on colony foundation success.

Over the 6-month period of the experiment, no additional neotenics emerged and almost no nymphs were produced (nymphs could potentially develop into brachypterous neotenics). These results are consistent with previous studies, in which limited numbers of nymphs were produced during a colony's first year (Grube and Forschler 2004; Janowiecki et al. 2013). Likewise, no soldiers were produced by either species during the experiment. It takes approximately two to four weeks for a worker to develop into a soldier (Büchli 1958; Lainé and Wright 2003; Darrouzet et al. 2014). Since, in our experiment, the first workers appeared after the first month, we would have expected to see development of soldiers. However, no

soldiers were observed in any colonies of either species. Of course, it could be that workers would have developed into soldiers later on, if the experiment had run longer than 6 months (Lainé and Wright 2003). In social insects, there is a trade-off between colony defense and colony maintenance. Thus, a lesser investment in soldiers could represent an adaptive strategy for increasing colony foundation success (Chouvenc et al. 2015) in the absence of predators, such as in our study.

We observed significant differences in colony foundation success between two sympatric species occurring in the same subterranean environment. Overall, our results show that *R. flavipes* colonies seem better at ICF than *R. grassei* colonies under laboratory conditions. In the field, *R. grassei* colonies are small and occupy disparate patches whereas *R. flavipes* colonies are bigger, more sprawling, and readily merge with neighboring colonies due to their lower aggressive behavior (DeHeer and Vargo 2004; Fisher et al. 2004; Perdereau et al. 2010). Altogether, it suggests that invasive populations more frequently employ budding (Perdereau et al. 2015). Aggressive interactions within a population are known to impact foundation success. It has been reported in invasive populations of the Argentine ant that non-aggressive colonies experienced lower mortality and better brood production than aggressive ones (Holway et al. 1998). This lack of aggression is also observed in other invasive species and result from a decrease of genetic variability due to a bottle neck effect induced by the introduction event (Tsutsui et al. 2000). It allows to reduce the cost of territoriality resulting in larger colonies like it was observed in the invasive Argentine ant (Holway et al. 1998) and in *R. flavipes* (Perdereau et al. 2010). *R. flavipes* differential demographic growth, observed here during foundation, corroborate the absence of simple families (i.e., a royal couple with its brood, defined by Thorne et al. (1999)) in French invasive populations of this termite already noticed in other studies (Dronnet et al. 2004, 2005; Perdereau et al. 2010, 2015; Vargo and Husseneder 2010). Along with our results, about the better ICF abilities of *R. flavipes*, it enforces the hypothesis that the invasive *R. flavipes* could ultimately competitively exclude *R. grassei* in areas where they co-occur (Perdereau et al. 2011) or at least displace it from its natural habitat as it was observed in populations of the Argentine ant (Erickson 1971).

Subterranean termites are excellent models with which to study foundation mechanisms as they perform both ICF and DCF, thanks to their three types of reproductives. In this study, we focused on ICF and shed light on variation in the growth dynamics of incipient colonies in an invasive and an indigenous termite species using a non-destructive experimental approach. ICF events were experimentally generated and the early colony development (over a 6-month period) was followed without disturbing the nest structure and the colony's social cohesion. To our knowledge, this study is the first to examine early colony development in the indigenous *R. grassei*. It has also contributed to a growing body of work on the invasive *R. flavipes*, notably showing that *R. flavipes* does a better job of carrying out ICF than does *R. grassei*. The latter result could partially explain the invasiveness of *R. flavipes*, one of the most widespread termite pests in the Americas and Europe.

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**Author contributions** The experiment was designed by CL, LB, AGB, SD. Fieldwork was performed by SD, LB, CL. The termite crosses and the transfer of the royal pairs to the glass cases were performed by LB, CL, SD, SB. LB followed colony development with the help of CL, SD, SB. The pictures were analyzed by AM, LB. The statistical analyses were conducted by LB, CL. The paper was written by LB, CL, AGB. All the authors read and approved the final version of the manuscript.

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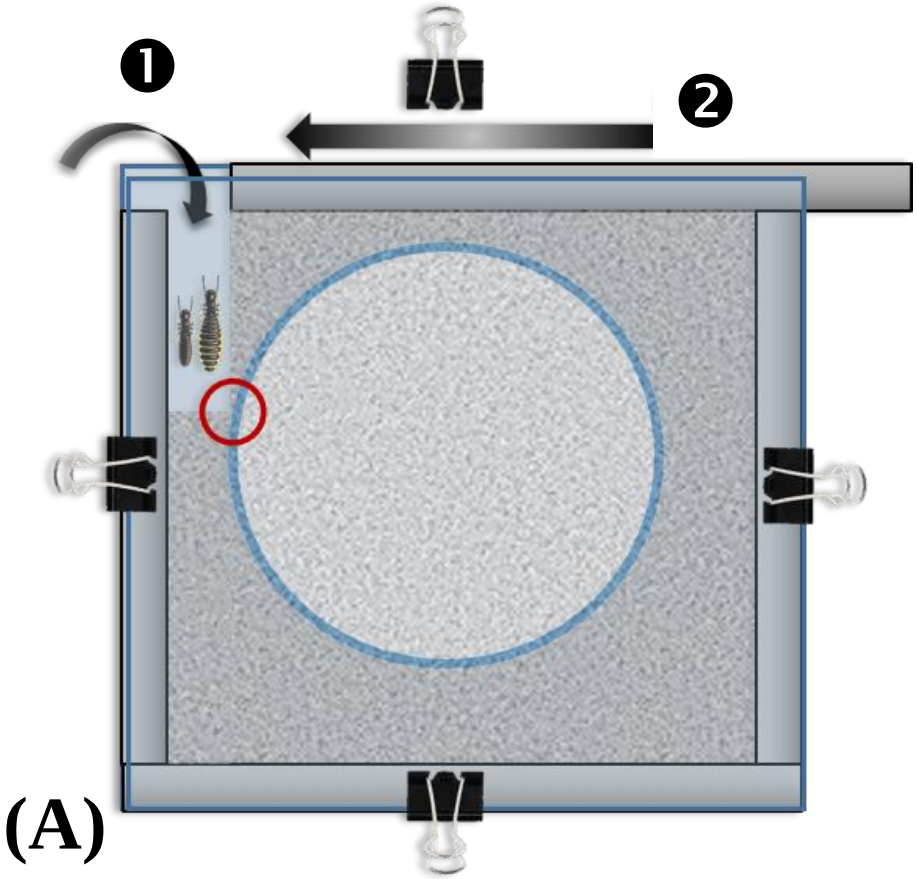
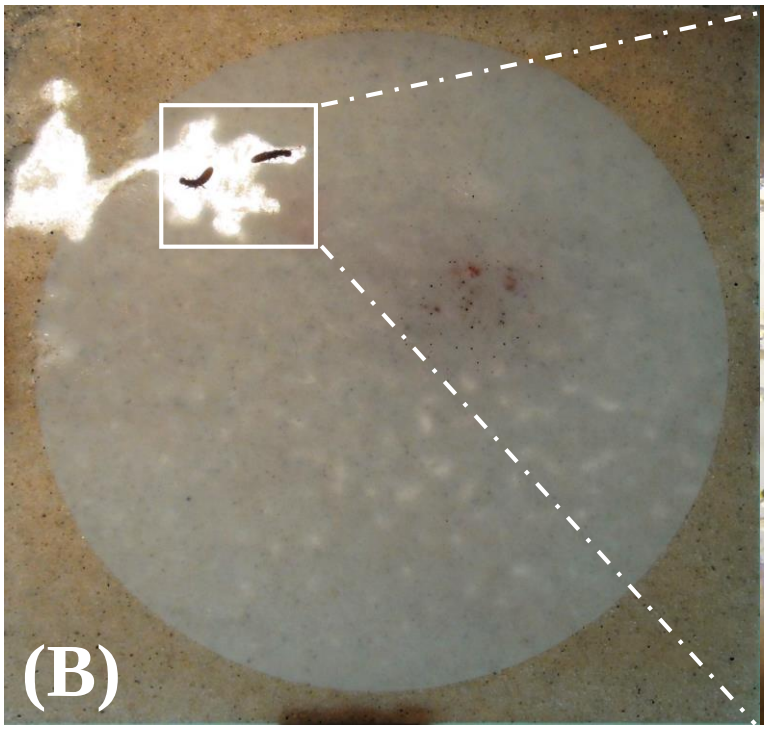
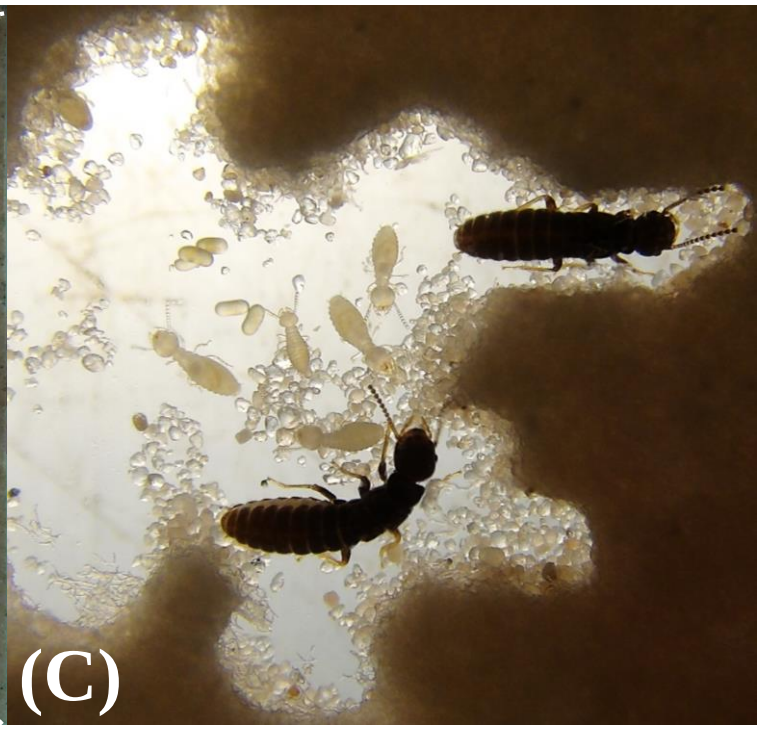
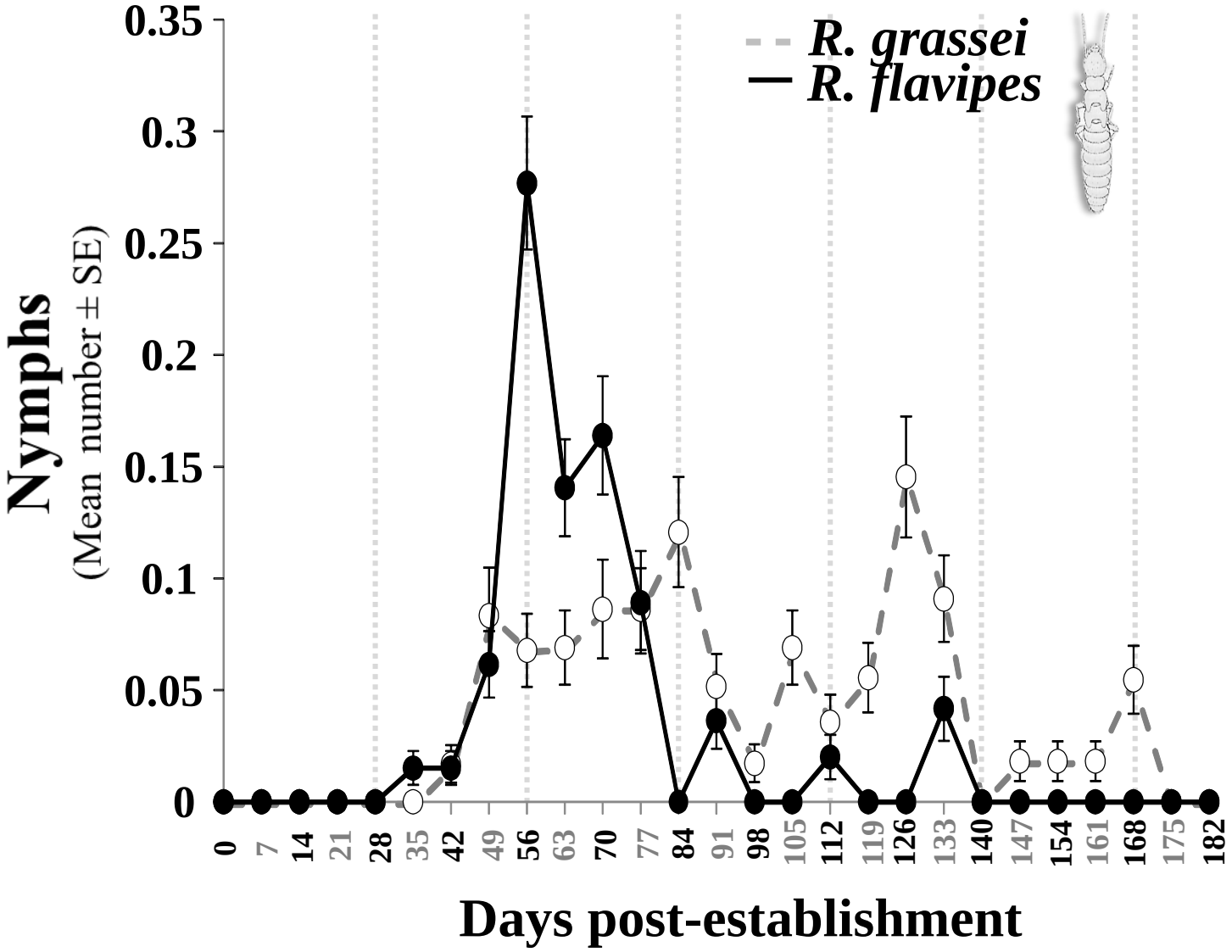
# Sup. Fig. 1



# Sup. Fig. 2


R. grassei
R. flavipes
Nymphs
(Mean number ± SE)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112 119 126 133 140 147 154 161 168 175 182
Days post-establishment

# Sup. Table 1

| Species | Cross ID | | N |
|---|---|---|---|
| | Male | Female | |
| *R. grassei* | A | B | 8 |
| | A | C | 6 |
| | A | D | 6 |
| | B | A | 9 |
| | B | C | 7 |
| | B | D | 6 |
| | C | A | 8 |
| | C | B | 8 |
| | C | D | 4 |
| | D | A | 9 |
| | D | B | 9 |
| | D | C | 7 |
| *R. flavipes* | E | F | 8 |
| | E | G | 8 |
| | E | H | 4 |
| | F | E | 8 |
| | F | G | 6 |
| | F | H | 7 |
| | G | E | 7 |
| | G | F | 7 |
| | G | H | 4 |
| | H | E | 8 |
| | H | F | 8 |
| | H | G | 3 |

# Sup. Table 2

| Time (Days) | Eggs | | Larvae | | Workers | |
|---|---|---|---|---|---|---|
| | W | P-value | W | P-value | W | P-value |
| 0 | - | - | - | - | - | - |
| 7 | 2701.5 | 0.1116 | - | - | - | - |
| 14 | 2643 | 0.7152 | - | - | - | - |
| 21 | 2524.5 | 0.9885 | - | - | - | - |
| 28 | 2714.5 | 0.0783 | 2018.5 | **0.0266** | 2278 | 0.3317 |
| 35 | 2600 | **0.0012** | 1702.5 | 0.1655 | 2016.5 | 0.263 |
| 42 | 2944.5 | **<0.0001** | 1858.5 | 0.6574 | 2059.5 | 0.3852 |
| 49 | 3234.5 | **<0.0001** | 2023.5 | 0.7137 | 2059.5 | 0.5435 |
| 56 | 3196 | **<0.0001** | 2181.5 | 0.1793 | 2366 | **0.0207** |
| 63 | 3092 | **<0.0001** | 2173.5 | 0.0974 | 2137 | 0.1444 |
| 70 | 2968 | **<0.0001** | 2505 | **<0.0001** | 2194 | **0.0223** |
| 77 | 2666.5 | **<0.0001** | 2383 | **<0.0001** | 1949 | 0.0631 |
| 84 | 2380.5 | **<0.0001** | 2417 | **<0.0001** | 2005.5 | **0.0175** |
| 91 | 2432.5 | **<0.0001** | 2561.5 | **<0.0001** | 2028 | **0.012** |
| 98 | 2279 | **<0.0001** | 2361 | **<0.0001** | 1973.5 | **0.005** |
| 105 | 2041 | **<0.0001** | 2131 | **<0.0001** | 1856 | **0.0119** |
| 112 | 1922 | **<0.0001** | 1651 | **0.007** | 2163.5 | **<0.0001** |
| 119 | 1633.5 | **0.0105** | 1846.5 | **<0.0001** | 2013.5 | **<0.0001** |
| 126 | 1618.5 | **0.0062** | 1690.5 | **<0.0001** | 2036.5 | **<0.0001** |
| 133 | 1306.5 | 0.8986 | 1536 | **0.0036** | 1974 | **<0.0001** |
| 140 | 1397 | 0.1943 | 1386.5 | 0.0619 | 1984.5 | **<0.0001** |
| 147 | 1328 | 0.4772 | 1357 | 0.0647 | 1953 | **<0.0001** |
| 154 | 1028.5 | **0.0155** | 1334 | 0.1122 | 1897.5 | **<0.0001** |
| 161 | 1093.5 | **0.0482** | 1182.5 | 0.2723 | 1821.5 | **<0.0001** |
| 168 | 1238.5 | 0.4851 | 1220 | 0.4397 | 1751 | **<0.0001** |
| 175 | 915 | **0.0084** | 1169.5 | 0.7339 | 1749.5 | **<0.0001** |
| 182 | 907.5 | **<0.0001** | 1148.5 | 0.8591 | 1684 | **<0.0001** |

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ORIGINAL RESEARCH

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# Unbalanced biparental care during colony foundation in two subterranean termites

Lou Brossette[1] | Joël Meunier[1] | Simon Dupont[1] | Anne-Geneviève Bagnères[1,2] | Christophe Lucas[1]

[1]Institut de Recherche sur la Biologie de l'Insecte (UMR7261), CNRS – University of Tours, Tours, France

[2]CEFE, CNRS UMR5175, Univ. Montpellier, Univ. Paul Valéry Montpellier 3, EPHE, IRD, Montpellier, France

**Correspondence**
Christophe Lucas, Institut de Recherche sur la Biologie de l'Insecte (UMR7261), CNRS – University of Tours, Tours, France.
Email: christophe.lucas@univ-tours.fr

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## Abstract

Parental care is a major component of reproduction in social organisms, particularly during the foundation steps. Because investment into parental care is often costly, each parent is predicted to maximize its fitness by providing less care than its partner. However, this sexual conflict is expected to be low in species with lifelong monogamy, because the fitness of each parent is typically tied to the other's input. Somewhat surprisingly, the outcomes of this tug-of-war between maternal and paternal investments have received important attention in vertebrate species, but remain less known in invertebrates. In this study, we investigated how queens and kings share their investment into parental care and other social interactions during colony foundation in two termites with lifelong monogamy: the invasive species *Reticulitermes flavipes* and the native species *R. grassei*. Behaviors of royal pairs were recorded during six months using a non-invasive approach. Our results showed that queens and kings exhibit unbalanced investment in terms of grooming, antennation, trophallaxis, and vibration behavior. Moreover, both parents show behavioral differences toward their partner or their descendants. Our results also revealed differences among species, with *R. flavipes* exhibiting shorter periods of grooming and antennation toward eggs or partners. They also did more stomodeal trophallaxis and less vibration behavior. Overall, this study emphasizes that despite lifelong monogamy, the two parents are not equally involved in the measured forms of parental care and suggests that kings might be specialized in other tasks. It also indicates that males could play a central, yet poorly studied role in the evolution and maintenance of the eusocial organization.

KEYWORDS

division of labor, foundation, parental care, social behavior, task allocation, termites

## 1 | INTRODUCTION

Parental care is a taxonomically widespread phenomenon across animals (Klug & Bonsall, 2010; Korb, Buschmann, Schafberg, Liebig, & Bagnères, 2012; Wong, Meunier, & Kölliker, 2013). It can last from a few days to several years, be performed before and/or after the emergence of juveniles and involve either the mother, the father or both parents (Smiseth, Kölliker, & Royle, 2012). From mammals

to insects, parental care can take multiple forms, such as egg and offspring attendance, nest building and burrowing and food provisioning (Smiseth et al., 2012). All these forms typically provide benefits to offspring by enhancing offspring survival, growth, and/or quality, as well as by improving their lifetime reproductive success (Klug & Bonsall, 2014; but see Kramer et al., 2017). However, investing into parental care may also go along with costs for parents. That is because it can entail an exaggerated loss of energy, as well as an increased risk of pathogen exposure and predation during offspring attendance, which all may ultimately curtail their survival rate and capability to invest into future reproduction (Alonso-Alvarez & Velando, 2012). The evolution of parental care therefore requires that its costs remain lower than its associated benefits for each family member.

For parents, one way to reduce the costs of parental care is to share them with the other parent. The presence of two parents with offspring has been reported in numerous birds, cichlid fishes, primates, and a few insects (Balshine, 2012; Trumbo, 2012). Although this mutual presence is typically associated with biparental care, a sexual conflict between mothers and fathers over their respective investment into care often emerges during family life, as each parent can maximize its own fitness by selfishly minimizing its investment into cares (Lessells, 2012). Such a selfish strategy allows males, for instance, to increase their investment into the search of additional partners and thus to maximize the number of offspring produced during a single reproductive season, while it allows females to reallocate their saved energy into future reproduction (Smiseth et al., 2012). The tug-of-war between mothers and fathers over parental investment has been shown to generally lead to a disequilibrium, during which one parent exhibits a lower investment compared to the other, while this latter does not fully compensate for this reduction (Harrison, Barta, Cuthill, & Szekely, 2009).

Although most studies on sexual conflict explore its resolution in species where parents can do extra pair copulations and/or have novel mating partners at each reproductive season (Jennions, Kahn, Kelly, & Kokko, 2012), the expression and organization of biparental care remain unclear when the lifetime fitness of each parent tightly relies on its partner's. This is the case, for instance, in the biparental family units often present in termites (Kramer & Meunier, 2018; Wilson, 1971). In this eusocial insect, mothers (queens) and fathers (kings) form pairs quickly after they reach adulthood and remain together during their entire lives, which can last decades (Boomsma, 2009). Each couple typically lives in a dark nuptial chamber at the center of the colony, where queens produce eggs and kings regularly inseminates queen(s); kings and queen having no direct contact with foreign individuals (Hartke & Baer, 2011). Because the termite royal couple has no opportunity of extrapair copulation, it has long been thought that parental care is equally shared between queens and kings especially at colony foundation (Bignell, Roisin, & Lo, 2011; Nalepa & Jones, 1991; Shellman-reeve, 1997). However, empirical support of this claim remains scarce (Rosengaus & Traniello, 1991; Shellman-Reeve, 1990).

In this study, we investigated how termite queens and kings share their investment into social interactions, as well as whether this share depends on the developmental stage of their offspring. Using an experimental setup allowing non-invasive and fine-scaled behavioral observations (Brossette et al., 2017), we analyzed the expression of grooming, antennation, trophallaxis (proctodeal and stomodeal), and body-shaking by queens and kings over the six first months of their colony foundation. Because we aimed at taking a broader perspective and exploring whether this biparental organization was species specific, we used two species of subterranean termites: the invasive *Reticulitermes flavipes* (Kollar, 1837) and the native *R. grassei* (Clément, 1978). If sexual conflict between males and females over their respective investment in parental care is absent in these species, we expected queens and kings to express a similar level of grooming, antennation, and trophallaxis toward their offspring. Note that body-shaking is a behavior that has been frequently reported in termites, but for which the role is still unclear (Funaro, Böröczky, Vargo, & Schal, 2018; Whitman & Forschler, 2007). Our study will thus also provide novel insights into our understanding of its expression and function during colony foundation.

## 2 | MATERIAL AND METHODS

### 2.1 | Sampling and crossings

We investigated the behaviors of newly produced queens and kings originating from a total of four colonies of *R. flavipes* and four colonies of *R. grassei*. The workers, nymphs (i.e., future queens and kings), and soldiers of each of these colonies were field sampled in March 2014 in pine forests on Oléron Island in France and immediately transferred into plastic boxes (18 × 24 × 9.5 cm) with their own nest material and moistened sand (Brossette et al., 2017). These colonies were 100 m away from each other for *R. grassei* and 300 m for *R. flavipes*, that is, distances that typically ensure colony independence (Perdereau, Bagnères, Dupont, & Dedeine, 2010). Back in the laboratory, these field-sampled colonies were maintained under standard conditions (80% relative humidity, 26°C, 13.5 L/10.5 D cycle) until nymphs became reproductive adults. To prevent uncontrolled sibmating, each colony was checked twice a day to collect the newly produced winged alates (females and males, i.e., future new queens and kings) and to transfer these individuals into sex-specific new plastic boxes (50 mm diameter; Starpack) containing moistened pure cellulose paper (47 mm diameter; Whatman, GE Healthcare; Brossette et al., 2017). Seven days after the emergence of the first winged alate, virgin males were paired with unrelated virgin females. To limit the risks of mating incompatibility between colonies, we paired individuals following 12 intercolonial combinations (later called cross ID), which were each replicated from 3 to 9 times (later called pair ID). This led to a total of 70 and 86 experimental pairs of *R. flavipes* and *R. grassei*, respectively. Each pair was then transferred to an experimental glass case allowing detailed behavioral observations (Brossette et al., 2017) and containing a food source composed of a pure cellulose disk (90 mm in diameter; Whatman,

GE Healthcare) supplemented with a solution composed of mineral salts, vitamins, and nitrogen (Argoud, Mocotte, & Sternalski, 1982). Over the subsequent six months of experiment, all pairs were maintained under standard laboratory conditions (80% relative humidity, 26°C) and complete darkness. Humidity was controlled with the use of potassium nitrate wells (35 ml $KNO_3$/100 ml $H_2O$; Thermo Fisher Scientific).

## 2.2 | Behavioral recording

Over the six months of the experiment (from May to October), 12 pairs per species were randomly selected every 2 weeks to be video-recorded (Sony HDR CX700V). The chambers where the royal couple were settled with eggs and larvae were video-recorded for 30 min (after a five-min resting time, as the experimental glass cases were moved to the recording setup), under controlled environment (80% RH, 26°C) and total darkness using infrared lights (940 nm wavelength, 15 LEDs of 26 mm diameter, Kingbright). The presence of eggs and larvae in the royal chamber were assessed. Note that because the parents were the focal individuals, we discarded three videos with missing reproductives from the statistical analyses. The resulting videos were analyzed with the freeware Boris v3.0 (Friard & Gamba, 2016) to quantify parental care behaviors between parents and between parents and descendants. This allowed us to disentangle behaviors that are specifically directed toward offspring (i.e., parental care) from behaviors that are directed toward all family members. In these analyses, donor individuals were defined as individuals expressing the behavior (queen or king), while recipients were defined as individuals receiving the behaviors (defined as either partners, eggs or descendants—this latter including larvae, nymphs, and workers). The recorded behaviors were (a) grooming and antennation (i.e., any contact from the head of a donor toward a recipient), (b) trophallaxis (either proctodeal or stomodeal, i.e., anal-to-mouth or mouth-to-mouth fluid transfer, respectively), and (c) body-shaking (rapid back and forth movement of the whole body with no contact with the substrate). Note that this latter behavior is not directed toward any recipient (Whitman & Forschler, 2007). Other behaviors were observed, but discarded from this study because they were not directly involved in parental care (e.g., dejections, selfgrooming, copulations, and food intake). For each video, queens and kings were discriminated by measuring the size of their seventh sternite (Zimet & Stuart, 1982). Videos were processed following a double blind process during recording and reading (Gamboa, Reeve, & Holmes, 1991).

## 2.3 | Statistical analyses

The total duration of antennation and grooming behaviors (together) was analyzed using a general linear mixed effects model (LMM), in which the explanatory factors were the donors (Queens/Kings), the recipients (Partner/Offspring), the species (*R. flavipes*/*R. grassei*), and the developmental stage of the offspring (Eggs/Descendants). To interpret the resulting significant triple interaction involving recipients, species, and offspring developmental stage (see Section 3), the dataset was then split per developmental stage and the two resulting subsets were used to conduct two additional LMMs with the same explanatory factors (without the developmental stage factor). The observation of at least one type of trophallaxis (presence/absence) and the total duration of trophallaxis (when observed) were then tested using a generalized linear mixed effect model (GLMM) with binomial error distribution and a LMM, respectively. In these models, the explanatory factors were the donor, the recipient, the species, and the type of trophallaxis (proctodeal/stomodeal). Note that these models were restricted to the dataset where descendants were present, because trophallaxis is not possible toward eggs. Finally, the observation of at least one body-shaking (presence/absence) and the total number of body-shaking (when observed) were tested using a GLMM with binomial error distribution and a LMM, respectively. In these models, the explanatory factors were the donors, the species, the presence of eggs, and the presence of descendants.

In all the above statistical models, the cross ID and the pair ID (nested into the cross ID) were included as random factors to control for the fact that several kings and queens came from the same field colonies. The date of each video were also included as a random factor to control for the fact that parental behaviors may change over time, while providing an overview of the different behaviors over the six-month recording (i.e., the main goal of this study). To fit with homoscedasticity and normal distribution of model residuals, the total duration of antennation and grooming behaviors were log(+1)-transformed, while the total duration of trophallaxis and the total number of body-shakings were log-transformed. All GLMMs with binomial error distribution were fitted using the "cloglog" link-function to correct for the unbalanced representation of 1 and 0 (Crawley, 2012). All models were first tested with all possible interactions among explanatory variables and were then simplified step-by-step by removing the non-significant interactions (all $p > 0.08$). Note that some non-significant interactions are reported in the results to allow direct comparison between analyses, but their removal induces no qualitative changes. When required, we conducted post hoc pairwise comparisons within each model using model contrasts based on estimated marginal means. When appropriate, non-significant factors were pooled in the presented figures. All analyses were performed using the software R v3.4.3 (www.r-project.org) loaded with the packages *lme4*, *car*, and *emmeans*.

# 3 | RESULTS

Grooming and antennation were overall present in 86.3% of the movies. The total duration of grooming and antennation depended on a triple interaction between species (*R. flavipes* or *R. grassei*), recipients (offspring or partner), and offspring developmental stage (eggs or descendants; LR $\chi^2_1 = 26.0$, $p < 0.0001$). In the presence of eggs, *R. grassei* and *R. flavipes* adults spent the same amount of time grooming and antennating their partners (Table 1; Contrast, $p = 0.9203$), whereas *R. grassei* adults spent more time grooming

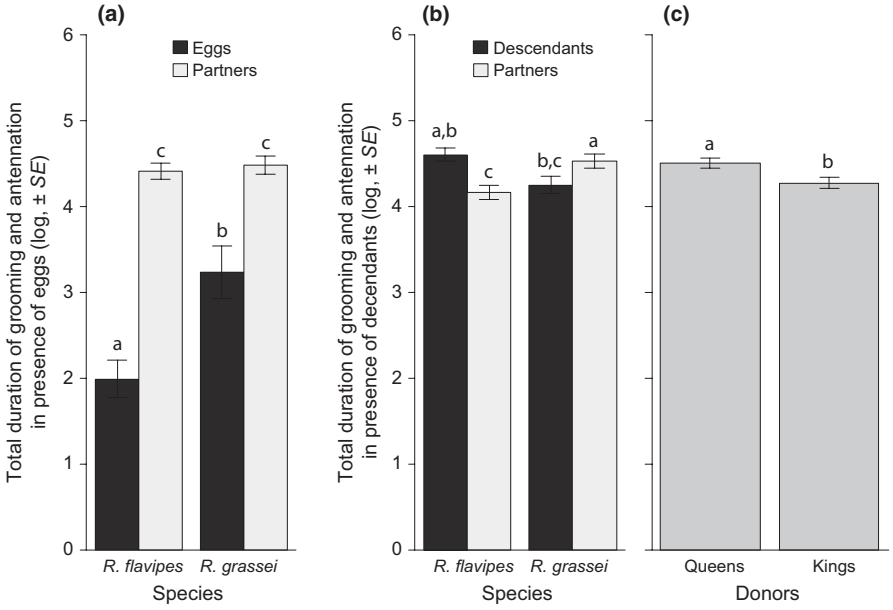


**FIGURE 1** Effects of species (*R. flavipes*/*R. grassei*), recipients (Partner/Offspring), and donors (Queens/Kings) on the total duration of allogrooming and antennation either in the presence of eggs (a) or in the presence of descendants (b, c). When the factors showed no significant interactions (see tables), they were pooled to better represent the statistical results. Bars represent mean values of the log(+1)-transformed total duration ±*SEM*. Different letters refer to $p < 0.05$

and antennating their eggs compared to *R. flavipes* adults (Contrast, $p = 0.0247$; Figure 1a). Nevertheless, both *R. grassei* and *R. flavipes* adults spent overall more time grooming and antennating their partner than their eggs (Table 1, Figure 1a). Conversely, in the presence of descendants, *R. flavipes* adults spent more time grooming and antennating descendants than partners (Table 1; Contrast, $p = 0.0002$), whereas *R. grassei* adults spent more time grooming and antennating partners than descendants (Contrast, $p = 0.0091$; Figure 1b). During that period, the total time spent grooming and antennating was higher in queens compared to kings, but independent of the species and the type of recipient (Table 1, Figure 1c).

At least one of the two types of trophallaxis (proctodeal and stomodeal) was present in 22.4% of the movies. Stomodeal trophallaxis was more likely to be observed in *R. flavipes* compared to *R. grassei* (Table 2; Contrast, $p = 0.0430$), whereas this difference was absent for proctodeal trophallaxis (Contrast, $p = 0.2655$; Figure 2a). Independent of the species and its type, trophallaxis was more likely to be expressed by queens than kings (Table 2; Figure 2b,e) and more likely to be received by descendants than partners (Table 2; Figure 2c,f). When at least one type of trophallaxis was observed, queens spent more time performing trophallaxis than kings (Table 2), descendants received trophallaxis for a longer total time compared to partners (Table 2) and proctodeal trophallaxis was overall expressed longer than stomodeal trophallaxis (Table 2). The total duration of trophallaxis was independent of any interaction among donors, recipient, and type of trophallaxis (all $p > 0.0975$).

Finally, body-shaking was observed in 39.2% of the movies. The observation of at least one body-shaking event depended on double interactions both between donors and eggs presence (Table 3) and between eggs and descendants presence (Table 3). In particular, queens were less likely to perform body-shaking in the presence compared to in the absence of eggs (Figure 3a; Contrast, $p = 0.0339$), whereas this effect was absent in kings (Figure 3a; Contrast: $p = 0.9146$). Conversely, queens and kings were overall more likely to perform body-shaking in the presence compared to absence of descendants, but only in the presence of eggs (Figure 3b; Contrasts: eggs presence, $p = 0.0023$; eggs absence, $p = 0.5270$). Finally, when body-shaking was observed, its total number was overall higher in *R. grassei* compared to *R. flavipes* (Figure 3c), whereas it was independent of eggs and descendants presence, as well as of the type of donor (Table 3b).

**TABLE 1** Effects of recipient, donor and species on total duration of allogrooming/antennation when (a) eggs or (b) descendants were present

| | (a) When eggs are present | | (b) When descendants are present | |
|---|---|---|---|---|
| | LR $\chi^2_1$ | *p* | LR $\chi^2_1$ | *p* |
| Recipient | 95.4 | **0.0000** | 0.4 | 0.5524 |
| Donor | 0.2 | 0.6516 | 8.7 | **0.0032** |
| Species | 1.6 | 0.2003 | 0.1 | 0.7681 |
| Recipient:Species | 9.7 | **0.0019** | 20.5 | **0.0000** |

*Note*. Significant *p*-values are in bold.

## 4 | DISCUSSION

In this study, we compared the involvement of queens and kings in social interactions during colony foundation in the invasive *R. flavipes* and the native *R. grassei* termites. Our results first reveal that queens invest more in the measured forms of parental care than kings, as they overall performed more trophallaxis, grooming, and antennation (when descendants are present) than their partner. This sex-specific effect was independent of the species. Secondly, we showed that differences in parental care are species specific. In particular, *R. flavipes* exhibited less grooming/antennation toward

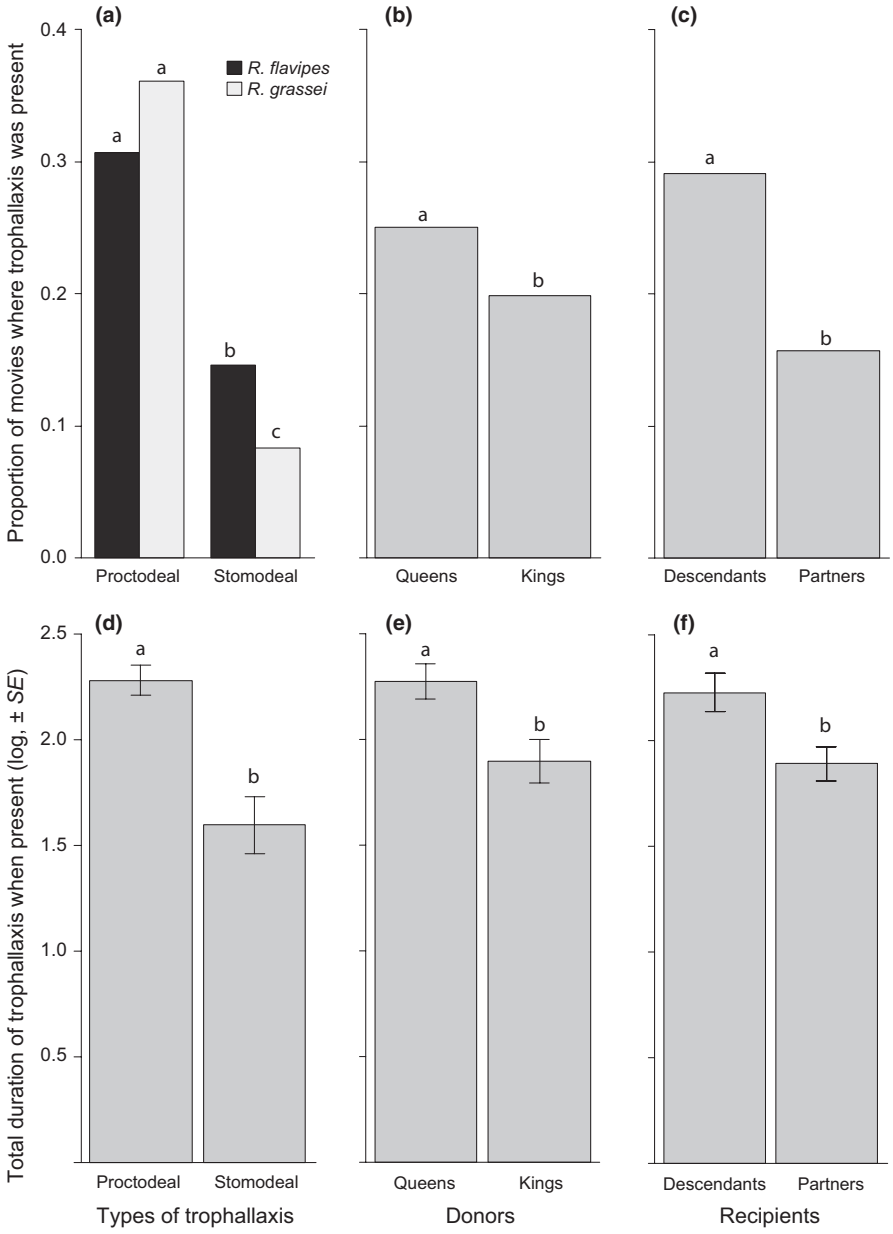


**FIGURE 2** Effects of species (*R. flavipes*/*R. grassei*), recipients (Partner/Offspring), donors (Queens/Kings), and types (Proctodeal/Stomodeal) on the observation (a–c) or total duration of trophallaxis (d–f). When the factors showed no significant interactions (see tables), they were then pooled to better represent the statistical results. Bars represent proportion of movies (a–c) or mean values of the log-transformed total duration ±*SEM* (d–f); Different letters refer to $p < 0.05$. Note that these models were restricted to the dataset where descendants were present, because trophallaxis is not possible toward eggs

**TABLE 2** Effects of recipient, donor, species and types of trophallaxis on (a) the presence of at least one trophallaxis event and on (b) the total duration of trophallaxis when present

| | (a) Presence/absence | | (b) Total duration when expressed | |
|---|---|---|---|---|
| | LR $\chi^2_1$ | *p* | LR $\chi^2_1$ | *p* |
| Recipient | 24.8 | **0.0000** | 10.4 | **0.0013** |
| Donor | 4.6 | **0.0321** | 7.9 | **0.0051** |
| Species | 0.0 | 0.9445 | 0.8 | 0.3762 |
| Types of trophallaxis | 56.2 | **0.0000** | 24.6 | **0.0000** |
| Species:Types of trophallaxis | 6.8 | **0.0091** | 1.9 | 0.1682 |

*Note*. Significant *p*-values are in bold.

eggs compared to partners, but more grooming/antennation toward descendants compared to partners. By contrast, *R. grassei* exhibited more grooming/antennation toward partners compared to both eggs and descendants. The two species also differed in their overall expression of stomodeal trophallaxis, which was more likely to occur in *R. flavipes* compared to *R. grassei*. This difference was absent in term of proctodeal trophallaxis. In both species, trophallaxis was preferentially directed toward descendants instead of partners and was overall more likely to involve proctodeal instead of stomodeal contacts. Finally, our results reveal that body-shaking depends on the species, the sex of the donor, and the developmental stage of the offspring. Body-shaking was overall more frequent in *R. grassei* compared to *R. flavipes*. Moreover, queens were more likely to perform body-shaking in the absence compared to presence of eggs, whereas this effect was absent in kings. When eggs were present, body-shaking was also more likely to occur in the presence compared to

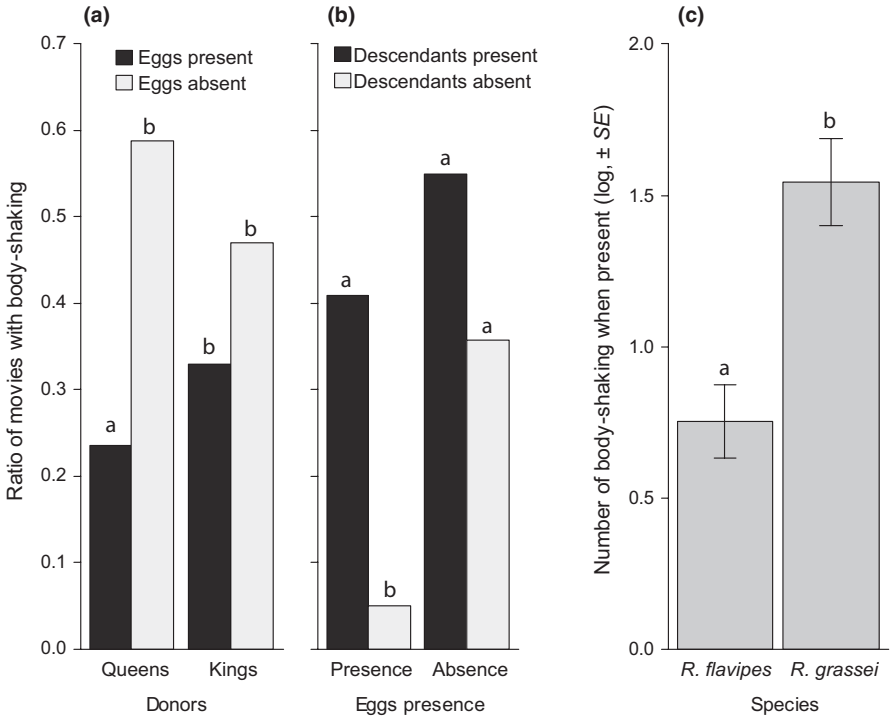


**FIGURE 3** Effects of donors (Queens/Kings), eggs presence, descendants presence, and species (*R. flavipes*/*R. grassei*) on the observation (a,b) and total number (c) of body-shaking. When the factors showed no significant interactions (see tables), they were then pooled to better represent the statistical results. Bars represent the ratio of movies with body-shaking (a,b) or the log-transformed total number of body-shaking when present ±*SEM* (c); Different letters refer to $p < 0.05$. Note that no recipients were assigned for this behavior

absence of descendants, whereas this effect was not found in the absence of eggs.

Somewhat surprisingly, our results reveal that queens and kings exhibit unbalanced investment in the measured forms of parental care during colony foundation in both *R. flavipes* and *R. grassei*. In particular, the involvement of queens into grooming/antennation and trophallaxis was overall higher than the one of kings. This finding both contrasts with the few results reporting an absence of sexual polyethism in incipient colonies of two other termite species, *Zootermopsis angusticollis* and *Z. nevadensis* (Rosengaus & Traniello, 1991; Shellman-Reeve, 1990), and provides no support for the general prediction of a tight association between lifelong monogamy and balanced investment of each parent into egg/offspring care (Boomsma, 2009). The higher investment of queens into direct interactions in both *R. grassei* and *R. flavipes* suggests that kings are either involved into other tasks and/or overall less active than queens during colony foundation. The success of colony foundation generally involves a broad set of tasks, such as nest construction and/or fights against predators and pathogens (Chouvenc, Efstathion, Elliott, & Su, 2013; Eggleton, 2010), or could be a dynamic response to local environmental changes (Shellman-Reeve, 1990), for which kings might indeed be more involved. The full task repertoire exhibited by termite queens and kings during colony foundation will be investigated in the future experiments taking into account other life traits like sexual size dimorphisms, physiological traits, or metabolite composition which might be part of the observed unbalanced biparental care.

**TABLE 3** Effects of donor, species, eggs and the presence of descendants on (a) the presence/absence and (b) total number of body-shaking

| | (a) Presence/absence | | (b) Number of event when expressed | |
|---|---|---|---|---|
| | LR $\chi^2_1$ | *p* | LR $\chi^2_1$ | *p* |
| Donor | 0.0 | 0.9390 | 0.3 | 0.5943 |
| Species | 0.0 | 0.9350 | 4.5 | **0.0347** |
| Eggs presence (Ep) | 0.3 | 0.6051 | 1.3 | 0.2457 |
| Descendants presence (Dp) | 4.5 | **0.0348** | 0.0 | 0.9506 |
| Ep:Dp | 5.0 | **0.0259** | 2.4 | 0.1189 |
| Donor:Ep | 6.8 | **0.0090** | 0.4 | 0.5395 |

*Note*. Significant *p*-values are in bold.

While both *R. flavipes* and *R. grassei* show unbalanced levels of parental care, we found species-specific levels of parental investment for grooming/antennation depending on the presence of eggs or descendants. In particular, if we compare the duration of grooming/antennation toward eggs/descendants with the one toward partners (for both species), then *R. grassei* exhibited identical durations in the presence of eggs and descendants, whereas *R. flavipes* exhibited less grooming/antennation in the presence of eggs compared to in the presence of descendants. In social insects, grooming and antennation typically increase the development and survival of offspring (larvae and eggs) by mediating the application of chemical compounds preventing the risks of desiccation or microbial infections (Bulmer, Denier, Velenovsky, & Hamilton, 2012; Fujita, Minamoto, Shimizu, & Abe, 2002; Matsuura et al., 2002), by mechanically removing external pathogens from the cuticles (Rosengaus, Maxmen, Coates, & Traniello, 1998) and by facilitating ecdysis or egg hatching (Whitman & Forschler, 2007). It also allows to directly assess the nestmates needs and also increase social cohesion through exchange of chemical cues (Blomquist & Bagnères, 2010; Hoffmann, Gowin, Hartfelder, & Korb, 2014; Lucas et al., 2018;

Lucas, Pho, Fresneau, & Jallon, 2004, 2005 ; Soroker et al., 2003). The reported differences of grooming/antennation between *R. flavipes* and *R. grassei* suggest a species-specific role of parental care in the success of colony foundation, which might explain the differences in the colony foundation success observed between those two species (Brossette et al., 2017; Leniaud et al., 2011). Further studies should nevertheless be conducted to investigate whether the intrinsic quality of eggs and juveniles differ between *R. flavipes* and *R. grassei*, and whether parental care can mitigate the costs of these intrinsic differences in terms of foundation success.

Our results also reveal that parents exhibited more trophallaxis with their descendants compared to their partners, independent of both the sex of the parent and the species. In subterranean termites such as *R. flavipes* and *R. grassei*, trophallaxis between adults and juveniles typically mediate the transmission of symbionts that are necessary to digest wood (Fujita, Shimizu, & Abe, 2001). These symbionts are present in adults, but generally absent in newborn descendants (Nalepa, Bignell, & Bandi, 2001). Our finding thus suggests that both queens and kings are equally involved into the transmission of symbionts to the descendants in *R. flavipes* and *R. grassei*. Interestingly, our results also shed light on the occurrence of trophallaxis between parents. In addition to its potential role in the homogenization of gut microbial community among parents (Nalepa et al., 2001), this occurrence may also mediate the regular exchange of nutrients (particularly for larvae instars which are unable to feed themselves; Nalepa & Jones, 1991), nestmate recognition cues (Kirchner & Minkley, 2003; Soroker et al., 2003), and/or immune defenses between colony members (Bulmer, Bachelet, Raman, Rosengaus, & Sasisekharan, 2009; Chouvenc, Su, & Robert, 2009; Mirabito & Rosengaus, 2016). Our results also suggest that the nature of the compounds exchanged could be driven by the mode of transfer (proctodeal vs. stomodeal) and/or the donor (queens vs. kings). The absolute quantities of the fluids transferred are unknown; thus, the exact investment of each parent is difficult to assess.

Although body-shaking has been described as a response to disturbance in a large number of termites (Bagnères & Hanus, 2015; Howse, 1965), the modality of its expression remained unclear (Funaro et al., 2018) and was not previously studied in incipient colonies (Rosengaus & Traniello, 1991; Shellman-Reeve, 1990). Our results reveal that body-shaking is a relatively frequent behavior exhibited by both parents at colony foundation and that its expression depends on the species, the sex of the parent and the presence/absence of eggs and descendants. In particular, body-shaking was overall more frequent in *R. grassei* compared to *R. flavipes*, in the presence compared to the absence of descendants, and finally less frequently expressed by queens in the presence compared to absence of eggs. Termites are known to use vibration communication to quickly transmit information thorough the entire colony (Hunt & Richard, 2013). Body-shaking might be part of this communication system and mediates the rapid spread of a social signal. The importance of egg presence on its expression suggests that the body-shaking might be used to transmit information on the reproductive state of the incipient colony to the other member of the colony, either independently or in complement with other potential chemical signals. Indeed, the presence of eggs or descendants could represent a proxy of the reproductive state of the incipient colony that could modulate social organization. More investigations are needed to fully explain the observed interactions between the body-shaking and the presence of eggs or descendants and to explore all factors possibly involved in its expression. Those studies would also allow to explain why this behavior was conserved over several termite species (Bagnères & Hanus, 2015).

Overall, this study sheds light on unbalanced investment into parental care by queens and kings during colony foundation, as well as on species-specific patterns of social interactions between the invasive *R. flavipes* and the native *R. grassei* termites. These findings emphasize that despite lifelong monogamy, the two parents are not equally involved in the measured forms of parental care and instead suggest that kings are specialized in other tasks and/or overall less active. Second, the presence of species-specific patterns of social interactions may provide important insights into our understanding of the invasive success of *R. flavipes* (Brossette et al., 2017; Perdereau et al., 2010; Perdereau, Dedeine, Christidès, Dupont, & Bagnères, 2011). More generally, the sex-specific organization of parental care during termites' colony foundation emphasizes that males could play a central, yet poorly studied role in the evolution and maintenance of the eusocial organization.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHOR CONTRIBUTIONS

The experiment was designed by CL, LB, and AGB. SD, CL, and LB performed the field work. LB, CL, and SD performed the termite crossings and transferred royal couples to the glass cases. LB followed colony development and analyzed the movies. JM, LB, and CL conducted the statistical analyses. JM, CL, LB, and AGB wrote the manuscript. All the authors read and approved the final version of the manuscript.

## DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.vs6md76.

## ORCID

*Joël Meunier* https://orcid.org/0000-0001-6893-2064
*Christophe Lucas* http://orcid.org/0000-0003-2839-7583

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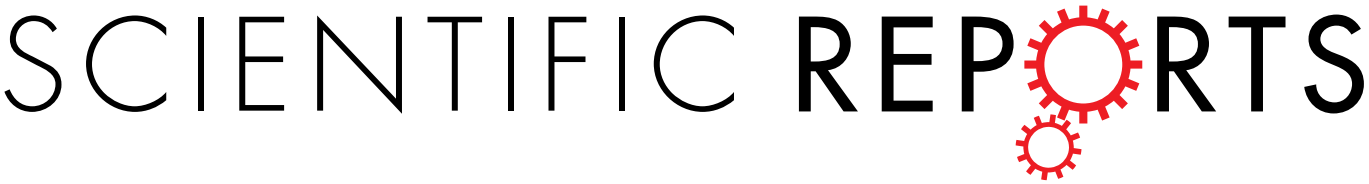

OPEN

# Lock-picks: fungal infection facilitates the intrusion of strangers into ant colonies

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Enikő Csata[1,*], Natalia Timuș[2,*], Magdalena Witek[3,*], Luca Pietro Casacci[4,*], Christophe Lucas[5], Anne-Geneviève Bagnères[5], Anna Sztencel-Jabłonka[3], Francesca Barbero[4], Simona Bonelli[4], László Rákosy[2] & Bálint Markó[1]

**Studies investigating host-parasite systems rarely deal with multispecies interactions, and mostly explore impacts on hosts as individuals. Much less is known about the effects at colony level, when parasitism involves host organisms that form societies. We surveyed the effect of an ectoparasitic fungus, *Rickia wasmannii*, on kin-discrimination abilities of its host ant, *Myrmica scabrinodis*, identifying potential consequences at social level and subsequent changes in colony infiltration success of other organisms. Analyses of cuticular hydrocarbons (CHCs), known to be involved in insects' discrimination processes, revealed variations in chemical profiles correlated with the infection status of the ants, that could not be explained by genetic variation tested by microsatellites. In behavioural assays, fungus-infected workers were less aggressive towards both non-nestmates and unrelated queens, enhancing the probability of polygyny. Likewise, parasitic larvae of *Maculinea* butterflies had a higher chance of adoption by infected colonies. Our study indicates that pathogens can modify host recognition abilities, making the society more prone to accept both conspecific and allospecific organisms.**

In natural ecosystems, relations among different organisms are characterized by a high degree of complexity which makes studies on multispecies systems difficult. For this reason, many investigations focus exclusively on pairwise relationships[1,2], although the outcomes of these interactions vary and depend on the context[3]. For instance, in the classical case of the brood parasitic giant cowbirds (*Scaphidura oryzivora*), where the presence of other parasites, as botflies, may change the cowbird's effect on the host from damaging to beneficial[4]. In the amphipod *Gammarus insensibilis* the parasite manipulation is less efficient when the host is infested with both nematodes and trematodes, compared to being exclusively parasitized by trematodes[5]. Increase in immune function caused by the presence of the mildly antagonistic ectosymbiont *Laboulbenia formicarum* might boost the survival chances of the host ant *Lasius neglectus* exposed to the lethal entomopathogen fungus *Metarhizium brunneum*[6].

Interaction between different organisms could have more complex consequences when social animals, as e.g. ants, are targeted. Changes induced by parasites at the individual level may also be reflected in the social system through the interaction of host individuals. Generally, the structure of social insect colonies, in which many similar individuals live together, makes them attractive for social parasites and various pathogens[7,8]. This is especially true in the case of ants due to the stability of their colonies and their mostly ground-based nesting habits, which make their colonies accessible to a plethora of organisms. Among these, fungi are the most important antagonists ants must constantly deal with, and they can occur inside the colony either as ectoparasites on the host cuticle, endoparasites, or endosymbionts[8,9]. Some fungi are even able to manipulate host ant behaviour to increase their own fitness[10]. The relationships between the hosts and these fungi often have spectacular outcomes, but as these parasitic species have quite a low prevalence[11], their interference with other parasites is hard to study. Certain fungi, like *Rickia wasmannii*[9] an ectoparasitic fungus of *Myrmica* ants, however, are common in some populations

[1]Hungarian Department of Biology and Ecology, Babeș-Bolyai University, Clinicilor 5-7, 400006 Cluj-Napoca, Romania. [2]Department of Taxonomy and Ecology, Babeș-Bolyai University, Clinicilor 5-7, 400006 Cluj-Napoca, Romania. [3]Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679, Warsaw, Poland. [4]Department of Life Sciences and Systems Biology, University of Turin, via Accademia Albertina 13, 10023 Torino, Italy. [5]Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 7261, Université François Rabelais, 37200 Tours, France. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to M.W. (email: mawitus@yahoo.co.uk) or B.M. (email: marko.balint@ubbcluj.ro)

with up to 50% of colonies infected, and with high within-colony prevalence[12]. Therefore, they can serve as easily accessible model organisms to test the effects of parasitic infections both on individuals and societies.

Since one of the main communication channels in ant societies is based on surface chemical compounds, including identification of non-kin conspecifics and nestmates[13], the composition of these chemical substances and the ways in which these substances are perceived may be affected by such epicuticular fungus. Nestmate recognition is a dynamic process, largely based on specific cocktails of cuticular hydrocarbons (CHC) covering the surface of all individuals within a society, the primary biological function of which is to prevent desiccation[7,14]. The template model suggests that each individual uses its own cocktail of CHCs to match against others[15]. Social interactions, such as allogrooming, ensure an exchange of these mixtures between nestmates and give rise to a collective CHC *gestalt* odour[1,15]. The *gestalt* may be relatively homogenous in small monogynous societies, but it may be blurred, to a certain extent, in large polygynous colonies due to higher genetic diversity[15] and the higher number of interactions needed to assemble the full colony[16]. Consequently, a margin of error is tolerated when a worker assesses the CHC of another ant, resulting in a context-dependent and dynamic threshold of similarity at which individuals distinguish nestmates from strangers[1,17]. Therefore, it is plausible to assume that epicuticular fungal infection may affect accuracy of recognition systems in ant colonies, especially because its prevalence within infected ant colonies can reach extreme values. In certain colonies almost 100% of workers are parasitized and fungal thalli may cover the entire body of its host[12]. Recently, it was demonstrated that the fungus *Rickia wasmannii* not only reduces the lifespan of infected ant workers[18,19], but also causes subtle changes in the host's behaviour by increasing worker allogrooming frequency, which may enhance parasite transmission[19]. As *Myrmica* ants interact with several other invertebrates and host diverse social parasites[20], fungus-induced behavioural changes as well as altered nestmate discrimination abilities could shape associations of *Myrmica* ants with other organisms. One example could be the adoption of new queens, since many *Myrmica* ants display facultative polygyny, as a consequence of which foreign queens might be adopted[21].

Finally, changes in ant chemical cues become essential in the case of the socially parasitic larvae of *Maculinea* butterflies, which infiltrate the ant colony by breaking the nestmate recognition code[22–24]. Various *Maculinea* species use different feeding strategies inside the host colony, preying directly on the ant brood (namely predatory) or being fed by throphallaxis (called cuckoo), and they also exhibit different levels of chemical adaptations to infiltrate and integrate into the ant colony[20,23,25]. During the adoption, cuckoo larvae, as *M. alcon*, are already chemically similar to their host ants[22,23,26], while predatory larvae, such as *M. teleius*, possess a less sophisticated chemical mimicry that they have to compensate for with complex behaviour[27].

The above multipartite system, involving distantly related organisms ranging from fungus to ants, is a perfect model for the study of how the presence of one partner could alter the infiltration chances of the others, which in turn potentially has consequences for the fitness of all the interacting entities, and for the society as a whole.

The main aim of our work was to assess the effect of *Rickia wasmannii* infection on *Myrmica scabrinodis* host ant behaviour and interaction with other organisms. Specifically, we investigated the differences between infected and uninfected ants (i) in CHC profiles, while exploring the (ii) genetic and social structure of colonies, and the differences (iii) in kin-discrimination ability towards non-nestmates and unrelated queens, and (iv) in adoption rate of two socially parasitic *Maculinea* species, *M. alcon* and *M. teleius*, having diverse feeding strategies, cuckoo and predatory, respectively.

## Results

**The effects of fungal infection on the CHC profile of ants.** *M. scabrinodis* possesses complex CHC blends that include 37 identified hydrocarbons; these are homologous series of linear alkanes, methyl-branched alkanes and linear alkenes between $C_{21}$ and $C_{31}$ (Table 1). Both infected and uninfected workers shared the same number of compounds but relative proportions and concentrations changed according to infection and age (Figs 1 and 2, Table S1).

*a. Quantitative differences of hydrocarbon composition.* We found significant differences in the overall hydrocarbon abundance according to infection status and age of workers (LMM: $F_{3,63} = 13.31$, $p < 0.001$; Fig. 1). A significant reduction was detected in young infected workers compared to uninfected ones, but no difference was found in the case of old individuals (Fig. 1A, Table S1). Generally, the total abundance was significantly lower for old individuals compared to young ones (Fig. 1A, Table S1).

When considering the three hydrocarbon classes separately, the infection status did not significantly modify the abundance of linear alkanes (LMM: $F_{1,63} = 0.69$, $p = 0.408$), only differences between the two age classes were revealed (Fig. 1B, Table S1). For methyl-branched alkanes, a significant decrease was detected for individuals coming from infected colonies (LMM infection: $F_{1,63} = 5.36$, $p = 0.024$; Fig. 1C), paired with the already observed decrease in old individuals (LMM age: $F_{1,63} = 32.84$, $p < 0.001$). Pairwise comparisons resulted in significant differences only in young workers (Fig. 1C, Table S1). Abundance of the alkenes was similar to that of the alkanes. It was also not influenced by fungal infection (LMM: $F_{1,63} = 1.11$, $p = 0.297$; Fig. 1D), but it was significantly influenced by age (LMM: $F_{1,63} = 28.04$, $p < 0.001$). Pairwise comparisons did not result in any significant differences either in young or in old workers (Fig. 1D, Table S1).

The relative proportion of linear alkanes (Fig. 2) significantly increased both in the chemical profile of young and old infected workers compared to their uninfected counterparts (Table S1). However, we detected a decrease of methyl-branched alkanes in the chemical profile of workers with age and infection (LMM: $F_{3,65} = 10.66$, $p < 0.001$) due to significant differences only in old individuals. Differences between age classes irrespective of infection status were also significant. For alkenes, no difference was detected either according to infection status (LMM: $F_{1,65} = 0.29$, $p = 0.596$) or age classes (LMM: $F_{1,65} = 0.08$, $p = 0.775$).

| Peak | Hydrocarbon | Relative proportion (±SEM) | F | Infection effect | | Age effect | |
|---|---|---|---|---|---|---|---|
| | | | | Young uninf vs. inf | Old uninf vs. inf | Uninfected young vs. old | Infected young vs. old |
| 1 | *n*-C21 | 0.65 (±0.41) | 1.80 | — | — | — | — |
| 2 | 3-MeC21 | 0.60 (±0.34) | 0.32 | — | — | — | — |
| 3 | *n*-C22 | 0.71 (±0.27) | 0.64 | — | — | — | — |
| 4 | 3-MeC22 | 0.58 (±0.22) | 0.50 | — | — | — | — |
| 5 | X-C23:1 | 1.09 (±0.21) | 7.84*** | −0.87 | 1.20 | 0.75 | ↓4.79*** |
| 6 | *n*-C23 | 12.71 (±1.81) | 12.07*** | ↑ −2.86** | ↑ −3.61*** | ↑ −2.80** | ↑ −4.05*** |
| 7 | 7-MeC23 | 0.14 (±0.10) | 0.14 | — | — | — | — |
| 8 | 5-MeC23 | 0.06 (±0.03) | 2.85* | −0.89 | −0.67 | ↑ −2.23* | −1.70 |
| 9 | 3-MeC23 | 15.21 (±1.21) | 10.23*** | ↓2.28* | ↓2.07* | ↓3.69*** | ↓3.25** |
| 10 | X-C24:1 | 0.29 (±0.11) | 0.57 | — | — | — | — |
| 11 | X'-C24:1 | 0.02 (±0.03) | 0.97 | — | — | — | — |
| 12 | *n*-C24 | 1.12 (±0.24) | 2.70* | ↑ −2.49* | ↑ −2.25* | 0.41 | 0.71 |
| 13 | 8-MeC24 | 0.51 (±0.24) | 1.64 | — | — | — | — |
| 14 | X,Y-C25:2 | 0.11 (±0.05) | 1.09 | — | — | — | — |
| 15 | 4-MeC24 | 16.06 (±4.65) | 4.66** | 0.51 | 1.41 | 0.99 | ↓3.77*** |
| 16 | X',Y'-C25:2 | 35.17 (±3.95) | 0.75 | — | — | — | — |
| 17 | X-C25:1 | 2.05 (±0.42) | 0.52 | — | — | — | — |
| 18 | X'-C25:1 | 0.13 (±0.06) | 2.08 | — | — | — | — |
| 19 | *n*-C25 | 6.44 (±0.82) | 8.16*** | −1.69 | −1.93 | ↑ −3.092** | ↑ −3.26** |
| 20 | 5-MeC25 | 0.62 (±0.32) | 18.92*** | −1.69 | −0.68 | ↓3.00** | ↓6.81*** |
| 21 | 3-MeC25 | 1.94 (±0.41) | 4.76** | 0.80 | 1.04 | ↓2.36* | ↓2.79** |
| 22 | 5,17 di-MeC25 | 0.11 (±0.06) | 1.64 | — | — | — | — |
| 23 | *n*-C26 | 0.12 (±0.05) | 0.58 | — | — | — | — |
| 24 | 3,9-diMeC25 | 0.09 (±0.05) | 0.15 | — | — | — | — |
| 25 | X,Y-C27:2 | 0.08 (±0.07) | 2.61 | — | — | — | — |
| 26 | X-C27:1 | 0.10 (±0.04) | 1.64 | — | — | — | — |
| 27 | X'-C27:1 | 0.85 (±0.20) | 1.35 | — | — | — | — |
| 28 | *n*-C27 | 0.57 (±0.13) | 5.23** | 1.58 | 1.54 | ↑ −2.53* | ↑ −2.43* |
| 29 | C28 + unknown | 0.03 (±0.01) | 3.17* | 0.13 | −2.62* | −1.61 | −0.50 |
| 30 | X-C29:1 | 0.43 (±0.26) | 3.86* | 0.88 | 1.29 | ↑ −2.65** | −1.77 |
| 31 | *n*-C29 | 0.49 (±0.19) | 11.87*** | 0.99 | 1.39 | ↑ −4.55*** | ↑ −3.62*** |
| 32 | 15-, 13-, 11-MeC29 | 0.15 (±0.10) | 2.02 | — | — | — | — |
| 33 | 5,17-diMeC29 | 0.14 (±0.11) | 2.76* | −0.46 | 0.16 | 0.81 | ↓2.75** |
| 34 | C30 + unknown | 0.02 (±0.01) | 0.78 | — | — | — | — |
| 35 | X-C31:1 | 0.40 (±0.24) | 4.47** | 0.93 | 1.75 | ↑ −2.92** | −1.58 |
| 36 | X'-C31:1 | 0.05 (±0.03) | 2.78* | 1.06 | 1.06 | −1.83 | 1.06 |
| 37 | *n*-C31 | 0.15 (±0.09) | 8.61*** | 0.49 | 0.91 | ↑ −3.97*** | ↑−3.07** |

**Table 1. Cuticular hydrocarbons of *Myrmica scabrinodis* with their relative proportion (±SEM) depending on their infection status (infected/uninfected) or their age classes (young/old).** *t*-values are represented when a significant difference was observed, with "↓" and "↑" that refer to decrease or increase of the examined CHC between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

*b. Principal component analyses of the hydrocarbon composition.* When we performed the PCA on the abundances of the CHCs, only the analysis on the alkenes yielded a component which was significantly explained by infection and also by age. In this case, five alkenes showed the highest component loadings (Fig. 3; X,Y-C25:2; X',Y'-C25:2; X,Y-C27:2; X-C27:1; X'-C27:1), and all showed a significant reduction in young infected workers compared to their uninfected counterparts (LMM: $t \leq 3.87$, $p < 0.041$). In the case of linear alkanes and the methyl-branched alkanes, only age proved to be an explanatory factor (Table 2).

On the basis of the PCA performed on the relative proportions of *n*-alkanes, only for PC2 the best LMM model included the factor infection besides age (Table 2), while PC1 was influenced only by the age of the workers. Among the three peaks with high loadings on the PC2 (Table 2) for which infection proved to have a significant effect, two linear alkanes (*n*-C23 and *n*-C24) had significantly higher relative proportions in infected colonies, and this effect was significant for both young and old workers (LMM: *n*-C23 $t \leq -2.86$, $p < 0.006$; *n*-C24 $t \leq -2.25$, $p < 0.028$; Fig. 3). In uninfected and infected colonies, changes in the third compound, *n*-C25, were only connected with the age of workers (LMM: $t \leq -3.09$, $p \leq 0.003$).

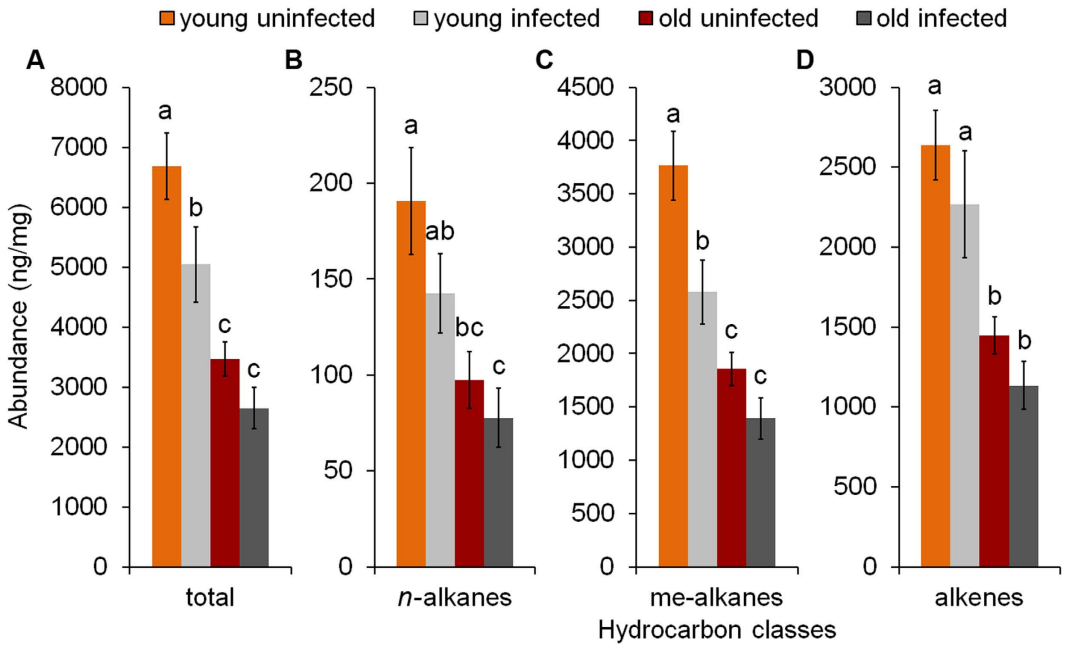


**Figure 1.** Abundance (±SE) of the CHC profile – (**A**) total, (**B**) alkanes, (**C**) methyl-branched alkanes and (**D**) alkenes – extracted from the body surface of young and old *M. scabrinodis* workers from uninfected and infected colonies. Bars with different letters are statistically different according to LMM pairwise comparisons.

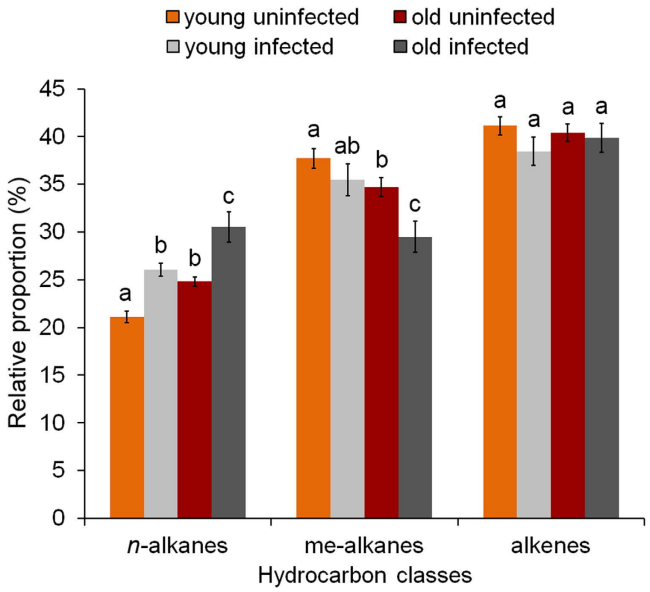


**Figure 2. Relative proportions (±SE) of different CHC classes (alkanes, branched alkanes and alkenes) extracted from the body surface of young and old *M. scabrinodis* workers from uninfected and infected colonies.** Bars with different letters are statistically different according to LMM pairwise comparisons.

In the case of methyl-branched hydrocarbons, the PCA returned three components that were significantly explained by the factors tested. The best model for PC3 was explained by both infection and age, while PC1 and PC2 were only explained by age (Table 2). Only the 3-MeC23 showed a significant decrease (Fig. 3) in the relative proportions of the infected colonies for both young (LMM: $t = -2.28$, $p = 0.026$) and old (LMM: $t = -2.07$, $p = 0.043$) workers.

The PCA on alkenes resulted in three PCs significantly explained by age only (Table 2).

When considering the relative proportion of alkanes, we found variations in the relative dispersion around the centroids for the four worker groups ($F = 3.61$, $p = 0.030$; Fig. 4A). A significant increase of variability in the CHC profile was observed for the old workers depending on their infection status (mean Euclidean distance: old UI = 0.817 vs. old I = 1.378).

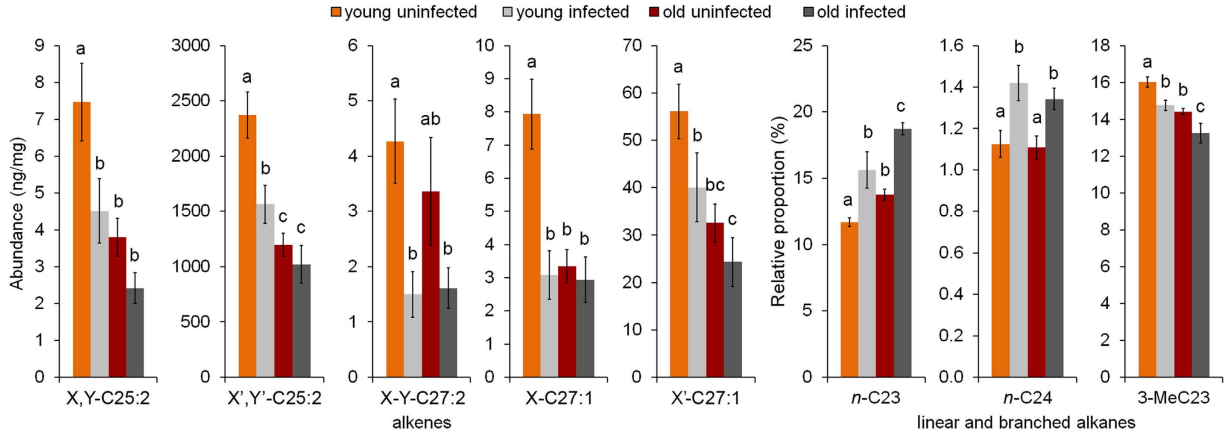


**Figure 3. Mean relative proportions (±SE) of specific CHCs of young and old *M. scabrinodis* workers from infected and uninfected colonies.** These specific CHCs were pointed out by the principal component analyses for which a fungal effect was detected. Bars with different letters are statistically different according to LMM pairwise comparisons.

| | Components (% variance) | Age | Infection | Compounds with component loadings ≥0.6 |
|---|---|---|---|---|
| **Abundance** | | | | |
| Linear alkanes | PC1 (77%) | 6.51* | 3.84(*) | *n*-C21, *n*-C22, *n*-C23, *n*-C24, *n*-C25, *n*-C26, *n*-C27, *n*-C29, *n*-C31 |
| Methyl-branched alkanes | PC2 (31%) | 30.51*** | 3.81(*) | 3-MeC23, 4-MeC24, 5-MeC25, 3-MeC25 |
| Linear alkenes | PC1 (57%) | 9.49** | 11.09*** | X-C23:1, X-C24:1, X,Y-C25:2, X',Y'-C25:2, X-C25:1, X,Y-C27:2, X-C27:1, X'-C27:1, X-C29:1, X-C31:1, X'-C31:1 |
| **Relative proportion** | | | | |
| Linear alkanes | PC1 (33%) | 21.37*** | — | *n*-C26, *n*-C27, *n*-C29, *n*-C31 |
| | PC2 (24%) | 14.59*** | 11.94*** | *n*-C23, *n*-C24, *n*-C25 |
| Methyl-branched alkanes | PC1 (30% | 9.58** | — | 3-MeC21, 3-MeC22, 7-MeC23, 5-MeC23, 8-MeC24 |
| | PC2 (28%) | 12.63*** | — | 5-MeC25, 3,9-diMeC25, 15-, 13-, 11-MeC29, 5,17-diMeC29 |
| | PC3 (12%) | 28.76*** | 8.85** | 3-MeC23, 4-MeC24, 3-MeC25 |
| Linear alkenes | PC1 (25%) | 8.27** | — | X'-C27:1, X-C29:1, X-C31:1, X'-C31:1 |
| | PC3 (13%) | 6.87* | — | X'-C24:1, X'-C25:1 |
| | PC4 (11%) | 15.91*** | — | X-C23:1, X,Y-C25:2 |

**Table 2. Results of the LMM models on the principal components obtained by PCA analyses of the abundances and the relative proportions of CHCs.** Only PCs explained by age, infection or both factors (retained by the best LMM models) are shown. F-values of the infections status and the age class are reported, as well as the variance explained by each PC. The CHCs whose component loadings resulted to be higher than 0.6 are also listed. (*)$p = 0.055$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The methyl-branched alkanes also showed significant differences in the relative dispersion of the samples ($F = 4.53$, $p = 0.010$; Fig. 4B). The fungal effect on the variability of methyl-branched alkanes was clear when comparing infected and uninfected workers regardless of their age (mean Euclidean distance: UI = 0.86 vs. I = 1.33) ($F = 7.69$, $p = 0.010$; Fig. 4B). These differences were only marginally significant (Fig. S2) when considering separate age classes both for young (mean Euclidean distance: young UI = 0.64 vs. young I = 1.16) and old workers (mean Euclidean distance: old UI = 0.88 vs. old I = 1.37).

This analysis was not performed for alkenes, since infection did not significantly affect either their abundance or their relative proportion.

**Colony structure.** The structure of the 18 assessed colonies was highly variable with regards to both worker number (mean no. of workers = 1298.89 ± 1104.03) and queen number (mean = 10.44 ± 16.39). The GLM analysis showed that larger colonies contained more queens (GLM: $z = 11.37$, $p < 0.001$), while also infection had a significant, positive effect on the degree of polygyny (GLM: $z = 5.013$, $p < 0.001$; Fig. S3).

In the case of the 11 colonies genotyped we found significant heterozygosity excess, after Bonferroni correction, for all loci. With the exception of 3 colonies, the HWE hypothesis was rejected, but significant heterozygosity excess was found in 8 out of 11 colonies. Stuttering and possible null alleles were not detected for any of the loci

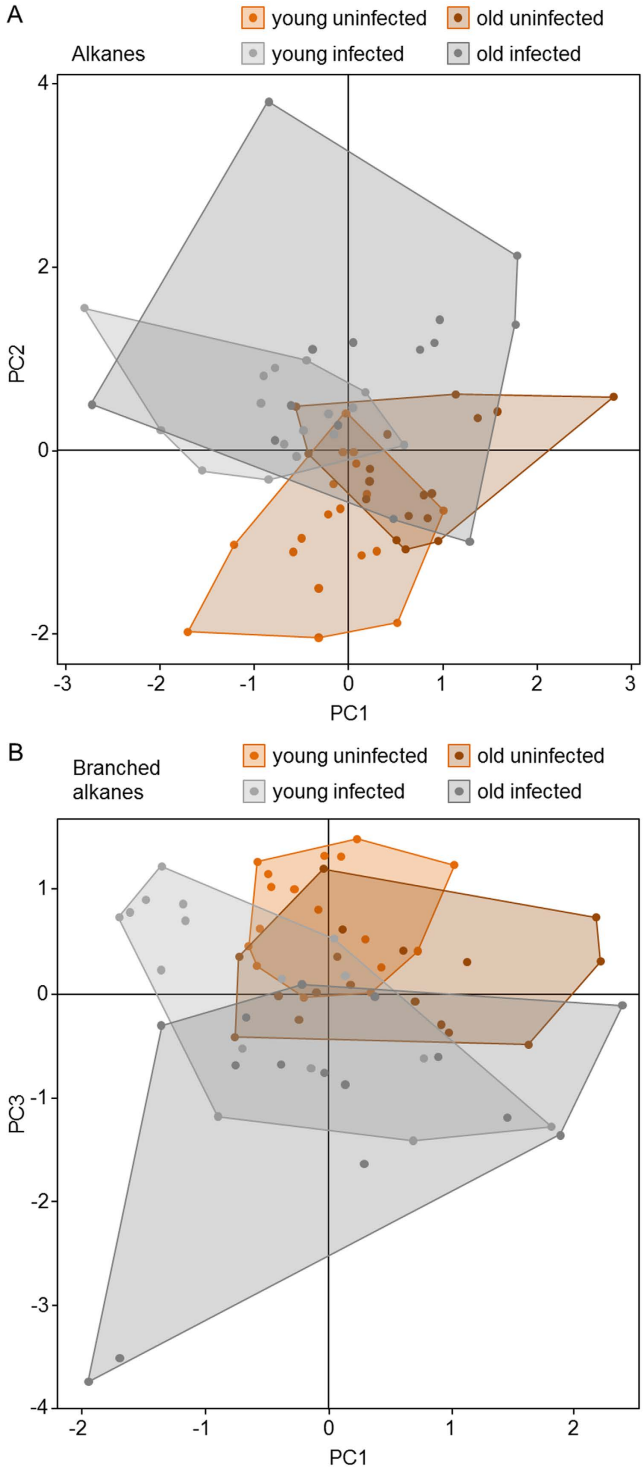


**Figure 4.** Principal component analysis plots of the CHCs extracted from young and old *M. scabrinodis* workers from uninfected and infected colonies: (**A**) linear alkanes (based on the first and second principal components), and (**B**) methyl-branched alkanes (based on the first and third principal components).

with the exception of the MP67 locus. The genotypes of this locus were double checked. However, due to almost binomial character of this locus and the general lack of HWE in our data we decided not to correct for possible null allele effect[28].

Two parameters concerning the genetic structure of the colonies, namely the mean fixation index ($F_{ST}$) and mean relatedness (Rel), did not differ significantly between infected and uninfected colonies. The $F_{ST}$ was 0.13 for infected and 0.21 for uninfected colonies; (two-sided 10000 permutation test: $p = 0.361$), whereas Rel was 0.26 for infected and 0.43 for uninfected colonies (two-sided 10000 permutation test: $p = 0.317$). The pairwise $F_{ST}$ values

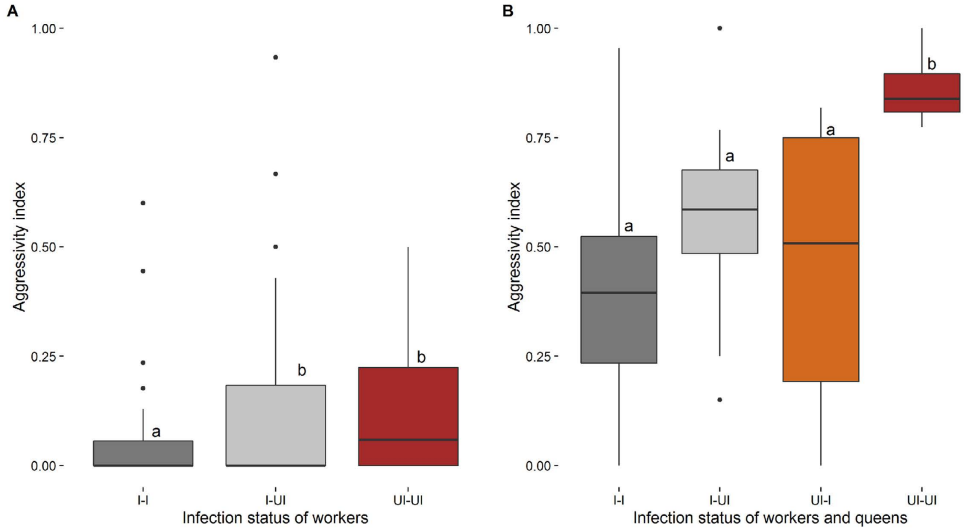


**Figure 5.** Aggression indices (**A**) between *M. scabrinodis* workers of different infection status, and (**B**) between *M. scabrinodis* workers (w) and queens (q) of different infection status: I – infected by *R. wasmannii*, UI – uninfected (median, quartiles, min-max values).

between colonies ranged from moderate to high (from 0.05 to 0.4), whereas the mean pairwise within colony relatedness value ranged from 0.1 to 0.7 in the studied colonies.

**The effects of fungal infection on ant behaviour.** *a. Aggression assays between ant workers.* No aggressive interactions occurred in the control experiments in which aggression between nestmates was tested; only neutral or positive behaviours were recorded. Aggression between non-nestmates was significantly lower when both ant workers were infected (I–I) in comparison to assays in which only one of the workers was infected (I–UI) or both were uninfected (UI–UI) (GLMM: $z \geq 2.47$, $p \leq 0.034$; Fig. 5A). There was no significant difference between the two latter groups (I–UI vs. UI–UI) (GLMM: $z = 0.64$, $p = 0.519$).

*b. Acceptance of foreign ant queens.* The aggression of workers towards foreign queens was significantly higher when both workers and queen came from uninfected colonies (UI–UI) compared to other trials (GLMM: $z \geq 3.27$, $p < 0.001$; Fig. 5B). No differences were found among groups out of which at least one partner (queen and/or workers) was infected by the fungus (I–UI, UI–I, and I–I groups) (GLMM: $z \leq 1.34$, $p = 0.178$).

When the aggression of queens towards workers was analysed, the UI–UI combinations also displayed significantly higher aggression than I–I combinations (GLMM: $z = 2.72$, $p = 0.039$), but there were no differences between groups in which at least one partner was infected (I–UI and UI–I groups) (GLMM: $z \leq 1.10$, $p = 0.267$).

*c. Adoption of Maculinea caterpillars.* Altogether 32 caterpillars were adopted during the experiments, while the rest were rejected (9) or undiscovered (22). Infected colonies adopted *Maculinea* larvae at a significantly higher proportion (Fisher's exact test: $p = 0.042$). Significant changes (e.g., higher rejection by uninfected) were recorded separately in *M. teleius* (Fisher's exact test: $p = 0.050$), whereas only marginally significant differences were found in *M. alcon* (Fisher's exact test: $p = 0.056$) (Fig. S4). Regardless of the status of the ant colony, the adoption success of *M. alcon* was significantly higher than that of *M. teleius* (Fisher's exact test: $p < 0.001$).

Infected ants adopted caterpillars at a significantly higher rate (Cox coeff $= 1.055$, $\chi^2 = 4.63$, $p = 0.031$; Fig. 6). *Maculinea alcon* larvae were adopted at a significantly higher rate than *M. teleius* (Cox coeff $= -1.27$, $\chi^2 = 17.52$, $p < 0.001$). The interaction of the ant infection status with the species of the caterpillar was not significant (Cox coeff $= -0.75$, $\chi^2 = 0.87$, $p = 0.350$).

There were no significant differences in inspection indices of workers discovering the caterpillars according to the ants' infection status (GLMM: $\chi^2 = 0.1$, $p = 0.743$). The inspection indices did not differ between the two *Maculinea* species (GLMM: $\chi^2 = 2.23$, $p = 0.135$), and the interaction between the infection status of the ants and the species of the caterpillar was also not significant (GLMM: $\chi^2 = 0.001$, $p = 0.990$).

## Discussion

Our findings indicated that the cues used for nestmate discrimination in *M. scabrinodis* ants were affected by the infection of the ectoparasitic fungus *R. wasmannii*. The CHC profiles of infected workers showed differences in relative proportions of linear and methyl-branched alkanes, and also higher variability, than in uninfected individuals, probably resulting in reduced foe discrimination abilities. Fungus-infected workers were thus less aggressive towards non-nestmates and unrelated queens. As a consequence, chemical and behavioural variations induced by the fungus strengthen the infiltration chances of 'intruders', making it easier for strangers (both social parasites and foreign queens) to gain acceptance into the colony (Fig. 7). Therefore, fungal infection, through induced behavioural modifications in individual ants, could affect the fitness of the whole society either positively by (i) increasing genetic heterogeneity, and, implicitly, colony lifespan through higher degree of polygyny, and/

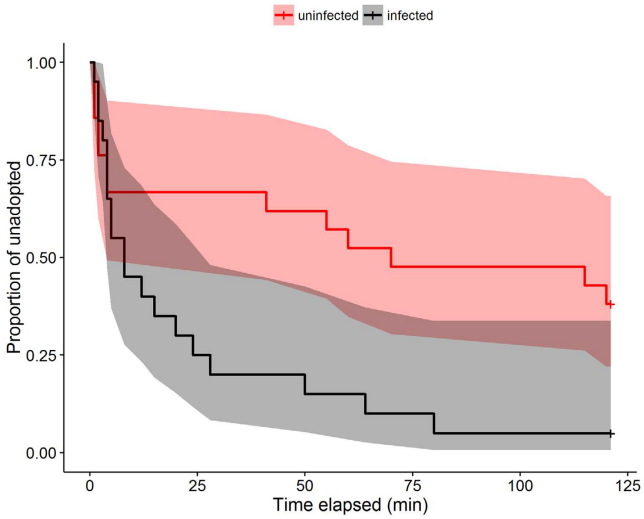


**Figure 6. Estimated functions for Cox regression of the adoption time for *Maculinea* caterpillars by ant colonies of different infection status with the point-wise 95% confidence interval for the corresponding functions.**

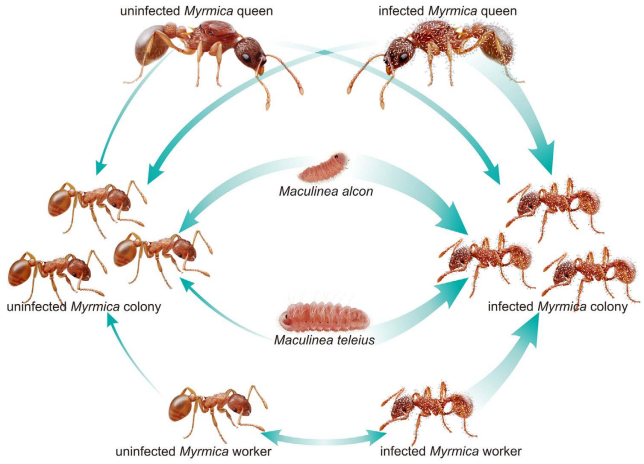


**Figure 7. Summary of the experimental setup and the findings of the study.** The thickness of arrows correlates with the degree of acceptance by ant colonies of different infection status (drawing by Natalia Timuș).

or negatively by (ii) decreasing brood quantity through the higher acceptance rate of socially parasitic *Maculinea* larvae. Some of these changes are consistent with the fungus' need to increase its reproductive and dispersal success, since successful adaptation to hosts with different genetic background, paired with potentially increased colony lifespan, could assist the long-term persistence of the fungus in an invaded host population. In addition, the reduced aggression towards infected queens by uninfected workers could also contribute to the queen's successful penetration in the new host colonies.

In ants, cuticular compounds play several crucial roles, protecting the animal from water loss and acting as signalling cues primarily as part of the nestmate recognition system[1,13]. While uninfected individuals shared a very similar CHC mixture, old infected workers showed much more quantitatively diversified CHC compounds and different proportions of particular linear alkanes and methyl-branched hydrocarbons. According to our findings, these kinds of differences in the CHC profiles cannot be attributed to genetic variation between infected and uninfected colonies, thus the presence or absence of the fungus seems to remain the only plausible explanation.

In general, the presence of fungal parasites can be linked to behavioural, mechanical, biochemical or even to physiological changes in ants[29–32]. Recently, it was demonstrated that *R. wasmannii* has an influence on the physiology of *M. scabrinodis*, and infected ants in particular spent significantly more time consuming water than uninfected ones[18]. Since the primary function of CHCs is to prevent insect desiccation[1,15], a modification in the abundances and mixture of epicuticular hydrocarbons could increase water loss, thus inducing the higher intake of water observed in infected individuals. Nonetheless, the modification of the CHC composition has the most significant impact on ant recognition abilities. We observed a higher variability in relative proportions of linear

and methyl-branched alkanes in old, infected workers, which also correlates with the age-related progress of infection, as old workers are the most infected ones[33]. If we interpret our result in terms of Reeve's[34] adjustable threshold model, in *Myrmica scabrinodis* colonies infected by *R. wasmannii* the chemical template gets broader, inducing the infected workers to move towards a more tolerant overall template. Therefore, more acceptance errors will occur in infected colonies than in uninfected colonies. This is consistent with results obtained from our behavioural observations, according to which the aggression level was interpreted as a proxy for acceptance towards non-nestmates. During encounters between non-nestmates which were infected by *R. wasmannii*, the level of aggression decreased compared to encounters in which at least one partner was uninfected, whereas aggression peaked between uninfected non-nestmate workers. This clearly cannot be attributed to any influence of the infection on the general activity of hosts, as recently demonstrated by Csata *et al.*[33]. Bos *et al.*[35], on the other hand, demonstrated that fungal infection by *Metarhizium brunneum*, which does not modify the CHC profile of hosts, increased the level of aggression towards non-nestmates. One possible explanation for the difference between the outcomes of our study and that of Bos *et al.*[35] lies in the differences in fungus virulence and host specificity. While *M. brunneum* is a virulent and generalist entomopathogen that kills its hosts quickly[8], *R. wasmannii* is confined to *Myrmica* ants, and inflicts only small damages to its hosts[18,19,36]. For *R. wasmannii*, which reproduces by spore transmission among ants, it would be more advantageous to make ants amiable and sociable, thus increasing the frequency of social interactions such as allogrooming, which could efficiently spread the spores. Increased allogrooming indeed occurs more often in *R. wasmannii* infected colonies, as demonstrated by Csata *et al.*[19].

Making the CHC template used by ants to recognize nestmates broader and less specific also causes a lower level of aggression between infected *Myrmica* workers and unrelated queens. These findings imply that infected colonies could be more open for polygyny, as new queens would be accepted more easily regardless of their infection status. Our data on colony structure indicated that, indeed, infected colonies contain more queens than uninfected colonies. We do not have any data on the effect of *R. wasmannii* on queen fecundity and longevity, but we can presume that, as in the case of workers[19], fungus infection may decrease life expectancy of queens as well, resulting in higher queen turnover in infected colonies compared to uninfected ones. With ants, the colony foundation success of individual queens is generally around 2% or less[14]. Paradoxically, for a young queen in a population in which some part of the colonies is infected, fungus infection can consequently be beneficial. Even if some life history parameters may decrease due to fungal infection, the reproduction chances due to being successfully adopted by an unrelated colony can be considerably higher when meeting an infected colony. Furthermore, it seems beneficial for the fungus, as it can spread horizontally through workers inside the same colony, but it can also be transmitted vertically by adoption of young infected queens into existing colonies, coming from the same population or even neighbouring populations. According to Hughes *et al.*[36], we could consider the ant colony rather than the ant individuals as host to *R. wasmannii* where the fungus exploits ant workers and gynes to spread among hosts.

Previous studies also reported modifications in cuticular profiles of organisms in the presence of parasites. In the honey bee/*Varroa* system, a higher production of cuticular hydrocarbons by parasitized compared to unparasitized adult honey bees was observed, while the cuticular profile of parasitized larvae contained higher relative proportions of two unsaturated hydrocarbons, than unparasitized larvae[37]. Modifications in the synthesis/release of 13 CHCs (six *n*-alkanes, five monomethylalkanes, and two dimethylalkanes) were also detected in the CHC profile of the ant *Temnothorax nylanderi* after the infection by the endoparasitic tapeworm *Anomotanoa brevis*, probably explaining the occasional aggressions which the parasitized ants suffer in their own society[38].

Likewise, in our study we observed a clear effect of *R. wasmannii* infection on two consecutive linear alkanes, *n*-C23 and *n*-C24, which were found in higher relative proportions, and a methyl-branched hydrocarbon, the 3-MeC23, which was negatively affected by the infection. While *n*-C24 represents 1–2% on the overall proportion of cuticular hydrocarbons, *n*-C23 and 3-MeC23 contribute to 25–30% of the chemical profile of workers, suggesting a considerable impact of the fungus on the CHC profile. It is worth noting that these changes are already evident in the chemical profile of young workers, suggesting that the fungus may begin to act even if a few thalli are present on the ant cuticle.

*Rickia wasmannii* infection not only influences intraspecific interactions but it also affects *M. scabrinodis*' relationships with other organisms, such as socially parasitic *Maculinea* butterfly larvae, which rely on chemical mimicry to get accepted into the host colony[22,23,26]. If we consider that the chemical profile of newly moulted fourth-instar larvae of all European *Maculinea* species have a simple combination of linear alkanes[22], we can assume that their adoption may be favoured when encountering *M. scabrinodis* infected foragers (which are all old individuals), less aggressive and characterized by a higher proportion of one or more linear alkanes[39]. Moreover, in a recent study[40] *n*-C23 and *n*-C24 were shown to represent common hydrocarbons to all cuticular extracts of both IV instar parasitic larvae of *Maculinea nausithous* and its host ant, *Myrmica rubra*, with *n*-C24 being much more abundant in ant brood extracts and able to promote the first contact of *Maculinea nausithous* with *Myrmica rubra* foragers[40].

According to previous studies[22,23] the quick retrieval of *Maculinea* larvae is supposedly mediated only by the chemical mimicry of surface hydrocarbons on the epicuticle of *Myrmica* workers. Cuckoo larvae, as *Maculinea alcon*, are commonly retrieved in just a few minutes due to higher degree of chemical similarity to their host ants[23]. On the other hand, the retrieval of predatory larvae, such as *M. teleius*, can take much longer[41]. It has been hypothesised that *M. teleius* larvae must exhibit more complex behaviours of adoption, including production of vibroacoustic signals[27] in order to compensate for the lack of a sophisticated chemical mimicry. Nevertheless, infection of the *Myrmica* host ants by *Rickia wasmannii* seems to assist the adoption of *Maculinea* larvae regardless of their species. Moreover, this effect seems to be quite strong for *M. teleius*, for which the rejection rate of the larvae was higher in uninfected colonies whose discrimination abilities were not compromised by the fungus.

It is important to point out that even though the presence of *R. wasmannii* affected the CHC compositions, we also detected changes in the chemistry of ant cuticles which were age-dependent. Old workers (foragers) have higher proportions of *n*-alkanes and lower proportions of branched alkanes than young ones (mostly nurses), similarly to what is known in other ant species, such as *Pogonomyrmex barbatus*[42] and *Formica exsecta*[43].

The scanty knowledge available on the feeding strategy of *R. wasmannii* allows only speculations about the mechanism used by the fungus to modify the ant surface chemistry. However, it is fair to assume that solely by attaching to the cuticle it could change the hydrocarbon profile of its host either by downgrading or by adding components[32,44,45]. On the other hand, the fungus could also hinder non-nestmate recognition by physically blocking the access of the antennae to the surface of the cuticle or, in advanced cases, even by residing on the antenna. Whatever the mechanism, physical or chemical, in infected colonies the intensity of infection is highly variable. Some individuals, mostly old ones, are covered entirely with fungi, while others, mostly young ones, have only a few or no thalli[12]. Therefore, in infected colonies considerable variation can be expected in sensory detection, and for this reason the acceptance thresholds in infected and uninfected colonies may differ.

Our findings indicated that fungal infection can modify intraspecific behaviour of ants and their interactions with other organisms. Thus, the presence of pathogens can change the outcomes of these interactions and influence the fitness of both the host and its guests. Host behavioural modifications are most probably the result of changes in the discrimination abilities of infected ants, although the precise mechanism responsible for this has not yet been investigated. As suggested by our results, the consequences of parasitic relationships in social contexts could be manifold. Whereas usually changes in individual behaviour are considered, as e.g. the extended phenotype syndromes caused by fluke worms[8] and *Ophiocordyceps* fungi[11], we suggest that certain parasites might cause alterations even in the social structure of colonial organisms by making it more susceptible to accept non-kins and other social parasites.

## Materials and Methods

**Study species and site.** Experimental *Myrmica scabrinodis* ant colonies, both infected (I) and uninfected (UI), *M. scabrinodis* queens, and socially parasitic *Maculinea* caterpillars were collected from the same grassland area (46.92N, 23.73E, 410–460 m a.s.l., Romania). The colony-level prevalence of the ectoparasitic fungus *Rickia wasmannii* was more than 50% in the *Myrmica scabrinodis* population, while the within-colony prevalence reached 100% in certain colonies[12]. This fungus, like other Laboulbeniales, has no mycelium and its thallus develops from a bicellular ascospore attached to the outer layer of the host cuticle[46]. The fungal infection status of the ants was assessed in the field and confirmed in the lab using an Olympus SZ51 stereomicroscope.

**Analyses of cuticular hydrocarbon profiles.** Altogether 6 uninfected and 6 infected *M. scabrinodis* colonies were brought to the laboratory for the analysis of the CHC profiles. Since CHCs can change during insect maturation[47] both young and old workers were selected randomly from their colonies on the basis of their cuticular pigmentation[48]. For each colony, 5 workers per age category were pooled into a clean glass vial and their CHCs were solvent-extracted using 200 μl of hexane (Sigma) for 20 min after having been weighed. Three replicates were performed for each age class per colony. The extracts were then stored at −20 °C until analysis. Then, workers were dried at 60 °C for 5 days and their dry mass was weighed individually to the nearest 0.0001 mg with an ultra-microbalance (Sartorius SC2).

Prior to chemical analyses, 800 ng of *n*-eicosane ($n$-$C_{20}$; Sigma E-9752) were added to each extract as an internal standard. Samples were then evaporated under a nitrogen flow before being suspended in a final volume of 20 μl of heptane (Sigma). Two μl of each sample were analysed in an Agilent 7890B gas chromatograph coupled with an Agilent 7000C mass spectrometer using a Gerstel MPS autosampler. The GC was equipped with a capillary column 30 m × 250 μm × 0.25 μm (Zebron ZB-5HT INFERNO) using helium as carrier gas at a flow rate of 1 ml/min. Initial program temperature was 70 °C and ramped at 30 °C/min to 150 °C. It was then increased to 320 °C at a rate of 5 °C/min and held for 10 min at 320 °C. Splitless injection (2 mins) was performed with the injector maintained at 280 °C. Mass spectra were acquired in full scan mode every 0.3 s with a scan lapse of 0.1 s over a range of 0 to 600 amu. Electron impact was setup at 70 eV. Mass spectra were analysed by compiling previous publications and comparing fragmentation patterns[49] with the help of injections of standard series of *n*-alkanes (Fluka, 94234) for calibration. The chromatograms were manually integrated to calculate the area of each peak of interest using the proportion of the sum over the area of all peaks[50]. We also calculated the quantity of CHCs (ng/mg of ant) per worker as a sum of the areas of all the peaks divided by the peak area of the internal standard ($n$-$C_{20}$) and multiplied by 800 (quantities in ng of the internal standard per sample); the resulting value was divided by the weight of the 5-ant sample.

**Colony structure.** The structure of colonies used for CHC analyses (with the exception of 1 uninfected) was examined by using microsatellite loci in order to determine whether there were genetic differences between infected and uninfected colonies that could be responsible for any differences in the CHC profiles. In total, 220 individuals from 11 colonies (20 individuals from each) were genotyped; 6 infected and 5 uninfected colonies. In each case the whole colony was collected from the field and workers and queens were counted. In addition to the colonies sampled for DNA analysis we also counted the workers and queens in 3 infected and 4 uninfected colonies. We isolated genomic DNA from the thorax and legs of workers using the Chelex 100 method[51]. Workers were assayed at eight microsatellite markers: Myrt4[52], MP 67, MP 84[53], Msca 43, Msca 47, Msca 64, Msca 78[54], and Msca1[55]. Two sets of multiplex reactions were used with the forward primers labelled with WellRed Dyes D2, D3 and D4 (multiplex 1 – D2: Msca 43; D3: Msca 64, Msca 78; D4: Msca 47; multiplex 2 – D2: MP 84; D3: Myrt 4, Msca 1; D4: MP 67). The PCRs were performed in a total volume of 10 μl composed of 10 ng of DNA template, 0.2 μM of each primer, 5 μl of Multiplex PCR Master Mix (Qiagen) and water. For PCR amplification, a thermal cycler (Applied Biosystems) was used with the following PCR profile: initial denaturation at 95 °C for 15 min (hot start), 40 cycles of 30 s at 94 °C, 90 s at 63 °C (first multiplex) and 60 °C (second multiplex), 90 s at 72 °C, followed

by a final elongation step at 72 °C for 10 min. PCR products were genotyped on a CEQ 8000 DNA fragment analyser (Beckman Coulter) and genotypes were scored using the fragment analysis software CEQ™.

**Behavioural assays.** Experimental *Myrmica scabrinodis* ant colonies, both infected (I, N = 21) and uninfected (UI, N = 11) were kept in a laboratory in plastic boxes (16 × 10 × 5 cm) with a wet foam brick under controlled conditions (20 °C, 12 L:12D cycle) with a daily food mixture of sugar and proteins[56].

*a. Aggression assays between workers.* Aggressiveness of workers was tested after four days of acclimatization under laboratory conditions. Ants from 21 infected and 11 uninfected colonies were used in this experiment. As a control, workers from four infected (I) and four uninfected (UI) colonies were used to test the aggressiveness at the intracolonial level. Six repetitions were performed for each tested colony, thus altogether 48 tests were carried out. Only old workers were selected for the purpose of the experiment, since they are mostly located in the periphery of the nest, and are the only which forage outside, thus having the chance of coming across potential intruders, and constituting the first line of colony defence. Therefore, by default their non-kin discrimination ability should be more formed. Selection of old workers was carried out on their within-nest location (in the arena), and on the basis of their cuticular pigmentation, which is traditionally used for age class estimation in *Myrmica* workers[48]. All ants coming from one particular colony were marked on the thorax with the same colour of fast-drying acrylic paint (Paint Royal Talens: ArtCreation essentials). To allow the ants to recover from marking, they were kept in Petri dishes for 30 minutes. Aggression assays were carried out with the use of two connected transparent plastic tubes (3 cm long), which were first separated by a small piece of red plastic foil. A single *Myrmica* worker was placed in each tube. Ants were allowed to acclimatize for one minute before the red plastic foil was removed. After each assay, the plastic tubes were rinsed with ethylic alcohol (98°), and with water, then wiped, and left to dry for at least 20 minutes. Assays were carried out with both infected and uninfected *Myrmica* workers with three replications per treatment (new individuals were used for each test). Altogether, 111 aggression tests were performed in a randomized order with 3 combinations: infected vs. infected (I–I, N = 39), infected vs. uninfected (I–UI, N = 57) and uninfected vs. uninfected (UI–UI, N = 15). The observations started from the first contact of the workers and lasted for three minutes. All interactions were recorded and categorized as: (1) allogrooming, (2) antennation, (3) frightened off, (4) mandible gaping, (5) biting, (6) dragging, and (7) stinging. Allogrooming was considered as positive event, antennation was considered neutral, and the last five behaviours were considered aggressive. An aggression index (AI) for each encounter was calculated as: AI = the total number of aggressive behaviours divided by the total number of interactions[57].

*b. Queen acceptance assays.* The queen acceptance experiments were carried out concurrently with aggression assays. Twenty-four old workers were collected per colony and put into a small plastic box (5 × 5 × 4 cm) with coated rims using paraffin to prevent ants from escaping. A wet foam brick was added to serve as a nest. Workers were kept in boxes for 24 hours prior to the experiments. Functional, old queens were separated from their original colonies five hours before being introduced into new colonies. During this time, they were kept in Petri dishes with a wet sponge. A single queen was introduced into each experimental colony. The queen was placed in a plastic tube connected to the plastic nest box from where she could freely enter the arena. The observations began with the first contact between the queen and the workers and lasted 15 minutes. All behavioural events, displayed separately by workers and queens, were recorded, and an aggression index (AI) as described above was calculated. The following combinations were tested according to the infection status of the workers and queens: acceptance of infected queens by (a) 10 infected ($I_{workers} - I_{queen}$) and (b) 10 uninfected ($UI_{workers} - I_{queen}$) colonies, and acceptance of uninfected queens by (c) 9 infected ($I_{workers} - UI_{queen}$) and (d) 8 uninfected ($UI_{workers} - UI_{queen}$) colonies.

*c. Maculinea adoption assays.* To assess whether there are differences in the adoption rate of *Maculinea* caterpillars between infected and uninfected *Myrmica scabrinodis* colonies, we chose caterpillars of two co-occurring *Maculinea* species *M. alcon* (the '*pneumonanthe*' ecotype) and *M. teleius*; two species with different feeding strategies and for which *Myrmica scabrinodis* is a primary host of the population under study[58,59]. *Maculinea alcon* and *M. teleius* larvae of pre-adoption stage were obtained by collecting their host plants, *Gentiana pneumonanthe* and *Sanguisorba officinalis*, respectively. Shoots were collected from the end of June until the middle of August and kept in the laboratory in water for 2–3 weeks until the caterpillars reached their pre-adoption maturity and fell off the host plant. Altogether, 63 caterpillars of *Maculinea* forms were used during the experiments: 31 *M. alcon*, and 32 *M. teleius* larvae. Thirteen infected and 9 uninfected *Myrmica scabrinodis* colonies were collected from the field. No *Maculinea* larvae were present in these colonies. They were then divided into 31 and 32 queen-less sub-colonies, each containing 50 workers in addition to 10–15 ant larvae and pupae. Ants were kept in transparent plastic boxes (16 × 10 × 5 cm) under the abovementioned laboratory conditions for at least a week prior the experiment.

*Maculinea* caterpillars were divided between infected and uninfected ant sub-colonies as follows: 15 vs. 16 for *M. alcon*, and 16 vs. 16 for *M. teleius* larvae, respectively. A single fourth-instar larva of *Maculinea* was presented to each ant sub-colony. The caterpillar was placed into the plastic box at the position opposite to the ant shelter. The time elapsed from the introduction of the caterpillar until its discovery by ants and its transportation to the shelter was recorded on a minute by minute basis for 120 minutes. Caterpillars were considered adopted when they were introduced into the nest by workers, whilst those discovered but not introduced into the nest were regarded as rejected. The behaviour of ants toward the caterpillar was also recorded and categorized as follows: (1) antennation, (2) licking the larval secretions and (3) picking up the caterpillar. Antennation is considered neutral behaviour in ants that is also connected to foe discrimination. Therefore, an inspection index was calculated for each caterpillar separately as: the number of antennation events divided by the sum of all behavioural events recorded between ants and caterpillar.

**Statistics.** *a. Cuticular hydrocarbon profiles.* The differences among the four groups (young I, young UI, old I, old UI) with regards to the total proportion of linear alkanes, methyl branched alkanes, and alkenes were tested with a linear mixed model (LMM, maximum likelihood fit) with colony ID as a random factor. The same analysis was carried out separately on the concentrations (ng/mg) of the linear alkanes, methyl-branched alkanes, alkenes, and the overall CHC profile.

Considering that the 3 classes of CHCs that compose the cuticular profile of *M. scabrinodis* workers may convey different information, we reduced the number of variables by performing separate Principal Component Analyses (PCA based on correlations, varimax rotation) on linear alkanes (9 variables), methyl-branched alkanes (13), and alkenes (13). We excluded 2 compounds (*n*-C28 + unknown and *n*-C30 + unknown) for which the CHC class was not clearly identified. For each analysis, we retained the principal components with eigenvalues $\geq$1. LMMs (maximum likelihood fit) were used to investigate the effect of age and infection, and their interaction, on each retained principal component, including the colony ID as a random factor. Best models were selected based on the lowest Akaike's information criterion (AIC) values. The same procedure was applied to both transformed relative proportions and overall concentrations using $\ln(x+1)$ formula. From the principal components for which an effect of the infection was discovered, we selected CHC peaks with correlation coefficients ranging between 0.6 and 1 (absolute values). We then tested whether the proportions and concentrations of those CHC peaks were influenced by infection and by age using LMMs (maximum likelihood fit), including the colony ID as a random factor. To compare the within-group data dispersion, non-Euclidean distances between objects and group centroids were handled by reducing the original distances to principal coordinates. To test for significance, we used F-tests based on sequential sums of squares obtained from permutations of the principal component scores (99 permutations). A set of confidence intervals on the differences among the mean distance-to-centroid of the levels of the grouping factor with the specified family-wise probability of coverage were created. The intervals were based on the Studentized range statistic, Tukey's 'Honest Significant Difference' method.

LMMs and PCA were carried out with SPSS 21 (IBM). Differences in dispersion of CHC profiles between I and UI ants were analyzed with R v. 3.2.5 (R Development Core Team) using the *betadisper* and *TukeyHSD.betadisper* functions in the *vegan* package[60], a multivariate analogous of the Levene's test for comparing group variances[61]. The graphs were carried out using the *ggplot2* R package[62].

*b. Colony structure.* The effect of colony size (no. of workers) and fungal infection on the number of queens was analysed using a generalized linear model approach (GLM, Poisson error, maximum likelihood fit) with number of workers as input variable and fungal infection as fixed factor.

The genotyped data were checked for amplification errors and presence of null alleles using Micro-checker Version 2.2.3[63]. The conformance with Hardy-Weinberg expectations (HWE) was calculated in Genepop on the Web (v.4.2)[64] using an exact probability test (Markov chain parameters: 10000 dememorizations, 100 batches, 1000 iterations per batch) with Bonferroni correction, followed by a heterozygosity excess test with same parameters due to found deviation from HWE. Fixation index ($F_{ST}$) and mean pairwise within colony relatedness (Rel; mean across all loci and colony with correction for sample size) were calculated for groups (infected and uninfected colonies), and tested regarding their differences with a two-sided 1000 permutation test in FSTAT (v.2.9.3)[65]. We calculated mean pairwise within colony relatedness for each colony according to the algorithm of Queller and Goodnight[66] in Kingroup[67], with allele frequency calculated from the whole dataset, to confirm the results obtained.

*c. Behavioural assays.* Worker-worker and worker-queen aggression indices were analysed using a generalized linear mixed model approach (GLMM, binomial error, maximum likelihood fit) with the colony ID as a random factor and with different fixed factors: I–I, I–UI and UI–UI for the worker aggression experiment and $I_w$–$I_q$, $I_w$–$UI_q$, $I_q$–$UI_w$, $UI_w$–$UI_q$ for worker-queen interactions. Inspection indexes resulting from the *Maculinea* adoption experiments were analysed with GLMM (binomial error, maximum likelihood fit) with the ant colony ID as a random factor and with different fixed factors: the infection status of the workers, the caterpillar species and their interactions. Only caterpillars discovered within 120 minutes were considered if at least one ant-caterpillar interaction occurred (N = 41). For the *Maculinea* adoption success the proportion of adopted, rejected and undiscovered caterpillars was compared with Fisher's exact test between I and UI and colonies, and then separately for each species.

*Maculinea* adoption rates were analysed with a Cox regression approach with mixed effects (Efron approximation, N = 63 caterpillars). The time elapsed until the adoption of the caterpillar was included as a dependent variable, whereas the infection status of the ant colony and the butterfly species and their interaction were included as dummy variables. Initial ant colony ID was included as a random factor to handle dependencies.

All statistics were performed using R (v. 3.1.2, R Development Core Team). For Cox regression analyses the *coxme* function in the *coxme* package[68] was used, while GLM and GLMMs were performed using *glm*, *glmer* and *glmer.nb* functions in the *lme4* package[69]. *Relevel* function was used in order to carry out sequential comparisons among factor levels when performing Cox regressions and GLMM analyses. Table-wide sequential Bonferroni-Holm correction revealed the exact significance levels among different factor levels in these cases, and also in the case of pairwise Fisher's exact tests. The graphs were carried out using the *ggplot2* R package[62].

**Data availability.** Data available from the Dryad Digital Repository: http://dx.doi.org/ 10.5061/dryad.dt226[70].

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## Author Contributions

B.M., E.C., N.T., M.W., L.P.C., S.B., L.R. designed the research. B.M., E.C., N.T., and M.W. collected the samples and conducted the behavioural assays. L.P.C., C.L., A.-G.B., F.B. carried out the CHC analysis. M.W., A.S.-J., E.C. conducted the genetic analysis. E.C., N.T., L.P.C., A.S.-J. and B.M. performed statistical analyses. The manuscript was written by E.C., N.T., M.W., L.P.C., F.B. and B.M. Further on all authors contributed to revisions of the manuscript and analyses.

## Additional Information

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# Sequential Learning of Pheromonal Cues Modulates Memory Consolidation in Trainer-Specific Associative Courtship Conditioning

**Aki Ejima,[1] Benjamin P.C. Smith,[2] Christophe Lucas,[2] Joel D. Levine,[2] and Leslie C. Griffith[1,*]**
[1]Department of Biology and
Volen Center for Complex Systems
Brandeis University MS008
415 South Street
Waltham, Massachusetts 02454-9110
[2]Department of Biology
University of Toronto at Mississauga
3359 Mississauga Road North
Mississauga, Ontario L5L 1C6
Canada

## Summary

**Background:** Associative memory formation requires that animals choose predictors for experiences they need to remember. When an artificial odor is paired with an aversive experience, that odor becomes the predictor. In more natural settings, however, animals can have multiple salient experiences that need to be remembered and prioritized. The mechanisms by which animals deal with multiple experiences are incompletely understood.
**Results:** Here we show that *Drosophila* males can be trained to discriminate between different types of female pheromones; they suppress courtship specifically to the type of female that was associated with unsuccessful courtship. Such "trainer-specific" learning is mediated by hydrocarbon olfactory cues and modifies the male's processing of those cues. Animals that are unable to use olfactory cues can still learn by using other sensory modalities, but memory in this case is not specific to the trainer female's maturation state. Concurrent and serial presentation of different pheromones demonstrates that the ability to consolidate memory of pheromonal cues can be modified by the temporal order in which they appear.
**Conclusion:** Suppression of memory by new learning demonstrates that the dynamics of memory consolidation are subject to plasticity in *Drosophila*. This type of metaplasticity is essential for navigation of experience-rich natural environments.

## Introduction

Predicting an outcome based on previous experience has huge survival value for an organism in the wild, and most species that have been examined demonstrate some form of learning. Associative learning has been elegantly demonstrated for *Drosophila melanogaster*. Association of a defined conditioned stimulus (CS) with a controlled unconditioned stimulus (US) in the odor-shock paradigm allowed the first genetic dissections of the molecular basis of learning and memory [1, 2]. In the natural environment, however, cues are rarely so clear cut. Animals are presented with complex chemosensory stimuli that they must deconvolute to extract salient information. Mammals, birds, and honeybees are able to process and assign varying levels of salience to multiple cues, and interactions between components of complex cues are commonly observed (for review, see [3]). The ability to evaluate and prioritize multiple experiences over time requires neural mechanisms that allow plasticity to be modulated. Mechanisms in this class have been broadly termed "metaplasticity" [4] and are common to all organisms, including humans. To investigate these issues in *Drosophila*, we have explored the ability of male flies to use and discriminate complex pheromonal signals in an associative-learning paradigm.

Male courtship in *Drosophila* is a stereotyped set of behaviors that are stimulated by chemical, visual, tactile, and auditory signals given off by female flies [5]. Although courtship appears to be a hard-wired aspect of the male nervous system, the gating of the behavior is plastic [6]. Exposure to a mated female has been shown to suppress subsequent courtship via an associative mechanism [7]. The US is believed to be an aversive substance that females produce after mating, whereas the CS is a courtship-stimulating chemical cue, or pheromone [8]. Learning in this paradigm must be driven primarily by chemosensory cues because visual input is not necessary [9]. How the learning-relevant substances are detected, via gustatory or olfactory pathways, is unknown (for review, see [10]).

Suppression of courtship after mated female training can be demonstrated with mature, immature, and mated female testers (e.g., [6, 11, 12]), suggesting that males are capable of extracting general information about the sex of another fly by using chemosensory input. The pheromones involved are thought to be cuticular hydrocarbons [13], which change in amount and type as adult animals mature [14–16]. Males can also extract more than just information about the sex of the courtship object from her pheromonal profile. Observations of basic courtship have shown that immature females generally elicit a higher level of initial courtship than do mature virgin females [17, 18]. Immature females and mature virgin females, therefore, do not present identical chemosensory cues, although males avoid them equally after training with a mated female.

Can males use their ability to discriminate between females of different ages in a learning situation? In this study we train males to specifically avoid females of a particular age and show that this associative learning is based on olfactory, and not gustatory, cues. Learning involves a specific modulation of the male's processing of these olfactory cues. Presentation of compound cues and sequential training experiments further suggest that males can modulate memory consolidation of a specific pheromonal cue based on its temporal relationship with the US and the association of other cues with the same US. The flexible processing of multiple stimuli demon-

*Correspondence: griffith@brandeis.edu

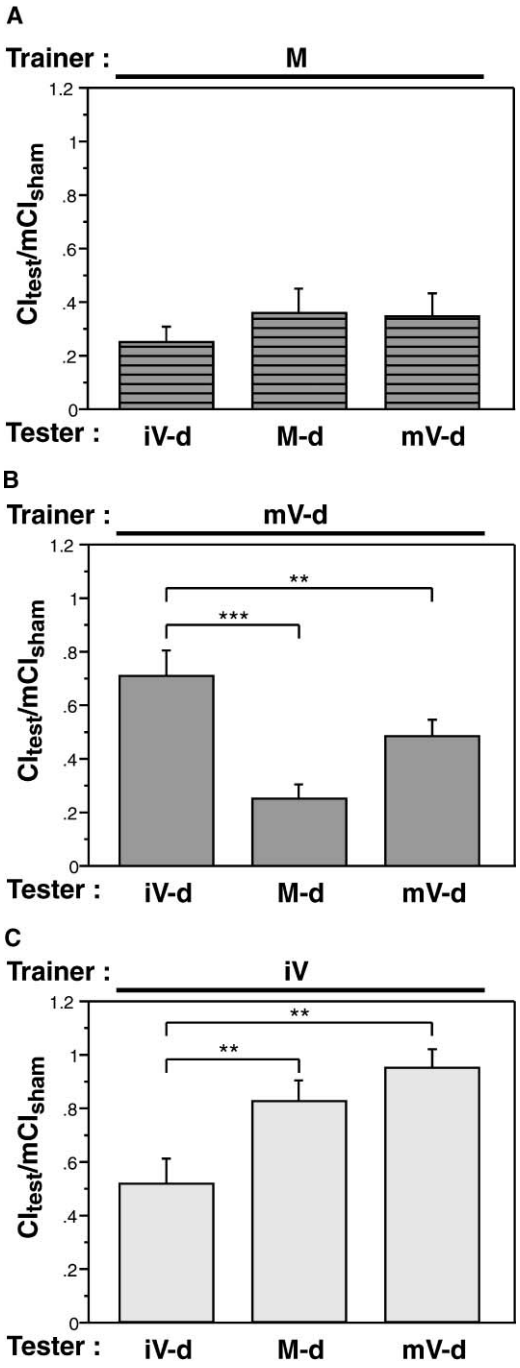


Figure 1. Differential Memory Formation with Trainers of Different Maturity

Males were trained for 1 hr with the indicated female type (M, mated female; mV, mature virgin; iV, immature virgin; -d, decapitated), and memory was assessed immediately after training. In all experiments, memory is expressed as the courtship index during the 10 min test period over the mean courtship index of sham-trained males of the same genotype ($CI_{test}/mCI_{sham}$). A value of 1 indicates no memory. Trainer type is indicated above each panel, and tester type is indicated below each bar. Data are presented as mean ± SEM.

(A) Mated female trainer. No statistical difference was seen for memory scores between tester types ($p > 0.05$).

(B) Decapitated mature virgin trainer. Testing with mature females (mated or virgin) revealed significantly stronger memory than if testing was done with an immature virgin (***$p < 0.001$, **$p < 0.05$).

(C) Immature virgin trainer. Memory with an immature virgin tester was significantly greater than with mature female testers (**$p < 0.05$).

strated by male flies in courtship learning implies the existence of metaplasticity mechanisms that gate memory consolidation in *Drosophila*.

## Results

### Trainer-Type-Specific Conditioning with Virgin Females

Exposure of a male to a mature mated female reduces the intensity of subsequent courtship toward mature virgins (4 days old) [6], mated females (4 days old, 24 hr post-mating) [12] and immature virgins (0–24 hr old) [11]. Different types of females are not, however, courted with identical vigor by naive males [17, 19], suggesting that the stimulatory chemosensory properties of these three types of females differ. The disparity in courtship toward virgins of different maturities is not understood at a chemical level, but it implies that maturation induces qualitative and/or quantitative changes in stimulatory pheromones. To determine if the suppression of courtship activity after training with a mated female might be biased toward mature females, we trained males with mature mated females and then tested those males with all three female types (Figure 1A). Memory was expressed as a ratio of the courtship index (CI) during the 10 min test period to the mean CI of a sham-trained male ($mCI_{sham}$; sham males have spent an hour alone in a courtship chamber) tested with the same type of female. The use of a ratio allows direct comparison of the strength of memory between conditions, with a value of $CI_{test}/mCI_{sham} = 1$ indicating no memory. The magnitude of courtship suppression for all female testers was statistically indistinguishable, showing that experience with a mated female causes an equal reduction in courtship of all types of females. This indicates that mated female training is generalized to pheromonally distinct female types.

Because males can distinguish mature and immature virgins, we tested the effects of training with these female types, neither of which is believed to give off an aversive chemical cue. Previous reports on courtship conditioning have claimed that virgin female trainers are not able to produce courtship suppression. In all cases [7, 8, 20–23], training was done with immature virgin females, which rarely copulate [24], and testing was performed with mature virgin females. No experiments in which the maturation of the tester was the same as the trainer were reported. To revisit this issue, we trained with either decapitated mature virgins or immature virgins. After the training, males were paired with decapitated immature virgins, mature virgins, or mated females. Decapitation serves to lower the probability of copulation with mature virgin trainers [25] and to eliminate maturation-specific differences in female rejection behavior during testing. No copulation was observed with decapitated females in our experiments.

Figures 1B and 1C show the effects of training with

virgins. Training with mature virgins produced a significantly greater suppression of subsequent courtship toward mature females (virgin and mated) compared with immature females ($p < 0.01$ for both virgin and mated females). Conversely, training with immature virgins caused a greater decrease in courtship toward immature virgins than toward mated females or mature virgins ($p < 0.01$ for both virgin and mated females). These data are consistent with previously published studies that failed to find modification of behavior toward mature virgins after training with immature virgins [7, 8, 20–23]. When the tester is of the same maturity (Figure 1B and 1C), however, learning is revealed. Our data indicate that males can use maturation-specific cues to learn about different females and that an aversive pheromone is not required for all types of courtship learning.

### Maturation Alters the Female Cuticular Pheromone Profile

Previous work on a number of Drosophilid species [14, 15, 26], houseflies [27], blowflies [28], and grasshoppers [29, 30] has suggested that maturation affects the type and quantity of cuticular hydrocarbons. To determine whether discrimination between immature and mature virgins might reflect differences in cuticular hydrocarbons, we qualitatively and quantitatively compared hexane washes of 0- to 1-day-old virgins and 4- to 5-day-old virgins by using gas chromatography-flame ionization detection and mass spectrometry. Five replicate washes, each from 20 flies, were analyzed (Figure 2; Table S1 in the supplemental data available with this article online). Comparison of the profiles shows that 63 peaks are found in common. Mature virgins contain 10 compounds that are not found in immature virgins, and immature virgins have 12 peaks, including a number of high-molecular-weight, complex peaks that are not found for mature females. In addition to these qualitative differences, the total amount of hydrocarbons significantly increased in mature virgins (mean amount hydrocarbons/fly $= 1885.5 \pm 52.9$ ng) compared to immature virgins (mean amount hydrocarbons/fly $= 896.7 \pm 50.4$ ng) ($p \leq 0.01$).

Immature and mature virgins differ in their proportion of saturated and unsaturated hydrocarbons, as shown in the Supplemental Data. A key difference is that unsaturated hydrocarbons are increased in mature virgins. We and others believe that such differences in hydrocarbon class correspond to differences in function on the cuticle (e.g., [31]). Due to their higher melting temperatures and more efficient organization on the cuticle, n-alkanes and externally branched monomethylalkanes are believed to prevent water loss [32–34]. In contrast, unsaturation lowers the melting point of hydrocarbons and consequently increases their fluidity and volatility [33], making them more likely candidates for olfactory signals [31].

Pheromonal activity has been attributed to many of the unsaturated hydrocarbons. C27 dienes on mature females have been shown to stimulate wing extension, a late courtship behavior [35]. Immature virgins express only trace amounts, if any, of this compound on their cuticle, implying that their ability to stimulate courtship is due to the presence of different pheromones. Interestingly, younger virgins express high levels of C33 and C35 dienes, which may act as an identifier of immaturity [36]. As will be demonstrated below, these hexane extracts contain molecules that are able to act as a maturation-specific CS, suggesting that the maturation level of *Drosophila* females can be identified by distinct compounds or sets of compounds on the cuticle.

### Trainer-Type-Specific Conditioning Is Not Habituation

Courtship learning can be either associative, as in the case of training with mated females [7, 8], or nonassociative, as in habituation after exposure to immature males [19, 37, 38]. Reduced courtship of immature males after prior experience was shown to be habituation based on the ability of pheromones themselves, either transferred to filter paper by the immature male [37] or obtained by hexane extraction of immature males ([38] and A.E., unpublished data], to be an effective trainer in the absence of a courtship object. To determine whether learning with virgin females is habituation, we tested the ability of pheromones transferred to wet filter paper and the hexane extracts characterized above to produce trainer-type specific courtship suppression (Figure 3A). A 1 hr training session with filters exposed for either 1 or 4 hr to a mature virgin or spotted with a hexane extract equivalent of 1.25 mature virgins failed to modify courtship of a mature virgin tester. These results are consistent with this type of learning requiring additional associative cues.

Another way in which habituation can be distinguished from associative learning is via dishabituation: the ability of nonspecific noxious stimuli to reset the response level. To determine if trainer-type-specific learning could be dishabituated, we subjected males to a 1 min vortexing immediately after training. As a positive control, we carried out the same protocol on males that had been habituated to immature males. Figure 3B demonstrates that immature-male habituation can be significantly reversed by vortexing ($p < 0.01$ in a comparison of vortexed with non-vortexed trained males), but mature virgin training is unaffected ($p > 0.05$). This is inconsistent with trainer-type-specific learning being simple habituation.

### Trainer-Type-Specific Learning Is Associative

Associative learning requires concurrent exposure to the two stimuli being associated. To test whether trainer-type-specific conditioning is a form of associative learning, we attempted to reconstitute learning by pairing pheromone filters with courtship objects. Filters that had been exposed to a mature virgin for 1 or 4 hr or which contained a hexane extract equivalent of 1.25 mature virgins were paired with either an immature virgin (Figure 4A) or an immature male (Figure 4B) as a courtship object. As controls, immature virgin courtship objects were paired with hexane extract from an immature male and vice versa. All trained males were tested with a mature virgin. As with immature female trainers (Figure 1C), immature male trainers failed to produce courtship suppression with mature female testers (Figure 4B, filled bar). Pairing of mature virgin pheromone with a courtship

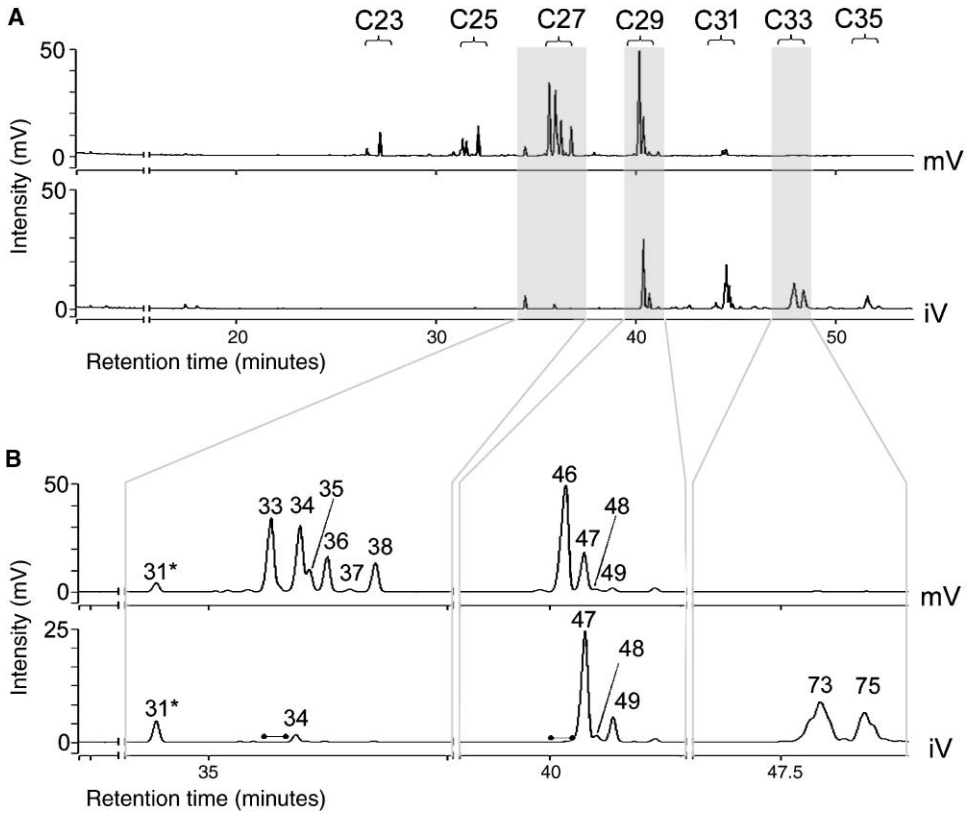


Figure 2. Hydrocarbon Profiles of Individual Extracts from Mature and Immature Virgins

The chromatogram plots the area of each peak associated with a compound in units of millivolts (mV) with column retention time on the *x* axis.

(A) Total hydrocarbon profiles for mature virgins (mV; top) and immature virgins (iV; immediately below). Bracketed peaks show areas of these chromatograms that contain compounds in the range of C23–C35. Shaded areas indicate regions of comparison shown in (B) below.

(B) Magnified details from the chromatograms in (A) illustrate the absence of the major dienes such as 7,11-nC27:2 (peak 33) and 7,11-nC29:2 (peak 46) in immature virgins (represented by bulleted lines) and the presence of large-chain alkenes in immature virgins (Xi-nC33:2, peak 73; and Xi-nC33:1, peak 75). These examples illustrate the more general developmental delay in the appearance of unsaturated compounds in female *Drosophila*. Immature (iV) samples typically show lower amounts of total hydrocarbons, and therefore the scale has been adjusted for better peak identification. Peak 31, indicated by an asterisk, is the reference standard nC26 [10 ng].

object produced suppression of mature virgin courtship (Figures 4A and 4B). No modification of mature virgin courtship was observed after training with object/filter combinations that did not include mature virgin pheromones, showing that conditioning depends on the type of pheromone. The 4 hr pheromone filters were more effective modifiers of male behavior, indicating that the effect was correlated with the amount of substance on the filter ($p < 0.06$).

To probe the temporal requirements for learning, we exposed males to the two cues sequentially. Males who spent 1 hr with an immature virgin courtship object followed by 1 hr with a mature virgin filter and 30 min rest, failed to demonstrate learning when tested with mature virgins. Reversing the order also failed to generate memory (Figure 4C). Memory decay over the course of the training protocol cannot account for the failure of these males to avoid mature virgins because training with a mature virgin and a subsequent 1.5 hr in an empty chamber produced robust memory ($p < 0.05$, for comparison with the null hypothesis, Figure 4C).

These experiments demonstrate that learning with virgin females requires association of a maturation-state-specific cuticular hydrocarbon pheromone with unsuccessful courtship and sheds light on the nature of the cues required to produce memory. The pheromone becomes a CS when paired with a courtship object. The US in this task must differ from that involved in mated-female courtship conditioning because virgins are not known to produce an aversive pheromone. We reasoned that in the case of virgin-female training, the failure of the male to complete the courtship program, i.e., to copulate, might be the US. In a number of learning situations, the failure to receive an expected reward is aversive [39–41]. To test this possibility, we exposed males either to an intact mature virgin (with whom they copulated) or to a decapitated mature virgin for 1 hr and assessed memory with a mature virgin tester 1 hr later. Males that had been allowed to copulate did not form memory (Figure 4D, $p > 0.1$), whereas males that had been paired with decapitated females and failed to copulate showed an obvious suppression of courtship ($p <$

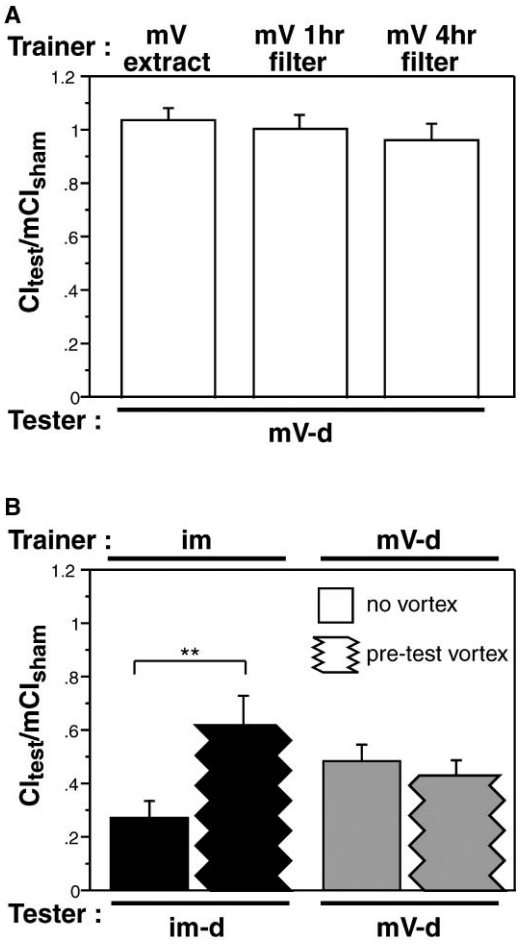


Figure 3. Trainer-Specific Learning Is Not Habituation

Trainer condition is indicated above the panel, and tester type is indicated below each set of bars (mV, mature virgin; im, immature male; -d, decapitated). Data are presented as means ± SEM.

(A) The ability of a 1 hr exposure to a filter on which mature virgins had been stored (for 1 or 4 hr) or a filter containing a hexane extract of mature virgins (1.25 fly equivalents) to produce trainer-specific memory was assessed. Males exposed to pheromone-containing filters in the absence of a courtship object failed to form memory when tested with mature virgins ($p > 0.05$).

(B) Male flies trained with either immature males or mature virgins were subjected to a 1 min dishabituating stimulus (vortexing) before the memory test. Habituation of the response to immature males was significantly reversed by vortexing (**$p < 0.05$). Memory for training with a mature virgin was unaffected by the dishabituating stimulus ($p > 0.05$).

0.01 for comparison with the null hypothesis). These data suggest that lack of copulation is a critical feature of the US that is provided by the courtship object.

### The Conditioned Stimulus Is Sensed by the Olfactory System

Early studies found that both olfactory [42] and gustatory [25] pathways provide courtship-relevant information to the male fly. Which sensory pathway provides the CS used for courtship suppression? In experiments shown in Figure 4, the CS was delivered on a filter that was paired with a courtship object. These filters contain both volatile and nonvolatile hydrocarbons (Figure 2). Males were allowed to touch the pheromone-containing filter, and cues could therefore have been detected by either the olfactory or the gustatory sensory system. To assess if the chemical cue used was volatile, we placed a mesh barrier between the male and the pheromone filter or decapitated mature female providing the CS. An immature virgin-female courtship object was placed with the male to elicit courtship behavior during training. Lack of direct contact with pheromone did not disturb the production of mature-female-specific courtship suppression (Figure 5A). Without a courtship object, presentation of a pheromone filter or a decapitated mature virgin over the mesh did not elicit any courtship behavior (data not shown). These results indicate the pheromones used to discriminate between mature and immature virgins, and to provide the trainer-specific CS, are volatile. This is consistent with our hypothesis that alkenes, due to their greater fluidity and volatility, are the important signaling molecules.

### Olfactory Input Is Necessary for Learning of Trainer Specificity

To determine whether olfaction is required for formation of trainer-specific memory, we prepared olfaction-impaired males by amputating the second and third antennal segments and the maxillary palps [43]. Males lacking these sensory organs were almost completely unable to initiate courtship under dim red lights, showing very low initial CIs during training (Figure 5B, left) due to a greatly increased latency to first courtship (406 ± 57 s versus 10 ± 4 s for intact males, both tested with decapitated virgins, $p < 0.0001$). Observing behavior under white lights (allowing these males to use visual cues to find the female) produced more normal levels of initial courtship (Figure 5B, right). Surprisingly, when trained with a mature virgin, these males were able to robustly suppress courtship (Figure 5C, left). The suppression produced, however, was not specific to the trainer type. Males trained with an immature virgin also suppressed courtship with a mature virgin tester. Intact males under the same white-light conditions were still able to form trainer-type-specific memory (Figure 5C, right). A two-way ANOVA shows that both trainer type ($p < 0.01$) and the presence of antennae ($p < 0.0001$) contribute to performance. Antenna-less males showed significantly lower courtship compared to intact males in both training conditions ($p \leq 0.0001$ for both). These data suggest that in the absence of information from volatile cues, males associate some other, more general, cue with unsuccessful courtship. This cue is likely to be visual because learning in the absence of olfaction is still expressed as an increased latency to initiation (data not shown), but the cue could also be a general chemical one sensed by receptors in other organs. The ability of males to switch to a non-olfactory cue as a predictor of the US suggests that unsuccessful courtship is a potent negative experience for males.

### Trainer-Type Learning Modulates Olfactory Processing

How does learning alter the courtship-behavior circuit? Male courtship in *Drosophila* is a series of innate behav-

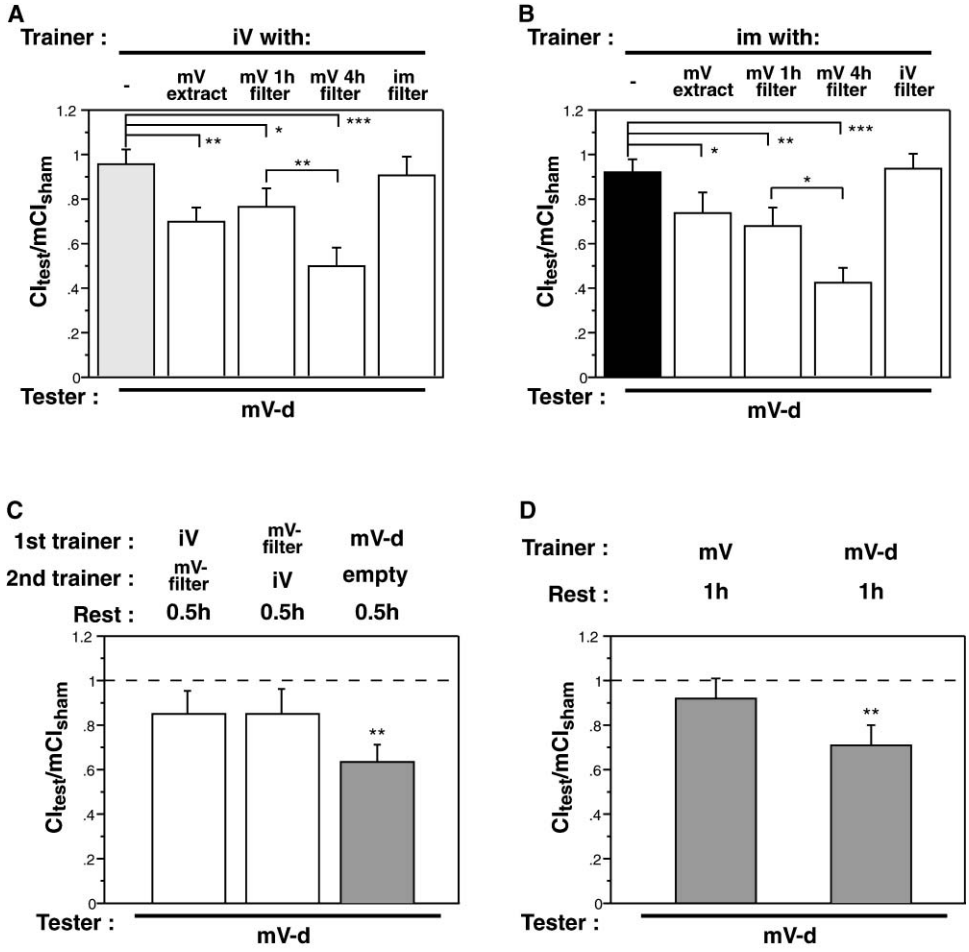


Figure 4. Trainer-Specific Learning Is Associative

Males were trained for 1 hr. Trainer condition is indicated above the panel, and tester type is indicated below each set of bars (mV, mature virgin; iV, immature virgin; im, immature male; -d, decapitated). Data are presented as means ± SEM.

(A) Learning requires a trainer-specific pheromone signal. Males were trained with an immature virgin courtship object in the presence of a pheromone-laced or control filter. Compared to males trained with a control filter, males that had been exposed to mature virgin pheromones in the context of an unsuccessful courtship suppressed subsequent courtship of mature virgins (*$p < 0.1$, **$p < 0.05$, ***$p < 0.001$). Filters that had been exposed to a mature virgin for 4 hr were more effective trainers than filters that had only been exposed for 1 hr (**$p < 0.05$). Pairing of the courtship object with an irrelevant pheromone (immature male) did not produce memory when tested with a mature virgin.

(B) Substitution of an immature male for the immature virgin courtship object produced the same results, indicating that the nature of the courtship object does not influence the specificity of the learning.

(C) Sequential presentation of cues does not support memory formation. Males were trained for 1 hr with an immature virgin, then exposed for 1 hr to a filter containing mature-virgin pheromone (left bar) or to the same cues in reversed order (middle bar). No memory was formed in comparison to time-matched control males who were trained for 1 hr with a mature virgin and tested after a 1.5 hr delay (right bar, **$p <$ 0.05 for comparison with the null hypothesis).

(D) Copulation during training blocks learning. Males were exposed to a mobile mature virgin (left bar) for 1 hr and copulation was confirmed visually. Control males were trained for 1 hr with a decapitated mature virgin and did not copulate. Males were tested for memory 1 hr after training to allow copulated males to recover from fatigue. Courtship vigor of males that had copulated was unaffected after training and rest ($p > 0.1$), whereas males that did not copulate showed significant suppression of courtship (**$p < 0.05$ for comparison to the null hypothesis).

iors that are triggered by external cues. Initiation and orientation toward the female are limited by the ability of the male to detect the female and are believed to be primarily under control of vision and olfaction ([44]; Figure 5). Subsequent courtship steps are enhanced by gustatory information the male receives by touching the female's abdomen and genitals [45]. Sub-behaviors are done in an ordered fashion, although the male may return to earlier stages multiple times before copulation. This results in courtship behaviors occurring in clusters, or "bouts" during the observation period.

Memory in courtship conditioning paradigms is expressed as CI decrease, which reflects total courtship, initiation through attempted copulation. A decreased CI could therefore result from either fewer initiations or decreased bout duration. To investigate these possibilities, we looked at the latency to first courtship activity (Figure 6). For males trained with an immature virgin and tested with a mature virgin, latency was very short. Males trained with a mature virgin and tested with the same type of female had a much longer latency, and latency was negatively correlated with memory index

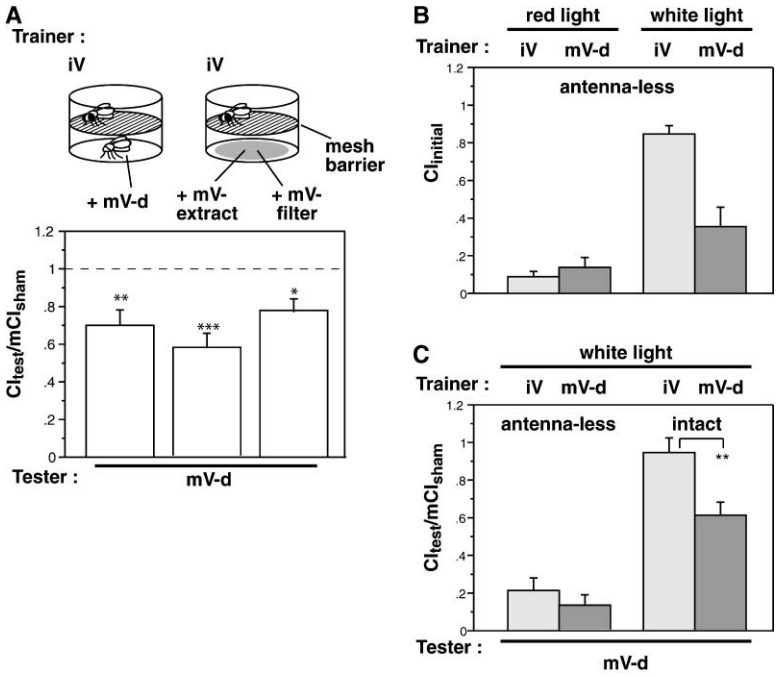


Figure 5. The Conditioned Stimulus for Trainer-Specific Learning Is a Volatile Odorant

Males were trained for 1 hr. Trainer condition is indicated above the panel, and tester type is indicated below each set of bars (mV, mature virgin; iV, immature virgin; -d, decapitated). Data are presented as means ± SEM.

(A) Males can sense trainer-specific pheromones without contacting them. Males were trained in a two-part chamber separated by a mesh. The courtship object was placed in the same chamber as the male, and the CS (either a mature virgin or a pheromone filter that had been in contact with a mature virgin or that contained a hexane extract equivalent to 1.25 mature virgins) was placed in the lower chamber. Volatile CS was an effective cue (*$p < 0.1$,**$p < 0.05$, or ***$p < 0.001$, for comparison with the null hypothesis).

(B) Males require olfaction to initiate courtship in the absence of visual cues. Antennae and maxillary palps were amputated at eclosion, and males were allowed to recover for 4 days. In dim red lights (a condition in which males cannot use visual cues [9]), males failed to initiate significant levels of courtship when presented with either an immature virgin or a mature virgin trainer (left bars). Observation of behavior under white lights shows that these males can court when allowed to see the female (right bars). Courtship of immature virgins is more vigorous than courtship of older females, as has been previously observed [17].

(C) Males with surgically ablated olfactory organs were trained with either immature or mature virgins and tested with mature virgins. Olfactory impairment suppressed courtship to both types of females to the same degree ($p > 0.05$ for comparison of immature virgin and mature virgin training), whereas intact males trained and tested under white lights showed trainer-specific memory (**$p < 0.05$, for comparison of immature virgin and mature virgin training).

($CI_{test}/CI_{sham}$) for individual animals. Average latency for males tested with a mature virgin after training with an immature virgin was 12.4 ± 2.7 s, compared with 128.7 ± 28.7 s after training with a mature virgin ($p < 0.001$). The 10-fold effect on latency suggests that change in initiation rate is the primary behavioral manifestation of learning.

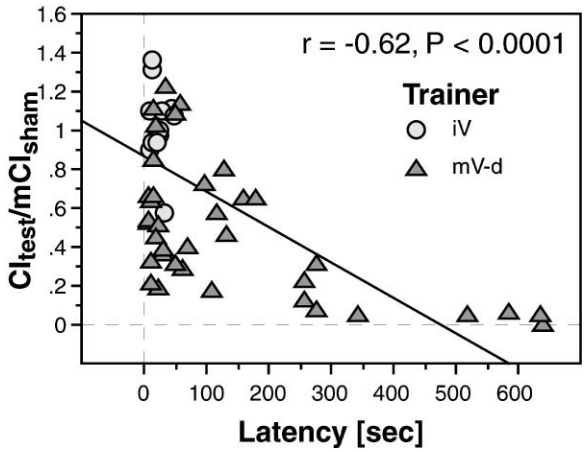


Figure 6. Trainer-Specific Learning Is Expressed by Modulation of Courtship Initiation

Memory scores ($CI_{test}/mCI_{sham}$) for individual animals tested with a mature virgin immediately after training were plotted against their latency to first courtship. Males trained with an immature virgin showed low latency and poor memory scores (light gray circles). Males trained with mature virgins (dark gray triangles) had longer latency, and length of latency was negatively correlated with memory score ($r = -0.62$, $p < 0.0001$).

Analysis of courtship bout length (the $CI_{test}$/number of bouts) showed that, for trained flies, bout length with a tester female of the same maturity as the trainer was 2-fold shorter than if the tester was of different maturity (for males tested with a mature virgin, 15.0 ± 1.4 s after training with an immature virgin versus 6.9 ± 1.2 s after training with a mature virgin, $p < 0.005$). This indicates that in addition to the substantial effect on latency, there is also a smaller effect on the intensity of courtship. In the absence of visual cues, olfactory input is the critical determinant of latency (see Figure 5). Our data support the idea that learning changes the ability of the male to respond to maturation-specific volatile pheromones but leaves the gustatory pathways that stimulate courtship, once it has started, largely intact.

### cAMP-Pathway Mutants Have Abnormal Trainer-Type-Specific Memory

Odor-shock learning has been used to dissect the molecular basis of associative memory. Two of the first mutants that were isolated with this paradigm are *dunce (dnc)* [46] and *amnesiac (amn)* [47], both of which encode proteins (a phosphodiesterase and a family of neuropeptides, respectively) believed to modulate cAMP signaling pathways. These mutants are also abnormal in mated-female courtship conditioning [6, 21]. To determine whether trainer-type-specific memory formation utilized similar biochemical machinery, we trained $dnc^1$ and $amn^{28A}$ males with both mature and immature virgins and tested them with mature virgins.

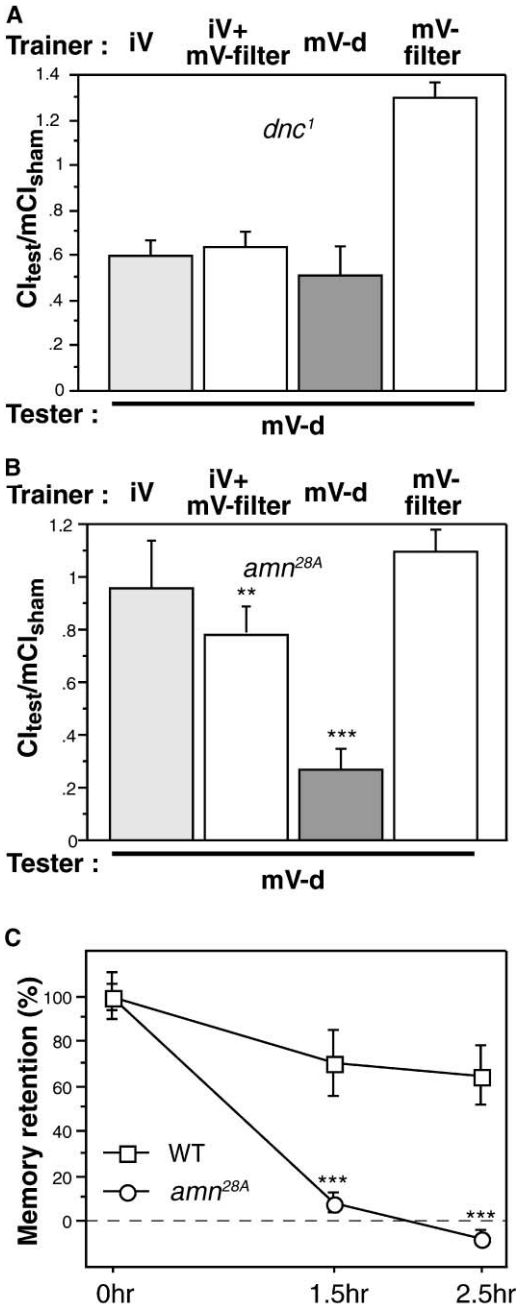


Figure 7. Trainer-Specific Learning Requires Intact cAMP Signaling Pathways

Males of the indicated genotypes were trained for 1 hr and tested at varyious times after training. The training condition is indicated above each panel, and the tester type is indicated below each panel (mV, mature virgin; iV, immature virgin; -d, decapitated). Data are presented as means ± SEM.

(A) *dnc*[1] males were trained and tested for memory immediately after training. These males failed to form trainer-specific memory and suppressed courtship of a mature virgin tester equally regardless of training condition ($p > 0.05$).

(B) *amn*[28A] males were trained and tested for memory immediately after training. They were able to form trainer-specific memory with either a mature virgin trainer (***$p < 0.001$, for comparison to the null hypothesis) or a pheromone filter and immature-virgin courtship object (**$p < 0.05$, for comparison to the null hypothesis).

(C) *amn*[28A] males failed to maintain memory. Memory retention [(1 − $CI_{test}/mCI_{sham}$)/m(1 − $CI_{test}/mCI_{sham}$ at t = 0)] is plotted against time after training. Mutant male memory decayed to zero by 1.5 hr, whereas wild-type memory was robust out to 2.5 hr (***$p < 0.001$ for comparison between wild-type and *amn*[28A]).

In contrast to results with odor-shock or mated-female training, *dnc*[1] males were able to learn to suppress courtship after training with a virgin. Interestingly, however, *dnc*[1] males failed to show trainer-specific suppression (Figure 7A), decreasing courtship toward a mature virgin after training with either mature or immature females. This defect is similar to the behavior of olfaction-impaired males in white light (Figure 5C). It is important to note that the ability to discriminate between mated and virgin females is intact in *dnc*[1] mutant males [21, 48], suggesting that the primary sensory neurons that process pheromonal signals are intact. The discrimination defect after training is consistent with these males having an olfactory-processing impairment. This has been demonstrated for the *dnc*[1] allele in other paradigms [49–51]. The ability to successfully suppress courtship suggests either that the *dnc*-encoded phosphodiesterase is not involved in initial association in this paradigm or that the anatomical locus of memory formation for trainer-type-specific learning is distinct from that of mated female and odor-shock learning.

*amn*[28A] males learned normally if tested immediately after training with a mature virgin and did not avoid mature virgins after training with an immature female (Figure 7B). Training with a pheromone filter paired with a courtship object produced memory, but it was significantly weaker. Introducing a delay between training with a mature virgin and testing uncovered a more rapid decay of memory in *amn*[28A] males (Figure 7C), consistent with their phenotype in other behavioral assays, including mated-female courtship conditioning [6]. By 1.5 hr after training, *amn*[28A] memory had decayed to baseline, whereas wild-type memory was at 65% of its initial level after 2.5 hr. This experiment indicates that trainer-type-specific associative learning requires *amn* peptide for consolidation.

### Males Can Learn Multiple Cues

In experiments shown in Figure 4A, we trained males with an immature female courtship object paired with a filter containing mature virgin pheromone and found that males could learn to avoid mature virgins. Had these males concurrently learned to avoid immature females? Training in the presence of two maturation-specific pheromones resulted in the male learning to avoid both types of females. Memory measured with an immature virgin tester for males trained with an immature virgin in the presence of a mature female filter ($CI_{test}/mCI_{sham} = 0.33 \pm 0.07$) was equivalent to that measured for males trained with an immature virgin alone ($p > 0.1$).

Does this ability to learn two simultaneously presented cues generalize to non-pheromonal odors? We trained males with a mature virgin in the presence of benzaldehyde (Bz). Using immature and mature virgin testers, we observed males in the presence or absence of Bz. We kept sham-trained males in a chamber with

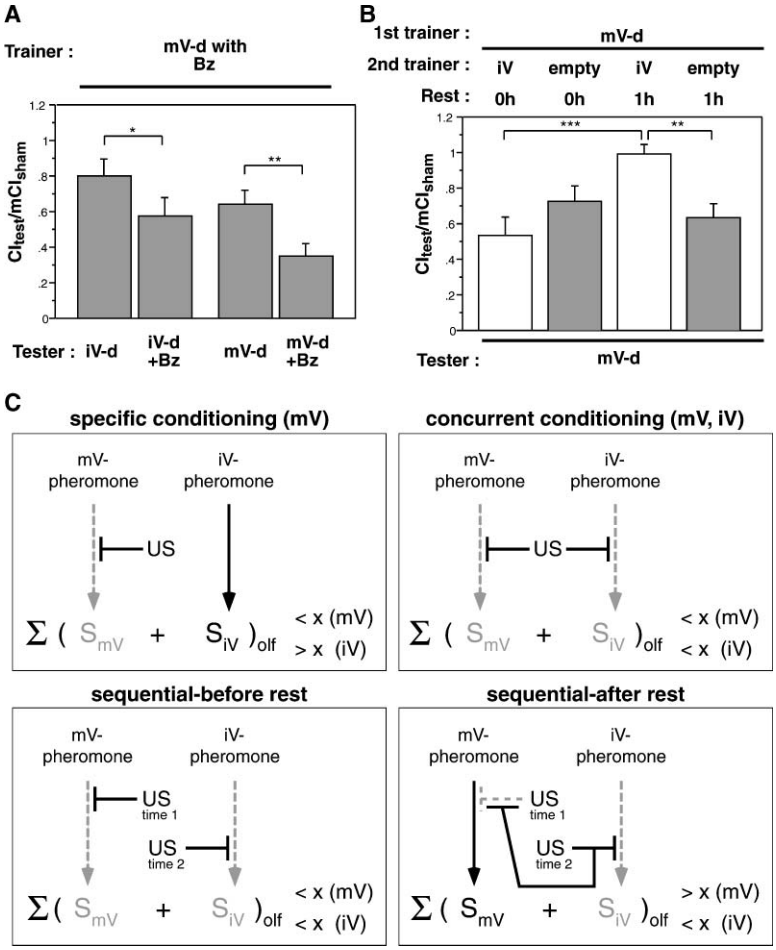


Figure 8. Multiple Associations and Cue Interactions in Trainer-Specific Learning

Cue interactions were investigated by concurrent and sequential presentation of multiple conditioned stimuli. Trainer conditions are indicated above each panel, and tester type is indicated below each panel (mV, mature virgin; iV, immature virgin; -d, decapitated; Bz, benzaldehyde). Data are presented as means ± SEM.

(A) Male flies can make multiple associations when trained in the presence of a non-pheromonal odorant. Males were trained for 1 hr with a mature virgin in the presence of Bz and tested ± Bz with the indicated female types. Males formed trainer-specific memory and additional Bz-specific memory that could be seen when testers were presented in the presence of Bz (*$p < 0.1$ and **$p < 0.05$, for comparisons ± Bz).

(B) Sequential training modulates memory retention. Males received two training sessions. In the first they were trained for 1 hr with a mature virgin. The second training session consisted of 30 min with an immature virgin or an empty chamber. Males were tested immediately after the second training session or after a 1 hr delay. Males who spent the second session in an empty chamber formed memory against mature virgins, and this memory was evident even if testing was delayed. Males that received a second training session with an immature virgin had memory when tested with immature virgins, even after a 1 hr delay (data not shown). When tested with a mature virgin, however, these males had normal memory immediately after training, but by 1 hr after the second session memory was gone (***$p < 0.001$ for comparison of memory tests for double-trained males 1 hr after the second session with double-trained males tested immediately after training and **$p < 0.05$ for comparison with males receiving an empty-chamber second session and a 1 hr delay before testing).

(C) Models of trainer-specific memory formation and consolidation. The decision to initiate courtship, in the absence of visual cues, is based on the strength of stimulatory olfactory cues that the female presents to the male. If the strength of the stimulatory cue exceeds some threshold, x, courtship will ensue. If the strength of stimulation is less than x, the male will not initiate courtship toward that female type. Solid lines indicate intact pathways, and dashed lines indicate blocked pathways. If the male is trained with a single type of female, e.g., a mature virgin, he will not initiate courtship when presented with that female type [summed stimulus $<$ x (mV)], but he will initiate with the other female type [summed stimulus $>$ x (iV)], in this example an immature virgin (top left panel). If both pheromonal cues are paired concurrently with the US, the male will fail to initiate with either female (top right panel). Sequential training (bottom panels) has time-dependent effects. The male initially forms memory for the cue that is trained first, in this case the mature-virgin pheromone, as can be seen if memory is assessed immediately after the second training session. The second training actively blocks retention of the memory for the first cue, resulting in apparently accelerated decay. If a rest is imposed after the two training sessions, memory for the first cue is lost. Memory for the cue that was trained second is seen both before and after the delay.

Bz for 1 hr as a control to test for nonspecific effects of Bz on courtship. Bz during training did not affect the ability of males to form trainer-type-specific memory; when trained with a mature virgin in the presence of Bz, males failed to suppress courtship toward Bz-free immature virgins but showed some suppression with a Bz-free mature virgin tester. Additionally, males tested in the presence of Bz could discriminate between tester types (Figure 8A, $p < 0.06$ for comparison of immature and mature virgin testers in the presence of Bz). Testing in the presence of Bz enhanced avoidance of the mature virgin ($p < 0.06$ for comparison ± Bz) and produced suppression of immature-virgin courtship ($p < 0.1$ for comparison ± Bz). These data are consistent with the male associating both mature virgin pheromone and Bz with unsuccessful courtship, such that the effects are roughly additive. Simultaneous memory formation for two distinct stimuli (either two pheromones or a pheromone and an odorant) suggests that each cue was of similar salience to the male. In situations where salience is unequal, the high salience cue can "overshadow" the low salience cue and block memory formation. This type of cue competition has been seen in many organisms (for review, see [3]).

### Memory Consolidation Can Be Altered by New Learning

What happens when training sessions with distinct cues are separated in time? We trained males with a mature virgin for 1 hr and then gave the male a second 30 min

training session with either an empty chamber or an immature virgin. Testing memory with a mature virgin immediately after the second training session demonstrated that males formed mature-virgin memory regardless of whether there was another female presented during the second training session (Figure 8B). If a 1 hr delay was imposed before testing, memory in males that had been trained with immature females during the second training period was undetectable ($p > 0.05$ for the comparison of sham and immature virgin second session training following 1 hr delay). This suggests that the temporal dissociation of the two learning experiences allowed the second experience to become dominant and block maintenance of the first memory. Because the memory of the first pheromone can be demonstrated immediately after training, the effect of the second training session must entail some active process that disrupts the consolidation of the first memory.

## Discussion

### Memory Consolidation in *Drosophila* Is Plastic

Consolidation of memory in *Drosophila* has been shown to occur in multiple, mechanistically discrete stages that occur in the hours following initial learning. Separation of these stages can be accomplished by physical and genetic manipulation for classical [52], operant [53, 54], and courtship conditioning [6, 55]. The ordered nature and slow time course of consolidation imposes a delay between learning and the time a memory achieves its ultimate long-term form. This delay provides a temporal window in which the animal can revise its initial assessment of the importance of an experience and reconcile it with subsequent experiences. The ability to alter priorities on the fly is important for organisms, including humans, in complex environments and is likely to have strong survival value.

The trainer-specific learning paradigm has given us an opportunity to catch this reordering occurring in a laboratory setting. The ability of males to use maturation-specific pheromones to discriminate between female types allowed us to investigate the mechanisms by which males can deal with multiple simultaneous or temporally separated experiences. The experiments lead to several conclusions. First, learning one cue does not prohibit learning of a second cue. In many learning paradigms, concurrent presentation of cues of unequal salience is associated with suppression of response to the weaker cue, a phenomenon termed "blocking" or "overshadowing" [3]. Males trained in the presence of both mature- and immature-virgin pheromone can remember both stimuli when they are individually presented during testing, implying that the two pheromone cues are of equivalent salience. More importantly, initial memory for cues trained sequentially is also normal; having learned one cue does not prevent the animal from learning another. We conclude that initial learning in this paradigm does not appear to be subject to metaplastic regulation.

Second, memories for simultaneously learned cues can coexist and do not appear to interact. The ability of concurrent training with either multiple pheromones or both pheromone and odorant to produce normal memory to both cues argues that the animal is capable of holding multiple memories at one time. Concurrent training with cues of equivalent salience should not invoke any sort of competitive mechanism because the animal has no reason to value one memory over another.

Third, new learning with the same US can block consolidation of old learning. Sequential presentation of each pheromonal CS in the context of unsuccessful courtship revealed that the maintenance of memory of a pheromone can be modified by its temporal relationship to the US. A model of how this might occur is shown in Figure 8C. Animals that are trained with a mature virgin and then with an immature virgin have memory for both if tested immediately after the last training session. If a 1 hr delay is imposed before testing, the second memory is still intact, but the first memory is completely gone. This loss cannot be due to normal decay over time because time-matched animals trained with a mature virgin have normal levels of memory. This type of phenomenon has been seen with conditioned taste aversion in rats for which the CS most closely associated in time with illness is preferentially remembered [56]. Modulation of consolidation is a form of behavioral metaplasticity in which new learning alters the rules by which the subsequent processing of an older memory is handled [57].

### Identification of Conditioned and Unconditioned Stimuli for Trainer-Specific Learning

The trainer-specific conditioning paradigm has also allowed us to define both conditioned and unconditioned stimuli relevant for courtship learning. The inability to copulate with a trainer female provides a very strong negative stimulus that can produce learning even in the absence of an aversive chemical substance such as the one postulated to be the US in mated-female conditioning. The ability of lack of copulation to act as a US implies that once the male has begun to court, he has an expectation that the behavior will end with mating. Furthermore, the culmination of this behavioral cycle must be associated with reward; preventing completion is aversive. Consistent with this hypothesis, we have found that the efficacy of learning can be modulated by dopaminergic transmission (A.E., unpublished results), which has been associated with both reward and aversive US pathways in *Drosophila* [58, 59].

Males can associate failure to copulate with several CS cues, but the dominant association in males that possess an intact olfactory system is with maturation-specific cuticular pheromones. A number of compounds are expressed only by one female type; most of these are dienes. These compounds are all candidates to be the maturation-specific CS, and given that the learning-relevant cue is volatile, it is likely that unsaturated compounds are playing this role. Previous studies of mature female compounds [35] have focused on C27 dienes such as 7,11-heptacosadiene and have used wing vibration as an assay. Wing vibration, or courtship song, occurs at a late stage of courtship and is under control of compounds sensed by the gustatory system [45]. In

addition, 7,11-heptacosadiene may act in this role; it has been reported to be incapable of stimulating courtship initiation; blind males were not stimulated to sing by its presence [35]. It is therefore unlikely that 7,11-heptacosadiene is the mature-virgin CS of trainer-specific learning. Our results suggest a more extensive role for hydrocarbons in recognizing the trainer. Precise identification of the candidate compounds that are behaviorally relevant will require substantial additional experimentation.

### Mechanisms of Trainer-Specific Learning

Manipulation of identified associative cues has also allowed us to define neural and molecular mechanisms for trainer-specific conditioning and its plasticity. Both smell and taste are important sensory modalities for courtship [25, 42]. Gustatory input is important for the progression of courtship once it has begun [45], but olfaction is used to initiate the behavior ([44] and Figure 5B). Our results demonstrate that olfaction is the predominant chemosensory system involved in plasticity of courtship behavior. The CS is a volatile hydrocarbon, and males require an intact olfactory system to learn to discriminate between young and old females.

The olfactory system is not just the driver of trainer-specific plasticity; it is also the target. The main effect of learning is to modify olfactory courtship drive. The courtship suppression exhibited after training is due mostly to a change in courtship initiation rate, with only small effects on the intensity of courtship, implicating the processing of olfactory information as a central focus of initial plasticity. The unique effects of the *dnc*$^{1}$ mutation in this assay, a loss of pheromone specificity, supports this idea because *dnc*$^{1}$ has previously been shown to have defects in olfactory processing [49–51].

### Mechanisms of Consolidation and Metaplasticity

Newly formed trainer-specific memory is likely resident in antennal lobes [60]. In *Drosophila* antennal lobes, as in the mammalian olfactory bulb, odor quality is encoded initially in local activity patterns [61]. For odor-shock learning, the CS and US can converge in projection neurons to alter odor representation immediately after training. These local changes do not last long, and information regarding association may be only transiently present in this structure before being moved up to lateral protocerebrum/mushroom bodies during consolidation [62].

Studies of mated female courtship conditioning also suggest that there are initial transient sites of memory formation that feed into mushroom bodies in this behavior. Memory recall immediately after training does not require intact mushroom bodies, but 30–60 min after training, recall is impaired in animals with damage in that region [55]. The involvement of *amn* in retention of mated-female [6] and trainer-specific memory is also consistent with late involvement of the mushroom body. The *amn* gene product, which is believed to be a PACAP-like neuropeptide [63], is expressed most predominantly in the dorsal paired medial neurons that innervate the mushroom body neuropil [64]. The fast memory decay in *amn* mutants may not reflect enhanced forgetting but rather may reflect failure of the memory trace to be transferred to the mushroom body [65].

The time delay between the end of the second training session and the loss of the first memory implies that loss of the initial memory is an active process: deletion of an already formed memory. The time course is remarkably close to that of the memory loss seen in *amn* mutants. One interpretation of these data is that formation of a second association with the US prevents the normal transfer of the memory trace for the first association to the mushroom body. Accordingly, the initial interaction of the two cues is probably occurring proximal to the mushroom body, perhaps in antennal lobes. These data suggest that the consolidation process is itself plastic, and new associations with a common US are preferentially maintained. At the cellular level, this may reflect alterations in antennal-lobe activity patterns generated by the second associative event [66]. Similar behavioral phenomena in mammals remain poorly understood, and the detailed mechanisms of this higher-order associative behavior will be approachable in flies if available genetic, electrophysiological, and functional imaging tools are used.

Learning from experience is crucial to survival, and animals have developed a multitude of well-characterized plasticity mechanisms to cope with the external world. Dealing with multiple related experiences demands that an animal prioritize its responses to external cues based on either their importance or their temporal proximity. Plasticity in the dynamics of memory formation in response to new learning provides one type of solution to this problem. We find that in *Drosophila*, this type of metaplasticity exists at the level of modulation of consolidation of newly formed memories.

## Experimental Procedures

### Fly Strains

Flies were raised on autoclaved cornmeal-yeast-sucrose-agar food in a 12 hr light/dark cycle at 25°C. Males and females were anesthetized with $CO_2$ and separated on the day of eclosion, then used immediately as immature flies or aged for 4–5 days. Experimental males were housed in individual tubes. For mated-female preparation, 3-day-old females were placed with males. Only females that copulated for $\geq$14 min were used the next day. For preparation of decapitated flies, heads were cut off with fine scissors just before use. Amputation of olfactory organs (second and third antennal segments and maxillary palps) was done with fine forceps at eclosion. Males were allowed to recover from surgery in a humidified food vial until testing. Canton-S was used as the wild-type strain. *dnc*$^{1}$ is described in Lindsley and Zimm (1992) [67]. *amn*$^{28A}$ is described in Moore et al. [68].

### Behavioral Assays

All behavior was done under dim red lights (except where noted) in a Harris environmental room (25°C, 80% humidity). A 4- or 5-day-old male was placed with a trainer in a single-pair-mating chamber (8 mm in diameter, 3 mm deep) for 1 hr. Wet filter paper (Whatman, 42 ashless) was put in each chamber to maintain humidity. So that direct contact with pheromone filters would be prevented (experiments in Figure 5), a fine nylon mesh (Tetko, 3-180/43) was introduced into a two-part chamber (8 mm diameter, 6 mm deep). The first 10 min of the training period were videotaped with a digital camcorder (SONY, DSR-PD150). Pairs that copulated during training or had initial CI $<$ 0.1 were eliminated from further analysis. Immediately after training, males were transferred into a clean chamber and paired with a decapitated tester and videotaped for 10 min. In some experiments, additional training or delays were imposed before testing. Sham-trained males were are kept alone in the mating chamber for the first hour and then paired with a tester for 10 min.

For each of the 10 min periods, a courtship index (CI) was calculated. CI is the fraction of time a male spent in courtship activity in the 10 min observation period (CI = courtship [s]/observation [s]). Memory index is calculated by dividing CI at test ($CI_{test}$) by the mean of sham CIs ($CI_{sham}$): $CI_{test}/mCI_{sham}$. If $CI_{test}/mCI_{sham} = 1$, it indicates that there has been no learning because the courtship level of trained males is equivalent to that of sham-trained males. At least 20 males were tested for each condition. Latency was scored as the time lag (in sec) to the first display of courtship after pairing with the courtship object. Males that failed to initiate courtship during the 10 min observation period received a latency score of 600 s.

### Statistics and Data Presentation

Each CI was subjected to arcsine-square-root transformation to effect an approximation of normal distribution; Statview software version 4.5 for the Macintosh was used. ANOVA with each indicated condition as the main effect was performed on the transformed data. Posthoc analysis was done with Fisher's PLSD test for behavioral data and with a 2-tailed Steel's rank test for hydrocarbon data. Bars in figures represent means ± SEM, with levels of significance indicated by *** p significant = $\alpha < 0.001$, ** p significant = $\alpha < 0.05$, and * p significant = $\alpha < 0.1$. Trainer and tester types are abbreviated as follows: M, mated female; mV, mature virgin; iV, immature virgin; im, immature male; -d, decapitated; Bz, benzaldehyde. Histogram bars for data in which males were trained with mated females are dark gray with stripes; with mature virgins, dark gray; immature virgins, light gray; and immature males, black. For more complex protocols involving pairing of filters and courtship objects, bars are white.

### Pheromone Extraction

For pheromone collection, a fly was put on a wet filter paper in a mating chamber for 1 or 4 hr to transfer odors to the filter. For preparation of hexane extracts, the bodies of 20 flies were washed with 80 μl of hexane (ALDRICH). For training, 5 μl of extract was applied to the filter paper in a mating chamber and evaporated for 2 min, after which 7 μl of water was added to the filter to add humidity. For chemistry, the extract was evaporated (passive evaporation at room temperature in a dust-free environment) and stored at −80°C until analyzed.

### Benzaldehyde Training

Benzaldehyde (0.1%, 10 μl; ALDRICH) was applied to each filter paper in a mating chamber immediately prior to training.

### Supplemental Data

Supplemental Results, Experimental Procedures, and a table are available with this article online at http://www.current-biology.com/cgi/content/full/15/3/194/DC1/.

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# Sequential Learning of Pheromonal Cues Modulates Memory Consolidation in Trainer-Specific Associative Courtship Conditioning

**Aki Ejima, Benjamin P.C. Smith, Christophe Lucas, Joel D. Levine, and Leslie C. Griffith**

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Table S1. Cuticular Hydrocarbons of Mature and Immature Virgins

| | | | | Immature Virgin (iV) | | | Mature Virgin (mV) | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound | Peak | Source | Time | Mean Amount (Area) | SEM | Percent | Mean Amount (Area) | SEM | Percent |
| nC19 | 1 | both | 17.42 | 0.278 | 0.107 | 0.924 | 0.362 | 0.064 | 0.573 |
| uic | 2 | both | 18.00 | 0.237 | 0.088 | 0.786 | 0.169 | 0.030 | 0.268 |
| nC21 | 3 | both | 22.07 | 0.041 | 0.016 | 0.135 | 0.123 | 0.014 | 0.195 |
| uic | 4 | both | 22.31 | 0.043 | 0.023 | 0.143 | tr | | |
| Xi-nC20:1 | 5 | both | 23.08 | tr | | | tr | | |
| nC22 | 6 | both | 24.64 | tr | | | 0.076 | 0.005 | 0.121 |
| 7,11-nC23:2 | 7 | mV | 25.87 | nd | | | 0.127 | 0.005 | 0.202 |
| 2-MeC22 | 8 | mV | 26.27 | nd | | | 0.101 | 0.006 | 0.160 |
| 9-nC23:1 | 9 | both | 26.34 | tr | | | 0.105 | 0.005 | 0.166 |
| 7-nC23:1 | 10 | both | 26.54 | 0.065 | 0.046 | 0.214 | 0.680 | 0.030 | 1.077 |
| 6-nC23:1 | 11 | mV | 26.67 | nd | | | 0.076 | 0.002 | 0.120 |
| 5-nC23:1 | 12 | both | 26.82 | tr | | | tr | | |
| nC23 | 13 | both | 27.19 | 0.034 | 0.022 | 0.114 | 2.241 | 0.100 | 3.549 |
| 9-nC24:1 | 14 | mV | 28.85 | nd | | | tr | | |
| 7-nC24:1 | 15 | mV | 28.94 | nd | | | tr | | |
| 5-nC24:1 | 16 | both | 29.04 | tr | | | tr | | |
| nC24 | 17 | both | 29.66 | tr | | | 0.186 | 0.006 | 0.295 |
| 9,13-nC25:2 | 18 | both | 30.67 | tr | | | 0.116 | 0.007 | 0.184 |
| 7,11-nC25:2 | 19 | both | 30.86 | tr | | | 0.457 | 0.020 | 0.724 |
| 2-MeC24 | 20 | mV | 31.21 | nd | | | 0.116 | 0.116 | 0.184 |
| 9-nC25:1 | 21 | both | 31.33 | 0.060 | 0.046 | 0.199 | 2.334 | 0.140 | 3.697 |
| 7-nC25:1 | 22 | both | 31.52 | 0.080 | 0.063 | 0.267 | 1.526 | 0.061 | 2.417 |
| 5-nC25:1 | 23 | both | 31.78 | tr | | | 0.143 | 0.016 | 0.226 |

(continued)

Table S1. Continued

| | | | | Immature Virgin (iV) | | | Mature Virgin (mV) | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound | Peak | Source | Time | Mean Amount (Area) | SEM | Percent | Mean Amount (Area) | SEM | Percent |
| nC25 | 24 | both | 32.12 | 0.034 | 0.024 | 0.112 | 2.888 | 0.104 | 4.573 |
| uic | 25 | both | 32.90 | 0.286 | 0.193 | 0.948 | tr | | |
| 7,11-nC26:2 | 26 | both | 33.27 | 0.294 | 0.197 | 0.975 | 0.116 | 0.004 | 0.184 |
| 2-MeC25 | 27 | both | 33.60 | tr | | | 0.131 | 0.003 | 0.207 |
| 9-nC26:1 | 28 | mV | 33.70 | nd | | | tr | | |
| 7-nC26:1 | 29 | both | 33.79 | tr | | | 0.069 | 0.003 | 0.109 |
| 5-nC26:1 | 30 | both | 33.89 | tr | | | 0.085 | 0.003 | 0.134 |
| nC26 | 31 | STD | 34.44 | 1.000 | 0.000 | 3.320 | 1.000 | 0.000 | 1.580 |
| 9,13-nC27:2 | 32 | both | 35.40 | tr | | | 0.341 | 0.015 | 0.540 |
| 7,11-nC27:2 | 33 | both | 35.69 | tr | | | 8.628 | 0.274 | 13.664 |
| 2-MeC26 | 34 | both | 35.91 | 0.392 | 0.042 | 1.304 | 7.883 | 0.210 | 12.484 |
| 9-nC27:1 | 35 | both | 36.09 | 0.044 | 0.030 | 0.146 | 2.310 | 0.091 | 3.658 |
| 7-nC27:1 | 36 | both | 36.21 | 0.124 | 0.069 | 0.412 | 3.998 | 0.147 | 6.332 |
| 5-nC27:1 | 37 | both | 36.48 | tr | | | 0.283 | 0.007 | 0.448 |
| nC27 | 38 | both | 36.72 | 0.069 | 0.021 | 0.229 | 3.119 | 0.036 | 4.940 |
| 8,12-nC28:2 | 39 | both | 37.78 | tr | | | 0.077 | 0.007 | 0.123 |
| 7,11-nC28:2 | 40 | both | 37.90 | tr | | | 0.408 | 0.013 | 0.646 |
| 2-MeC27 | 41 | mV | 38.01 | nd | | | 0.060 | 0.002 | 0.100 |
| 6,10-nC28:2 | 42 | both | 38.15 | 0.084 | 0.011 | 0.277 | 0.116 | 0.003 | 0.184 |
| uic | 43 | both | 38.58 | 0.052 | 0.035 | 0.173 | tr | | |
| nC28 | 44 | both | 38.94 | 0.033 | 0.002 | 0.111 | 0.093 | 0.005 | 0.148 |
| 9,13-nC29:2 | 45 | mV | 39.90 | nd | | | 0.328 | 0.042 | 0.519 |
| 7,11-nC29:2 | 46 | both | 40.23 | tr | | | 13.987 | 0.326 | 22.151 |
| 2-MeC28 | 47 | both | 40.37 | 5.073 | 0.484 | 16.846 | 4.839 | 0.121 | 7.663 |
| 9-nC29:1 | 48 | both | 40.48 | 0.072 | 0.022 | 0.238 | 0.108 | 0.013 | 0.172 |
| 7-nC29:1 | 49 | both | 40.66 | 0.868 | 0.142 | 2.881 | 0.483 | 0.036 | 0.765 |
| 5-nC29:1 | 50 | both | 40.88 | tr | | | tr | | |
| nC29 | 51 | both | 41.10 | 0.168 | 0.008 | 0.558 | 0.477 | 0.011 | 0.756 |
| uic | 52 | both | 41.78 | 0.126 | 0.008 | 0.419 | 0.019 | 0.005 | 0.031 |
| uic | 53 | iV | 41.89 | 0.040 | 0.016 | 0.132 | nd | | |
| uic | 54 | both | 41.99 | 0.158 | 0.026 | 0.526 | 0.071 | 0.004 | 0.113 |
| uic | 55 | both | 42.44 | 0.160 | 0.012 | 0.532 | tr | | |
| uic | 56 | both | 42.67 | 0.312 | 0.064 | 1.037 | tr | | |
| uic | 57 | iV | 42.87 | 0.029 | 0.005 | 0.100 | nd | | |
| nC30 | 58 | both | 43.18 | tr | | | tr | | |
| uic | 59 | both | 43.87 | 0.042 | 0.042 | 0.138 | tr | | |
| 9,13-nC31:2 | 60 | both | 44.00 | 0.699 | 0.064 | 2.321 | 0.110 | 0.020 | 0.174 |
| uic | 61 | both | 44.16 | 0.125 | 0.011 | 0.414 | 0.066 | 0.008 | 0.105 |
| 7,11-nC31:2 | 62 | mV | 44.33 | nd | | | 0.673 | 0.023 | 1.065 |
| 2-MeC30 | 63 | both | 44.52 | 4.905 | 0.275 | 16.287 | 1.044 | 0.067 | 1.653 |
| 9-nC31:1 | 64 | both | | tr | | | tr | | |
| 7-nC31:1 | 65 | both | 44.67 | 1.365 | 0.253 | 4.534 | 0.097 | 0.016 | 0.153 |
| 5-nC31:1 | 66 | both | 44.82 | 0.337 | 0.052 | 1.120 | 0.053 | 0.013 | 0.083 |
| nC31 | 67 | both | 45.21 | 0.219 | 0.010 | 0.728 | 0.068 | 0.008 | 0.108 |
| uic | 68 | both | 45.84 | 0.187 | 0.015 | 0.622 | tr | | |
| uic | 69 | both | 45.96 | 0.247 | 0.020 | 0.820 | tr | | |
| uic | 70 | iV | 46.42 | 0.399 | 0.138 | 1.326 | nd | | |
| uic | 71 | iV | 47.53 | 0.035 | 0.035 | 0.115 | nd | | |
| 11,17-nC33:2 | 72 | both | 47.53 | 0.104 | 0.054 | 0.345 | tr | | |
| Xi-nC33:2 | 73 | both | 47.91 | 4.496 | 0.585 | 14.928 | 0.204 | 0.069 | 0.323 |
| uic | 74 | iV | 48.16 | 0.091 | 0.020 | 0.304 | nd | | |
| Xi-nC33:1 | 75 | both | 48.38 | 2.716 | 0.338 | 9.018 | 0.146 | 0.040 | 0.232 |
| uic | 76 | iV | 48.50 | 0.060 | 0.037 | 0.198 | nd | | |
| uic | 77 | both | 49.09 | 0.141 | 0.068 | 0.469 | tr | | |
| uic | 78 | iV | 49.73 | 0.464 | 0.082 | 1.542 | nd | | |
| uic | 79 | iV | 50.22 | 0.343 | 0.198 | 1.137 | nd | | |
| uic | 80 | iV | 50.36 | 0.148 | 0.099 | 0.491 | nd | | |
| uic | 81 | iV | 51.18 | 0.307 | 0.117 | 1.020 | nd | | |
| Xi-nC35:2 | 82 | iV | 51.31 | tr | | | nd | | |
| Xi-nC35:2 | 83 | both | 51.58 | 2.256 | 0.152 | 7.492 | tr | | |
| Xi-nC35:1 | 84 | both | 52.14 | 0.577 | 0.062 | 1.917 | tr | | |
| uic | 85 | iV | 53.64 | 0.158 | 0.030 | 0.524 | nd | | |
| uic | 86 | both | 54.19 | 0.068 | 0.023 | 0.226 | tr | | |

(continued)

Table S1. Continued

Compound Xi: unknown position of double bond(s); uic = unidentified compound (absent from vial control). The peak numbers refer to peaks that showed up in all samples. Other peaks were not included in this analysis. Source indicates which samples included the designated compound: both = present in both; iV = present in immature virgins; mV = present in mature virgins. Mean amount is expressed as peak area counts normalized to the area of the standard (peak 36; nC26 [10 ng]). n = 5 independent samples. nd = not detected (limit of detection 10 pg on-column) tr= trace $< 0.1\%$. Percent refers to the proportion that each compound comprises of the total amount of hydrocarbons extracted.

**Results:** We and others have suggested that although *n*-alkanes and externally branched monomethylalkanes perform a structural function, alkenes act as signals (e.g., [S1]). Consistent with this hypothesis, the proportion of alkanes (externally branched monomethylalkanes + *n*-alkanes) to total hydrocarbons did not significantly differ between immature and mature virgins. No internally branched alkanes were detected in our samples. The proportion of alkenes (monoenes + dienes) did significantly increase with age (immature virgins: $47.8 \pm 1.7\%$. Mature virgins: $61.2 \pm 0.4\%$, $p < 0.01$). Although no difference was observed in the proportion of "total" alkanes, the proportion of monomethylalkanes was increased in the immature virgins ($34.7 \pm 1.3\%$) compared to the mature virgins ($22.5 \pm 0.3\%$; $p < 0.01$). The proportion of *n*-alkanes significantly increased in the mature virgins (immature virgins: $3.0 \pm 0.2\%$. Mature virgins: $15.3 \pm 0.3\%$, $p < 0.01$). With respect to the alkenes, the proportion of dienes significantly increased in the mature virgins ($26.5 \pm 1.0\%$ and $40.8 \pm 0.2\%$, respectively; $p < 0.01$). The proportion of monoenes did not differ between immature and mature virgins.

**Methods:** Hexane extracts, prepared as described, were reconstituted in 60 μl of hexane containing 10 ng/μl hexacosane (nC26) as an injection standard. A 1 μl sample of the extract was then injected on a Varian CP3800 gas chromatograph with a flame ionization detector and PTV injector (cool-on-column mode), fitted with 0.25 mm × 15 m Varian CP8510 fused silica capillary column with a 0.25 μm film thickness and a 2.5 m deactivated silica retention gap (Varian, Mississauga, Ontario, Canada). Carrier gas was Helium at a flow rate of 1 ml/min.

Analysis of the extract was carried out with a column temperature profile that began at 50°C (held for 1 min) and was ramped at 15°C/min to 150°C and then at 3°C/min to 280°C, where it was held for 5 min. The injector oven was programmed at 50°C for 0.1 min and then ramped to 280°C at 200°C/min. Varian Star Integrator software was used to calculate the retention time and total area of each peak for subsequent analysis.

Compound identification was conducted on a Shimadzu GC-17A gas chromatograph fitted with a HP-5MS fused silica capillary column (0.25 mm × 30 m, 0.25 μm film thickness) linked to a mass analyzer (Shimadzu QP5050A mass spectrometer). The injector was used in splitless mode with a splitless time of 0.5 min, and the carrier gas was helium at 1 ml/min. Injector temperature was held constant at 280°C. An oven program that began at 60°C (1 min) and was ramped at 6°C/min to 225°C and then 3°C/min to 310°C(10 min) and a pressure program of 57 kPa (1 min) to 185 kPa (1.83 min) at 2 kPa/min were employed. Electron impact positive ions at 70 eV were recorded in the scanning mode (mass range scanned 45–550 amu). The mass spectra were interpreted by fragmentation analysis and comparison to published criteria [S2, S3–S8]. Retention indices, based on a series of n-alkane standards (C10–C32; extrapolation to C36), were used to match GC-FID and GC-MS data and to obtain approximate comparisons to published data.

Statistical analysis of the proportion of hydrocarbon types (i.e., straight and branched alkanes and mono- and dienes) was conducted by Steel's 2-tailed Rank Test ($p \leq 0.01$) following Bartlett's F-test for equality of variance with ToxStat v3.4 software (Western Ecosystems Technology Inc., Cheyenne, WY). All calculations were made after removal of the standard area from the data matrix.

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Report

# Generalization of Courtship Learning in *Drosophila* Is Mediated by *cis*-Vaccenyl Acetate

**Aki Ejima,[1] Benjamin P.C. Smith,[2,4] Christophe Lucas,[2,5] Wynand van der Goes van Naters,[3] Carson J. Miller,[3] John R. Carlson,[3] Joel D. Levine,[2] and Leslie C. Griffith[1,*]**

[1] Department of Biology and Volen Center for Complex Systems
Brandeis University
MS008
415 South Street
Waltham, Massachusetts 02454

[2] Department of Biology
University of Toronto at Mississauga
3359 Mississauga Road North
Mississauga, Ontario L5L 1C6
Canada

[3] Department of Molecular, Cellular, and Developmental Biology
Yale University
New Haven, Connecticut 06520

## Summary

**Reproductive behavior in *Drosophila* has both stereotyped and plastic components that are driven by age- and sex-specific chemical cues. Males who unsuccessfully court virgin females subsequently avoid females that are of the same age as the trainer. In contrast, males trained with mature mated females associate volatile appetitive and aversive pheromonal cues and learn to suppress courtship of all females. Here we show that the volatile aversive pheromone that leads to generalized learning with mated females is (*Z*)-11-octadecenyl acetate (*cis*-vaccenyl acetate, cVA). cVA is a major component of the male cuticular hydrocarbon profile, but it is not found on virgin females. During copulation, cVA is transferred to the female in ejaculate along with sperm and peptides that decrease her sexual receptivity. When males sense cVA (either synthetic or from mated female or male extracts) in the context of female pheromone, they develop a generalized suppression of courtship. The effects of cVA on initial courtship of virgin females can be blocked by expression of tetanus toxin in *Or65a*, but not *Or67d* neurons, demonstrating that the aversive effects of this pheromone are mediated by a specific class of olfactory neuron. These findings suggest that transfer of cVA to females during mating may be part of the male's strategy to suppress reproduction by competing males.**

*Correspondence: griffith@brandeis.edu

[4] Present address: FIRMENICH SA, Route des Jeunes 1, PO Box 239, CH-1211 Genève 8, Switzerland.

[5] Present address: Department of Ecology and Evolution, University of Lausanne, Biophore, UNIL-Sorge, Room 3106, CH-1015 Lausanne, Switzerland.

## Results

In *Drosophila,* unsuccessful courtship decreases subsequent courtship [1, 2]. When the initial courtship object (trainer) is a virgin female, suppression has been shown to be the result of formation of an associative memory linking the failure to copulate with volatile stimulatory courtship cues specific to the age of the female trainer [1]. Exposure to a mated female, on the other hand, results in a suppression of courtship toward all types of females [1, 2] and is believed to require an aversive pheromone [3]. The cuticular hydrocarbon profiles of mature and immature females differ significantly [1, 4, 5], but these types of females also differ behaviorally. Mature virgins are receptive to courtship, while immature virgins and mated females show characteristic rejection behaviors. Immature females kick, fend, and run away, while mated females extrude their ovipositors [6, 7]. To determine whether female behavior or appearance had any role in the development of age-specific or general courtship suppression, we trained and tested males with decapitated females in dim red light (Figure 1). Memory index was expressed as a ratio of the courtship index (CI) during the 10 min test period to the mean CI of a sham-trained males tested with the same type of female. The use of a ratio allows direct comparison of the strength of memory between conditions, with a value of $CI_{test}/mCI_{sham} = 1$ indicating no memory.

We find that, consistent with results with mobile trainer females [1], decapitated virgins provoke an age-specific suppression, while decapitated mated female trainers cause general suppression of courtship. These data indicate that the specificity of learning with different trainer types does not stem from behavioral differences in the trainer female's response to courtship or from visual cues specific to the trainer type. Generalization of learning with a mated female trainer is therefore the result of chemosensory cues. In all subsequent experiments, decapitated trainers and testers were used, except where noted.

In the previous experiment, males were placed in the same chamber as the trainer female and therefore could obtain both olfactory and gustatory information about that female. To investigate the nature of the generalization cue, we attempted to reconstitute generalized learning with virgin trainers and mated female extracts. Placing a filter containing a hexane extract of mated female in the chamber with either a mature or immature trainer female caused a generalization of learning, as demonstrated by the ability of mature trainers to generate memory against immature testers and vice versa (see Table S1 in the Supplemental Data available online). To determine whether the active component of the mated female bouquet was volatile, we used a two-compartment courtship chamber (Figure 2A) and placed a pheromone source (fly corpse or filter with extract) across a mesh from the side of the chamber containing the male and the trainer female. For both mature and

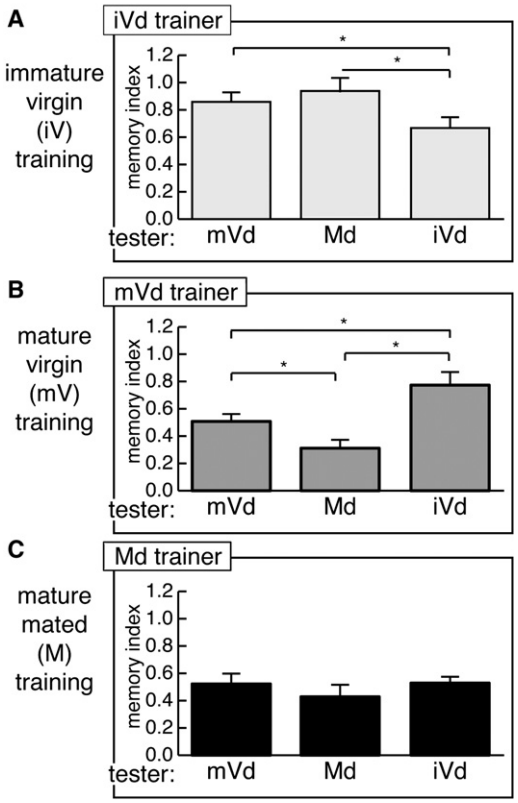


Figure 1. Courtship Suppression Learned with a Mated Female Trainer Is Generalized to All Types of Females

(A) Training with an immature decapitated virgin female produces suppression only when the tester is also an immature female.

(B) Training with a mature decapitated female produces suppression of courtship with mature (virgin and mated) testers.

(C) Training with a decapitated mated female produces a generalized suppression to all female testers.

Data were analyzed as described in Experimental Procedures; *p < 0.05 for the indicated comparison. Trainer and tester types are abbreviated as follows: Md, decapitated mated female; mVd, decapitated mature virgin; iVd, decapitated immature virgin. Histogram bars for data in which males were trained with mated females are black; with mature virgins, dark gray; immature virgins, light gray.

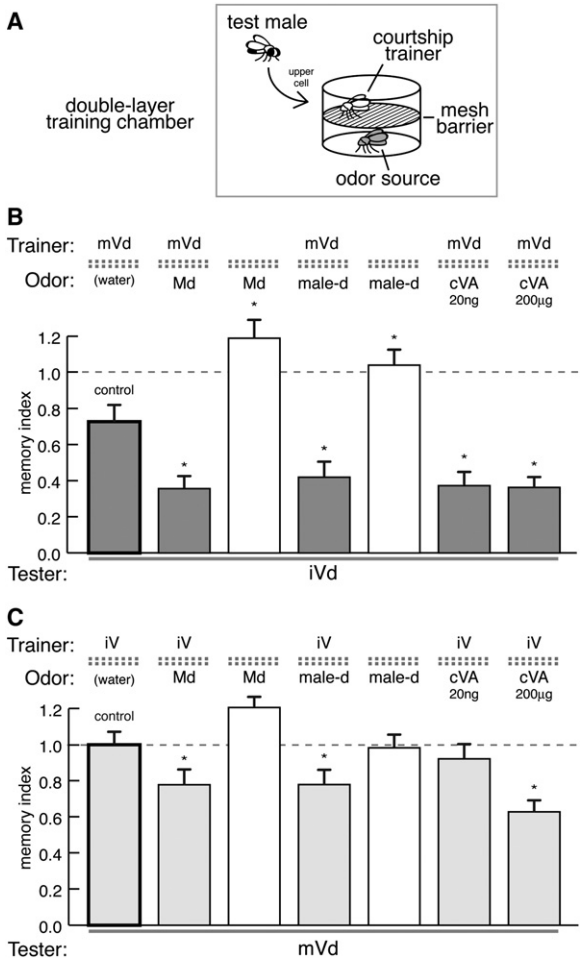


Figure 2. The Generalizing Cue Is a Volatile Pheromone

Histogram bars for data in which males were trained with mature virgins, dark gray; immature virgins, light gray; no courtship objects, white. *p < 0.05 for comparison to control.

(A) Males were trained in a courtship arena with two chambers separated by a mesh as shown in the diagram. The male was placed in the top chamber with or without a courtship object. The bottom chamber contained a decapitated fly on a filter paper soaked with either water or cVA. Mesh position vis-à-vis cues is indicated over each histogram bar in (B) and (C) by hatching.

(B) Training with a mature decapitated female (mVd) produces very little suppression of immature virgin (iVd) courtship (left bar, labeled "control"). Addition of a decapitated mated female (Md), a decapitated male (male-d), or pure cVA to the bottom chamber causes the male to generalize his unsuccessful courtship experience and suppress courtship of the immature tester. Presentation of a mated female or a male across the mesh in the bottom chamber in the absence of courtship object does not alter courtship of the immature tester.

(C) Training with an immature decapitated female produces very little suppression of mature virgin courtship (left bar, labeled "control"). Addition of a decapitated mated female (Md), a decapitated male (male-d), or a high dose of cVA to the bottom chamber causes the male to generalize his unsuccessful courtship experience and suppress courtship of the mature tester. Presentation of a mated female or a male across the mesh in the absence of courtship object does not alter courtship of the mature tester.

immature trainers, the presence of volatile compounds from either a mated female or a male was sufficient to cause generalization of courtship suppression, although the effects of these pheromones appeared more potent with mature trainers (Figures 2B and 2C). In the absence of a courtship object, the presence of a filter with extract (Table S1) or a corpse (Figures 2B and 2C) did not generate suppression of courtship toward tester females.

We next addressed the identity of the generalization cue. The mated female and mature virgin trainers we use are of the same age (4–5 days old) and might be expected to have similar cuticular hydrocarbon profiles, so any compound that differed between these two classes of females might have a role in generalization. We compared hexane washes of 4- to 5-day-old virgins and 4- to 5-day-old mated females that had been mated 24 hr before extraction by using gas chromatography-flame ionization detection and mass spectrometry (Figure 3A, top two traces). Qualitatively, the two types of females appear identical with the exception of one peak, *cis*-vaccenyl acetate (cVA), which is undetectable in virgins but present at significant levels in mated females. cVA is a major component of mature male cuticular hydrocarbon (Figure 3A, lower trace) and is not synthesized by females [8, 9]. Its presence in both males and mated

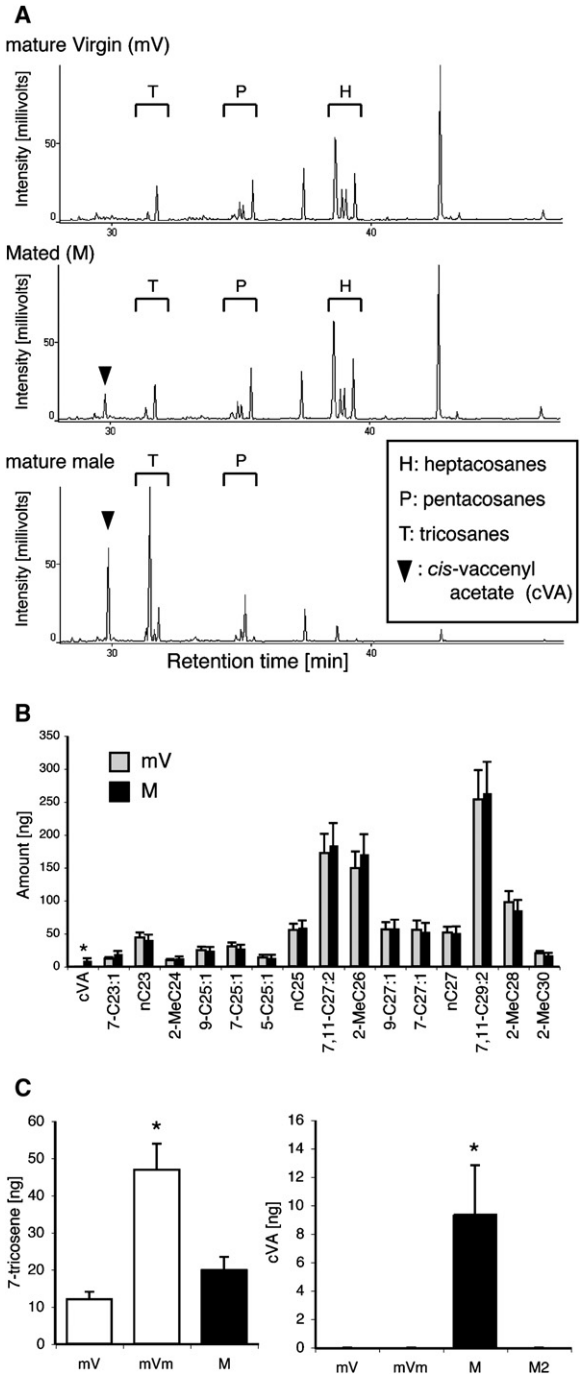


Figure 3. Cuticular Hydrocarbons of Mature Virgins and Mated Females Differ in Levels of *cis*-Vaccenyl Acetate

Cuticular hydrocarbons were extracted in hexane and analyzed by gas chromatography-flame ionization detection and mass spectrometry.

(A) Total hydrocarbon profiles for mature virgin (top), mated female (middle), and male (bottom) extracts. Each profile is from a hexane extract of 20 age-matched virgin or mated (24 hr before extraction) females.

(B) Quantification of major compounds for mature virgin (light bars, n = 28) and mated female (black bars, n = 29) single-fly extracts. Mated females were extracted 24 hr after copulation. *p < 0.05 for comparison between mated and virgin female.

(C) 7-tricosene (left) and cVA (right) levels from decapitated mature virgins (mV, n = 28), decapitated mVs that were courted, but not mated, and extracted immediately (mVm, n = 22), mated females who copulated >14 min (M, n = 29) and mated females who had copulation interrupted after 2 min (M2, n = 16). Mated females were extracted 24 hr after copulation. *p < 0.05 for comparison of the marked female type to all other types. Other types were not significantly different from one another (p > 0.05).

females makes it a good candidate for being the generalization cue for courtship learning.

Quantitation of mature virgin and mated female hydrocarbon levels shows a significant difference in cVA levels (Figure 3B). There is also a small, but statistically insignificant (p > 0.05), increase in 7-tricosene (7-C23:1). 7-tricosene is a major component of the male cuticular hydrocarbon and is believed to have inhibitory effects on male-male courtship [10]. Transfer of 7-tricosene to females has been shown to occur via cuticular contact during copulation, but it is largely gone by 8 hr after mating [11]. Consistent with this, we see larger amounts of 7-tricosene on virgins that have been courted, but not copulated, when they are extracted immediately after the courtship (Figure 3C, left). Mature virgins and mated females that have been aged 24 hr after copulation have lower and statistically indistinguishable levels of 7-tricosene. The loss over time (presumably through passive transfer and grooming) of 7-tricosene and the nonvolatile nature of this compound make it an unlikely candidate for the generalizing cue. It is also significant to note that we do not see any decreases in mated females of hydrocarbons such as 7,11-heptacosadiene (7,11-nC27:2), 7,11-nonacosadiene (7,11-nC29:2), and 9-pentacosene (9-C25:1) that are believed to be stimulatory pheromones for courtship conditioning [12, 13]. Thus, the only consistent mated female-specific difference in hydrocarbon content we find is in cVA.

How does cVA, a male lipid, become part of the mated female pheromonal profile? Like 7-tricosene, cVA could be transferred directly by contact during courtship and/or copulation. Alternatively, the presence of cVA in the male ejaculatory bulb suggests that it can be transferred with sperm during copulation [8]. To determine the major mode of cVA transmission, we measured cVA levels on virgin females, virgin females that were courted in a small chamber and extracted immediately, and females that were extracted 24 hr after complete (>14 min) copulation or disrupted ($\leq$2 min) copulation. We find that only females that copulated long enough to receive ejaculate [14] have significant levels of cVA (Figure 3C, right). Females that did not copulate and were merely courted by the male had virtually no cVA, even though they had significant amounts of passively acquired 7-tricosene (Figure 3C, left). This suggests that transfer of cVA occurs via ejaculate and that mated females store cVA.

These data support a role for cVA as a generalizing cue, but the presence of other volatile compounds in mated female and male extracts might still be required. To test the sufficiency of cVA, we applied varying amounts of purified cVA to filters across the mesh in a two compartment courtship chamber, trained males with either mature or immature virgins, and then tested with a virgin of the other age. In both cases, cVA was sufficient to generalize memory (Figures 2B and 2C). With mature virgin testers, 0.2 ng of cVA was enough to generalize memory (Table S2). The average amount of cVA

present on a mated female 24 hr after mating is 9.3 ± 3.5 ng, so the effects of synthetic cVA are occurring in the biologically relevant dose range. In contrast to results with mature virgins, pairing cVA with immature trainers is less effective. Only large amounts of cVA (200 μg) produce generalized learning. cVA alone (with no trainer female) is ineffective, as is *cis*-vaccenol (cVOH), a putative metabolite of cVA [15] with either trainer type (Table S2).

The circuitry underlying generalization is of great interest for understanding this behavior. As a first step, we sought to identify the olfactory receptor neurons that carry the aversive cVA signal. cVA has been shown to be sensed by a subset of trichoid sensilla [16, 17] in the *Drosophila* antenna, which includes the T1 type sensillum [18] that expresses *Or67d* [19]. By using the "empty neuron" preparation [20, 21], which allows the decoding of odor specificity for *Drosophila* olfactory receptors (ORs), we found that there is an additional cVA-responsive receptor, Or65a, and that Or65a and Or67d differ in their response to cVOH, with Or67d responding strongly and Or65a not responding [22]. Or65a is one of the several ORs expressed in neurons of the T3 sensilla [19].

With this information, we investigated the role of the olfactory receptor neurons that express these two receptors in sensing the aversiveness of cVA. Initial courtship levels provide a simple assay for this property of cVA. Naive males show lower levels of courtship toward mated females than toward virgins of the same age [11, 23–25]. This effect can be reproduced by addition of a cVA-laced filter across the mesh in the two-compartment courtship chamber with a mature virgin courtship object in the upper chamber with the male (Figure 4A). Expression of tetanus toxin (TNT), which blocks synaptic release, under control of *Or65a-GAL4*, but not *Or67d-GAL4*, abolished the ability of cVA to inhibit initial courtship (Figures 4A and 4B). Males heterozygous for *Or65a-GAL4* or the *UAS-TNT* transgene showed cVA-dependent courtship suppression as did males expressing inactive toxin (TNT-VA) under control of *Or65a-GAL4*. These results indicated that ORNs expressing *Or65a-GAL4*, but not *Or67d-GAL4*, are required for sensing cVA as an aversive cue.

*Or65a* has been reported to be expressed solely in ORNs that innervated the DL3 glomerulus of the antennal lobe with anti-GFP immunohistochemistry in animals expressing GFP under control of *Or65a-GAL4* promoter fusions. [19, 26]. Our *Or65a-GAL4*, while it has strong expression in DL3, shows a somewhat broader pattern, with significant expression in VA1v, DC1, and DA4m (Figure S1). By using confocal microscopy to directly visualize GFP from a *UAS-mCD8-GFP* transgene in unfixed brains, we compared our GAL4 line to the published *Or65a-GAL4* lines. We found that our line was many times stronger than that published by Fishilevich et al. [26], which has predominant expression in DL3 (Figure S2). GFP fluorescence in the Couto et al. [19] GAL4 line was barely detectable (data not shown). To determine whether the weak, but more DL3-specific, Fishilevich et al. driver (which we designate as *V-Or65a-GAL4* in Figure 4B) would also block cVA effects, we used it to express active and inactive tetanus toxin. Consistent with the results with our *Or65a-GAL4*, active tetanus toxin significantly abrogated the ability of cVA to suppress initial courtship (Figure 4B), although the

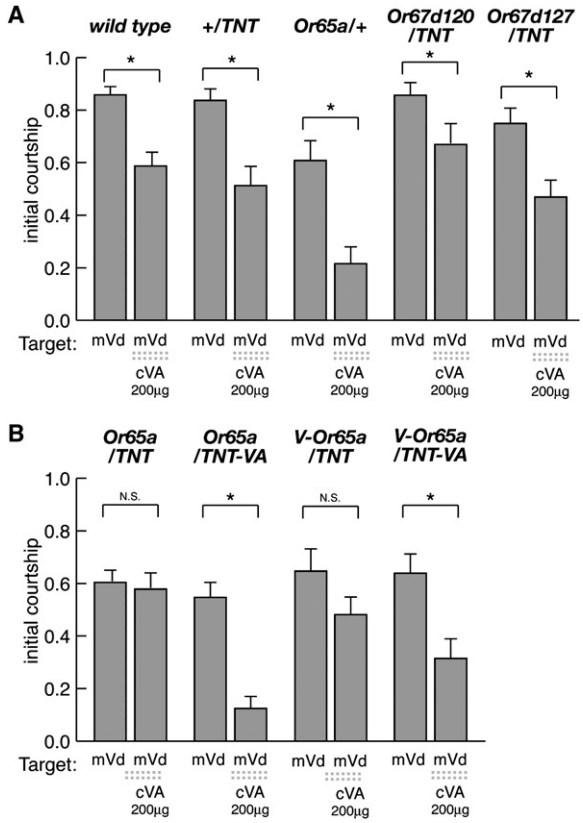


Figure 4. Modulation of Courtship by cVA Requires *Or65a-GAL4*, but Not *Or67d-GAL4* Olfactory Sensory Neurons

Initial courtship level of naive males was measured with a mature virgin courtship object in a two-chambered courtship arena with a filter containing either cVA or water in the lower chamber. *$p < 0.05$ for indicated comparison; N.S. means not significant.

(A) Wild-type males court mated females with less vigor than they court mature virgins. This courtship decrement can be reproduced by the odor of cVA. *UAS-TNT*/+ and *Or65a-GAL*/+ males are indistinguishable in cVA sensitivity from wild-type. Expression of tetanus toxin under control of two independent *Or67d* promoter GAL4 drivers does not eliminate cVA-dependent modulation of initial courtship.

(B) Animals expressing active tetanus toxin under control of our *Or65a-GAL4* or a published *Or65a* line [26] (*V-Or65a-GAL4)* fail to suppress initial courtship in the presence of cVA. The difference in strength of blockade is likely to be due to the fact that *Or65a-GAL4* is a much stronger driver than *V-Or65a-GAL4* (see Figure S2). Expression of mutant tetanus toxin (TNT-VA) does not block the courtship-suppressing effects of cVA with either driver.

effect appeared weaker than with our line. Inactive tetanus toxin had no effect on cVA-mediated suppression. We conclude that the aversive effects of cVA on initial courtship are most likely mediated by ORNs expressing *Or65a.*

## Discussion

Since its identification as a male-specific lipid in the *Drosophila* ejaculatory bulb [8], there has been interest in cVA as a potential modifier of reproductive behavior. In this study, we show that pairing of cVA and a virgin female trainer is sufficient to reproduce the unique effects of exposure to a mated female: generalized suppression of male-female courtship. We identify

neurons expressing *Or65a-GAL4* as responsible for the aversive effects of cVA. These results provided the first molecular/genetic insights into the pheromones responsible for courtship learning.

Our results also provide some insight into the controversial nature of cVA's role in *Drosophila* behavior. The literature on cVA has posited roles for this lipid as both as an attractant [17, 27] and as an antiaphrodisiac [15, 28, 29], although this last function has been disputed [30, 31]. The social attractant role of cVA makes sense because it is deposited on eggs at feeding sites by females, and congregation at such sites is advantageous in terms of finding food and mates. The aversive role is equally plausible in light of cVA's transfer to females during mating, which would make it a marker of previous copulation. Understanding the molecular basis of cVA function and the circuitry subserving its behavioral effects will be necessary to completely unravel its multiple roles, but several important findings have emerged.

First, there are multiple cVA receptors, and they appear to have different behavioral roles. Or67d, which is expressed in T1, singly innervated sensilla, has a role in sensing the attractive properties of cVA [17, 18]. This receptor is not required for the courtship inhibitory role of cVA; this function appears to be served by Or65a, which is expressed in one of the three neurons of the T3 trichoid sensilla [19]. These data suggest that Or67d is an "appetitive" cVA receptor while Or65a is an "aversive" receptor. Segregating the hedonic effects of this lipid by activating two independent receptors is an interesting way of establishing, at an early step, independent behavioral circuits for attraction and repulsion. The lack of behavioral redundancy between *Or65a* and *Or67d* neurons is also interesting in light of findings with the nonpheromonal olfactory receptor *Or43b*, where elimination of the receptor does not change the behavioral response to its preferred odorant [32]. The interpretation of this study was that other olfactory receptors that recognized the odorant, but projected to different antennal glomeruli, could signal the same behavioral response. Our results suggest that for some pheromone odorants the antennal lobe circuitry they connect to is critical to the behavioral output they engender.

Second, responses to cVA appear to be context dependent. Having multiple sensory channels for cVA does not itself help the animal decide how to respond to this chemical; there must be some mechanism by which the environment or other cues can tell the animals which sensory channel is relevant for a particular situation. One way to achieve this would be to have the cVA channels be linked to other, situation-relevant, odor cues. In the case of both attraction and aversion, this appears to be the case. The first report of cVA as an attractant, by Bartelt et al. in 1985, found that cVA was not attractive unless presented with food or food-associated odors [27]. This group's assay set-up was designed to measure fast (minutes) attractive responses in an open arena, as opposed to the long-term (days) maze/trap assays used by Smith's group [17], who did not uncover a role for food odor. The two paradigms may differ in sensitivity and relevance to particular behaviors, but the issue remains to be fully explored.

Context also appears to be important for the aversive effects of cVA. Synthetic cVA is a very effective, and completely sufficient, generalizing cue when applied in small doses to mature virgin trainers, but is not very effective, requiring $10^4$ times more, when used with immature virgin trainers (Table S1). The potency of mated female extracts with immature trainers is also less than with mature trainers, but the difference is not as exaggerated. This strongly suggests that some component of the mature female hydrocarbon profile that is not shared with immature virgins acts in concert with cVA to generalize learning. With the immature trainer, the mated female extract is supplying a low dose of cVA, but it also may supply a mature female compound that enhances the cVA effect. The identity of the compound(s) is unknown, but given that mature male extract is also able to allow generalization with immature trainers, it may be a hydrocarbon that is shared between mature males and females.

The requirement for concurrent mature fly chemical signals for cVA to be an effective aversive cue and generalizer of learning is not unreasonable from an evolutionary point of view. Under normal circumstances, cVA is only found on males or mated females. The meaning of cVA in the presence of male hydrocarbons is clear: males should suppress courtship of other males because it is wasted reproductive energy. If a male in the wild sees cVA in the context of an immature female pheromone profile, however, it is likely that he has encountered a virgin at a feeding site where cVA-laced eggs have been deposited, and he should not suppress courtship.

The underlying logic of suppressing courtship when presented with cVA in the context of a mature (and theoretically receptive) female is less obvious from a male's point of view. Copulation with a previously mated female is not ideal because she is already storing sperm from her previous mate, but there is still marginal gain; the second male's sperm can displace the first male's sperm [33]. From the female's reproductive point of view, remating might also be advantageous because she will have more genetically diverse offspring, but it comes at a cost: it is correlated with reduced life span [34]. The only player for whom remating does not have some advantage is the first male. It has been well documented that components of seminal fluid in *Drosophila* alter female behavior and reproduction to decrease remating [35]. Transfer of cVA may be another facet of the successful male's strategy to decrease reproduction by competitor males. The effect of cVA on initial courtship decreases a second male's chances of success with that particular mated female, but the aftereffect, generalized suppression of his courtship drive, eliminates him as a competitor for other virgin females. The ability of cVA to engage the intrinsic plasticity machinery that allows animals to adapt to and learn from change to bring about a long-lasting change in another male's behavior could provide selective advantage to successfully copulating males.

## Experimental Procedures

### Fly Strains

Flies were raised on autoclaved cornmeal-yeast-sucrose-agar food in a 12 hr light/dark cycle at 25°C. Males and females were anesthetized with $CO_2$ on the day of eclosion, and then used immediately as immature flies or separated by sex and aged for 4 or 5 days. Experimental males were housed in individual tubes. Mated females were

prepared by putting 3-day-old females with males. Only females that copulated for ≥14 min were used the next day. Decapitated flies were prepared by cutting their head off with fine scissors just before use. *Canton-S* was used as the wild-type strain. Descriptions of the generation and characterization of promoter-GAL4 fusions for *Or65a* and *Or67d* are provided in Supplemental Experimental Procedures. V-*Or65a-GAL4* was a gift of L. Vosshall (Rockefeller University, New York, NY). *Or-GFP* transgenic lines used for identification of specific glomeruli were a gift of B. Dickson (IMP, Vienna). *UAS-TNT* flies (active TNT-E and inactive IMPTNT-VA, obtained from the Bloomington Stock Center) were crossed to each GAL4 line for targeted expression of tetanus toxin [36].

### Behavioral Assays

All behavior experiment was done under dim red lights in a Harris environmental room (25°C, 70% humidity). A 4- or 5-day-old male was placed with a decapitated female "trainer" in a single-pair-mating chamber (8 mm in diameter, 3 mm deep) for 1 hr. In the experiments presented in Figure 2C, an intact immature virgin was used as a trainer for convenience, but this should not affect training outcome (compare our Figure 1 and Figure 1 in [1]). Wet filter paper (Whatman, 42 ashless) was put in each chamber to maintain humidity. To block direct contact with pheromone filters or odor-source flies, a fine nylon mesh (Tetko, 3-180/43) was introduced into a two-part chamber (8 mm in diameter, 6 mm deep). The first 10 min of the training period were videotaped with a digital camcorder (Sony, DSR-PD150). Pairs that copulated during training or that showed courtship lower than CI < 0.1 (see below) were eliminated from further analysis. Immediately after training, males were transferred into a clean chamber and paired with a decapitated female "tester" and videotaped for 10 min. Sham trained males were kept alone in the mating chamber for the first hour and paired with a tester for 10 min.

For each of the 10 min periods, a courtship index (CI) was calculated. CI is the fraction of time a male spent in courtship activity in the 10 min observation period (CI = courtship [s]/observation [s]). Initial courtship is CI of the first 10 min when a naive male was put together with a female. Memory index is calculated by dividing CI at test ($CI_{test}$) by the mean of sham CIs ($CI_{sham}$): $CI_{test}/mCI_{sham}$. If $CI_{test}/mCI_{sham} = 1$, it indicates that there has been no learning since the courtship level of trained males is equivalent to that of sham trained males. ≥20 males were tested for each condition.

### Statistical Analysis

Each CI was subjected to arcsine square root transformation to effect an approximation of normal distribution, with JMP software version 5.0.1.2 for the Macintosh. ANOVA with each indicated condition as the main effect was performed on the transformed data. Posthoc analysis was done with Fisher's PLSD test. Bars in figure represent means ± SEM, with levels of significance indicated by *p significant = $\alpha < 0.05$.

### Pheromone Extraction

Hexane extracts were prepared by washing the bodies of 20 flies with 80 μl of hexane (Aldrich). In order to collect male pheromones, a mature male was put on a wet filter paper in a mating chamber for 1 hr to transfer odors to the filter. For training, 5 μl of extract, containing pheromones of about one donor fly, was applied to the filter paper in a mating chamber and evaporated for 2 min, after which 7 μl of water was added to the filter to add humidity. For chemistry, the extract was evaporated (passive evaporation at room temperature in a dust-free environment) and stored at −80°C until analyzed. Synthetic cVA was purchased from Pherobank. Detailed methods for hydrocarbon analysis are provided in Supplemental Experimental Procedures.

### Supplemental Data

Supplemental Data include two figures, two tables, and Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/7/599/DC1/.

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# Supplemental Data



# Generalization of Courtship Learning in *Drosophila* Is Mediated by *cis*-Vaccenyl Acetate

**Aki Ejima, Benjamin P.C. Smith, Christophe Lucas, Wynand van der Goes van Naters, Carson J. Miller, John R. Carlson, Joel D. Levine, and Leslie C. Griffith**

## Supplemental Experimental Procedures

### Generation of Or65a- and Or67d-Promoter GAL4 Fusion Lines

The *Or65a-GAL4* line was generated by inserting a 4665 bp fragment upstream of the predicted translational start of *Or65a* into the pG4PN vector [S1]. The promoter fragment, with added Kpn1 and Not1 ends, was obtained from genomic DNA extracted from a male Canton S wild-type fly with the Expand High Fidelity PCR system (Roche) with primers 5′-ATAGGTACCGGCACCATCACTTCGAAC-3′ and 5′-ATAGCGGCCGCTTTCAATCCGATCCAATGC-3′. 19 independent lines were generated. Multiple lines crossed to *UAS-GFP* showed a similar expression pattern; neurons expressing GFP were found to innervate trichoid sensilla in a broad region of the distal-lateral side of the antenna (the T3 region by the Shanbhag et al. designation [S2]). In the adult brain, expression was limited to the DL3, DC1, VA1v, and DA4m glomeruli in the antennal lobe, with DL3 showing the strongest expression (see Figure S1). *Or67d120.1-GAL4* and *Or67d127.1-GAL4* were generated similarly, with 6139 bp of DNA immediately upstream of the predicted *Or67d* translational start site to drive expression of GAL4. The *Or67d* promoter fragment was amplified from Canton S genomic DNA with the PCR primers 5′-TAC CTAGGCTTTATTGCTCTTAAATATTTGAACAATCCA-3′ and 5′-TAG CGGCCGCTTGTTTGTTAGCTATGCAACTTAAAGGAG-3′. These oligonucleotides carry AvrII and NotI restriction sites, respectively, which were used subsequently to clone the resulting promoter fragment into pG4PN+. pG4PN+ is identical to pG4PN, except that it has a slightly modified cloning site upstream of GAL4 to allow cloning of AvrII/NotI fragments. The lines *Or67d120.1-GAL4* and *Or67d127.1-GAL4* carry independent insertions of the *Or67d* promoter GAL4 construct, and both lines show similar patterns of expression in trichoid sensilla in the central region of the antenna when crossed to *UAS-GFP* reporter lines. This pattern is consistent with expression in singly-innervated T1 sensilla [S3], as reported by Couto et al. [S4], but also includes a small number of basiconic sensilla. In the adult brain, this line expressed in at least 5 glomeruli, most prominently in DA1 and VA6. None of the glomeruli appeared to overlap those found in the *Or65a-GAL4* pattern (data not shown).

### Hydrocarbon Analysis

Hexane extracts, prepared as described, were reconstituted in 60 μl of hexane containing 10 ng/μl hexacosane (nC26) as an injection standard. A 1 μl sample of the extract was then injected on a Varian CP3800 gas chromatograph with a flame ionization detector and PTV injector (cool-on-column mode), fitted with 0.25 mm × 15 min Varian CP8510 fused silica capillary column with a 0.25 μm film thickness and a 2.5 m deactivated silica retention gap (Varian Inc, Mississauga, ON, Canada). Carrier gas was helium at a flow rate of 1 ml/min.

Analysis of the extract was carried out with a column temperature profile that began at 50°C (held for 1 min), ramped at 15°C/min to 150°C, and then 3°C/min to 280°C where it was held for 5 min. The injector oven was programmed at 50°C for 0.1 min and then ramped to 280°C at 200°C/min. Varian Star Integrator software was used to calculate the retention time and total area of each peak for subsequent analysis.

Compound identification was conducted on a Shimadzu GC-17A gas chromatograph fitted with a HP-5MS fused silica capillary column (0.25 mm × 30 min, 0.25 μm film thickness) linked to a mass analyzer (Shimadzu QP5050A mass spectrometer). The injector was used in splitless mode with a splitless time of 0.5 min and the carrier gas was helium at 1 ml/min. Injector temperature was held constant at 280°C. An oven program that began at 60°C (1 min) and was ramped at 6°C/min to 225°C and then 3°C/min to 310°C (10 min) and pressure program of 57 kPa (1 min) to 185 kPa (1.83 min) at 2 kPa/min was employed. Electron impact positive ions at 70 eV were recorded in the scanning mode (mass range scanned 45–550 amu). The mass spectra were interpreted by fragmentation analysis and comparison to published criteria [S5–S11]. Retention indices, based on a series of n-alkane standards (C10–C32; extrapolation to C36), were used to match GC-FID and GC-MS data and to obtain approximate comparisons to published data.

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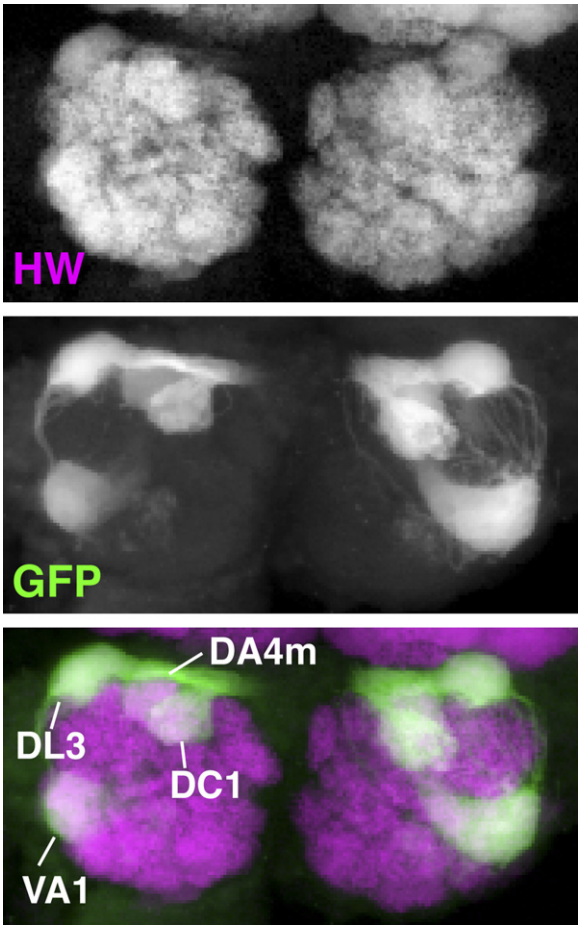


Figure S1. Antennal Lobe Expression of *Or65a-GAL4*

Adult brains from *UAS-mCD8-GFP;Or65a-GAL4* animals were fixed for 45 min in 8% formaldehyde in PBS, rinsed with PBT, and incubated in 1:200 rabbit anti-GFP TP401 (Torrey Pines Biolabs) and 1:200 mouse anti-Hiw 6H4 antibody (Developmental Studies Hybridoma Bank) at 4°C overnight. After washing 3 times with PBT, brains were incubated with 1:200 goat anti-rabbit FITC (Jackson ImmunoResearch Laboratories) and 1:200 donkey anti-mouse Cy5 (Jackson ImmunoResearch Laboratories). After incubation with secondary detection reagents, brains were washed for 20 min in PBT and mounted in Vectashield for analysis. Confocal fluorescence microscopy was performed on a Leica TCS SP2 mounted on a Leica DMIRE2 inverted microscope. Anti-Hiw 6H4, which stains an unknown pan-neuropil antigen [S12], is shown in magenta and GFP in green. Where the two overlap is represented in white. Identification of the *Or65a-GAL4* glomeruli was done by crossing *Or65a-GAL4;UAS-RFP* animals to lines carrying *Or-GFP* promoter fusion transgenes [S4].

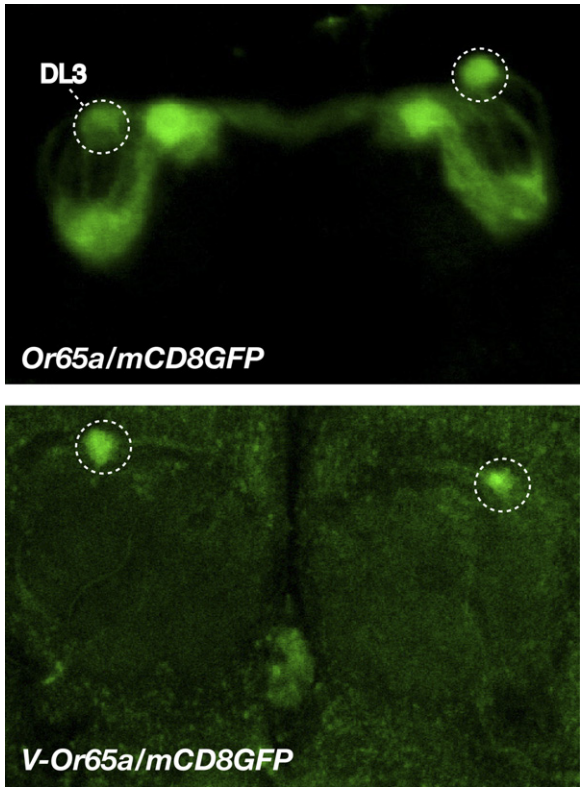


Figure S2. Comparison of *Or65a-GAL4* and *V-Or65a-GAL4*

Brains from animals expressing *UAS-mCD8-GFP* under control of the indicated driver were dissected and imaged on a Leica TCS SP2 mounted on a Leica DMIRE2 inverted microscope without fixation. Images shown were taken at different laser intensities, contrasts, and gains in order to optimize visualization. Background in the *V-Or65a-GAL4* image may be due to these factors as opposed to ectopic GFP expression. As an indirect comparison of the strength of the two lines, we found that the contrast ratio of DL3 in *Or65a-GAL4* (the ratio of mean DL3 intensity to total signal) was 5.9, while for *V-Or65a-GAL4* it was 2.9.

Table S1. Courtship Suppression Is Enhanced by Pheromone Extract

| Training Condition | | | | Memory Index | | |
|---|---|---|---|---|---|---|
| Object | Mesh | Odor Source | Tester | Mean | SE | |
| Data Group 1 | | | | | | |
| mVd | | water | iVd | 0.76 | 0.09 | control * |
| none | yes | mVd+Md | iVd | 0.96 | 0.08 | * |
| mVd | | M-extract | iVd | 0.51 | 0.10 | * |
| none | | M-extract | iVd | 1.33 | 0.06 | N.S. |
| none | yes | mVd+male-d | iVd | 1.13 | 0.08 | * |
| mVd | | male-filter | iVd | 0.29 | 0.07 | * |
| none | | male-filter | iVd | 1.04 | 0.07 | * |
| mVd | yes | male-filter | iVd | 0.25 | 0.07 | * |
| Data Group 2 | | | | | | |
| iV | | water | mVd | 1.00 | 0.06 | control + |
| iV | | M-extract | mVd | 0.72 | 0.08 | + |
| none | | M-extract | mVd | 1.09 | 0.03 | N.S. |
| none | yes | iVd+male-d | mVd | 0.91 | 0.07 | N.S. |
| iV | | male-filter | mVd | 0.65 | 0.08 | + |
| none | | male-filter | mVd | 1.14 | 0.05 | N.S. |
| iV | yes | male-filter | mVd | 1.03 | 0.09 | N.S. |

Pheromone extracts enhance courtship suppression. Males were trained in a courtship arena with two chambers separated by a mesh. The male was placed in the top chamber with or without a courtship object, as indicated (mVd, decapitated mature virgin; iV, immature virgin). Hexane extracts of mated females on filters (M-extract) or filters that had been exposed to a mature male for 1 hr (male-filter) were placed either in the top chamber with the male or in the bottom chamber ("yes" in mesh column). In some cases a fly corpse, iVd, mVd, or decapitated male (male-d) was presented in the bottom chamber as an odor source. Odors presented in the top chamber could be sampled by both gustatory and olfactory sensory systems. Odors presented in the bottom chamber provided only olfactory cues. A memory index with either an immature or mature decapitated virgin tester was calculated. A value of 1 indicates no memory was formed. * or + means significantly different from training condition control ($p < 0.05$). N.S. means not significant. These data suggest that the generalization of learning requires some volatile substance(s) found on mated female and male cuticle; the experimental male does not have to touch the compound to respond to it. This compound does not by itself cause suppression of courtship, as shown by the fact that it requires the presence of a trainer female in the top chamber with the experimental male.

Table S2. Courtship Suppression Is Enhanced by Purified cVA

| Training Condition | | | | Memory Index | | |
|---|---|---|---|---|---|---|
| Object | Mesh | Chemical | Tester | Mean | SE | |
| Data Group 1 | | | | | | |
| mVd | | water | iVd | 0.76 | 0.09 | control * |
| mVd | yes | cVA 0.2 ng | iVd | 0.52 | 0.08 | * |
| mVd | yes | cVA 20 ng | iVd | 0.37 | 0.07 | * |
| mVd | yes | cVA 200 ng | iVd | 0.35 | 0.08 | * |
| mVd | yes | cVA 2 μg | iVd | 0.52 | 0.07 | * |
| mVd | yes | cVA 20 μg | iVd | 0.35 | 0.06 | * |
| mVd | yes | cVA 200 μg | iVd | 0.36 | 0.06 | * |
| none | yes | cVA 200 μg | iVd | 1.32 | 0.09 | * |
| mVd | yes | cVOH 200 μg | iVd | 0.73 | 0.15 | N.S. |
| mVd | yes | hexane | iVd | 0.66 | 0.12 | N.S. |
| Data Group 2 | | | | | | |
| iV | | water | mVd | 1.00 | 0.06 | control + |
| iV | yes | cVA 20 ng | mVd | 0.92 | 0.08 | N.S. |
| iV | yes | cVA 200 ng | mVd | 0.85 | 0.07 | N.S. |
| iV | yes | cVA 2 μg | mVd | 0.94 | 0.08 | N.S. |
| iV | yes | cVA 20 μg | mVd | 0.90 | 0.06 | N.S. |
| iV | yes | cVA 200 μg | mVd | 0.63 | 0.06 | + |
| none | yes | cVA 200 μg | mVd | 1.10 | 0.06 | N.S. |
| iV | yes | cVOH 200 μg | mVd | 0.81 | 0.11 | N.S. |

Synthetic cVA can act as a generalizing cue. Males were trained in a courtship arena with two chambers separated by a mesh, as indicated by "yes" in mesh column. The male was placed in the top chamber with or without a courtship object, as indicated (mVd, decapitated mature virgin; iV, immature virgin). The bottom chamber contained filter paper with the indicated chemical. A memory index with either an immature or mature decapitated virgin tester was calculated. * or + means significantly different from training condition control ($p < 0.05$). N.S. means not significant. These data demonstrate that cVA acts in a dose-dependent manner to generalize learning with virgin trainers. cVA alone does not cause courtship in the absence of a courtship object. cVA is more effective in this role when it is presented with a mature virgin trainer, suggesting that its actions are dependent on the particular stimulatory pheromone context. cVOH, a closely related compound that is sensed by Or67d but not Or65a, is not capable of generalizing learning.

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RESEARCH ARTICLE

# Nest signature changes throughout colony cycle and after social parasite invasion in social wasps

**Marta Elia[1,2]*, Giuliano Blancato[1], Laura Picchi[1], Christophe Lucas[2], Anne-Geneviève Bagnères[2,3☯], Maria Cristina Lorenzi[1,4☯]**

**1** Department of Life Sciences and Systems Biology, University of Turin, Torino, Italy, **2** I.R.B.I. – UMR 7261 CNRS – Université de Tours, Faculté des Sciences, Parc Grandmont, Tours, France, **3** CEFE UMR 5175, CNRS – Université de Montpellier – Université Paul Valéry Montpellier – EPHE, Montpellier, France, **4** LEEC-Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris 13, Sorbonne Paris Cité, Villetaneuse, France

☯ These authors contributed equally to this work.
* marta.elia@outlook.com





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## Abstract

Social insects recognize their nestmates by means of a cuticular hydrocarbon signature shared by colony members, but how nest signature changes across time has been rarely tested in longitudinal studies and in the field. In social wasps, the chemical signature is also deposited on the nest surface, where it is used by newly emerged wasps as a reference to learn their colony odor. Here, we investigate the temporal variations of the chemical signature that wasps have deposited on their nests. We followed the fate of the colonies of the social paper wasp *Polistes biglumis* in their natural environment from colony foundation to decline. Because some colonies were invaded by the social parasite *Polistes atrimandibularis*, we also tested the effects of social parasites on the nest signature. We observed that, as the season progresses, the nest signature changed; the overall abundance of hydrocarbons as well as the proportion of longer-chain and branched hydrocarbons increased. Where present, social parasites altered the host-nest signature qualitatively (adding parasite-specific alkenes) and quantitatively (by interfering with the increase in overall hydrocarbon abundance). Our results show that 1) colony odor is highly dynamic both in colonies controlled by legitimate foundresses and in those controlled by social parasites; 2) emerged offspring contribute little to colony signature, if at all, in comparison to foundresses; and 3) social parasites, that later mimic host signature, initially mark host nests with species-specific hydrocarbons. This study implies that important updating of the neural template used in nestmate recognition should occur in social insects.

## Introduction

Social insect colonies are closed societies in which intruders are rejected, and thus, the integrity and the resources of the colonies are maintained [1]. Social insects are able to discriminate

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between nestmates and non-nestmate [2, 3], and the cuticular hydrocarbons are the main cues used for the discrimination [4].

Cuticular hydrocarbons cover the body of individuals and compose the chemical signature [2]. Typically, qualitative differences in the chemical signatures discriminate between species, while quantitative differences are found among colonies of the same species in ants, bees and wasps [5, 6, 7, 8, 9]. Cuticular hydrocarbons produced by the insects are also found on their nests, including the wall of the underground nests of ants, e.g. [10], the waxy comb of bees [11] or the paper nests of social wasps [12].

The paper nest, and the hydrocarbons that cover its surface, are especially relevant in the social biology of *Polistes* social wasps. During nest foundation, wasp foundresses mark their nests by stroking the abdomen on the nest surface [5, 13]. Possibly as a result of this marking behaviour, which might imply foundresses depositing their signature on the nest, foundresses and nests share the same hydrocarbon blends [5, 12, 14]. Wasps recognize their nests using nest odors [15, 16].

Further support for the crucial role of the nest—and the chemicals on its surface—in wasp social biology comes from the observations on the behaviour of the social parasites of paper wasps. When *inter-* and *intra*specific social parasites invade their colonies, parasites spend time rubbing their abdomens on the host nest surface [17, 18]. The host nest-marking by *intra*-specific social parasites has been associated with changes in host nest odors; as parasites establish in host colonies, the chemical signature of the host nests becomes biased towards that of the *intra*specific parasites themselves [5, 19, 20]. Similarly, obligate social parasite *Polistes sulcifer* contaminated artificial host nests made of plastic and aluminium with parasite-specific compounds [21]. These marking activities by both foundresses and social parasites are reasonably correlated with the role that the paper nest has in relation to the learning of colony odor. Indeed, there is a large amount of experimental evidence that proves that social wasps use the nest—and the chemicals therein—as a reference for their colony odor [22].

The exposure of newly emerged wasps to the colony odor deposited on the nest material is a fundamental step in the development of the recognition abilities and in the formation of the "template" for the nestmate recognition, that is, the neural representation of the colony odor stored in the memory [6]. Social wasps tolerate individuals with a chemical signature similar to the template formed, and attack those that are different [22, 23]. Young wasps exposed to their natal nests soon after the eclosion developed sufficiently good nestmate discrimination abilities, and the learning process was completed four hours later (reviewed in [22]), but was functional only if the nest was covered by hydrocarbons [24] (although the timing and other details of the learning process has been recently debated [25, 26]). In the lab, where the homogenous environment fades variations and colonies end up more similar than they were initially [27], the template formed at the emergence is still effective after months [28]. Studies on the learning of recognition cues often assume that colony odor is stable and it does not change over time, so that the learning mechanisms involved in the formation of the neural template have also been referred to as imprinting-like learning, e.g. [22].

However, colony odor might change over time as a function of variations in the physiological state of the foundress(es), and in the social structure and demography of the colony, or because diet, meteorological conditions, and natural enemies vary throughout colony cycle (e.g., [29, 30, 31]). In turn, the variation in colony odor may reflect in the signature deposited on the nest material and affect nestmate recognition mechanisms and the way the neural template is maintained and/or updated. Indeed, in the ant *Camponotus aethiops*, swapping soil between colonies caused the ants exposed to the soil from another colony to become chemically more similar (as for their cuticular hydrocarbon profile) to the foreign colony, and to receive less aggression from ants from the colony supplying the soil [10]. In the honeybee *Apis*

*mellifera*, swapping wax-combs between colonies, demonstrate that bees exposed to non-nestmate colony odor became more tolerant towards non-nestmates i.e., they modified their template [11].

In the present research, we aimed to study whether and how the nest signature changed throughout the colony cycle as residents lived undisturbed in the wild, foraged freely and their colonies were exposed to natural enemies. Moreover, we investigated whether the changes in the social life of the colonies (e.g., offspring emergence, social parasite invasion) played a role in the variation of the nest signature. We also aimed to understand whether the foundresses were mainly responsible for nest signature, and whether the offspring contributed as well. Furthermore, the invasion of host colonies by obligate social parasites offered the opportunity to test—via a natural experiment—whether parasites interfered with the nest signature and its variations. In order to do this, we tracked the nest signature of the social paper wasp *P. biglumis* in the wild from nest foundation until the end of the nesting season, when residents abandoned the nests. We extended our analyses to both colonies controlled by their legitimate foundresses (free-living colonies) and to those invaded by the social parasite *P. atrimandibularis* (parasitized colonies).

## Methods and materials

### Model species

*Polistes biglumis* is a social paper wasp with a boreo-montane distribution [32] that in southern Europe lives in high mountain habitats. Due to the harsh environmental conditions, the colonies of *P. biglumis* have a short colony cycle (3–4 months) [33], which makes it easy to follow the variations of the colony signature from the foundation until the colony declines. The colony cycle begins at the end of May-beginning of June, when overwintered *P. biglumis* foundresses solitarily found their colonies in open mountain meadows above 1000 m a.s.l. [33]. Foundresses build their paper nests by chewing plant fibers from weathered wood, mixing it with saliva, and shaping this material in a comb, which usually consists of less than 100 cells at the peak of colony growth [33]. Foundresses use nest cells to rear their offspring, but in this species, the peripheral ring of cells is always left empty, possibly as a way to protect brood from harsh weather conditions [33, 34]. Each foundress is the only adult on her colony for about 40 days (founding phase) and the only individual responsible for nest construction and brood care, before her brood emerge starting in mid-July; adults continue to emerge throughout the summer, until the end of August/mid-September. Colonies are small, and the brood is composed of few workers, if any, and few reproductive males and females [33, 35]. Due to the short nesting season (100 days approximately), an average of less than 20 wasps per colony emerge during the whole summer [33]. Newly emerged wasps often desert the colony soon after eclosion, so that colonies are rarely inhabited by more than 10 individuals [36].

*P. biglumis* colonies seldom survive intact until the end of the summer, because predators, *intra*specific and *inter*specific social parasites destroy or invade them [34]. Specifically, at the end of the founding phase and before brood emergence, some colonies are invaded by the rare, obligate social parasite *P. atrimandibularis*. Social parasites enter the colony peacefully, enslave the host foundress and exploit the hosts for rearing their own brood [18]. Soon after host-nest invasion, social parasites eat the youngest host brood while sparing the oldest ones (more likely to become workers) [34, 37]. Parasites start to lay eggs, and then the host foundresses care for the parasite brood, helped by the host descendants, later in the season [38].

The chemical signature of the host species *P. biglumis* is composed of 72 saturated hydrocarbons: *n*-alkanes, laterally and centrally branched monomethylalkanes, dimethylalkanes, and trimethylalkanes, ranging from 23 to 35 carbon atoms [39, 40, 41].

The chemical signature of *P. atrimandibularis* social parasites includes up to 46 saturated and unsaturated hydrocarbons with chain length up to 28 carbon atoms before host nest invasion, and extends up to 35 carbon-atoms one month after host nest invasion, when parasites chemically mimic their hosts [42]. After that, hosts recognize the resident parasites as nestmates [43]. The *pre*-invasion chemical signature of the social parasites is rich in several unsaturated compounds, such as (*Z*)-9-pentacosene, (*Z*)-9-nonacosene and (*Z*)-9-heptacosene (the latter being the most abundant), and nonacosene being the only compound with 29 carbon atoms in the *pre*-invasion chemical signature, which disappears in the *post*-invasion chemical signature during mimicry [40, 42].

## Ethical standards

The collection of colonies and the experiments performed comply with the current laws in Italy and France. No specific permits were required for the collection, nor for collection location. The species used in the experiments are not endangered or protected in Italy and France.

## Sample collection

In order to describe the chemical variation of *P. biglumis* colony signature over time and identify whether parasitized nests differ in this respect from free-living ones, we followed the fate of 129 *P. biglumis* colonies in the wild from their foundation at the beginning of June until their decline at the end of August.

The study was carried out in two populations in the Western Alps (Montgenèvre: Hautes Alpes, France, 44˚55'N, 6˚43'E; Ferrere: Valle Stura di Demonte, Cuneo, Italy 44˚22'N, 6˚57'E) in the summer 2014 (5 June– 25 August 2014).

The first time we checked a nest, the foundress was marked on the thorax with enamel paint for individual identification (n = 129). Then, we visited colonies weekly until their decline. At each census, we checked whether the foundress was present and whether a *P. atrimandibularis* parasite female was also present, which would indicate that the colony was parasitized. If present, we marked the parasite for individual identification. Invasions by *P. atrimandibularis* parasites occurred in 40 colonies out of 129 between 10 June and 10 July. At each census, we also counted the number of brood (eggs, larvae and pupae) and we noted whether the offspring had emerged in order to identify the colony phase (before or after brood emergence). Finally, at each visit, we cut a fragment of nest paper from the external lateral wall of each nest (approx. 1x1 $cm^2$) using scissors (scissors were washed with solvent before each use). Each week, we took the fragment from a different side of the nest, to reduce disturbance on the nests. For the same reason, we did not collect nest fragments when nests were smaller than 15 cells. To test whether nest signature was homogenous on the nest surface, we collected two fragments simultaneously from nine nests chosen at random from the 129 nests: one fragment from the right side and one from the left side.

At sampling, the nest fragments were kept separately in glass vials, brought to the laboratory and frozen at -20˚C.

Sample size varied during the summer because some nests were discovered later during the season, and others were killed by enemies (e.g., birds, small mammals but also humans and cows) at different time during colony cycle. Finally, some nests were missing in some surveys for logistical reasons. In total, excluding 74 nests invaded by *intra*specific social parasites, or where we were uncertain whether the female was the foundress or an *intra*specific parasite, we included in our analyses 15 free-living nests (i.e., nests controlled by the foundress for the entire colony cycle) and 40 parasitized nests (i.e., nests invaded by the obligate social parasites).

From these 55 nests, we collected 193 samples (66 fragments from free-living nests, 1–8 time points per nest; 127 fragments from parasitized nests, 1–9 time points per nest).

Nest fragments were weighed with a precision balance (Precisa 125A) (fragment weight: 3.01 ± 0.05 mg) to correct the concentrations of hydrocarbons by the weight of fragments.

## Chemical analyses

We extracted hydrocarbons by dipping each nest fragment separately in 500 μl of heptane for 60 sec twice, thus obtaining 1000 μl of solution for each nest fragment. Extracts were dried under a gentle nitrogen stream and re-dissolved in 100 μl of heptane containing *n*-C20 as an internal standard.

We injected 2 μl of each extract (which included 800 ng of *n*-C20) into a gas chromatograph with the flame ionization detection (GC-FID: Agilent Technologies 6850 equipped with a J&W HP-1 nonpolar-capillary-column, 30 m L x 0.32 mm ID x 0.25 μm PT) for the quantification of the hydrocarbons.

We injected another 2 μl of the extracts into a gas chromatograph-mass spectrometer (Agilent Technologies 7000C GC-MS Triple Quad equipped with a Zebron ZB-5HT capillary-column, 30 m L x 0.25 ID x 0.25 μm PT) for the identification of the hydrocarbons.

We used the same oven temperature program for both GC-FID and GC-MS (from 70˚C to 150˚C at 30˚C/min and from 150˚C to 320˚C at 5˚C/min; 10 min at the 320˚C final temperature; carrier gas was helium at 1 bar and 50 ml/min flux; injector temperature was 250˚C). Results were registered with the Agilent ChemStation program for GC-FID and Agilent Mass Hunter Workstation program for GC-MS.

## Identification and quantification of compounds

Most of the compounds have been previously described in Bagnères et al. [42] and Lorenzi et al. [39] using GC-MS in EI and CI modes; determination was then completed in Uboni et al. [40] and Bonelli et al. [41]. Verification of their presence was performed comparing the diagnostic ions and the M-15 of each peak by the GC-MS apparatus described above. For more details on structure and analysis of insect hydrocarbons see [44]. For quantification, peak areas were integrated using the GC ChemStation software and corrected manually.

## Statistical analyses

Following Bagnères et al. [45] and Lorenzi et al. [31], we quantified the total concentration of hydrocarbons on the nest fragments as the sum of all peak areas relative to the area of the internal standard (*n*-C20), which corresponded to 800 ng, and we divided this value by nest fragment weight (ng of hydrocarbons/mg of nest-fragment).

Then, we tested whether the nest hydrocarbon concentrations on the nest varied over time in free-living and parasitized nests using Generalized Linear Mixed Models (GLMM) (normal distribution, log link). Time was used as a fixed effect (each time period included 14 days and two nest samplings). The other fixed effects were nest status, population (Montgenèvre and Ferrere), brood (as a measure of the nest size), and colony phase (before or after brood emergence). Nest identity was used as a random factor; the random slope for time was included in the model as a random effect (nest identity as a subject; time as an effect).

In order to investigate the variation in the nest signature, we transformed the relative proportions of hydrocarbons using the log-ratio-transformation (natural log of the proportion of each peak divided by the geometric mean of the proportions of alkanes) according to Aitchison [46]. We reduced the number of variables (log-ratio-transformed hydrocarbon proportions) by performing a Principal Component Analysis (PCA) on all the 67 hydrocarbons

extracted from the nest fragments (PCA was based on correlation matrix, Varimax rotation). The PCA produced 14 Principal Components (PCs) (eigenvalues $> 1$; cumulative variance explained 80.94%). Then, we tested whether there were variations over time in the first three PCs (which were the PCs that cumulatively explained at least 51% of the variance) and by nest status using GLMMs (normal distribution, identity link). The other fixed effects were population, brood, and colony-cycle phase. A random factor with a random slope was included in the model (nest identity as a subject; time as an effect).

Finally, we tested whether the proportion of longer-chain hydrocarbons and that of branched hydrocarbons varied with time and nest status. We classified hydrocarbons as shorter-chain hydrocarbons if they had a chain length from C22 to C28 carbon atoms (chain length $<$ 29 carbon atoms: peak 1–25; these hydrocarbons are present in the chemical signature of hosts and *pre*-invasion parasites). We classified hydrocarbons as longer-chain hydrocarbons if they had a chain length $\geq$ 29 carbon atoms (peak 26–63; these hydrocarbons are present in the chemical signature of hosts and *post*-invasion parasites). We built two different GLMMs to test the variation in the (arcsine-square-root transformed) proportion of longer-chain hydrocarbons and that of branched hydrocarbons (normal distribution and identity link), which were entered as the response variables. Time, nest status, population, brood and colony-cycle phase were the fixed effects in both models; nest identity was included as a random factor. In the GLMM on the proportion of longer-chain hydrocarbons we also included time as a random effect (this was not done in the other model because the model failed to converge).

The preliminary GLMMs included all biologically reasonable interactions and terms. Then, we dropped the least non-significant interactions and terms and calculated the reduced models.

Because most colonies were killed by predators during the summer, we could only sample few colonies in August (n = 12 fragments). In order to test whether the variation in the chemical traits was driven by the values in late August, we also ran all Models using a reduced dataset that excluded late August data. These analyses yielded approximately similar statistical results as those of the complete data set, which we report in the Results section.

Preliminary analyses showed that nest side did not explain the variation in the traits describing nest signature, and, therefore, this factor was not included in the models presented in the paper. This result suggests that the signature was spatially homogeneous on the nest surface, as supported by the consistent results that we obtained by sampling nests once on the right side, and once on the left side.

Descriptive statistics are mean ± S.E. Statistical analyses were performed in IBM SPSS Statistic 22.0.

## Results

The nest signature was composed of 67 peaks and was a mixture of linear alkanes, monomethylalkanes, dimethylalkanes, trimethylalkanes and alkenes, ranging from 23 to 35 carbon-chain length. The hydrocarbons found on the nest paper of free-living colonies were the same as those previously reported for *P. biglumis* wasps [39, 40, 41], whereas additional hydrocarbons were found on the nest paper of parasitized colonies at certain sampling times during the season. These additional compounds were unsaturated, parasite-specific, and previously identified [40, 42] (see below) (Table 1).

### Analyses of the concentration of hydrocarbons on the nests

Nests had a relatively high concentration of hydrocarbons on their surface, with an average of 4.85 ± 2.57 μg of hydrocarbons per mg of nest paper (n = 193 nest fragments). The hydrocarbon

**Table 1. List of the hydrocarbons on free-living and parasitized nests.**

| PEAKS | HYDROCARBONS | FREE-LIVING NESTS | PARASITIZED NESTS (SEE TEXT) | PEAKS | HYDROCARBONS | FREE-LIVING NESTS | PARASITIZED NESTS (SEE TEXT) |
|---|---|---|---|---|---|---|---|
| 1 | *n*-Tricosane | ✓ | ✓ | 30 | 11,15-+9,15-+9,17-+9,19-Dimethylnonacosane | ✓ | ✓ |
| 2 | *n*-Tetracosane | ✓ | ✓ | 31 | 3-Methylnonacosane | ✓ | ✓ |
| 3 | *n*-Pentacosane | ✓ | ✓ | 32 | 5,11-+5,13-Dimethylnonacosane | ✓ | ✓ |
| 3a | 9-Pentacosene | | ✓ | 33 | 5,9-Dimethylnonacosane | ✓ | ✓ |
| 4 | 11-+13-Methylpentacosane | ✓ | ✓ | 34 | *n*-Triacontane | ✓ | ✓ |
| 5 | 7-Methylpentacosane | ✓ | ✓ | 35 | 3,9-+3,11-+3,13-Dimethylnonacosane | ✓ | ✓ |
| 6 | 5-Methylpentacosane | ✓ | ✓ | 36 | 9-+10-+11-+12-+13-+14-Methyltriacontane | ✓ | ✓ |
| 7 | 11,15-Dimethylpentacosane | ✓ | ✓ | 37 | 2-Methyltriacontane | ✓ | ✓ |
| 8 | 3-Methylpentacosane | ✓ | ✓ | 38 | 9,19-Dimethyltriacontane | ✓ | ✓ |
| 9 | *n*-Hexacosane | ✓ | ✓ | 39 | 4,12-Dimethyltriacontane | ✓ | ✓ |
| 9a | 3,11-+3,13-+3,15-Dimethylpentacosane | ✓ | ✓ | 40 | *n*-Hentriacontane | ✓ | ✓ |
| 10 | 12-Methylhexacosane | ✓ | ✓ | 41 | 9-+11-+13-+15-Methylhentriacontane | ✓ | ✓ |
| 11 | 2-Methylhexacosane | ✓ | ✓ | 42 | 7-Methylhentriacontane | ✓ | ✓ |
| 11a | 3-Methylhexacosane | ✓ | ✓ | 43 | 11,15-Dimethylhentriacontane | ✓ | ✓ |
| 11b | 9-Heptacosene | | ✓ | 44 | 9,17-+9,19-+9,21-Dimethylhentriacontane | ✓ | ✓ |
| 12 | *n*-Heptacosane | ✓ | ✓ | 45 | 5,11-+5,13-Dimethylhentriacontane | ✓ | ✓ |
| 13 | 9-+11-+13-Methylheptacosane | ✓ | ✓ | 46 | *n*-Docotriacontane | ✓ | ✓ |
| 14 | 7-Methylheptacosane | ✓ | ✓ | 47 | 3,9-+3,11-+3,13-Dimethylhentriacontane | ✓ | ✓ |
| 15 | 5-Methylheptacosane | ✓ | ✓ | 48 | 10-+11-+12-+13-+14-Methydocotriacontane | ✓ | ✓ |
| 16 | 11,15-Dimethylheptacosane | ✓ | ✓ | 49 | 11,15-+11,19-+11,21-Dimethyldocotriacontane | ✓ | ✓ |
| 17 | 3-Methylheptacosane | ✓ | ✓ | 50 | 2-Methyldocotriacontane | ✓ | ✓ |
| 18 | 5,11-+5,13-Dimethylheptacosane | ✓ | ✓ | 52 | *n*-Tritriacontane | ✓ | ✓ |
| 19 | *n*-Octacosane | ✓ | ✓ | 53 | 9-+11-+13-+15-+17-Methyltritriacontane | ✓ | ✓ |
| 20 | 3,9-+3,11-+3,13-Dimethylheptacosane | ✓ | ✓ | 54 | 13,21-+11,15-Dimethytritriacontane | ✓ | ✓ |
| 21 | 8-+11-+12-+13-+14-Methyloctacosane | ✓ | ✓ | 55 | 9,19-+9,21-+9,23-Dimethyltritriacontane | ✓ | ✓ |
| 22 | 4-Methyloctacosane | ✓ | ✓ | 56 | 11,17,21-+11,17,23-Trimethyltritriacontane | ✓ | ✓ |
| 23 | 2-Methyloctacosane | ✓ | ✓ | 57 | 9,13,17-+9,15,19-+9,15,21-+9,15,23-Trimethyltritriacontane | ✓ | ✓ |
| 24 | 3-Methyloctacosane | ✓ | ✓ | 58 | *n*-Tetratriacontane | ✓ | ✓ |
| 25 | Unknown | ✓ | ✓ | 59 | 3,9-+3,11-+3,13-+3,15-Dimethyltritriacontane | ✓ | ✓ |
| 26a | 9-Nonacosene | | ✓ | 60 | 10-+11-+12-+13-+15-Methyltetratriacontane | ✓ | ✓ |
| 26 | *n*-Nonacosane | ✓ | ✓ | 61 | 2-Methyltetratriacontane | ✓ | ✓ |

(*Continued*)

**Table 1.** (*Continued*)

| PEAKS | HYDROCARBONS | FREE-LIVING NESTS | PARASITIZED NESTS (SEE TEXT) | PEAKS | HYDROCARBONS | FREE-LIVING NESTS | PARASITIZED NESTS (SEE TEXT) |
|---|---|---|---|---|---|---|---|
| 27 | 9-+11-+13-+15-Methylnonacosane | ✓ | ✓ | 62 | 11-+13-+15-+17-Methylpentatriacontane | ✓ | ✓ |
| 28 | 7-Methylnonacosane | ✓ | ✓ | 63 | 9,23-+11,15+11,17-Dimethylpentatriacontane | ✓ | ✓ |
| 29 | 5-Methylnonacosane | ✓ | ✓ | | | | |

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concentration had a huge and significant increase (> 600%) during the 80-days sampling season (Fig 1). Initially, hydrocarbon concentration was similar in free-living and parasitized nests, but differed later on, so that parasitized nests had higher amounts of hydrocarbons than free-living nests at the end of the season (GLMM: nest status*time $F_{1,165} = 6.452$, $P \leq 0.01$) (Fig 1). There were no significant effects of the number of brood (GLMM: $F_{1,165} = 0.786$; P = 0.377), population (GLMM: $F_{1,165} = 0.229$; P = 0.633) and colony phase ($F_{1,165} = 0.013$; P = 0.908).

## Analyses of the chemical signature of the nests

Interestingly, PCA showed that almost no linear alkanes (only two quantitatively minor ones) were involved in the 3 first components.

PC1 explained 28% of variance and was strongly (positively) correlated with longer-chain hydrocarbons, that overall represented the 67% of the relative proportions of the longer-chain hydrocarbons in the signature; the only relatively lighter hydrocarbon loading on PC1 was 7-methylnonacosane. PC1 varied with time in different ways in free-living and parasitized nests, as shown by the significant interaction between nest status and time (GLMM: nest status*time $F_{1,175} = 6.496$; P = 0.012) (Fig 2a). PC1 values were relatively high during the first half of the colony cycle in free-living nests, then sharply declined, and increased again in August.

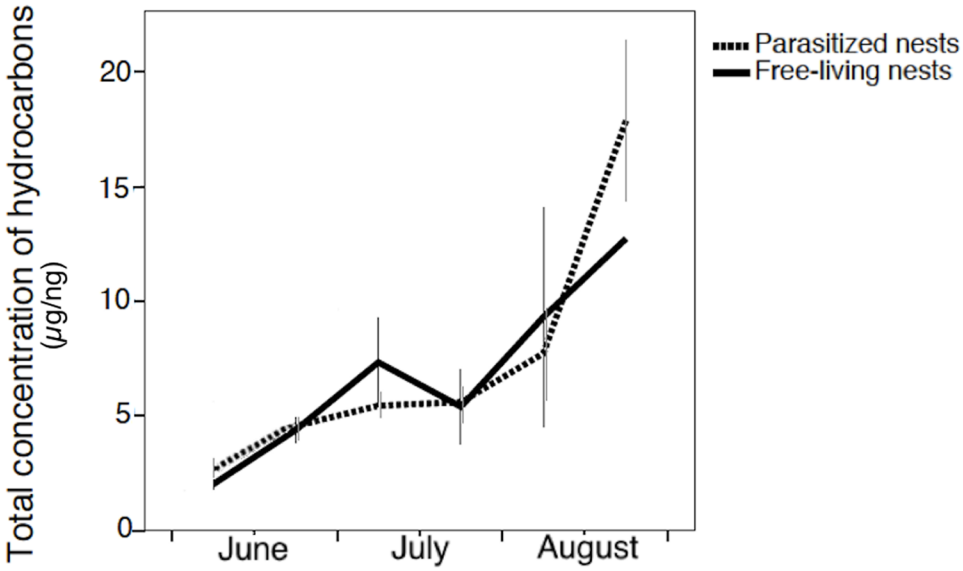


**Fig 1. The variation in the concentration of hydrocarbons on the nest paper throughout the season in free-living nests controlled by the *P. biglumis* foundresses and in parasitized nests controlled by the social parasite *P. atrimandibularis* (in µg/mg of nest paper).**

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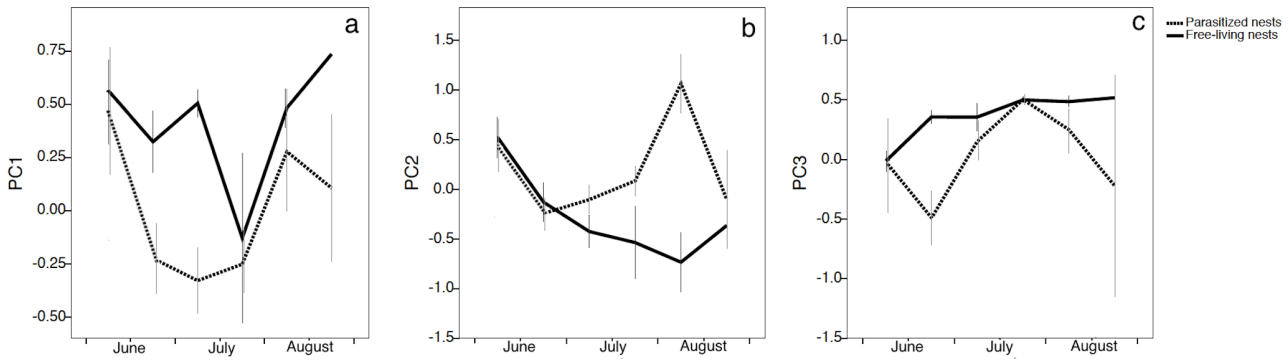


**Fig 2. The variation in PC1 (a), PC2 (b) and PC3 (c) throughout the season in free-living nests controlled by the *P. biglumis* foundresses and in in parasitized nests controlled by the social parasite *P. atrimandibularis*.**

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In parasitized nests, PC1 values decreased as social parasites invaded, and were lower than those of free-living nests throughout the season (Fig 2a). These results suggest that the chemical signature of free-living nests was characterized by a relatively larger proportion of longer-chain hydrocarbons and 7-methylnonacosane (Peak 28), compared to the parasitized ones. There was no significant effect of the population, the number of brood and the colony phase on PC1 (brood $F_{1,175} = 0.624$; P = 0.431; population $F_{1,174} = 0.048$; P = 0.827; colony phase $F_{1,175} = 2.661$; P = 0.105).

PC2 (which explained 16% of variance) was strongly (positively) correlated with hydrocarbons with intermediate chain-length (i.e., 27–32 carbon atoms). PC2 values decreased regularly in free-living nests across the season, whereas they increased (after an initial decrease) in parasitized nests. This variation with time and nest status was significant (GLMM nest status*time: $F_{1,176} = 9.839$; P = 0.002) (Fig 2b). There were no significant effects of the population, the number of brood and the colony phase (brood $F_{1,176} = 0.613$; P = 0.435; population $F_{1,174} = 0.152$; P = 0.697; colony phase $F_{1,175} = 0.307$; P = 0.580).

The fate of PC3 (which explained 7% of variance) is especially interesting because the parasite-specific compounds loaded the most on this component. PC3 was strongly, negatively correlated with the alkenes (*Z*)-9-pentacosene (Peak 3a), (*Z*)-9-heptacosene (Peak 11b), the main alkene, and (*Z*)-9-nonacosene (Peak 26a), and positively correlated with 2-methylhexacosane (Peak 11) (Table 2). Fluctuations in PC3 values over time were mainly due to the changes in the proportions of these compounds (Fig 2c). Indeed, PC3 values varied significantly over time ($F_{1,177} = 18.385$; $P < 0.0001$), as a function of nest status ($F_{1,177} = 13.170$; $P < 0.0001$), and of the amount of brood in the nest ($F_{1,177} = 4.511$; P = 0.035). There was no significant effect of other factors (population $F_{1,176} = 0.201$; P = 0.655; colony phase $F_{1,175} = 0.021$; P = 0.886). PC3 values increased slightly during the summer in free-living nests. In parasitized nests, instead, PC3 values rapidly decreased when parasites invaded the nests, as we expected since the parasite-specific compounds were negatively correlated with this PC. Therefore, parasite-specific compounds contaminated nest paper. PC3 values in parasitized nests rapidly increased the next four weeks, indicating–because of the negative correlation–that parasite-specific compounds decreased in these nests towards mid-July (i.e., at the time when parasites are expected to mimic the chemical signatures of their hosts) (Fig 2c). When the parasite brood started to emerge, PC3 values changed again, suggesting that alkene proportions increased once more at the time the parasite offspring emerged. Indeed, alkenes were detectable in the gas chromatograms of parasitized nests at parasite invasion and in August (as shown for (*Z*)-9-heptacosene in Fig 3), when their relative proportions, although very small, were different from zero (Fig 4).

**Table 2. The loading matrix (rotated component matrix) for the PC1, PC2 and PC3 (only peaks loading > 0.7 are shown).** Peaks are sorted by loading size.

| PEAK | HYDROCARBONS | PC1 | PC2 | PC3 |
|---|---|---|---|---|
| 61 | 2-Methyltetratriacontane | 0.907 | | |
| 63 | 9,23-+11,15+11,17-Dimethylpentatriacontane | 0.884 | | |
| 53 | 9-+11-+13-+15-+17-Methyltritriacontane | 0.882 | | |
| 42 | 7-Methylhentriacontane | 0.851 | | |
| 41 | 9-+11-+13-+15-Methylhentriacontane | 0.851 | | |
| 54 | 13,21-+11,15-Dimethytritriacontane | 0.829 | | |
| 55 | 9,19-+9,21-+9,23-Dimethyltritriacontane | 0.829 | | |
| 44 | 9,17-+9,19-+9,21-Dimethylhentriacontane | 0.808 | | |
| 43 | 11,15-Dimethylhentriacontane | 0.808 | | |
| 49 | 11,15-+11,19-+11,21-Dimethyldocotriacontane | 0.797 | | |
| 28 | 7-Methylnonacosane | 0.740 | | |
| 62 | 11-+13-+15-+17-Methylpentatriacontane | 0.728 | | |
| 32 | 5,11-+5,13-Dimethylnonacosane | | 0.926 | |
| 33 | 5,9-Dimethylnonacosane | | 0.926 | |
| 46 | n-Docotriacontane | | 0.918 | |
| 47 | 3,9-+3,11-+3,13-Dimethylhentriacontane | | 0.918 | |
| 35 | 3,9-+3,11-+3,13-Dimethylnonacosane | | 0.896 | |
| 15 | 5-Methylheptacosane | | 0.840 | |
| 17 | 3-Methylheptacosane | | 0.835 | |
| 31 | 3-Methylnonacosane | | 0.826 | |
| 18 | 5,11-+5,13-Dimethylheptacosane | | 0.798 | |
| 45 | 5,11-+5,13-Dimethylhentriacontane | | 0.714 | |
| 58 | n-Tetratriacontane | | 0.713 | |
| 59 | 3,9-+3,11-+3,13-+3,15-Dimethyltritriacontane | | 0.713 | |
| 29 | 5-Methylnonacosane | | 0.710 | |
| 3a | 9-Pentacosene | | | -0.896 |
| 11b | 9-Heptacosene | | | -0.890 |
| 26a | 9-Nonacosene | | | -0.884 |
| 11 | 2-Methylhexacosane | | | 0.833 |

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## Analyses of the relative proportions of longer-chain and branched hydrocarbons

The proportion of longer-chain hydrocarbons on the nest surface changed with time and nest status (GLMM: time $F_{1,177} = 86.319$; $P < 0.0001$; nest status $F_{1,177} = 15.573$; $P < 0.0001$). It increased during the season, but was consistently higher in free-living than in parasitized nests (Fig 5). The number of brood had a marginally significant effect (GLMM: brood $F_{1,177} = 5.237$; $P \leq 0.05$), while population and colony phase (i.e., the presence or absence of adult offspring) had no significant effect (population $F_{1,175} = 0.093$; $P = 0.761$; colony phase $F_{1,176} = 0.157$; $P = 0.692$).

The proportion of branched hydrocarbons increased over time as well (GLMM: time $F_{1,188} = 67.572$; $P < 0.0001$), and did not vary significantly between free-living and parasitized nests (nest status $F_{1,188} = 2.129$; $P = 0.146$) (Fig 6). Moreover, there were no significant effects of the population, the number of brood and the colony phase (brood $F_{1,175} = 0.927$; $P = 0.337$; population $F_{1,188} = 1.629$; $P = 0.203$; colony phase $F_{1,188} = 1.255$; $P = 0.264$).

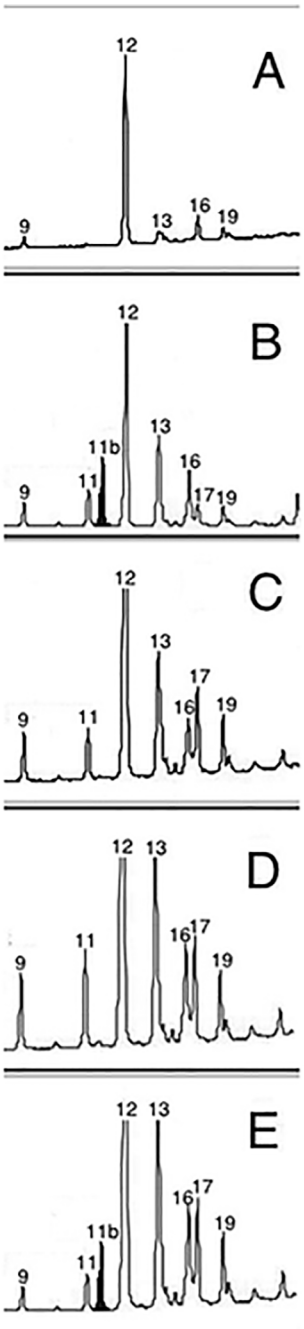


**Fig 3. Representative gas chromatograms of a parasitized nest of *P. biglumis* at five different time points during the colony cycle (a: GC before parasite invasion; b: one week after parasite invasion; c: two weeks after parasite invasion d: three weeks after parasite invasion; e: six weeks after parasite invasion).** Only hydrocarbons between peak 9 and peak 19 are shown. The picture shows the presence/absence of (Z)-9-heptacosene (the black peak), the most abundant alkene on parasitized nests. Peak 9: hexacosane; Peak 11: 2-methylhexacosane; Peak 11b: (Z)-9-heptacosene; Peak 12: n-heptacosane; Peak 13: 9-+11-+13-methylheptacosane; Peak 16: 11,15-dimethylheptacosane; Peak 17: 3-methylheptacosane; Peak 19: n-octacosane.

https://doi.org/10.1371/journal.pone.0190018.g003

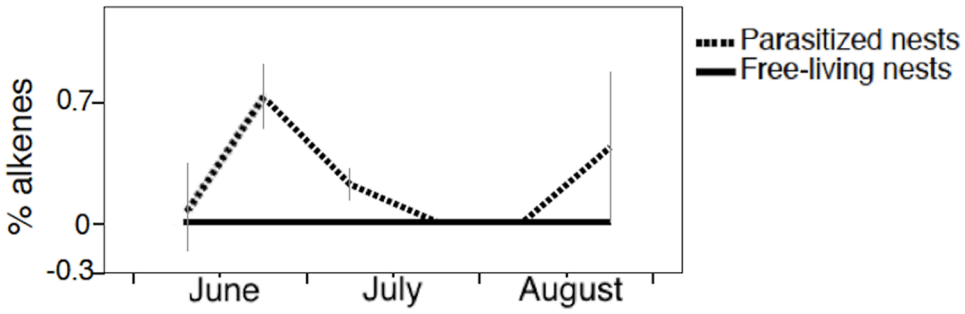


**Fig 4. The variation in the proportion of alkenes on the nest paper throughout the season in free-living nests controlled by the *P. biglumis* foundresses and in parasitized nests controlled by the social parasite *P. atrimandibularis*.**

https://doi.org/10.1371/journal.pone.0190018.g004

## Discussion

Our results show that nests of the paper wasp *P. biglumis* were covered by a rich layer of hydrocarbons, which matched the composition and the relative proportions of the blends of cuticular hydrocarbons reported for the wasps of the same species [39, 40, 41]; (Elia unpubl. data). By tracking nest signature throughout colony cycle in two different populations, we found that the concentration of hydrocarbons, as well as the proportions of longer-chain and branched hydrocarbons, increased from the moment colonies were founded at the beginning of June to their decline at the end of August. These seasonal changes were modified when *P. biglumis* nests were invaded by obligate social parasites.

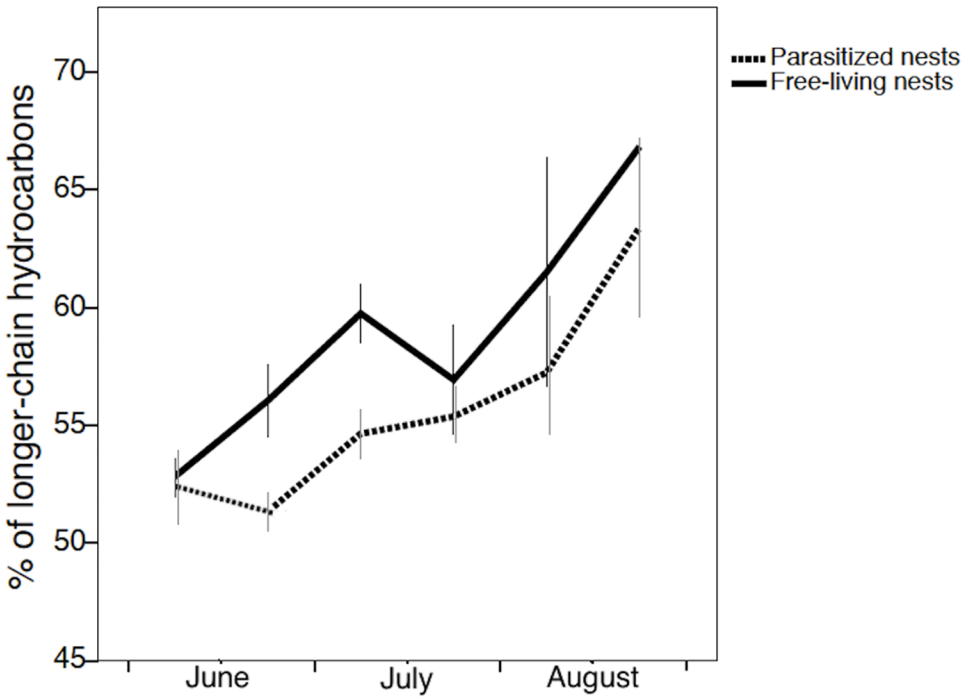


**Fig 5. The variation in the proportion of longer-chain hydrocarbons on the nest paper throughout the season in free-living nests controlled by the *P. biglumis* foundresses and in parasitized nests controlled by the social parasite *P. atrimandibularis*.**

https://doi.org/10.1371/journal.pone.0190018.g005

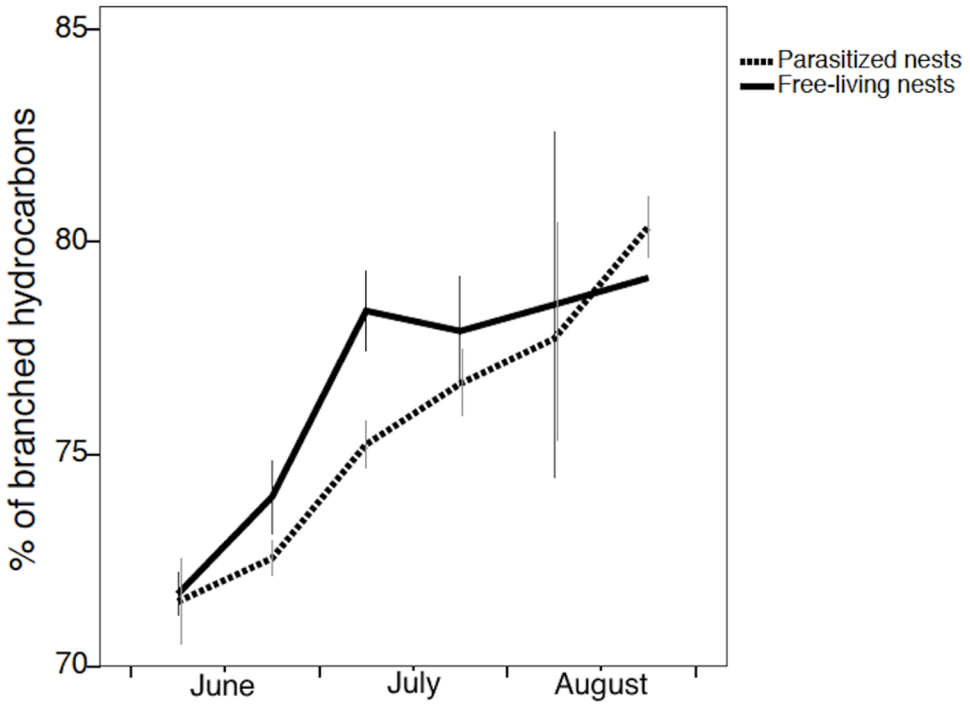


**Fig 6. The variation in the proportion of branched hydrocarbons on the nest paper throughout the season in free-living nests controlled by the *P. biglumis* host foundresses and in parasitized nests controlled by the social parasite *P. atrimandibularis*.**

https://doi.org/10.1371/journal.pone.0190018.g006

We analysed the chemical blend on the nest paper by repeated sampling. By doing this, we were able to follow for the first time how nest signature varied during the season in field colonies, where neither the adult wasp population, nor the brood were altered during the sampling procedure.

*P. biglumis* colony signature changed over time: on average, the amount of hydrocarbons on the nests increased seven times during about 80 days, which indirectly reinforces the hypothesis that the nest has a central role in the mechanisms of nestmate recognition in social wasps and open the question whether nests are similarly rich in hydrocarbons in hover wasps, which prioritizes visual cues over colony odor cues in nestmate recognition [47, 48]. A strong increase in hydrocarbon concentration with colony cycle has also recently found in bumblebee wax, where quantitative and qualitative variations in wax characteristics may affect worker reproductive decisions [49]. In contrast, in some ant, like *Camponotus aethiops*, the role of nest odor is not so crucial, and the colony signature on the nest walls is quantitatively scarce [10]. However, colony odor changes in ants as well, as shown in the weaver ant *Oecophylla smaragdina* [50] or in the ant desert *Cataglyphis niger*, where 25% of the hydrocarbons in the colony signature changed in relative proportions over a period of 3 months [51]. Similar results were shown by Provost et al. [52] in the ant *Temnothorax* (formerly *Leptothorax*) and in termites [53]. However, these studies tested changes in the colony signature of laboratory colonies, and here we test for the first time such changes in colonies in the wild throughout the nesting cycle.

It is unclear at the moment how the large quantitative changes in *P. biglumis* nest odor occurred, and focused experiments are needed. Since *Polistes* foundresses seem to be the main source of nest signature [13, 19], they may promote these variations through both active

marking (as they often do by rubbing their abdomen on the nest surface) and repeated passive contacts with their nests. Similarly, Lenoir et al. [54], in experiments with the ant *Lasius niger* and Bos et al. [10] with the ant *Camponotus aethiops*, have shown that the hydrocarbon blends extracted from the soil covering ant-nest tunnels and chambers are deposited passively by ants via repeated contacts with the substrate. One of the main physico-chemical properties of cuticular hydrocarbons of social insects is their low volatility [9, 55, 56] and their long-term stability; cuticular hydrocarbons were still present on 20-year-old hornet specimens kept in a museum [57], as well as on 70-year-old specimens of scolytid cone beetles [58]. This may explain why the hydrocarbon concentration increased markedly over time on the *P. biglumis* nests; nest-marking and passive contacts of wasps with the nest surface continued throughout the summer. If the hydrocarbons were continuously deposited and did not volatilize, they accumulated and their concentration inevitably increased on the nest surface over time. The hypothesis that foundresses were mainly responsible for the production and maintenance of the nest signature in free-living colonies is further supported by the fact that the variations in nest signature during summer were not significantly associated with the emergence of the offspring in the nest (which began approximately in mid-July). Offspring emergence marks the end of the solitary founding phase in *P. biglumis* colonies and the beginning of a true social life, but the presence of newly emerged individuals did not significantly contribute to the increase in the amount of nest surface hydrocarbons.

As for the quality of the nest hydrocarbons, the values of PC1 (which represents mainly longer-chain hydrocarbons), the proportion of longer-chain hydrocarbons, and the proportion of branched hydrocarbons increased over the season. The values of PC2 (which represents mainly hydrocarbons with a chain length between 27–32 carbon atoms) decreased in the same period, as these hydrocarbons were overwhelmed by the important increase in the relative proportions of longer-chain hydrocarbons. Possibly, these changes on the nest were associated with similar changes on the foundress cuticle and/or in glands associated with nest-marking (e.g., Dufour's or sternal glands). There is evidence that in newly emerged wasps, the relative proportions of some longer-chain hydrocarbons increased soon after emergence [59]. Similarly, mature wasps had higher relative proportions of branched and longer-chain hydrocarbons than younger ones [60, 61].

The variations in the colony signature over time, as represented by the nest signature, suggest that wasps constantly adjust their template to confine the acceptance range for reliable nestmate discriminations [2, 62]. Social insects may update their template via habituation to the updated signature; indeed, individuals exposed to non-nestmate signatures reduce their aggression to non-nestmates [11, 62, 63, 64].

The invasion of colonies by social parasites had many other important consequences on the nest signature in *P. biglumis* colonies, and produced several qualitative and quantitative changes, which suggests that parasites actively contribute to host nest signature.

The hydrocarbon concentration fluctuated in parasitized nests for most of the season, but it was smaller than in free-living nests at the end of the season. This is reasonable, because, although *P. atrimandibularis* parasites stroke their abdomen on host nests (at least soon after invasion) [18], they are chemically insignificant, and have approximately 20% the concentration of cuticular hydrocarbons of their hosts at. Parasite females at the end of their life cycle are no more chemically insignificant [65] and, if they mark, they may contribute to enrich the host nests in hydrocarbons.

While free-living and parasitized nests had similar proportions of branched hydrocarbons throughout the season, the proportion of longer-chain hydrocarbons was lower in parasitized than in free-living nests. Focused experiments are needed to investigate the causes of this difference. However, parasitized foundresses were subdued and inhibited by parasites in their

egg-laying activity [18, 66], which might imply a decrease in the proportions of longer-chain hydrocarbons on their cuticular signature, as it occurs to subordinate foundresses relative to dominants [67].

Finally, parasitized nests differed strikingly from free-living nests in the presence of parasite-specific alkenes, as shown by the variations in PC3 values. We identified three alkenes on the nest surface, (*Z*)-9-pentacosene, (*Z*)-9-heptacosene, and (*Z*)-9-nonacosene, which are typical of the signature that parasites exhibit prior to mimicking their hosts [42]. We show that alkenes appeared on host nests the week after host-nest invasion, disappeared seven days later (Fig 3), and appeared again at the end of the season (Fig 4). Indeed, parasites may have deposited alkenes on the host nests through stroking [18] at invasion, when their chemical signature have alkenes, and again at the end of the season, when both the old parasites and their daughters possess them on their cuticle [5, 42]. We do not know if spreading the alkenes on the nest surface may serve any function, and if there is a function, why it is limited to short time periods (soon after host nest invasion, and at colony decline). Parasites interfere with the process of nestmate recognition of their host, because hosts make more recognition errors in parasitized than in living colonies (i.e., hosts are more tolerant towards non-nestmates and less tolerant towards non-nestmates)[43, 68]. The presence of alkenes might disrupt the learning of a "correct" template or modify the recognition threshold [43] and facilitates the acceptance of the parasite and/or her progeny in host colonies. Alternatively, alkenes might blurr parasite recognition cues, as it has been proposed for the incipient leaf-cutting ant *Acromyrmex echinatior* [69]. Another paper wasp social parasite, *P. sulcifer*, marks also the host nests with parasite specific compounds [21]. Such marking activity may diminish the chemical distance between the nest signature and the parasite signature [21]. Similarly, *intra*specific parasites cover host nests with their own signature, possibly to avoid unmasking by host workers and regulate host worker egg production [19, 20, 70].

Other social parasites produce compounds that reduce host aggression. For example, the males of the social parasite *Bombus vestalis* bumblebees excrete cephalic secretions that repel host workers [71], and the slave-maker ant *Polyergus rufescens* sprays ester decyl butyrate to reduce host aggression during colony usurpation [72]. The alkenes of *P. atrimandibularis* social parasites may have a similar role: even if strongly chemically insignificant [65], parasites are attacked by hosts when they land on host colonies, but then very rapidly submit their hosts [18]. Alkenes might reduce host aggression in the early stages of parasite invasion, and again at the end of colony cycle, when parasite offspring emerge. However, focused studies are needed to clarify the function of alkenes on parasitized nests and test whether they are an adaptive trait. Preliminary observations suggest that foundresses exposed to alkenes diminish their alarm response (Elia, unpubl. data.).

Overall, these findings may indicate that *P. atrimandibularis* social parasites, have a very elaborate chemical strategy of integration, based on marking host nests with parasite-specific hydrocarbons (this paper), being chemically insignificant [65] and achieving chemical mimicry [42].

We still lack a full comprehension of the hydrocarbon-mediated mechanisms that regulate interactions in social insect colonies. We have not yet revealed all of the complexities of how hydrocarbons function to regulate worker reproduction and to discriminate between intruders and legitimate colony members. Our study brings new and strong evidence that the nest signature changes in the wild, which has important consequences on the mechanisms of the learning of recognition cues, and their updating, and on the way we study learning and memory in social insects in general, and in parasitic context also. Our results bring new and original information about the dynamics of colony odor in undisturbed colonies in the wild and on the

chemical-mediation of the behavior of hosts and parasites, and pose new areas of exploration on these topics.

## Supporting information

**S1 Fig. The chemical signature of nests of *P. biglumis* parasitised by the social parasite *P. atrimandibularis* or unparasitized (mean values +- s.e.obtained by pooling data across the nesting season, n = 193 nest fragments.**
(DOCX)

**S2 Fig. The two sampling site: Montgènevre in red, Ferrere in purple.**
(DOCX)

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## Author Contributions

**Conceptualization:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Data curation:** Marta Elia, Giuliano Blancato, Laura Picchi, Christophe Lucas, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Formal analysis:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Funding acquisition:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Investigation:** Marta Elia, Giuliano Blancato, Laura Picchi, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Methodology:** Marta Elia, Christophe Lucas, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Project administration:** Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Resources:** Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Software:** Marta Elia, Christophe Lucas, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Supervision:** Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Validation:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Visualization:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Writing – original draft:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

**Writing – review & editing:** Marta Elia, Anne-Geneviève Bagnères, Maria Cristina Lorenzi.

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Article

# Social Experience Modifies Pheromone Expression and Mating Behavior in Male *Drosophila melanogaster*

**Joshua J. Krupp,[1,2] Clement Kent,[1,2] Jean-Christophe Billeter,[1] Reza Azanchi,[1] Anthony K.-C. So,[1] Julia A. Schonfeld,[1] Benjamin P. Smith,[1,3] Christophe Lucas,[1,4] and Joel D. Levine[1,*]**

[1]Department of Biology
University of Toronto at Mississauga
Mississauga, ON L5L1C6
Canada

## Summary

**Background:** The social life of animals depends on communication between individuals. Recent studies in *Drosophila melanogaster* demonstrate that various behaviors are influenced by social interactions. For example, courtship is a social interaction mediated by pheromonal signaling that occurs more frequently during certain times of the day than others. In adult flies, sex pheromones are synthesized in cells called oenocytes and displayed on the surface of the cuticle. Although the role of *Drosophila* pheromones in sexual behavior is well established, little is known about the timing of these signals or how their regulation is influenced by the presence of other flies.

**Results:** We report that oenocytes contain functional circadian clocks that appear to regulate the synthesis of pheromones by controlling the transcription of *desaturase1* (*desat1*), a gene required for production of male cuticular sex pheromones. Moreover, levels of these pheromones vary throughout the day in a pattern that depends on the clock genes and most likely also depends on the circadian control of *desat1* in the oenocytes. To assess group dynamics, we manipulated the genotypic composition of social groups (single versus mixed genotypes). This manipulation significantly affects clock gene transcription both in the head and oenocytes, and it also affects the pattern of pheromonal accumulation on the cuticle. Remarkably, we found that flies in mixed social groups mate more frequently than do their counterparts in uniform groups.

**Conclusions:** These results demonstrate that social context exerts a regulatory influence on the expression of chemical signals, while modulating sexual behavior in the fruit fly.

## Introduction

Several recent studies of the fruit fly *Drosophila melanogaster* have shown that social context, defined as the size and genotypic composition of the social group, affects various behaviors including locomotor activity [1], aggregation [2], aggression [3], avoidance [4], feeding [5], reproductive behavior [6–9], and sleep [10]. Moreover, these studies emphasize an important role for chemical signals, particularly olfactory cues, in mediating these social effects.

Courtship and mating behaviors are perhaps the best known examples of social interactions involving the exchange and interpretation of various chemical signals in *D. melanogaster*. Some of these signals, like sex peptide [11] or cis-vaccenyl acetate [12–14], are transmitted along with sperm during copulation, whereas others, such as the cuticular hydrocarbons, are transmitted externally [7]. Several of these cuticular hydrocarbons are known to act as sex pheromones and modulate the probability of copulation. In *D. melanogaster*, sex pheromones are sexually dimorphic, i.e., males and females differentially express and respond to these cues. The *Drosophila* courtship ritual involves a set of pheromones for species recognition and sexual attraction that together define "sex appeal," as well as signals that inhibit male-male courtship [15, 16].

The circadian timing system influences social behavior [17]. The importance of circadian clocks for social interactions may be understood intuitively; in order to cooperate in a group activity, individual participants must know not only what to do, but also when to do it. In this way, the synchronization of behavior is necessary to organize the activity of the group. This influence of circadian rhythms is well documented in *Drosophila* where it temporally regulates the level of courtship and the probability of mating [8, 18–20].

The circadian timing system of adult *Drosophila* consists of specialized clock cells in various tissues [21, 22]. For example, certain clock cells in the brain (i.e., central clock cells) regulate the timing of locomotor activity, whereas antennal clock cells, residing outside of the central nervous system (i.e., peripheral clock cells) are thought to modulate responses to odorants, including social cues [23]. Peripheral clock cells have also been shown to exist in many other tissues including the eyes, wings, forelegs, guts, Malpighian tubules, and testes, but the function of clock cells in these tissues remains unclear [21, 22]. To date, little is known about the influence of circadian clocks on the production of chemical signals that define social responses, such as the sex pheromones. Based on the circadian nature of courtship behavior, we hypothesized that oenocytes, the pheromone-producing cells, contain functional peripheral clocks that regulate pheromone synthesis.

Here, we report that the expression of sex pheromones is clock regulated. We provide evidence that a circadian clock mechanism is present in the oenocytes and that this oenocyte clock regulates the production and emission of sex pheromones. In addition, manipulating the genotypic composition of social groups affects the circadian clock mechanism such that genotypic heterogeneity within the group reduces the amplitude and mean accumulation of clock gene transcripts in the head and in the oenocytes and alters the display of sex pheromones on the cuticle. Consistent with the modulation of pheromone productions, social interactions also alter the temporal profile of mating behavior, resulting in an increase in the daily total number of matings performed by wild-type males. The data demonstrate that the social environment can modify the endogenous clock mechanism governing sex-pheromone synthesis and influence sexual behavior in *Drosophila melanogaster*.

*Correspondence: joel.levine@utoronto.ca
[2]These authors contributed equally to this work
[3]Present address: Firmenich S.A., Route des Jeunes 1, P.O. Box 239 Genève CH-1211, Switzerland
[4]Present address: Department of Ecology and Evolution, University of Lausanne, Biophore, Lausanne, CH-1015, Switzerland

### Oenocytes Contain a Peripheral Clock

In insects, a specialized cell type referred to as oenocytes (Figure 1) is thought to be required for the production of cuticular hydrocarbons [24–28]. In *D. melanogaster,* feminization of male oenocytes results in the expression of female hydrocarbons on the male body surface [26]. These studies and others have demonstrated that cuticular hydrocarbons represent the output of the oenocytes [29].

The adult *Drosophila* oenocytes are located in the abdomen and are organized in metameric stripes extending along the inner cuticular surface of each tergite from the dorsal midline, adjacent to the dorsal vessel, laterally to the spiracles (Figures 1A–1E). These dorsal oenocyte clusters are composed of tightly grouped mononucleated cells, which form ribbon-like monolayers (Figure 1B and inset). Other, more ventral, oenocyte clusters exist on the inner surface of the abdominal sternites (Figure 1A) [30]. A basal lamina ensheaths the oenocytes, as has been described for ectoderm-derived tissues in other insects, and may account for the compact appearance of the monolayer (Figures 1D and 1E) [31]. When viewed in cross section, the interface between the oenocytes and the cuticle becomes apparent (Figures 1F and 1G). Positioned at the posterior edge of each segment, the oenocytes lay near to the flexible intersegmental membrane joining adjacent cuticle plates (Figures 1F and 1G). Although the mechanism by which hydrocarbon compounds are deposited onto the surface of the cuticle is not known, the close association of the oenocytes with the cuticle and intersegmental membrane suggests a route for their release to the outer body surface.

To determine whether oenocytes are circadian clock cells, we investigated whether these cells display features of the molecular clock mechanism defined by the cyclical expression of the core clock genes, *period* (*per*), *timeless* (*tim*), and *Clock* (*Clk*) [21]. In archetypal clock cells, the temporal profiles of *per* and *tim* RNA accumulation peak during the early night, whereas *Clk* peaks at an approximately opposite time in the daily cycle. This temporal pattern of clock gene expression defines the molecular signature of the circadian clock for both central and peripheral clock cells. The expression of these core clock genes are expected to be abnormal in the arrhythmic $per^0$ mutant. Consistent with these features of the clock, all three core clock genes are expressed in oenocytes and their temporal patterns of wild-type expression are significantly sinusoidal ($p \leq 0.0001$), whether in constant darkness (DD) or in a light-dark cycle (LD) (Figures 2A and 2B; Figure S2 and Table S1 available online). In addition, the phase relationships between *per*, *tim*, and *Clk* are consistent with the canonical *Drosophila* wild-type circadian clock mechanism in DD and LD [21, 22]. The peak in *per* RNA occurs about 1.5 hr earlier than *tim*, whereas the peak in *Clk* RNA occurs about 12 hr before *per* (Figures 2A and 2B; Table S2). Similarly, $per^0$ oenocytes display a disruption of the temporal pattern of expression in DD (Figure 2C; Tables S1 and S3). Together, these findings indicate that the oenocytes are peripheral clock cells.

### A Peripheral Clock Regulates Cuticular Pheromones

Because oenocytes have functional molecular clocks, we hypothesized that the expression profile of cuticular pheromones may display circadian rhythmicity. Of the 23 hydrocarbon compounds found on the surface of the male cuticle, 4 are thought to act as male sex pheromones: 5-tricosene or 5-C23:1 (5-T), 7-tricosene or 7-C23:1 (7-T), 7-pentacosene or 7-C25:1 (7-P), and 9-pentacosene or 9-C25:1 (9-P) (Figure 3A) [7]. We observed that these compounds cycle with a circadian rhythmicity. Although not all are identical in pattern, wild-type levels of these compounds in DD are generally lower at times during the subjective day (times 0–11) than

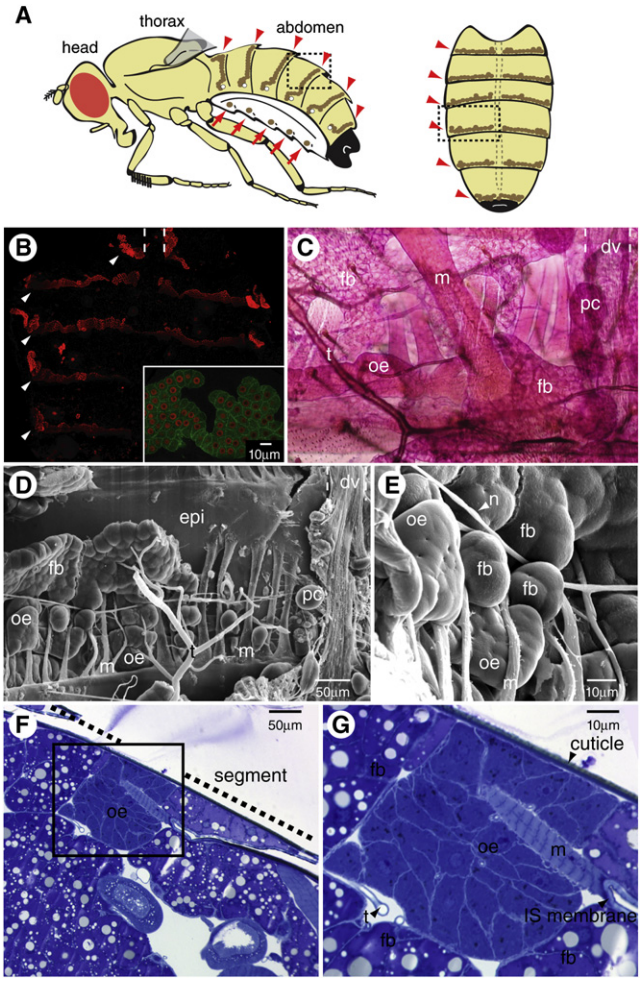


Figure 1. Cytology of Adult *Drosophila* Oenocytes

(A) Cartoons showing the location of the oenocytes in the abdomen of an adult fly. The profile of a fly is shown on the left. The dorsal aspect of a fly abdomen is shown on the right. The oenocytes lie in the posterior region of each abdominal segment, immediately beneath the surface of the cuticle. Dashed box in the left image frames the position of the sagittal section shown in (F) and (G). Dashed box in the right panel frames the location of the images shown in (B)–(E). Oenocytes, dark brown; dorsal oenocyte clusters, red arrowhead; ventral oenocyte clusters, red arrow.

(B–E) Filet preparations of adult male abdomens.

(B) Individual oenocytes are marked by a nuclear form of β-gal (UAS-NZ.lacZ; red) and a cell-membrane-targeted form of GFP (UAS-mCD8::GFP; inset only, green), each misexpressed via the Gal4 driver, 1407-Gal4 (Figure S1). Abdominal segments A1 to A5 are shown. Arrowheads demarcate the posterior edge of each segment. The dorsal vessel is marked by dashed lines.

(C) Hematoxylin and eosin staining of a single abdomen hemisegment. Oenocytes are strongly eosinophilic and appear as a dark pink band of cells extending along the posterior edge of the segment. A4 hemisegment is shown.

(D and E) Scanning electron microscope images of a single abdominal hemisegment.

(E) Increased magnification of oenocytes shown in (D).

(F and G) Sagittal section of an adult male abdomen stained with toluidine blue.

(G) Increased magnification of oenocytes shown in (F). Dotted lines bound the anterior and posterior aspects of the dorsal vessel located at the midline. dv, dorsal vessel; epi, epidermis; fb, fat body; m, muscle; n, nerve; pc, pericardial cell; oe, oenocytes; t, trachea; IS membrane, intersegmental membrane.

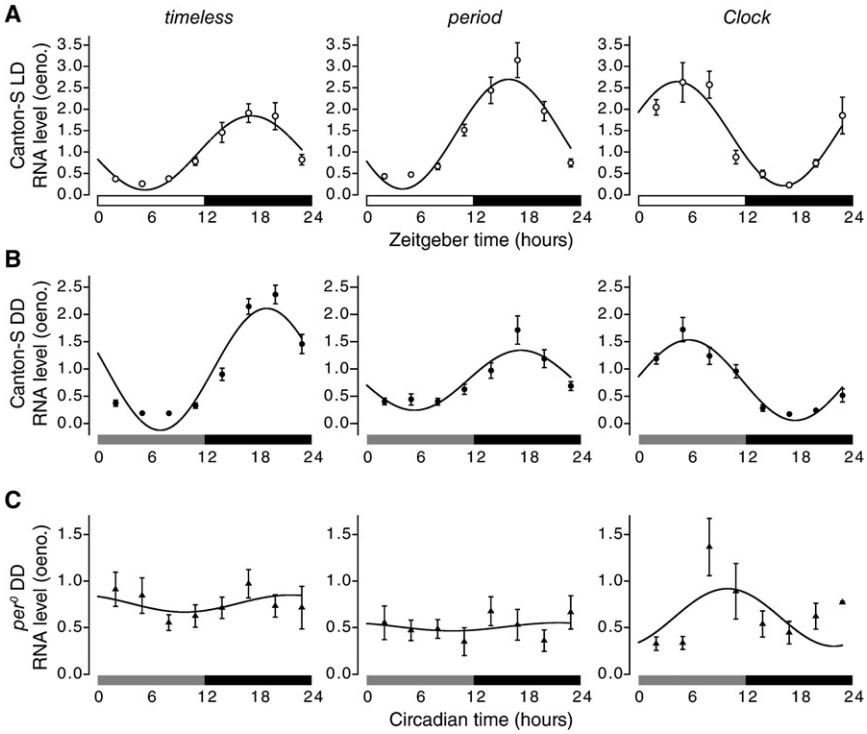


Figure 2. Oenocytes Contain a *per*-Dependent Clock

The temporal patterns of *tim*, *per*, and *Clk* RNA expression in wild-type and $per^0$ oenocytes as determined by quantitative RT-PCR. These patterns indicate a functional clock in the oenocytes for the wild-type in LD (A) and DD (B). The functional pattern is disrupted for $per^0$ in DD (C). Total RNA was isolated from dissected oenocyte preparations. Best-fit cosine curves (solid lines) are fitted to RNA expression values ± SEM (Tables S1–S3). Each time point represents the average of three replicate qPCR reactions from three independent experiments (n = 9). WT, wild-type; DD, first day constant darkness; WT LD, open circles; WT DD, filled circles; $per^0$ DD, filled triangles.

during the subjective night (times 12–23) (Figure 3B). Furthermore, the pheromone quantities on wild-type males compared with $per^0$ males differed at most times throughout the day (Figure 3B).

Hour-by-hour temporal patterns of hydrocarbon accumulation of wild-type and $per^0$ males were determined to evaluate whether the two genotypes displayed similar patterns of expression, albeit at different levels. Similarity between temporal patterns was assessed by evaluating the strength of the correlation between $per^0$ and wild-type temporal profiles in DD. No significant correlation was evident in DD for three of the four compounds: 5-T, 7-T, 7-P (9-P was correlated between the two male genotypes $p = 0.0014$). The daily temporal pattern of accumulation for these three courtship cues is thus *per* dependent in DD and at least partially dependent on a circadian clock.

Although constant darkness is used to assess whether an endogenous mechanism drives daily rhythms in the absence of environmental timing cues, we also evaluated the effect of such cues by comparing the wild-type levels of the four pheromones in DD to those in a more natural LD cycle (Figure 3C). Significant differences between DD and LD in compounds 7-P and 9-P are evident only around the transition from day to night, whereas compounds 5-T and 7-T are significantly elevated in DD compared to LD during subjective midday as well as during the subjective night. Further, correlation analysis of wild-type temporal patterns in LD and DD reveal a strong association for 5-T ($r = 0.671$, $p < 0.001$), 7-P ($r = 0.756$, $p < 0.001$), and 9-P ($r = 0.689$, $p < 0.001$), but not 7-T ($r = 0.419$, $p = 0.05$). A striking reduction in amplitude of the daily pattern is evident in LD when compared to DD for 5-T ($p < 0.003$) and 7-T ($p < 0.05$), although no difference is apparent for 7-P ($p = 0.63$) and 9-P ($p < 0.13$) (Figure 3C). In summary, male courtship pheromones accumulate on the body surface in a *per*-dependent pattern in DD (Figure 3B), and this pattern is light sensitive (Figure 3C).

We considered the possibility that the *per*-dependent pattern of hydrocarbon accumulation might be influenced by the central clock and may represent an indirect consequence of behavioral rhythmicity. For example, rhythmicity in feeding behavior could influence the temporal availability of metabolites required for hydrocarbon production. The transgenic *per7.2:2* strain was employed to determine whether the temporal profile of hydrocarbon accumulation requires a peripheral clock [32]. In this strain, rhythmic expression of PER protein is restricted to a subset of clock neurons in the brain essential for generating locomotor behavior rhythms; in peripheral tissues, PER is completely absent [33]. In the *per7.2:2* strain, the oenocyte clock is arrhythmic (Figure S3A). If the display of cuticular hydrocarbon pheromones is driven by PER-positive neurons in the brain or by behavioral rhythmicity, then the temporal pattern in the *per7.2:2* strain, even in the absence of a functioning oenocyte clock, should be the same as that of wild-type control. In constant darkness, the wild-type control and *per7.2:2* males displayed significantly different amounts of 5-T, 7-T, 7-P, and 9-P at all time points (Figure S3B). Moreover, no significant correlation was evident between *per7.2:2* and wild-type in the temporal patterns of these compounds. These observations strengthen the hypothesis that cuticular hydrocarbon accumulation is regulated by a *per*-dependent peripheral clock mechanism in the oenocytes and is not driven or significantly affected by central clock cell functions.

### *desat1* Is a Clock-Controlled Gene in the Oenocytes

We next investigated a potential mechanism by which the oenocyte clock may regulate pheromone expression patterns. The metabolic pathways for hydrocarbon synthesis yield three chemical classes: straight chain saturated compounds, unsaturated compounds, and methyl-branched compounds. All of the pheromones studied here are monounsaturated hydrocarbon compounds (Figure 3A). We assessed the oenocyte expression of *desaturase1* (*desat1*), a gene that encodes a Desaturase enzyme [34, 35]. DESAT1 activity directly affects only those compounds with double bonds at the 7- position, e.g., 7-T and 7-P [34]. However, consistent with previous reports [36], we have observed that a mutation affecting *desat1* broadly alters the expression of all unsaturated compounds as well as other classes of compounds. Manipulation of *desat1* can alter

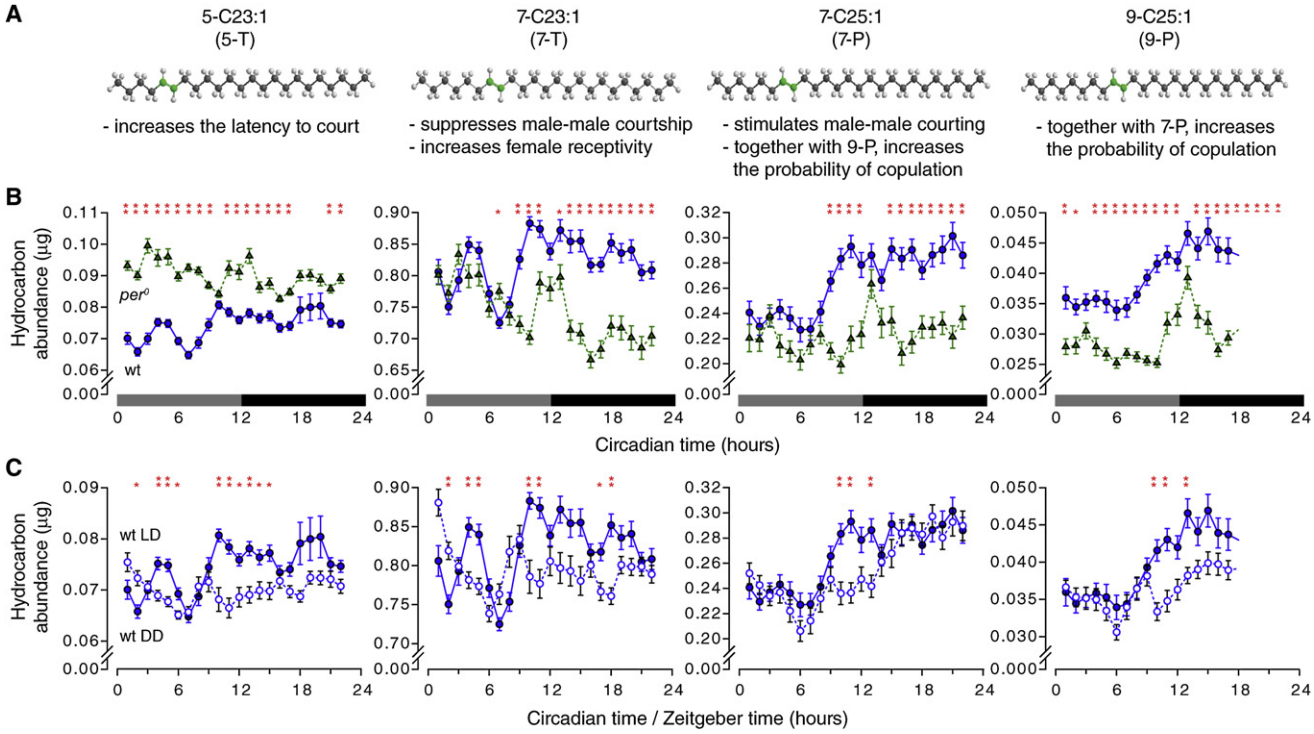


Figure 3. Cuticular Hydrocarbon Accumulation Is Regulated by a *per*-Dependent Clock and Is Influenced by Exposure to Light

(A) Structural representations of hydrocarbon compounds 5-T, 7-T, 7-P, and 9-P. Each consist of a carbon chain (23C or 25C) with a single double-bond at position 5, 7, or 9 (green).

(B and C) Comparisons in the temporal profile of the indicated hydrocarbon compound between wild-type and $per^0$ males in DD (B) and between wild-type males in DD and in LD (C). Shown are values calculated from a 3 hr moving average ± SEM (n = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two-sided Student's t test (**p < 0.01; *p < 0.05; data corrected with Benjamini-Hochberg False Discovery Rate $q$ = 0.1). WT, wild-type; WT DD, filled blue circles; WT LD, open blue circles; $per^0$ DD, filled green triangles.

both the blend of cuticular pheromones and male courtship behavior in *Drosophila* [7, 36]. We predicted that *desat1* would be expressed in the oenocytes and regulated by the oenocyte clock, and thereby may control the cyclic expression of cuticular hydrocarbons.

Consistent with these hypotheses, we observed that *desat1* is expressed in oenocytes and a sinusoidal expression pattern is evident under both DD and LD conditions ($p < 0.0001$) (Figure 4A). Like *Clk*, the temporal profile of *desat1* mRNA peaks during the subjective day with a trough at night. A fixed phase relationship occurs between *Clk* and *desat1* in oenocytes; on average, peak expression of *desat1* occurs approximately 4 hr after the peak in *Clk* expression ($p = 0.023$; Table S2). In addition, the circadian pattern of *desat1* expression is disrupted in $per^0$ flies in DD (Figure 4A). In these arrhythmic mutant flies, the *desat1* expression pattern is not significantly different from a flat line.

Although the above results demonstrate that *desat1* is expressed in oenocytes and may be clock regulated, the peak-to-trough variation in *desat1* expression, though statistically significant, is less than 2-fold in DD and in LD (Figure 4A). Similar results were obtained when we examined the temporal patterns of DESAT1 protein extracted from oenocytes at different times in the circadian cycle (Figure S4). Despite the small change, we hypothesized that circadian regulation of *desat1* expression is relevant to the daily pattern of pheromone accumulation. To further investigate this, we obtained a *desat1* hypomorphic mutant stock, $desat1^{1573\text{-}1}$, containing a *P*-element insertion in the *desat1* locus, and a control stock, $desat1^{1573\text{-}N2}$, formed by a precise excision of the insert [36]. Comparison of transcript, protein, and hydrocarbon levels in flies homozygous or heterozygous for the insertion and homozygous for the excision showed a monotonic relationship between levels of *desat1* transcript, protein, and the accumulation of both 7-P and 7-T (Figures 4B–4D; Figure S5). The degree of the difference seen among these flies reveals that a 50% change (i.e., less than a fold change) in transcript yields detectable changes in hydrocarbon accumulation.

The above observations support the interpretation that *desat1* is under clock control in the oenocytes and that the temporal lability of *desat1* transcript and protein affects the levels of pheromonal expression. It is clear that the observed relationship between *desat1* transcript, protein, and hydrocarbon expression encompasses the variation of wild-type values observed within a day (Figure 4D; Figure S5). This relationship between relatively subtle changes in *desat1* mRNA and the consequent effects on 7-P and 7-T accumulation provides suggestive evidence that low-amplitude rhythms in *desat1* RNA are functionally relevant.

## Social Context Affects Clock Gene Expression and Pheromone Output

Pheromonal communications influence the physiology and behavior of interacting individuals within a group. As such they play an important role in the organization of social interactions [37]. However, social interactions are dynamic. Next, we evaluated whether social experience affects the temporal control of pheromone accumulation by comparing circadian clock

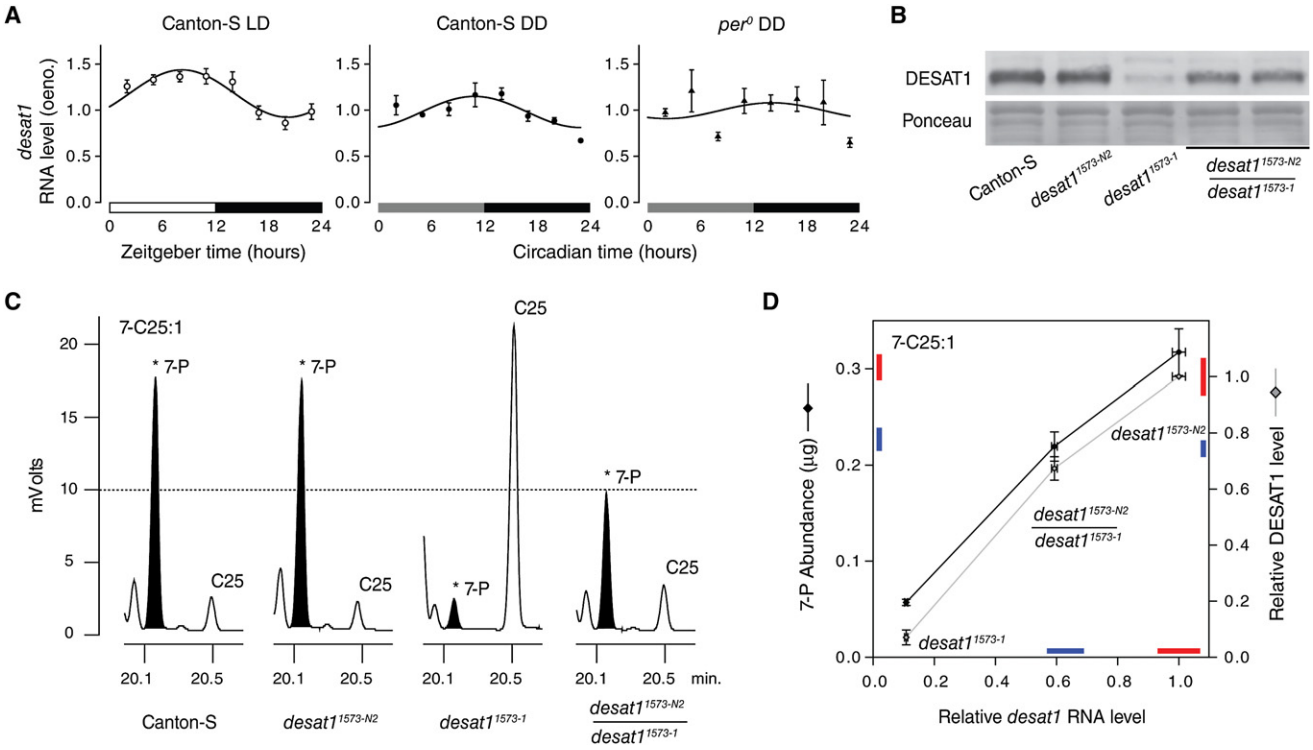


Figure 4. Cuticular Hydrocarbon Accumulation Is Regulated by the Expression Level of *desat1*

(A) The temporal patterns of *desat1* RNA expression in wild-type and $per^0$ oenocytes as determined by quantitative RT-PCR. Genotype and lighting conditions as indicated. Best-fit cosine curves (solid lines) are fitted to RNA expression values ± SEM (Tables S1–S3). RNA samples were the same as that used in Figure 2. Each time point represents the average of three replicate qPCR reactions from three independent experiments (n = 9), except for wild-type *desat1* expression in DD (n = 6). WT, wild-type; DD, first day constant darkness; WT LD, open circles; WT DD, filled circles; and $per^0$ DD, filled triangles.

(B) Western blot analysis of oenocyte extracts isolated from the indicated genotypes and labeled with an DESAT1 antibody. $desat1^{1573\text{-}N2}$ and $desat1^{1573\text{-}1}$ refer to control and homozygous *desat1* mutant genotypes, respectively. $desat1^{1573\text{-}N2}$ / $desat1^{1573\text{-}1}$ refers to the heterozygous *desat1* mutant genotype. Ponceau staining of the blot served as the loading control.

(C) Representative gas chromatographs displaying the amounts of 7-P (asterisks) and C25. Genotypes as in (B). The area under each peak corresponds to amount of each compound. The output of the flame ionization detector (FID) displaying the height of each peak (mVolts) and the corresponding retention times (min) is shown.

(D) Standard curve relating the level of *desat1* RNA to the level of 7-P (black line) and Desat1 (gray line) in genotypes shown in (B) and (C). The plotted lines may be compared to daily high and low values observed for *desat1* RNA (see [A]; LD), 7-P (see Figure 3C; WT LD), and Desat1 protein (see Figure S4; LD) as indicated by red and blue bars, respectively, positioned next to the indicated axis (bar length corresponds to ±SEM).

gene expression in the heads and oenocytes of wild-type males from homogeneous social groups with that of wild-type males from genotypically mixed groups (containing both wild-type and $per^0$ flies). In these tests, homogenous social groups consisted of 40 genotypically similar individuals per vial per time point. Heterogeneous groups consisted of a majority of 32 wild-type males, called "hosts," housed with a minority of 8 $per^0$ males, the "visitors." This 32:8 host:visitor ratio has been previously shown to maximize the effects of heterogeneous groups in this assay [1].

The overall temporal pattern of expression was similar in oenocytes and heads for both hosts and controls (Figures 5A and 5B; Tables S4 and S5). Interestingly, however, the social manipulation led to pronounced differences in the level and amplitude of gene expression. The amplitude of *tim* and *Clk* profiles, as well as the relative level of expression for *per*, *tim*, and *Clk*, were significantly reduced in the oenocytes of hosts compared to wild-type controls from homogeneous groups (Figure 5A; Tables S4 and S6). Like the clock genes, *desat1* expression was also reduced in host oenocytes relative to controls (Figure 5A; Tables S4 and S6). In heads, *per* and *Clk* expression were reduced in hosts compared to controls at each time point, as well as in the overall mean (taken across the day). The shape and amplitude of the respective expression curves, however, did not change in heads (Figure 5B; Tables S4 and S6). No significant difference was detected in the levels or pattern of *tim* expression. Thus, social context alters the patterns of clock gene expression within the oenocytes and heads, as well as the temporal relationship between the two tissues (Table S7). The mechanism by which social interactions produce this modulation of the molecular processes underlying such circadian timing systems remains to be elucidated.

The effects of social context on *Clk* and *desat1* expression in male oenocytes led to the prediction that patterns of cuticular hydrocarbons would also be altered by social context. To test this hypothesis, we compared sex-pheromone levels males from mixed versus homogeneous groups in DD (Figure 5C) and LD (Figure S6A). In hosts, the levels of 5-T and 7-T were significantly elevated over that of the controls during the second half of the subjective night and the latter parts of the subjective day in DD. Host levels of pheromones 7-P and 9-P, however, differed from controls mainly around the transition from subjective day to night (Figure 5C). The overall patterns of accumulation were not significantly correlated, indicating differences in expression patterns between hosts and controls

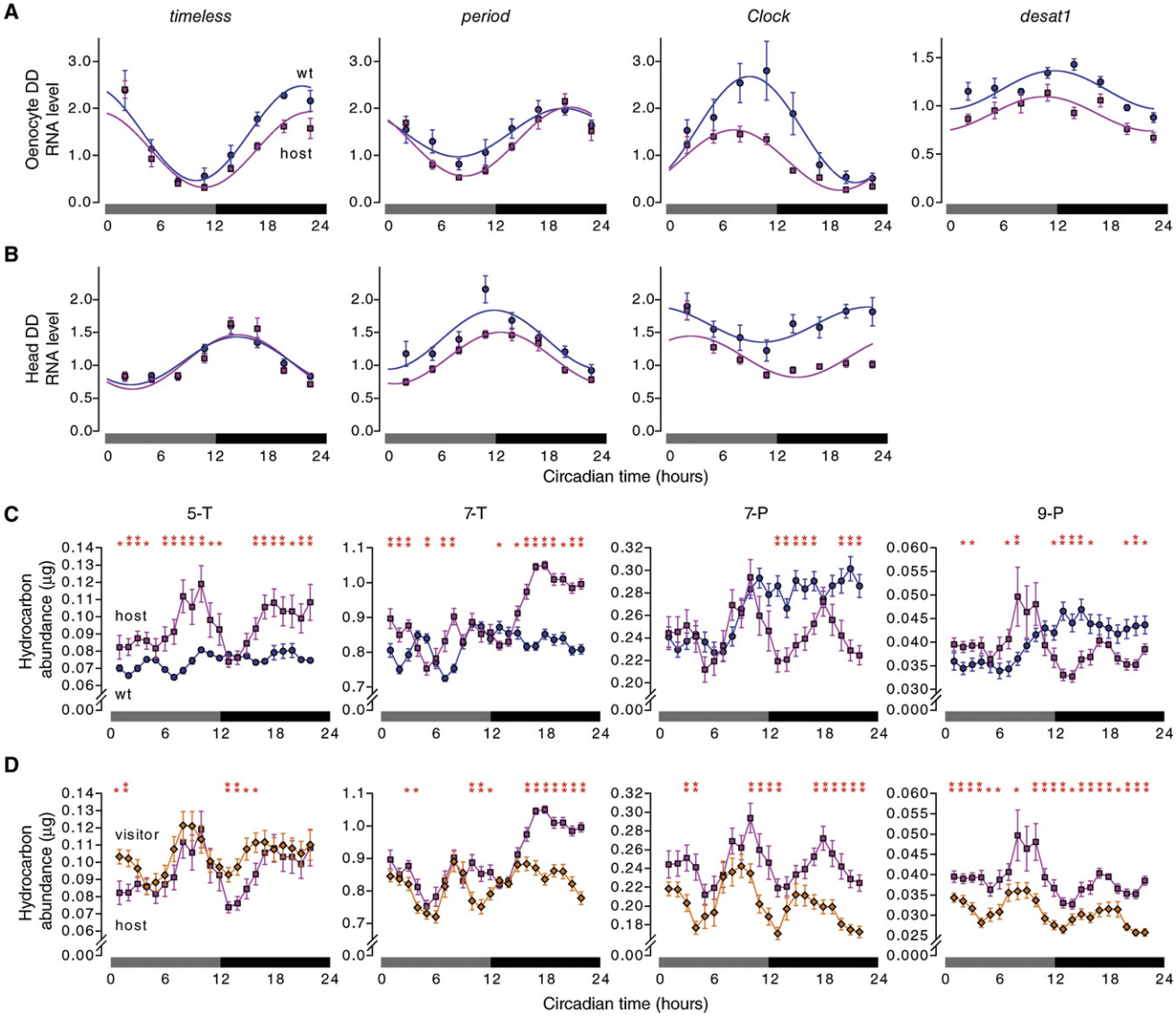


Figure 5. Social Interactions Affect the Temporal Profile of Gene Expression and Cuticular Hydrocarbon Accumulation

(A and B) The temporal patterns of *tim*, *per*, *Clk*, and *desat1* expression in wild-type control and host oenocyte preparations (A) and whole-head extracts (B) as determined by quantitative RT-PCR analysis. Oenocytes and heads were isolated from the same flies. Best-fit cosine curves (WT, wild-type; WT, blue line; host, magenta line) are fitted to RNA expression values ± SEM (WT DD, blue circles; host DD, magenta squares). Expression was assayed on the second day of constant darkness. Each time point represents the average of three replicate qPCR reactions from three separate experiments (n = 9).

(C and D) Comparison of the temporal profiles of 5-T, 7-T, 7-P, and 9-P between wild-type control and host males in DD (C) and between host and visitor males in DD (D). Shown are values calculated from a 3 hr moving average ± SEM (n = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two-sided Student's t test (**p < 0.01; *p < 0.05). WT, wild-type; WT DD, filled blue circles; host DD, filled magenta squares; visitor DD, filled orange diamonds.

for all four compounds: $r = 0.33$ (n.s.) for 5-T, $r = 0.07$ (n.s.) for 7-T, $r = 0.22$ (n.s.) for 7-P, $r = 0.22$ (n.s.) for 7-P, and $r = -0.39$ (n.s.) for 9-P. In LD, the effects of social interactions on the level of pheromone expression in hosts persist but are subtler, predominately occurring around the time of light transitions (Figure S6). Thus, these findings demonstrate that social context has significant effects on both the overall levels of these cuticular pheromones, as well as on their temporal patterns.

Notably, the relationship between these pheromones in visitors versus homogeneous $per^0$ controls (Figure S7) is similar to that of the hosts and their controls (Figure 5C; Figure S6A). In particular in DD, visitor levels of 5-T and 7-T were elevated during the late subjective night and late subjective day, whereas 7-P and 9-P differed around the transitions (Figure S7A). The visitor pattern correlated inversely with $per^0$ controls in all three compounds: $r = -0.63$ ($p < 0.002$) for 5-T, $r = -0.44$ ($p < 0.042$) for 7-T, $r = -0.48$ ($p < 0.023$) for 7-P, and $r = -0.66$ ($p < 0.001$) for 9-P. Thus, these data show that visitors are also affected by social context.

Pheromone patterns in the wild-type hosts and $per^0$ visitors correlate more strongly with each other than they do with their genotypic controls in DD: $r = 0.69$ ($p < 0.001$) for 5-T, $r = 0.52$ ($p < 0.015$) for 7-T, $r = 0.46$ ($p < 0.03$) for 7-P, and $r = 0.74$ ($p < 0.0001$) for 9-P (Figures 5C and 5D; Figure S7A). One possibility is that the similarity between hosts and visitors is due to transfer by contact. However, physical contact alone cannot

explain the altered pheromone profiles shown in heterogeneous groups (Figures 5C and 5D) because both hosts and visitors display similar changes in pheromone accumulation over time; i.e., loss or gain of a compound cannot be solely explained by contact-mediated transfer if both groups are increasing or decreasing relative to controls.

Interestingly, although the levels of *desat1* transcript are reduced in hosts compared to controls, the pheromonal patterns we observed are variable: patterns of 7-P and 9-P accumulation are consistent with the reduced level of the *desat1* transcript but 5-T and 7-T are higher in hosts than controls. Despite this apparent inconsistency, the total accumulation of monounsaturated compounds is reduced in hosts [38]. Thus, the level of *desat1* expression accurately predicts the accumulation of the combined total of all monounsaturated compounds. This is consistent with the broad effect on pheromone expression observed in hypomorphic *desat1* mutant flies.

These results demonstrate that heterogeneous and homogeneous male groupings differentially affect the production of male pheromones as well as their expression on the cuticle surface, thereby illustrating the sensitivity of these traits to social context. Thus, the pheromonal blend expressed on the cuticle appears to reflect social experience.

### Social Context Affects Mating Frequency

As shown above, wild-type male expression levels of specific cuticular hydrocarbons change when the flies are housed with $per^0$ mutants, but what is the behavioral significance of these changes? We next investigated the effect of heterogeneous social context on mating behavior. We developed an assay in which six virgin males are housed with six virgin females and allowed to interact continuously over a 24 hr period (Figure 6A). We tested both homogeneous groups composed of six wild-type males with six wild-type females and mixed groups of four wild-type host and two $per^0$ visitor males with six wild-type females. Both the temporal distribution and overall number of matings (copulations) were recorded in these different contexts.

We plotted the cumulative matings of the homogeneous and mixed groups over the 24 hr observation period (Figure 6B). Both groups mated at nearly the same rate until around CT21, when the mating frequency began to slow in the homogeneous group but not among the heterogeneous group. The two groups expressed similarly shaped temporal distributions of mating, but their levels of mating are significantly different and this difference becomes evident around dawn (CT0; Figure 6B).

After 24 hr, the total number of matings in the heterogeneous groups was 22% higher than in the homogenous groups (Figure 6C). In the heterogeneous groups, the two $per^0$ visitor males performed a mere 5% of the matings (Figure 6C), meaning that the four wild-type host males individually performed nearly twice as many matings (1.7 times more) as their counterparts in the homogenous groups (Figure 6D). A corresponding increase in the average number of matings per female within the mixed groups was also observed (Figure 6E). Interestingly, the four wild-type males in a mixed group mated significantly more (2 more matings per male over 24 hr) than did four wild-type males in a homogeneous group of four overall (Figure 6D). Thus, the increased wild-type mating frequency is dependent on the presence of the $per^0$ males and does not result from a difference in the number of wild-type males present in the mating chamber.

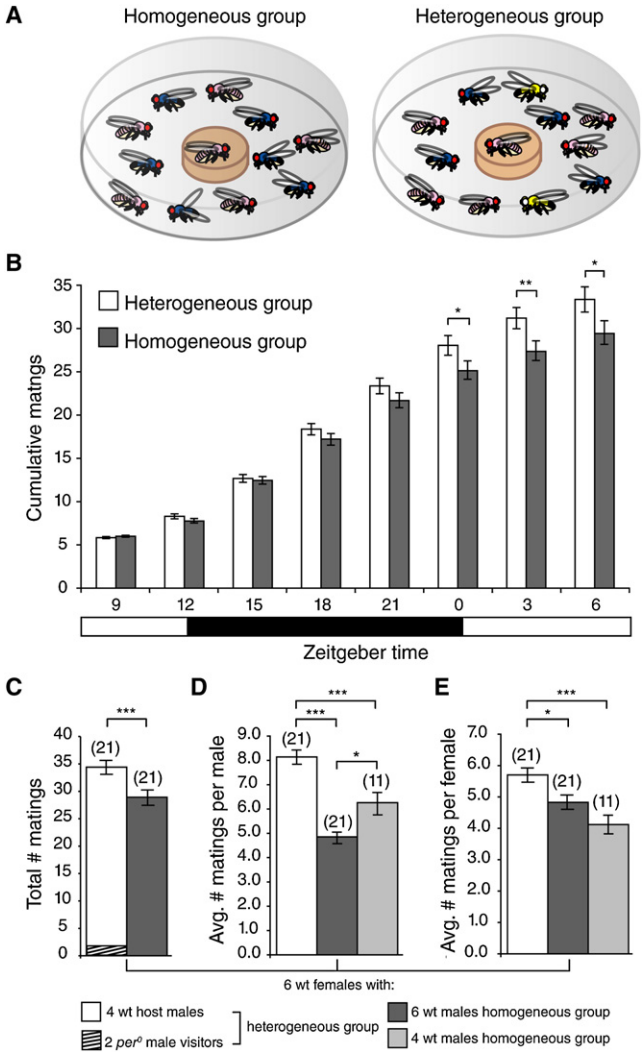


Figure 6. Social Context Changes the Amount and Temporal Distribution of Mating

(A) Schematic representation of the social-mating assay. The homogeneous group consists of six wild-type (WT) females (pink) and six WT males (blue). The heterogeneous group consists of six WT females, four WT host males, and two $per^0$ mutant visitor males (yellow).

(B) Cumulated number of matings over a 24 hr observation period shown at 3 hr interval time points. n = 13 for both heterogeneous (white) and homogeneous (gray) groups. (ANOVA: *p < 0.05, **p < 0.01; after False Discovery Rate correction at $q = 0.1$.)

(C) Total number of matings in a heterogeneous genotype (white) or homogeneous (gray) group. ***p < 0.001, paired t test. Number of repeats (n) is between parentheses.

(D) Mean number of matings *per* wild-type male in the indicated social context. Values represent the number of wild-type male matings over 24 hr divided by the number of wild-type males present (either 4 or 6) averaged across independent experiments. ANOVA: *p < 0.05; ***p $\leq$ 0.001.

(E) Mean number of matings per wild-type female in the indicated social context. ANOVA: **p < 0.01; ***p < 0.001. Error bars indicate ±SEM in all panels.

### Discussion

Social experience can influence behavior in *Drosophila*. In many cases, this influence is communicated via chemical cues, possibly in the form of pheromones [1–4, 8, 10]. Individuals sense these social cues and respond to one another as

they participate in group activities. Understanding how individuals send, receive, analyze, and respond to these signals is key to understanding how group dynamics affect behavior. By using measures of gene expression within the head and oenocytes, sex-pheromone accumulation on the outer cuticular surface, and mating behavior, we have identified aspects of a mechanism driven by social experience that influences sexual behavior in *D. melanogaster*. Our approach to studying group dynamics has reduced questions about social experience to questions about how molecular and cellular mechanisms mediate the effects of social interactions on individuals.

### Regulation of Pheromone Synthesis and Display by a Circadian Mechanism

We demonstrate that *desat1* is a circadian output gene of a peripheral clock contained in the oenocytes (Figures 2 and 4). The presence of *desat1* RNA and protein in the oenocytes supports the role of these cells as the primary site for the production of sex pheromones. Circadian fluctuations in the temporal display of many cuticular hydrocarbon compounds have been previously observed [39]. Here we examine four of these compounds that act as sex pheromones. Consistent with the cyclic expression of *desat1*, the temporal profile of these pheromones are *per* dependent (Figure 3) and appear to be influenced by a peripheral clock (Figure S3). Given the unique role oenocytes play in the production of sex pheromones [6], we suggest that the temporal fluctuations in male sex-pheromone production are regulated by the oenocyte clock via the circadian regulation of *desat1*.

Environmental inputs such as humidity and temperature are known to regulate hydrocarbons in *Drosophila* [40, 41]. We show here that social interactions regulate hydrocarbon physiology. Such input from the physical and social environment may be mediated by hygro-, thermo-, and olfactory receptors in the antennae [42] or by gustatory receptors in tarsi and proboscis [43]. Whereas circadian clocks regulate olfactory [44] and possibly gustatory input, as well as oenocyte output (this study), it will be important to determine whether these clocks are required for the regulation of cuticular hydrocarbon as the fly adjusts to its environment in general.

In particular, why should sex-pheromone display be under circadian regulation? In *Drosophila melanogaster*, sex pheromones differ between the sexes and are involved in mate recognition and preference [6]. As such, these compounds represent sex-specific characters that may provide a fitness benefit when displayed at a particular concentration, in a particular blend, at specified times, and/or under certain conditions [7, 35]. All of these factors have been shown in various species to come into play when attempting to attract a mate [37, 45]. That sex-pheromone production and display are clock controlled implies that courtship and mating may have a temporal structure. Indeed, daily rhythmicity in courtship behavior was demonstrated by Hardeland some years ago [19]. More recently, others have extended these studies and have shown a temporal pattern in both courtship and mating [8, 18, 46].

Sexually dimorphic and species-specific pheromones allow for mate recognition and effective mating strategies, and so may the circadian regulation of pheromones. In this way circadian changes in pheromonal profiles may represent the means of creating a temporal niche by influencing the probability of copulation In this regard, *per* has been associated with the temporal control of when different *Drosophila* species prefer to mate, thus creating a temporal barrier in mating [18]. With this in mind, it will be important to determine whether the sex pheromones of other *Drosophila* species are also under circadian regulation, and whether the patterns differ from that of *D. melanogaster*. This might represent a mechanistic basis to the proposal of Tauber et al. [18], whereby populations exhibiting different pheromonal levels at different times might become reproductively isolated.

### Social Effects on Pheromone Synthesis and Reproductive Behavior

We show that social interactions influence the circadian regulation of male sex pheromones and the mating behavior (Figures 5 and 6). The overall levels of male sex pheromones as well as their temporal patterns are affected by social context. The mixture of two genotypes (WT and $per^0$) within the group produced a general decrease in the total amount of monounsaturated hydrocarbon (except for 5-T and 7-T, which increase; Figure 5) and an increase in the frequency of mating. Given the prominent role male sex pheromones play in courtship behavior, it is possible that these changes in male pheromones directly relate to the change in mating behavior. 7-T is the most abundant of male sex pheromones in *D. melanogaster* and has been shown to increase female receptivity and repress male-male courtship. Males with elevated levels of 7-T show decreased latency to copulation and a higher mating success rate [47, 48]. Although an increase in 7-T may account for the increase in the frequency to copulate in our experiments, we favor an alternative explanation that a blend of hydrocarbons is associated with both the communication of social information and the influence on sexual behavior. We infer this because of the broad changes in cuticular hydrocarbons we observe between different social contexts. It remains to be determined how a change in pheromone expression in response to social interactions affects mating behavior in our social assay.

Consistent with the circadian changes in sex-pheromone expression, social interactions also affected the molecular rhythm of the oenocyte clock and the expression of *desat1*. The amplitude of *tim* and *Clk* expression decreased, whereas that of *per* increased in response to the heterogeneous social grouping; the period and the phase of the molecular rhythms remained unchanged. Although the oenocyte clock remains rhythmic, the relationship between the expression levels of these core clock genes appeared altered. Transcriptional rhythms of the clock genes contribute to clock function [49], and changes in the amplitude of transcription have been shown to affect daily locomotor activity rhythms [21]. Likewise, a change in the amplitude of clock gene expression within the oenocytes as a response to social interactions may drive the observed changes in sex-pheromone display. Correlated to the affect on *Clk* expression, the level of *desat1* RNA was also reduced, thereby providing a putative mechanism whereby social experience can affect the circadian production of sex pheromones.

The patterns of clock gene expression within tissues of the head were also affected by social interactions. The expression level of *per* and *Clk* was decreased, *tim* remained unchanged, and again, the period and phase of clock gene expression was unaffected. The head contains multiple circadian oscillators, including the central clock cells, which are required for the generation of locomotor rhythms. Although whole-head preparations prevent us from localizing this effect to only the central clock, the changes in gene expression are intriguing in the context of our previous findings, demonstrating an effect of social experience on locomotor activity rhythms [1].

Together with the effect on the oenocytes, it would appear that social interactions affect multiple circadian systems, including both the central and peripheral clocks. Notably, these observations indicate that the amplitude of clock gene expression plays an important role in modulating both physiological (e.g., pheromone production) and behavioral (e.g., locomotor activity) rhythms.

A direct demonstration of the mechanistic links between the individual observations presented in this study (i.e., oenocyte clock > *desat1* rhythm > pheromone rhythm > influences mating behavior) requires a means to manipulate the oenocyte clock specifically and the rhythmic expression *desat1*. This could be achieved through the use of the Gal4/UAS system targeting the oenocytes. However, it must be noted that the expression patterns of Gal4 driver lines used previously to examine the function of oenocytes are not restricted to only the oenocytes [26, 28, 29]. In some cases, expression was observed in the brain and/or fat body, two tissues known to affect courtship and circadian behavior [50]. Although there is no doubt about the use of these reagents to manipulate pheromones, we conclude that the available oenocyte drivers are not adequate to discriminate oenocyte-specific effects on behavior or the temporal pattern of pheromone accumulation (Figure S1).

## Indirect Genetic Effects

It is unlikely that social communication in *Drosophila melanogaster* is limited to chemical signaling; indeed, tactile and auditory displays are important features of reproductive behavior [51], and it seems likely that a variety of sensory modalities are linked to pathways that mediate social responses [52]. However, our data suggest that pheromonal responses are extremely sensitive to the social environment.

The possibility that behavioral feedback regulates molecular physiology in the fruit fly was proposed nearly two decades ago in the context of circadian rhythms [53]. Interestingly, the same idea has been proposed in a quantitative theory that views the social environment as a selective pressure. This theory of indirect genetic effects relies on the idea that social interactions, occurring over generations, may direct the distribution of alleles within a population [54]. According to this view, the relationship between an individual's phenotype and genotype is shaped in part by social context. We have observed this here. Given the increase in mating associated with a mixed social grouping (Figure 6), the influence of social interactions on clock gene and *desat1* expression may represent a detailed example in which an indirect genetic effect represents a modification in gene expression. Our data may offer a glimpse into the effects of social interactions on the mechanisms of inheritance. The ability to quantify the effects of social and physical environmental influences on behavior, together with the powerful tools available for studying inherited mechanisms of behavior, suggests that *Drosophila melanogaster* will be an important model organism for understanding the evolution of sociality.

## Experimental Procedures

### Fly Strains

Flies were reared on medium containing agar, glucose, sucrose, yeast, cornmeal, wheat germ, soya flour, molasses, propionic acid, and Tegosept in a 12 hr light/dark cycle (LD 12:12) at 23°C and 70% humidity. Male and female flies were anesthetized with $CO_2$ and separated within 8 hr of eclosion. For all quantitative PCR analyses, with the exception of those performed in the context of the social assay, male pairs (setup in multiple) were raised in individual glass vials (10 mm diameter × 75 mm height) containing 1 ml medium, entrained for 4 days in LD 12:12 conditions, and sacrificed the fourth day after eclosion. To examine free-running oscillations in gene expression, flies were placed in constant dark (DD) conditions at the end of the third day of entrainment, and sacrificed on the first full day of DD conditions.

Experiments examining the effects of social interactions utilized a modified version of the assay described by Levine et al. [1]. Social treatments consisted of 32 Canton-S (host) males housed with 8 *y,per$^{01}$,w* (visitor) males. Control groups consisted of either 40 Canton-S or 40 *y,per$^{01}$,w* males. Flies were housed in plastic vials (25 mm diameter × 92 mm height) containing 10 ml medium and were entrained for a total of 6 days under LD 12:12 conditions; on the fourth day, 32 Canton-S flies were mixed with 8 *y,per$^{01}$,w* flies, maintained in LD 12:12 for 2 additional days, and placed into DD on the sixth day. For quantitative PCR experiments, flies were sacrificed on the second full day of DD. For analysis of pheromone levels, flies were sacrificed on the first full day of DD conditions.

Canton-S strain was used as the wild-type strain. The *y, per$^{01}$,w,* and *per7.2:2* are described in Levine et al. [1]. The 1407-Gal4 line is described in Ferveur et al. [26]. The *desat1$^{1573\text{-}1}$* and *desat1$^{1573\text{-}N2}$* lines are described in Marcillac et al. [36]. UAS-mCD8::GFP and UAS-NZ.lacZ were obtained from the Bloomington Stock Center.

### Quantitative RT-PCR

Oenocytes were collected from five male flies over a period of ~2 hr, at eight scheduled time points spanning a 24 hr period. RNA was isolated from dissected oenocyte preparations (see Supplemental Experimental Procedures) with the RNeasy Micro kit (QIAGEN), and total RNA was reverse transcribed with the StrataScript QPCR cDNA Synthesis kit (Stratagene). Quantitative PCR reactions were performed with the qPCR MasterMix Plus for SYBR Green I kit (Eurogentec), on an ABI PRISM 7700 Sequence Detection System. The relative level of gene transcript expression was determined separately for *timeless*, *period*, *Clock, desat1,* and *Rp49* from cDNA prepared from a common pool of dissected oenocytes. *Rp49* served to normalize gene expression. qPCR reactions were performed in triplicate, and the specificity of each reaction was evaluated by dissociation curve analysis. Each experiment was replicated three times.

### Pheromone Extraction

Pheromone extracts were obtained from Canton-S and *y,per0,w* flies every hour over a 24 hr period. Extracts were obtained from three individuals per time point, selected from a vial containing 40 flies. Flies were removed under either light or red-light conditions depending on light schedule, and anesthetized with ether. Each fly was placed into an individual glass microvial containing 50 μl of hexane containing 10 ng/μl of octadecane (C18) and 10 ng/μl of hexacosane (nC26) as injection standards. To achieve efficient extraction, the microvials were gently agitated for 5 min. Flies were removed with a thin wire probe, and the extracts were stored at −20°C prior to analysis. Experiments were repeated from 3 to 5 times per treatment. Identical methods were used to obtain pheromone extracts from *per7.2:2* and the *desat1$^{1573}$* lines. Extracts were examined by gas chromatography (see Supplemental Experimental Procedures).

### Mating Assay

Testing arenas consisted of 60 × 15 mm plastic Petri dishes. A cylinder of fly flood (22 × 5 mm diameter) was placed in the center of the testing arena. Six Canton-S virgin females were aspirated into the testing arena followed a few minutes later by six males (six *Canton-S* males or four *Canton-S* plus two *y,per$^{01}$,w* males). Flies were from 5- to 6-day-old. Group interactions were recorded with Northern Eclipse software (Version 7.0 from Empix Imaging Inc.) set to take time-lapse images at 2 min intervals for 24 hr. The collected images were scored for the time at which copulation events occurred and strain of the male involved. Experiments were started at CT8 and groups were tested in LD 12:12 with constant red light (680 nm) illumination, allowing for copulation events to be monitored in darkness.

### Statistical Analyses

To determine whether RNA expression patterns are cyclic, the *R* statistical language was used to perform nonlinear best cosine curve fitting on combined RNA relative expression data (see Supplemental Experimental Procedures). Relative expression amounts were calculated with the REST relative expression method [55] with *Rp49* as an internal reference gene. Pheromone expression levels were determined as previously described [39]. To determine whether pheromone levels differed at a given time interval, we

performed two-tailed Student's t tests. ANOVA followed by the post-hoc Tukey-Kramer test was used to assess significance in the mating behavior.

### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/18/1373/DC1/.

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# Supplemental Data

# Social Experience Modifies Pheromone Expression and Mating Behavior in Male *Drosophila melanogaster*

**Joshua J. Krupp, Clement Kent, Jean-Christophe Billeter, Reza Azanchi, Anthony K.-C. So, Christophe Lucas, Benjamin P. Smith, Julia A. Schonfeld, and Joel D. Levine**

## Supplemental Experimental Procedures

### Hematoxylin and Eosin Staining, and Immunohistochemistry

Filleted cuticle preparations were fixed for 20 min with 4% [v/v] formaldehyde/$NaPO_4$, and washed with PBS while pinned flat to a Sylgard (Dow Corning) dissection plate. Cuticle fillets were stained with Eosin-Hematoxylin solution according to Ehrlich (Fluka), and developed using tap water.

Immunohistochemistry was performed according to standard techniques. Cuticle preparations were washed with PBS/0.5% [v/v] Triton X-100 (PBST), and blocked with PBST/4% [w/v] BSA. Antibodies were used at the following dilutions: α-βGal (1:40; 40-1a, DSHB), and goat α-mouse Cy5 conjugate (1:200; Jackson ImmunoResearch Laboratories, Inc.).

### Tissue Preparation and Fixation for SEM and Thin Sections

Filleted abdomen preparations were fixed for 3 hr at room temperature in 0.5% [v/v] glutaraldehyde/4% [v/v] paraformaldehyde in 0.1 M phosphate buffer pH 7.2, washed with 0.1 M phosphate buffer pH 7.2, and dehydrated through a graded ethanol series. Specimens were treated with 1% osmium tetraoxide in 0.1M phosphate buffer pH 7.2, dried with carbon dioxide in a Tousimis Autosamdri-810 critical point dryer, and mounted flat on double-sided carbon adhesives adhered to aluminum stubs. To enhance image quality samples were gold coated using a Polaron SC7640 sputter coater. Specimens

were viewed using a Hitachi S-570 scanning electron microscope. Specimens for thin sections were fixed as described above, after which the samples were embedded in resin and sectioned using an ultramicrotome. Sections were stained with toluidine blue.

### Oenocyte Dissection

Adult male abdomens were filleted from the ventral midline and oenocytes were dissected from the dorsal abdominal segments 2-5 while maintained in Shields and Sang M3 insect medium (Sigma). A fine tungsten needle was used to manually remove the oenocytes. Dissected oenocytes were immediately placed into the appropriate cell lysis buffer for either RNA or protein extraction.

### Quantitative PCR Analysis

The following gene specific primer sets were used in quantitative PCR reactions: *Rp49* (F1) 5'-atcggttacggatcgaacaa-3', and *Rp49* (R1) 5'-gacaatctccttgcgcttct-3'; *timeless* (F11) 5'-cctatgtggtcaacccgaat-3' and *timeless* (R11) 5'-tacatcacgtccacggagaa-3'; *period* (F12) 5'-ggttgctacgtccttctgga-3', and *period* (R12) 5'-tgtgcctcctccgatatctt-3'; *Clock* (F3) 5'-ggataagtccacggtcctga-3', and *Clock* (R3) 5'-ctccagcatgaggtgagt gt-3'; *desat1* (F2) 5'-ctacggagcctacctcatgg-3', and *desat1* (R2) 5'-gccacttggcc ttgtagga-3'.

### Antibody Production and Western Analysis

For the generation of protein-specific antibodies, DNA sequences encoding a unique region of DESAT1 (33-aa) was amplified and cloned into the pGEX-3X expression vector (GE Healthcare). Oligonucleotides used for PCR amplification of recombinant protein coding sequences are as follows: *desat1*f1 5'-tggatccacatgccgccc-'3 and *desat1*r1 5'agaattcctacagcttggtgg-3'. Introduced restriction sites (underlined) in the primer sequence facilitated cloning. Purified recombinant GST-DESAT1 was used to immunize rabbits. The following antibody dilutions were used for Western analysis: 1:2000 dilution of rabbit α-DESAT1, and 1:10,000 of horseradish peroxidase-conjugated goat anti-rabbit

IgG (Jackson Immunological). Western blots were developed using the ECL Plus Western Blotting Detection kit and imaged using the Storm 840 Imaging System (GE Healthcare). Densitometry of the immunoreactive 43 kDa DESAT1 was performed using the ImageQuant software package (GE Healthcare), and normalized to the Ponceau staining of the blot.

**Gas Chromatography**

Gas chromatographic analysis was carried out as previously described [2]. A 0.5 µl sample of each hexane extract was injected on a Varian CP3800 gas chromatograph with a flame ionization detector and PTV injector (cool-on-column mode), fitted with DB-1 20 m x 0.18 mm Agilent 100-2000 fused silica capillary column connected to a 5 m x 0.25 mm deactivated silica retention gap (Agilent Technologies, Mississauga, Ontario, Canada). Carrier gas was Helium at a flow rate of 1 ml/min. Analysis of the extract was carried out with a column temperature profile that began at 50˚C (held for 1 min) and was ramped at 36.6˚C/min to 150˚C and then at 5˚C/min to 280˚C, where it was held for 8 min. The injector oven was programmed at 50˚C for 0.1 min and then ramped to 280˚C at 200˚C/min. Varian Star Integrator software was used to calculate the retention time and total area of each peak for subsequent analysis.

Compound identification was conducted on a Shimadzu GC-17A gas chromatograph fitted with a HP-5MS fused silica capillary column (0.25 mm x 30 m, 0.25 µm film thickness) linked to a mass analyzer (Shimadzu QP5050A mass spectrometer). The injector was used in splitless mode with a splitless time of 0.5 min, and the carrier gas was helium at 1 ml/min. Injector temperature was held constant at 280˚C. An oven program that began at 60˚C (1 min) and was ramped at 6˚C/min to 225˚C and then 3˚C/min to 310˚C (10 min) and a pressure program of 57 kPa (1 min) to 185 kPa (1.83 min) at 2 kPa/min were employed. Electron impact positive ions at 70 eV were recorded in the scanning mode (mass range scanned 45–550 amu). The mass spectra were

interpreted by fragmentation analysis and comparison to published criteria. Retention indices, based on a series of n-alkane standards (C10–C32; extrapolation to C36), were used to match GC-FID and GCMS data and to obtain approximate comparisons to published data.

### Statistical Analyses

To perform non-linear best cosine curve fitting on combined RNA relative expression data the following equation was used: (n) $y_{i,j} = a + b \cdot \cos(2\pi(x_i - h)/\tau)$ - where $y_{i,j}$ is the average normalized RNA value at time $x_i$ from experiment j. Parameters *a, b, h* were fitted using non-linear least-squares regression using the *nls* program from the *R* language [3, 4]. Regression fits were checked using different starting estimates; in all cases fitted parameter values and statistics were identical to 5 significant digits independent of starting estimates. Fitted parameter values and associated *t* statistic and *p* values are as reported by *nls*. 95% confidence intervals for the 3 parameters *a,b,h* were found using the *confint.nls* function based on profile likelihoods [5]. The parameter *b* is the amplitude of the cycle; significance of cycling is tested against the null hypothesis $b = 0$ using a t-test, which is justified since the equation is linear in *b*. The parameter $\tau$ is the day or cycle length. We used the fixed day length of 24 hours since fitting with lengths longer or shorter than this produced lower $r^2$ values.

The equation is non-linear in *h*, the phase. To test whether phase differs in two treatments, we tested the null hypothesis that a single *h* could fit both sets of data against the alternative $h_1 \neq h_2$. We calculated $p_i(h)$ = prob (phase for dataset i is *h*) using F=(residual mean squares for the best fit of *a,b* at $h = h_i$)/(residual mean squares fit at $h = h_i$). The probability is 1 at $h=h_i$ and diminishes with distance from $h_i$. The joint probability of the pair $p(h)=prob(p_1(h), p_2(h))$ was calculated using Fisher's $\chi^2$ method of combining tests [6]. We report the maximum value $p_{max}$ of the $p(h)$ over all *h*. A $p_{max}$ value greater than 0.05 indicates there is a single *h* which fits both data sets; smaller

values indicate we may reject the hypothesis of a single *h* and conclude the phase differs between data sets.

We tested whether phase relationships between genes were consistent by calculating the phase difference (lag) for a pair of genes within one treatment and the significance of the lag. When all lags were of similar magnitude over several treatments, we report the mean value of the lag and the joint significance (using Fisher's $\chi^2$). As equation (n) is linear in the amplitude estimate *b*, the amplitude of two treatments can be compared using Student's t-test.

For tests of relative expression levels between treatments, we present relative expression levels of the gene of interest to the calibrating gene *Rp49.* Relative expression values were not normalized to a peak expression of 1. This allowed direct comparison of relative expression levels between treatment such as LD and DD or control vs. social. Our *a priori* hypothesis, based on prior literature, was that expression of clock genes should be lower in DD than in LD, so we tested for differences using one-sided Student t tests at each time point, and combined the resulting *p* values using Fisher's $\chi^2$. Thus, reported *p* values test the hypothesis that relative expression in the first treatment was higher than in the second treatment, over all hours tested.

## Supplemental References

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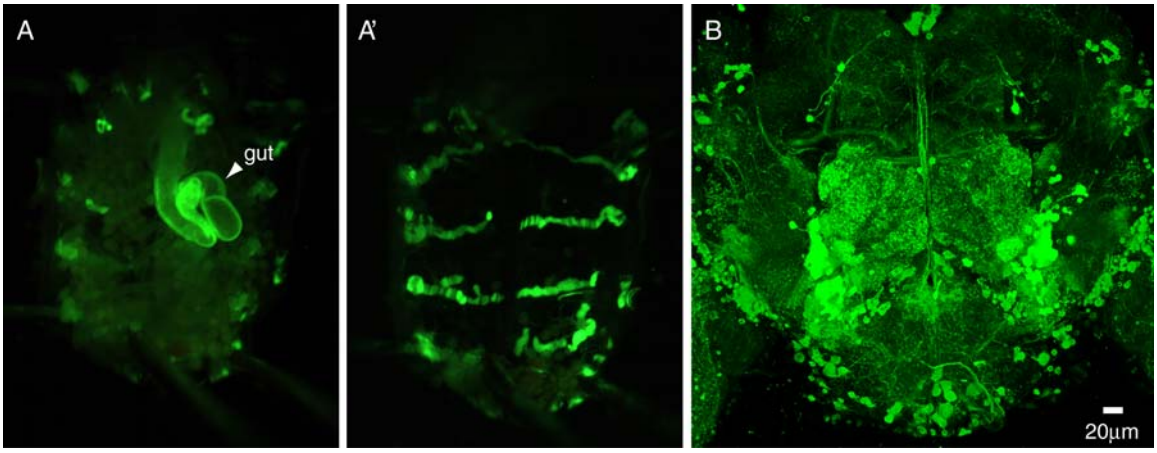


**Figure S1. Expression Pattern of the Gal4 Driver, 1407-Gal4.**

1407-Gal4 driving expression of UAS-mCD8::GFP in a 36hr old adult *D. melanogaster* male. (A-B) 1407-Gal4 drives expression within tissues of the male abdomen (A and A') and brain (B). (A) Before removing the abdominal organs, expression is found in the gut as well as in the remnants of the larval fat body. (A') After removing the gut and fat body, expression is seen in the underlying oenocytes.

(B) 1407-Gal4 is broadly expressed throughout the male brain. Other available oenocyte-Gal4 lines display similar broad, albeit partially non-overlapping, expression patterns in the nervous system and other organs [1]. Because these broad expression patterns are evident in the brain as well as other tissues with peripheral clocks, the available oenocyte drivers are not adequate to discriminate oenocyte-specific effects on behavior in this study.

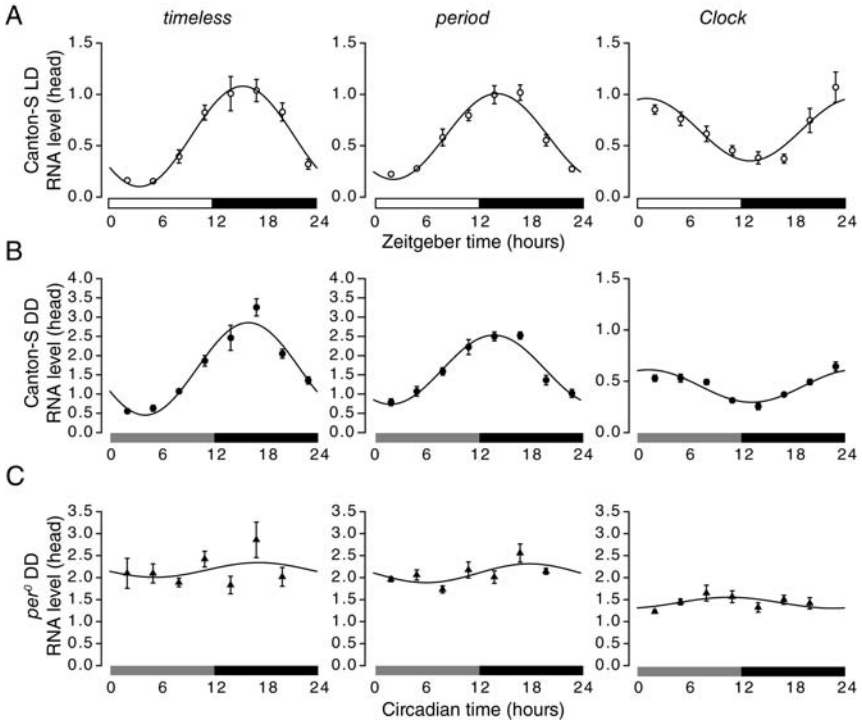


**Figure S2. Clock Gene Expression is Cyclic in Heads with an Advanced Phase Relationship Relative to that of the Oenocytes.**

(A-C) The temporal patterns of *tim*, *per*, and *Clk* RNA expression in wild type and $per^0$ heads as determined by quantitative RT-PCR. Genotypes and lighting conditions as indicated. Total RNA was isolated from the heads of the same animals used to isolate oenocyte RNA (see Figure 2). (A and B) In wild type heads isolated from flies under LD and DD conditions, the temporal patterns of expression are significantly sinusoidal for *tim*, *per*, *Clk*. (C) The temporal expression patterns of these genes are disrupted in $per^0$ heads isolated from flies under DD conditions. The phase relationships between the clock genes in heads in DD and LD conditions are stable, however the amplitude of expression is greater for *tim* and *per* in DD as compared to LD. Note that the phases of *tim*, *per* and *Clk* expression are slightly delayed in oenocytes relative to heads in both LD and DD (see Figure 2). Best-fit cosine curves (solid lines) are fitted to RNA expression values ±s.e.m. (see Statistical Methods). Each time point represents the average of 3 replicate qPCR reactions from 3 independent experiments ($n$ = 9). wt, wild type; wt LD, open circles; wt DD, filled circles; $per^0$ DD, filled triangles.

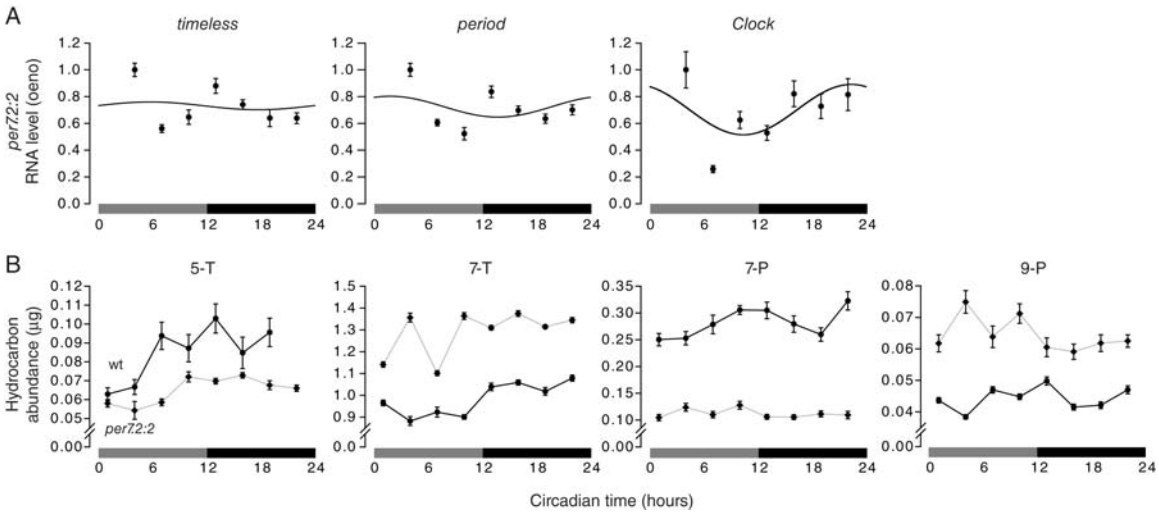


**Figure S3. A Peripheral Clock Mechanism is Required for Gene Expression and Cuticular Hydrocarbon Accumulation.**

(A) The temporal pattern of clock gene expression in *per7.2:2* oenocytes as determined by quantitative RT-PCR. The expression profiles of *tim, per,* and *Clk* are disrupted in *per7.2:2* oenocytes isolated from flies maintained in DD (compare to Canton-S DD Figure 2B). Best-fit cosine curves (solid lines) are fitted to RNA expression values (filled circles) ±s.e.m. Relative expression values for each time point were determined from three replicate quantitative PCR reactions representing one experiment.

(B) Comparison of the temporal profiles of 5-T, 7-T, 7-P, 9-P between wild type control (black circles with black line) and *per7.2:2* (black diamonds with gray line) males in DD. Wild type control and *per7.2:2* males displayed significantly different amounts of 5-T, 7-T, 7-P, and 9-P at all time points ($p < 0.05$). Independent of level differences, no significant correlation was evident between the genotypes in the temporal patterns of these compounds ($p > 0.2$). $n = 15$ flies for each time point; error bars indicate ±s.e.m.

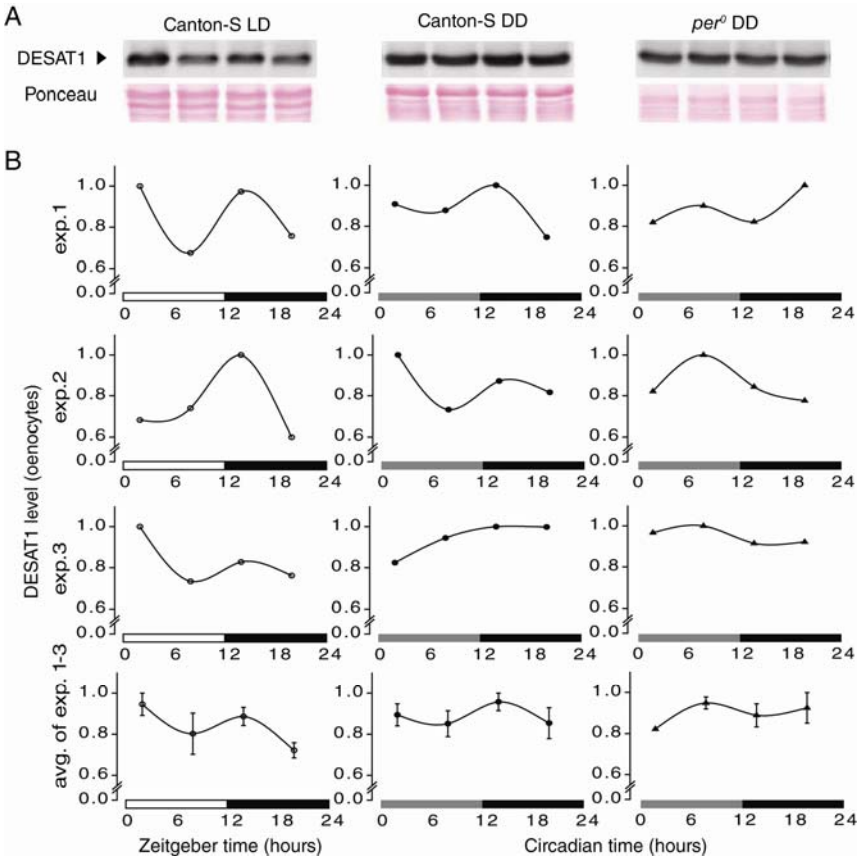


**Figure S4. Temporal pattern of DESAT1 expression in the oenocytes.**

(A) Temporal pattern of DESAT1 expression in wild-type (DD and LD) and *per*$^{0}$ (DD) oenocytes as determined by Western analysis. Blots from one representative experiment are shown (exp.1; see below)

(B) Relative quantification of the level of DESAT1 expression as normalized to the Ponceau stained blot and individually calibrated to the time of peak expression (i.e. peak expression = 1.00). Each time point represents the oenocytes of *n* = 24 flies. The quantification from three independent experiments is shown (exp.1-3), as well as the average across the three experiments. The expression of DESAT1 fluctuates in wild-type oenocytes under LD and DD conditions. In LD and DD DESAT1 expression appears to be bimodal with peaks occurring around the day to night and the night to day transitions. These fluctuations appear to be disrupted in *per*$^{0}$ oenocytes, with small peaks occurring at opposite times from that observed in wild type oenocytes. wt, wild-type; wt DD, filled circles; *per*$^{0}$ DD, filled triangles; wt LD, open circles.

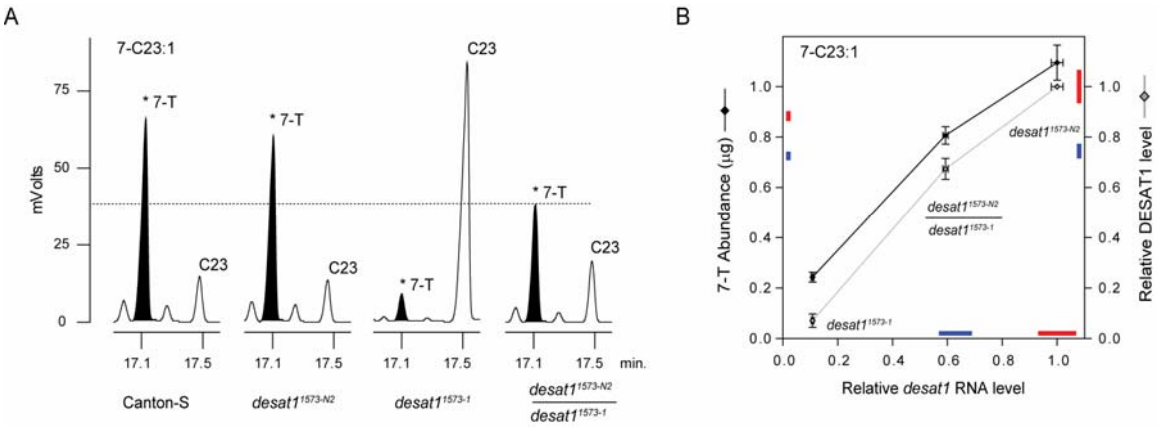


**Figure S5. 7-Tricosene Accumulation is Regulated by the Expression Level of *desat1*.**

(A) Representative gas chromatographs displaying the amounts of 7-T (asterisks) and C23. Genotypes as indicated. The area under each peak corresponds to amount of each compound. The output of the Flame Ionization Detector (FID) is shown displaying the height of each peak (mVolts) and the corresponding retention time (min.).

(B) Standard curve relating the level of *desat1* RNA to the level of 7-T (black line) and Desat1 (gray line) protein. High and low values observed for *desat1* RNA (see Figure 5A; wt LD), 7-T (see Figure 3C; wt LD), and Desat1 protein (see Figure S4; wt LD avg. of exp.1-3 recalibrated such that the peak expression level is equal to 1.0) are indicated by red and blue bars, respectively, positioned next to the indicated axis (bar length corresponds to ±s.e.m.).

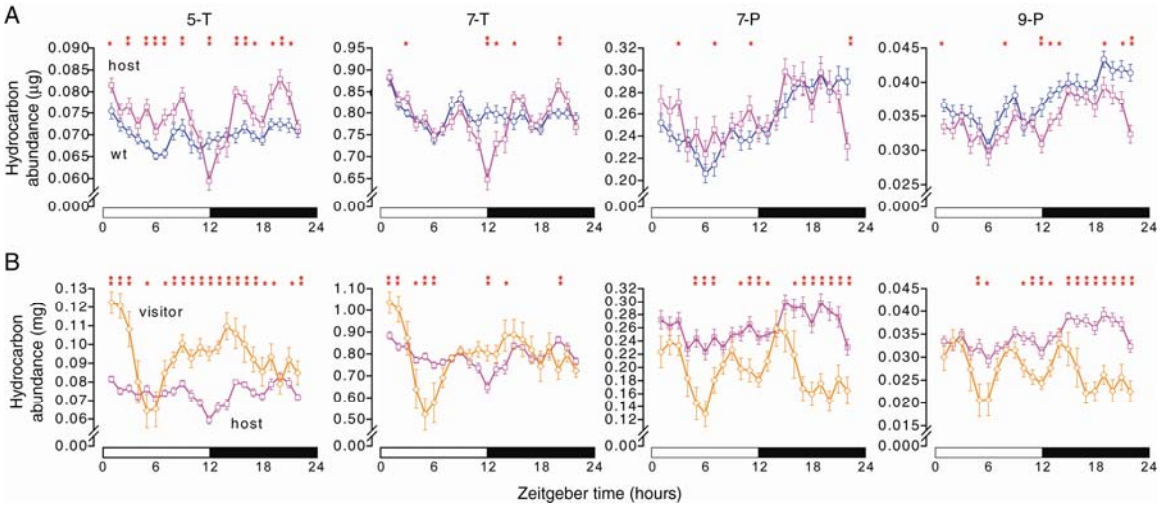


**Figure S6. The Effects of Social Interactions on Cuticular Hydrocarbon Profiles are Reduced in LD.**

(A) Comparison of the temporal profiles of 5-T, 7-T, 7-P, 9-P between wild type control and host males in LD. The temporal patterns of cuticular hydrocarbon (CH) accumulation are strongly correlated between wild type controls and hosts ($p < 0.04$). Although differences in 5-T, 7-T, 7-P, 9-P persist in LD, the differences are weaker than those found in DD (see Figure 5C), and occur predominately at LD transitions.

(B) Comparison of the temporal profiles of 5-T, 7-T, 7-P, 9-P between $per^0$ visitor and wild type host males in LD. The temporal patterns of CH accumulation are not correlated between hosts and visitors in LD, and the level of expression significantly differ at most times of the day. This differs from the comparison of hosts and visitors maintained in constant darkness (see Figure 5D). Shown are values calculated from a 3-hour moving average ±s.e.m. ($n$ = 27 to 45). Asterisks (red) designate points at which significant differences exist (**, $p < 0.01$; *, $p < 0.05$). wt, wild type; wt LD, open blue circles; host LD, open magenta squares; visitor LD, open orange diamonds.

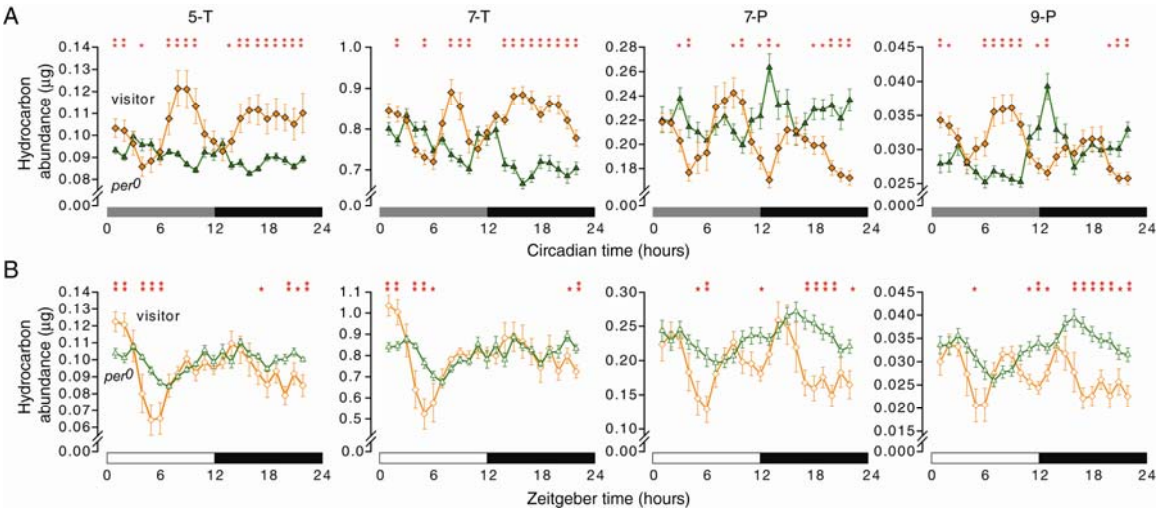


**Figure S7. Visitors are Affected by Social Context.**

(A and B) Comparison of the temporal profiles of 5-T, 7-T, 7-P, 9-P between *per*$^{0}$ control and visitor males in DD and LD. Shown are values calculated from a 3-hour moving average ±s.e.m (*n* = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two sided Student's t-test (**, $p < 0.01$; *, $p < 0.05$). *per*$^{0}$, filled triangles; visitor, orange diamonds.

**Table S1. Fitted Cosine Curves for Normalized Relative RNA Expression Levels from Oenocytes.**

| treatment | gene | *h* | *b* | S.E.(*b*) | *p*(*b*≠0) | *t*(*b*) | Pearson *r* | *p*(*r*≠0) | *t*(*r*) | df |
|---|---|---|---|---|---|---|---|---|---|---|
| wt LD | *per* | 16.02 | 1.28 | 0.109 | **6.4E-18** | 11.74 | 0.820 | **0.0E+00** | 11.83 | 67 |
| wt DD | *per* | 17.36 | 0.55 | 0.075 | **5.5E-10** | 7.34 | 0.682 | **3.9E-10** | 7.40 | 62 |
| $per^0$ DD | *per* | 21.64 | 0.04 | 0.086 | 6.2E-01 | 0.50 | 0.071 | 6.1E-01 | 0.51 | 49 |
| wt LD | *tim* | 17.33 | 0.86 | 0.084 | **2.5E-15** | 10.32 | 0.788 | **1.6E-15** | 10.40 | 65 |
| wt DD | *tim* | 19.02 | 1.12 | 0.069 | **1.9E-24** | 16.16 | 0.895 | **0.0E+00** | 16.28 | 65 |
| $per^0$ DD | *tim* | 21.77 | 0.09 | 0.079 | 2.5E-01 | 1.16 | 0.141 | 2.5E-01 | 1.17 | 66 |
| wt LD | *Clk* | 4.39 | 1.22 | 0.140 | **1.3E-12** | 8.70 | 0.728 | **9.1E-13** | 8.76 | 67 |
| wt DD | *Clk* | 5.66 | 0.74 | 0.062 | **8.2E-18** | 11.80 | 0.826 | **0.0E+00** | 11.89 | 65 |
| $per^0$ DD | *Clk* | 10.03 | 0.31 | 0.130 | 2.2E-02 | 2.37 | 0.330 | 2.1E-02 | 2.40 | 46 |
| wt LD | *desat1* | 8.26 | 0.26 | 0.039 | **5.7E-09** | 6.66 | 0.628 | **4.4E-09** | 6.71 | 68 |
| wt DD | *desat1* | 10.91 | 0.17 | 0.039 | **1.0E-04** | 4.26 | 0.536 | **8.5E-05** | 4.31 | 45 |
| $per^0$ DD | *desat1* | 14.12 | 0.09 | 0.077 | 2.7E-01 | 1.11 | 0.135 | 2.7E-01 | 1.12 | 67 |

Shown are the values for the fitted cosine curve parameters *h* and *b* for each of the indicated genes and treatments. The data corresponds to the quantitative RT-PCR data presented in Figure 2, where relative RNA expression levels for each time point represent the mean value of triplicate quantifications performed separately for each of three independent experiments (i.e. extractions), and for each of the treatments wt LD, wt DD, and $per^0$ DD ($n$ = 9 for each treatment). In all cases significant cycling was found, except for flies with the arrhythmic $per^0$ mutation, where no significant cycling was found for any gene. *h* = hour of peak expression, *b* = amplitude of curve, S.E.(*b*) = standard error of *b*, *p*(*b*>0) = probability that *b* exceeds 0 by Student's t-test, *t*(*b*) = *t* value for *b*, *r* = Pearson's correlation coefficient, *p*(*r*≠0) = probability *r* not equal 0, *t*(r) = *t* value for *r*. Note that some *p*(*r*≠0) values are reported as 0 due to round off in the statistical program, in such cases $p < 1E\text{-}16$. Red notes *p* values < 0.001; wt, wild type.

**Table S2. Phase Differences Between Clock Genes in Oenocytes.**

| treatment 1 | 1st gene | 2nd gene | $p(h_1=h_2)$ | Chisq (df=2) | Lag (hours) | *p* (group) | Average lag |
|---|---|---|---|---|---|---|---|
| wt LD | *per* | *tim* | 3.5E-01 | 2.09 | 1.31 | 6.4E-01 | 1.73 |
| wt LD | *tim* | *Clk* | **6.6E-11** | 46.88 | 11.06 | **5.7E-19** | 10.90 |
| wt LD | *Clk* | *desat1* | **1.0E-02** | 9.12 | 3.87 | 2.3E-02 | 3.92 |
| wt DD | *per* | *tim* | 5.2E-01 | 1.32 | 1.66 | | |
| wt DD | *tim* | *Clk* | **2.0E-05** | 21.61 | 10.64 | | |
| wt DD | *Clk* | *desat1* | 1.0E-01 | 4.53 | 5.25 | | |
| wt DD2 | *per* | *tim* | 6.4E-01 | 0.88 | 2.21 | | |
| wt DD2 | *tim* | *Clk* | **3.4E-07** | 29.81 | 11.01 | | |
| wt DD2 | *Clk* | *desat1* | 5.9E-01 | 1.06 | 2.65 | | |

Shown are the phase differences in the time of peak expression between *per* and *tim, tim* and *Clk*, and *Clk* and *desat1* in oenocytes. The data corresponds to the quantitative RT-PCR data presented in Figure 2, Figure 4A, and Figure 5A, where relative RNA expression levels for each time point represent the mean value of triplicate quantifications performed separately for each of three independent experiments (i.e. extractions), and for each of the treatments wt LD, wt DD, and wt DD2 ($n$ = 9 for each treatment). The *Clk-desat1* difference averaged 3.9 hours, $p < 0.023$. Note that average phase of oenocyte clock genes shifted by several hours from LD to DD2, as expected due to cycle lengthening, but the relationship between *desat1* and other clock genes remained constant. We conclude that *desat1* expression in oenocytes is synchronized to the endogenous clock, and lags *Clk* by approximately 4 hours. Probability $h_1 = h_2$ calculated from Fisher's $\chi^2$ as described. Lag is $h_1 - h_2$ in hours. $p$ (group) is joint Fisher's $p$ over 3 treatments. Red denotes $p$ values < 0.001; wt, wild type; DD, first-day DD; DD2 second-day DD.

**Table S3. RNA Expression Level Differences Between LD and DD for Wild Type Oenocytes, and Between Wild Type and *per*$^0$ Oenocytes in DD.**

| treatment 1 | treatment 2 | Gene | Chisq | df | $p(L_1>L_2)$ |
|---|---|---|---|---|---|
| wt LD | wt DD | *Clk* | 70.10 | 16 | **9.57E-09** |
| wt LD | wt DD | *desat1* | 74.47 | 16 | **1.63E-09** |
| wt LD | wt DD | *per* | 67.08 | 16 | **3.22E-08** |
| wt LD | wt DD | *tim* | 51.73 | 16 | **1.21E-05** |
| wt DD | *per*$^0$ DD | *Clk* | 47.48 | 16 | **5.73E-05** |
| wt DD | *per*$^0$ DD | *desat1* | 13.64 | 16 | 6.26E-01 |
| wt DD | *per*$^0$ DD | *per* | 37.43 | 16 | **1.82E-03** |
| wt DD | *per*$^0$ DD | *tim* | 58.86 | 16 | **8.13E-07** |

Shown are the probability values that the RNA expression level of treatment 1 ($L_1$) is greater than that of treatment 2 ($L_2$). The values correspond to quantitative PCR data presented in Figure 2, where relative RNA expression levels for each time point represent the mean value of triplicate quantifications performed separately for each of three independent experiments (i.e. extractions), and for each of the treatments wt LD, wt DD, and *per*$^0$ DD ($n$ = 9 for each treatment). For each comparison, one-sided Student t-test probabilities were calculated at each time point; the joint probability was combined for all 8 time points using Fisher's $\chi^2$. Comparison between LD and DD expression levels in wild type flies show that LD levels were greater than DD levels for all 4 genes, $p$ < 1.0E-5. Comparisons between wild type and *per*$^0$ expression levels DD show that wild type levels were greater than *per*$^0$ levels for all 3 clock genes, $p$ < 2.0E-3. The *desat1* difference was not significant. Probability $L_1 > L_2$ calculated from Fisher's $\chi^2$ as described in Supplement Experimental Procedures. Red denotes $p$ values < 0.001; wt, wild type.

**Table S4. Fitted Cosine Curves for Wild Type Controls and Hosts in Oenocytes and Heads.**

| treatment | Gene | *h* | *b* | S.E.(*b*) | *p*(*b*≠0) | *t*(b) | Pearson *r* | *p*(*r*≠0) | t(*r*) | df |
|---|---|---|---|---|---|---|---|---|---|---|
| wt Oeno DD2 | *Per* | 19.86 | 0.51 | 0.102 | **3.6E-06** | 5.05 | 0.525 | **3.1E-06** | 5.09 | 67 |
| Host Oeno DD2 | *Per* | 20.57 | 0.73 | 0.072 | **3.2E-15** | 10.26 | 0.786 | **2.0E-15** | 10.34 | 65 |
| wt Head DD2 | *Per* | 12.00 | 0.45 | 0.071 | **2.2E-08** | 6.34 | 0.609 | **1.7E-08** | 6.38 | 68 |
| Host Head DD2 | *Per* | 12.72 | 0.39 | 0.033 | **5.8E-18** | 11.71 | 0.818 | **0.0E+00** | 11.80 | 68 |
| wt Oeno DD2 | *Tim* | 22.07 | 1.01 | 0.113 | **5.3E-13** | 8.92 | 0.737 | **3.5E-13** | 8.99 | 67 |
| Host Oeno DD2 | *Tim* | 22.93 | 0.80 | 0.081 | **1.0E-14** | 9.92 | 0.774 | **6.4E-15** | 10.00 | 66 |
| wt Head DD2 | *Tim* | 14.70 | 0.36 | 0.039 | **5.1E-14** | 9.42 | 0.750 | **3.3E-14** | 9.49 | 69 |
| Host Head DD2 | *Tim* | 14.83 | 0.41 | 0.047 | **7.8E-13** | 8.80 | 0.730 | **5.3E-13** | 8.86 | 68 |
| wt Oeno DD2 | *Clk* | 9.08 | 1.13 | 0.178 | **2.2E-08** | 6.35 | 0.613 | **1.7E-08** | 6.40 | 67 |
| Host Oeno DD2 | *Clk* | 7.31 | 0.64 | 0.058 | **9.4E-17** | 11.01 | 0.800 | **0.0E+00** | 11.09 | 68 |
| wt Head DD2 | *Clk* | 22.55 | 0.27 | 0.083 | 2.1E-03 | 3.20 | 0.364 | 2.0E-03 | 3.22 | 67 |
| Host Head DD2 | *Clk* | 2.42 | 0.31 | 0.050 | **3.3E-08** | 6.26 | 0.610 | **2.6E-08** | 6.31 | 66 |
| wt Oeno DD2 | *Desat1* | 11.73 | 0.20 | 0.036 | **2.2E-06** | 5.47 | 0.641 | **1.6E-06** | 5.54 | 43 |
| Host Oeno DD2 | *Desat1* | 10.66 | 0.18 | 0.037 | **1.2E-05** | 4.74 | 0.507 | **1.0E-05** | 4.78 | 65 |

Shown are the values for the fitted cosine curve parameters *b* and *h* for Host flies in second-day DD as well as their wild type controls. Cycling was significant ($p < 3.1 \times 10^{-6}$) in both heads and oenocytes for all clock genes in both social and non-social flies. No significant phase differences were found between Hosts and controls (see Table S5). However, the amplitudes of the cycles differed between Hosts and controls in oenocytes for *tim* ($p = 0.046$) and *Clk* ($p = 0.02$). Interestingly, social environment had no significant effect on amplitude for any single clock gene in heads ($p > 0.21$). Joint probabilities of social effects on amplitude for the 3 clock genes are highly significant in oenocytes ($p < 0.0002$) and marginally significant in heads ($p < 0.043$). *h* = hour of peak expression, *b* = amplitude of curve, S.E.(*b*) = standard error of *b*, *p*(*b*>0) = probability that *b* exceeds 0 by Student's t-test, *t*(*b*) = *t* value for *b*, *r* = Pearson's correlation coefficient, *p*(*r*≠0) = probability *r* not equal 0, *t*(*r*) = *t* value for *r*. Note that some *p*(*r*) values are reported as 0 due to round off in the programs used, in such cases $p < 1.0 \times 10^{-16}$. Red denotes *p* values < 0.001; wt, wild type.

**Table S5. Phase Differences Between Wild Type and Hosts in Oenocytes and Heads.**

| treatment 1 | treatment 2 | gene | *p*(*h1=h2*) | Chisq (df=2) | Lag (wt-Host) |
|---|---|---|---|---|---|
| wt Oeno DD2 | Host Oeno DD2 | *per* | 9.7E-01 | 0.05 | 0.71 |
| wt Oeno DD2 | Host Oeno DD2 | *tim* | 8.6E-01 | 0.29 | 0.86 |
| wt Oeno DD2 | Host Oeno DD2 | *Clk* | 8.0E-01 | 0.44 | -1.77 |
| wt Oeno DD2 | Host Oeno DD2 | *desat1* | 9.4E-01 | 0.12 | -1.07 |
| wt Head DD2 | Host Head DD2 | *per* | 9.8E-01 | 0.05 | 0.72 |
| wt Head DD2 | Host Head DD2 | *tim* | 1.0E+00 | 0.00 | 0.13 |
| wt Head DD2 | Host Head DD2 | *Clk* | 5.3E-01 | 1.26 | 3.87 |

Shown are the phase differences in the time of peak expression *per, tim*, *Clk* and *desat1* in oenocytes and heads. No phase differences were found. Probability $h_1 = h_2$ calculated from Fisher's $\chi^2$ as described. Lag is $h_1 - h_2$ in hours. *p* (group) is joint Fisher's *p* over 3 treatments. wt, wild type.

**Table S6. Expression Level Differences Between Wild Type and Hosts in Oenocytes and Heads.**

| treatment 1 | treatment 2 | Gene | Chisq | df | *p* |
|---|---|---|---|---|---|
| wt Oeno DD2 | Host Oeno DD2 | *Clk* | 44.61 | 16 | **1.59E-04** |
| wt Oeno DD2 | Host Oeno DD2 | *desat1* | 56.95 | 16 | **1.69E-06** |
| wt Oeno DD2 | Host Oeno DD2 | *per* | 29.63 | 16 | 2.00E-02 |
| wt Oeno DD2 | Host Oeno DD2 | *tim* | 48.29 | 16 | **4.28E-05** |
| wt Head DD2 | Host Head DD2 | *Clk* | 81.76 | 16 | **8.00E-11** |
| wt Head DD2 | Host Head DD2 | *per* | 50.22 | 16 | **2.11E-05** |
| wt Head DD2 | Host Head DD2 | *tim* | 23.89 | 16 | 9.19E-02 |

Shown are comparisons between wild type control and Host expression levels in the oenocytes and in heads. The wild type expression level was greater than the host level for all 4 genes in oenocytes ($p < 0.02$). In heads, the wild type *per* and *Clk* expression levels were greater than that observed in Hosts ($p < 3x10^{-5}$). *tim* was not significantly different. Probability $L_1 > L_2$ calculated from Fisher's $\chi^2$ as described. Red denotes *p* values < 0.001; wt, wild type.

**Table S7. Phase Differences in Clock Gene Expression Between Oenocytes and Heads.**

| treatment 1 | treatment 2 | gene | *p(h1=h2)* | Chisq (df=2) | Lag (Oeno-Head) | *p* (group) |
|---|---|---|---|---|---|---|
| wt Oeno DD2 | wt Head DD2 | *per* | 8.4E-02 | 4.96 | 7.86 | |
| wt Oeno DD2 | wt Head DD2 | *tim* | **7.4E-05** | 19.03 | 7.38 | **4.3E-06** |
| wt Oeno DD2 | wt Head DD2 | *Clk* | 1.6E-01 | 3.67 | 10.53 | |

Significant cycling for *period, timeless,* and *Clock* was detected in RNA from heads of wild type flies in the second-day of DD ($p = 1.9 \times 10^{-6}$; see Table S4). We therefore asked whether the cycles in oenocytes and heads were synchronized or differed in phase. Shown are the phase differences for *period, timeless*, and *Clock* between oenocytes and heads in the second day of DD. Oenocyte genes peaked 7.8 hours later than in heads; the joint significance of the difference is $p = 4.3 \times 10^{-6}$. We conclude that the clock cycle in oenocytes is not synchronized with the cycle in heads; this difference in phases between head and peripheral clocks is the largest reported in *Drosophila*. Probability $h_1 = h_2$ calculated from Fisher's $\chi^2$ (see Supplemental Experimental Procedures). Lag is $h_1 - h_2$ in hours. *p*(group) is joint Fisher's *p* over 3 genes. Red denotes *p* values < 0.001; wt, wild type.

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# When predator odour makes groups stronger: effects on behavioural and chemical adaptations in two termite species

CHRISTOPHE LUCAS,[1] LOU BROSSETTE,[1] LAURIANE LEFLOCH,[1] SIMON DUPONT,[1] JEAN-PHILIPPE CHRISTIDÈS[1] and ANNE-GENEVIÈVE BAGNÈRES[1,2] [1]Institut de Recherche sur la Biologie de l'Insecte (UMR7261) CNRS – University of Tours, Tours, France and [2]Centre d'Écologie Fonctionnelle et Évolutive, CNRS, Université de Montpellier, Université Paul-Valéry Montpellier 3, EPHE, IRD, Montpellier, France

**Abstract.** 1. Being able to detect a predator before any physical contact is crucial for individual survival. Predator presence can be detected thanks to several types of signal, such as chemical cues. Chemical signals are produced by predators for their protection against desiccation, for their communication, or possibly as a side-effect of their activity. In insects, chemical communication plays a key role in diverse biological processes, including prey-predator or plant-insect interactions, courtship behaviour, and kin or species recognition.

2. Cuticular hydrocarbons (CHCs) are specifically involved in recognition processes and social organisation (division of labour, caste ratios) in social insects. Here, the questions raised are whether termites can detect a predator with their cuticular compounds and whether the predator-produced compounds can influence their prey.

3. The responses of termites *Reticulitermes grassei* (Clément, 1978) and *Reticulitermes flavipes* (Kollar, 1837) to the presence of the cuticular compounds produced by a predator, the ant species *Lasius niger* (Linnaeus, 1758), were investigated. More specifically, the study quantified termite traits such as caste ratios, mortality rates, CHC profile homogeneity and aggressiveness of workers after 2 months' exposure to predator-produced compounds.

4. The results show that the predator odour did affect the aggressiveness of the native species *R. grassei* but not of the invasive *R. flavipes*. The caste ratios and the mortality rates were not affected for both species.

5. Differences between species are discussed around the native or invasive status of each species, along with the role played by chemical cues on behavioural and chemical adaptations.

**Key words.** Aggressive behaviour, competitive tests, cuticular compounds, *Reticulitermes flavipes*, *Reticulitermes grassei*, subterranean termite.

## Introduction

In insects, cuticular chemical signatures are mostly composed of long-chain hydrocarbons called cuticular hydrocarbons (CHCs). They help to reduce desiccation (Gibbs & Rajpurohit, 2010) and, more importantly, are a key to chemical communication (Blomquist & Bagnères, 2010). Indeed, they mediate prey–predator and plant–insect interactions, courtship behaviour, dominance behaviour, species recognition, chemical mimicry, and fertility cues (Blomquist *et al.*, 1987; Cobb & Jallon, 1990; Bagnères *et al.*, 1996; Monnin *et al.*, 1998; Lucas *et al.*, 2002; Krupp *et al.*, 2008; Meunier *et al.*, 2011). In social insects, CHCs have been integral to the evolution and

Correspondence: Christophe Lucas, Institut de Recherche sur la Biologie de l'Insecte (UMR7261), CNRS – University of Tours, Tours, France. E-mail: christophe.lucas@univ-tours.fr

maintenance of sociality because they are crucial to kin and nestmate recognition (Vander Meer *et al.*, 1998; Lenoir *et al.*, 1999; Soroker *et al.*, 2003; Lucas *et al.*, 2004, 2005; Martin & Drijfhout, 2009*;* Martin *et al.*, 2013). They can also convey social origin (Meunier *et al.*, 2011) or the daily tasks carried out by an individual (*G*reene & Gordon, 2003). Recognition processes involve matching a neural representation of the colony's template (called the 'gestalt') with the odours exhibited by a given individual (Blomquist & Bagnères, 2010). Depending on the match, the conspecific individuals may be accepted or rejected (excluded from the colony or even killed) (Vander Meer & Morel, 1998; Lucas *et al.*, 2005).

However, CHCs produced by some predator species are also a tag that could be used by prey to detect their predators (Relyea, 2003). Being able to detect predators is a crucial part of individual and group survival. Predators sometimes use detection processes to misdirect naive prey (Bagnères *et al.*, 1996; Lorenzi *et al.*, 2014). Certain predators' chemical signatures mimic that of their prey or are chemically insignificant, so that the chemical degree of matching between the two chemical signatures elicits no reaction in prey species (Uboni *et al.*, 2012). As a result, predators may enter colonies without triggering defensive behaviour.

However, prey species can also use chemical communication to protect themselves. They can build a defence against predators by detecting predator-specific chemical signatures and then warning conspecifics. Indeed, in social insects, alarm signals are the most commonly produced chemical signals after sex pheromones (Blum, 1985). Termites, ants, wasps, and bees have highly developed alarm signal systems, which are composed of multiple compounds emitted from various glands (Aquiloni & Tricarico, 2015; Bagnères & Hanus, 2015). These alarm systems can be used to simultaneously recruit nestmates to fend off nest attacks and elicit aggressive behaviours. However, not all social insects show aggressive alarm responses. Indeed, some species panic and flee, just like *Lasius alienus* (Regnier & Wilson, 1969). In contrast, *Pheidole dentata* initiates nest defence by recruiting major workers (worker caste specialised in defence), if ever a fire ant gets too close (Hölldobler & Wilson, 1990). These examples underscore the major importance of chemical signals in predator–prey dynamics and the role they play in detection, a crucial process. They also highlight, through the diversity of the behavioural responses observed, the necessity of assessing how predator signals are detected by prey and the necessity of characterising any ensuing responses.

In social insects, one adaptation to predation pressure is developmental flexibility in the caste system. It has been hypothesised that the distribution of individuals across castes varies to enhance colony fitness in accordance with variation in ecological factors such as predation or competition (Wilson, 1985). Passera *et al.* (1996) demonstrated for the first time caste ratios variations when colonies of the ant *Pheidole pallidula* were submitted to high competition pressure. After 3 weeks, the number of soldiers had increased in colonies 'exposed' to competitors, raising questions about the role played by the chemical cues. These questions are the objects of the present study.

The aim of our study is to examine the impact of the odour of a predator on the two main termite species found in Europe: *Reticulitermes grassei* and *Reticulitermes flavipes*. The termite *R. grassei* (Clément) can be found in France along the Atlantic coast where it is considered as a native species (Lefebvre *et al.*, 2016). *Reticulitermes flavipes*, by contrast, was introduced to France around the 18th century by boats coming from Louisiana. This species is currently found throughout Europe and is considered to be invasive (Perdereau *et al.*, 2013). Both of these subterranean termite species are xylophagous and play an essential role in pine forest ecosystems, thanks to their ability to recycle organic matter (Davies *et al.*, 2003). They live and forage in pieces of dead wood in which ants are also nesting. Ants are the greatest predators of termites worldwide, resulting in an intriguing predator–prey system (Bignell *et al.*, 2011). The ant *Lasius niger* is largely spread in France and particularly in anthropised area like *Reticulitermes* termites. The cuticular profiles of *L. niger* are stable in several European countries and they are known to deposit their odour on various substrates like their nest or their foraging area (Lenoir *et al.*, 2009). In our study, termite species were sampled from sympatric populations, where they could have been experiencing similar predation pressure from the same predators, making their comparison even more interesting. Because we were dealing with one native and one invasive species, we expected interspecific differences. In particular, we tested whether the presence of a predator odour affected caste ratios, mortality ratios, aggressiveness, and CHC profiles.

## Materials and methods

### *Colony collection and rearing conditions*

Studied colonies of *R. grassei* and *R. flavipes* were collected in forested habitat on Oléron Island, where both species live sympatrically (Brossette *et al.*, 2017). Colonies were collected at least 300 m from each other to avoid collecting fused colonies (for both species) (Perdereau *et al.*, 2010a). Distances were confirmed using GPS data (Twonav Aventura, CompeGPS). Three colonies of the ant *L. niger* were collected near Azay-le-Rideau (47.261829, 0.465720) (France). They were also reared under controlled conditions (26 °C, 60% RH) in plastic boxes until chemical extractions were performed (see later).

### *Experimental setup used to measure caste ratios, mortality rates and to conduct predator odour tests*

Termites were sampled from 15 field colonies for each species. Those colonies were kept in their original nesting material (e.g. wood, sand, soil) and brought to the laboratory under constant conditions (26 °C, 60% RH) and a light cycle based on La Rochelle (46.160862, −1.149139). Individuals were then transferred to a 100-mm-diameter plastic container (Star-pack, product no. 112218, Boissy-l'Aillerie, France) with several paper discs made of pure cellulose (90 mm diameter; Whatman 42, Maidstone, U.K.). These 'field' nests were moistened weekly. The paper discs were impregnated with

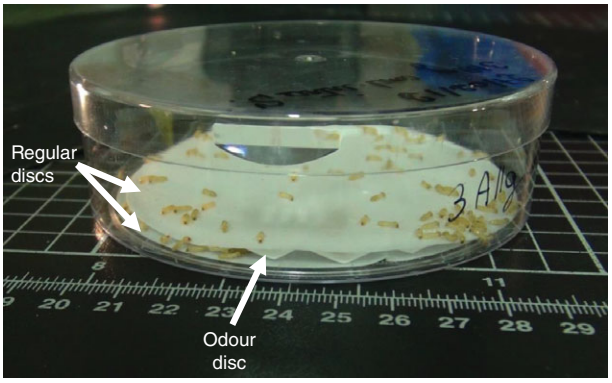


**Fig. 1.** Profile view of the experimental nest setup used for odour tests. [Colour figure can be viewed at wileyonlinelibrary.com].

a nutritive solution containing vitamins and inorganic salts (Argoud *et al.*, 1982). This nest design made it easy to quickly access individuals without harming the colony as we could have if the colonies had been set up in wood pieces. It also facilitates colony experimentations, surveys of mortality rates, surveys of caste ratios, and behavioural observations within controlled environmental conditions. Mortality and caste ratio surveys were performed by counting the number of individuals on weekly pictures of the colonies (after each picture, papers were placed back in the same order).

*Odour treatments*

Two sets of 350 workers per colony were collected from five field colonies (nests) of each species and sorted on a $CO_2$ pad. One of each paired set of 350 workers was used to establish 'predator odour treatment' group, while the other was used in the control 'solvent treatment' group. Thus, the total setup consisted of five 'predator odour treatment' groups and five control 'solvent treatment' groups for each species. In contrast with the field nests, the odour nests contained only three paper discs (Fig. 1; each paper disc represented a quantity of food that is sufficient for 350 workers for a period of months). The middle paper disc was the 'odour' disc. For the 'predator odour treatment', predator extracts were deposited on the 'odour' disc. For the 'solvent treatment', only the solvent used for extractions was deposited on the 'odour' disc. The odour disc was located in between the two other paper discs (Fig. 1). The odour disc was freshly prepared at the beginning of the experiment and was replaced once, after 1 month (Fig. 2a). The two other discs were changed weekly and were impregnated with the same nutritive solution used for the field nests.

The predator odour sample comprised a pool of three solvent extractions of 500 workers each, collected from three *L. niger* colonies. Collected individuals were sorted on a $CO_2$ pad then placed in a glass tube. Cuticular compounds were solvent-extracted for 1 min using 37.5 ml of heptane (Sigma) with three-dimensional agitation. The extraction process was repeated once. A total volume of 75 ml was obtained. Then, for the predator odour treatment, 50 workers equivalent (2.5 ml) was deposited on the predator odour discs using a 1-ml glass syringe (Hamilton). For the solvent treatment, the same volume (2.5 ml) of the same solvent was deposited on the solvent odour discs.

*Chemical extractions and analyses*

For each termite species, 20 workers were taken from the same five field nests on day 0 and then on day 56 (2 months) from each odour nest (predator odour and solvent treatments). The 20 workers were pooled and solvent-extracted for 1 min using 250 $\mu$l of heptane (Sigma) with three-dimensional agitation. The extraction process was repeated once more. The samples were evaporated under a nitrogen flow until dry evaporation was achieved and then stored at −20 °C. Before the analyses were performed, 10 $\mu$l of *n*-eicosane (*n*-C20; Sigma E-9752) at a concentration of $10^{-5}$ mg $\mu l^{-1}$ in heptane were added to each sample as an internal standard. Two microlitres of each sample were injected into a Perkin Elmer Auto System XL gas chromatograph coupled with a Perkin Elmer Turbomass mass spectrometer (Roissy-en-France, France) using an HTA model 110 automatic sampler (Alpha MOS, Toulouse, France). To confirm chemical identifications, samples were also injected into an Agilent 7890B gas chromatograph coupled with an Agilent 7000C mass spectrometer (Les Ulis, France) using a Gerstel MPS autosampler (Mülheim an der Ruhr, Germany). Injectors were used in splitless mode (splitless time of 2 min) at a constant temperature of 250 °C. The oven programme temperature started at 50 °C and was ramped at 5 °C $min^{-1}$ to 320 °C (held for 5 min). The carrier gas was helium at a flow rate of 1 ml $min^{-1}$. Electron impacts at 70 eV were recorded in scanning mode every 0.3 s with a scan lapse of 0.1 s and a mass scan range of 40–600 amu. Mass spectra were interpreted by fragmentation analyses and compared with previous publications (McLafferty & Tureček, 1993; Clément *et al.*, 2001). Retention indices were based on a series of *n*-alkane standards (Fluka, 94234). Comparisons with the chemical profile of *L. niger* were performed using information from a previous publication (Lenoir *et al.*, 2009). Data analyses were carried out using TURBOMASS software (v. 5.4.2, Perkin Elmer, Roissy-en-France, France) for the Perkin Elmer machine and MASSHUNTER software (v. B07, Agilent, Les Ulis, France) for the Agilent machine.

Cuticular compounds were analysed, and the relative areas of all the identified chromatogram peaks were calculated (Tables 1 and 2). To compare changes in chemical profiles over time and between treatments, we calculated a matrix of Nei's genetic distances (*D* index) (Nei, 1972). Traditionally, Nei's distance has been used to quantify differences in chemical composition among individuals or colonies (Lihoreau & Rivault, 2009; Perdereau *et al.*, 2010b; LeConte *et al.*, 2015). Nei's distance is an index of dissimilarity and ranges between 0 and 1, with low values indicating that cuticular profiles are more homogeneous. In this study, within each of the two treatments, all established groups were compared to make up indices of chemical profile homogeneity on day 56, which were then compared between the two treatments (10 pairwise comparisons per treatment and per species were analysed, built up from five field nests). They were calculated for each species after 2 months of treatment (predator odour and solvent treatments).

 

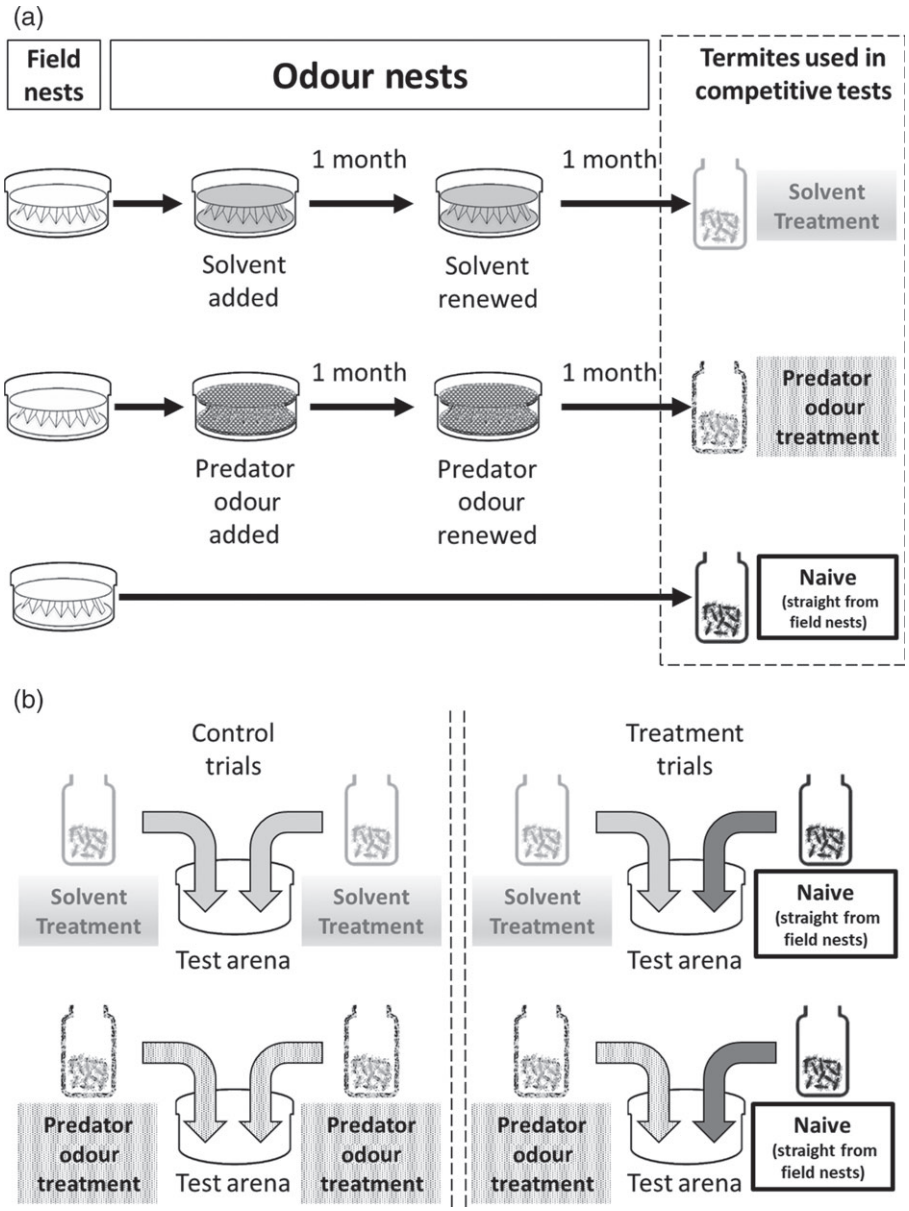


**Fig. 2.** (a) Experimental design of the chemical exposure experiments using solvent and predator odour to produce treated termites. For both treatments, five field nests were used (per species). Naive workers were extracted directly from five other field nests per species (used for both treatments) and were not submitted to any chemical treatments. Treated and naive workers were then used in competitive tests (b). Control trials involved only termites from the same treated box. In treatment trials encounters were between treated and naive termites.

*Competitive tests*

Competitive tests involved trials between individuals from three different groups per species (Fig. 2a): (i) individuals exposed to the predator odour for 2 months (predator odour treatment); (ii) individuals exposed to the solvent for 2 months (solvent treatment); and (iii) individuals taken directly from different field nests that had been kept in the same rearing room during the same time period but which had not experienced any treatments (hereafter referred as naive individuals). The tests were performed for each species with the five odour nests of each treatment (predator odour and solvent; both were prepared from the same five field nest as described earlier). Treated and naive termites were collected from different field nests but were associated by pairs. Treated individuals were sorted on a $CO_2$ pad and divided up equally into three subgroups according to the treatments. Naive individuals were divided into two subgroups only, with a total number of termites equal to the number of individuals they will face during the trials.

Two types of trials were conducted (Fig. 2b): (i) control trials, in which individuals from two identical subgroups encountered each other, meaning that they were nestmates that had

**Table 1.** Compound identification of the cuticular profiles of *Reticulitermes grassei*.

| | | | Field nests (day 0) | | Solvent (day 56) | | Predator odour (day 56) | |
|---|---|---|---|---|---|---|---|---|
| Peaks | Compounds | KI | Mean | SE | Mean | SE | Mean | SE |
| IS | *n*-C20 | 2013 | – | – | – | – | – | – |
| 1 | Unknown | 2195 | 0.06 | 0.01 | 0.09 | 0.03 | 0.10 | 0.02 |
| 2 | 9-C25:1 | 2471 | 0.06 | 0.01 | 0.07 | 0.02 | 0.06 | 0.01 |
| 3 | *n*-C25 | 2497 | 0.22 | 0.02 | 0.17 | 0.06 | 0.21 | 0.03 |
| 4 | 13-, 11-MeC25 | 2531 | 0.29 | 0.02 | 0.13 | 0.05 | 0.21 | 0.03 |
| 5 | 7-MeC25 | 2538 | 0.07 | 0.01 | 0.13 | 0.07 | 0.10 | 0.01 |
| 6 | 5-MeC25 | 2547 | 1.41 | 0.14 | 0.83 | 0.20 | 1.18 | 0.09 |
| 7 | Unknown | 2559 | 0.06 | 0.01 | 0.08 | 0.03 | 0.07 | 0.02 |
| 8 | Unknown | 2569 | 0.03 | 0.01 | 0.04 | 0.02 | 0.03 | 0.01 |
| 9 | Unknown | 2578 | 0.03 | 0.01 | 0.03 | 0.01 | 0.03 | 0.01 |
| 10 | 5,17-dimeC25 | 2583 | 0.06 | 0.03 | 0.04 | 0.01 | 0.06 | 0.01 |
| 11 | *n*-C26 | 2594 | 0.76 | 0.05 | 0.44 | 0.10 | 0.68 | 0.10 |
| 12 | 13-, 12-, 11-MeC26 | 2629 | 0.67 | 0.07 | 0.43 | 0.08 | 0.51 | 0.06 |
| 13 | 6-, 5-MeC26 | 2642 | 1.20 | 0.14 | 0.80 | 0.14 | 1.12 | 0.05 |
| 14 | 4-, 2-MeC26 | 2654 | 0.44 | 0.10 | 0.40 | 0.05 | 0.46 | 0.01 |
| 15 | 9-C27:1 | 2674 | 0.59 | 0.12 | 0.39 | 0.12 | 0.38 | 0.07 |
| 16 | *n*-C27 | 2701 | 21.26 | 1.27 | 19.51 | 1.32 | 22.27 | 1.87 |
| 17 | 13-, 11-MeC27 | 2733 | 14.84 | 0.66 | 11.32 | 2.07 | 12.45 | 0.23 |
| 18 | 7-MeC27 | 2740 | 1.76 | 0.08 | 2.24 | 0.60 | 2.53 | 0.26 |
| 19 | 5-MeC27 + 11,15-diMeC27 | 2752 | 19.36 | 0.81 | 18.39 | 1.80 | 20.04 | 1.60 |
| 20 | 3-MeC27 | 2770 | 0.85 | 0.12 | 1.45 | 0.16 | 1.26 | 0.15 |
| 21 | 5,17-diMeC27 | 2781 | 4.86 | 0.53 | 4.15 | 0.41 | 4.52 | 0.55 |
| 22 | *n*-C28 | 2796 | 3.15 | 0.17 | 3.64 | 0.30 | 4.01 | 0.15 |
| 23 | 14-, 13-, 12-MeC28 | 2827 | 0.78 | 0.05 | 0.68 | 0.08 | 0.93 | 0.13 |
| 24 | 6-, 5-MeC28 | 2841 | 1.32 | 0.08 | 1.31 | 0.12 | 1.63 | 0.16 |
| 25 | 4-MeC28 | 2854 | 0.35 | 0.04 | 0.34 | 0.05 | 0.41 | 0.03 |
| 26 | 2-MeC28 | 2859 | 0.37 | 0.04 | 0.83 | 0.12 | 0.74 | 0.05 |
| 27 | 9-C29:1 | 2873 | 0.45 | 0.08 | 2.00 | 0.59 | 1.09 | 0.26 |
| 28 | Unknown | 2886 | 0.03 | 0.01 | 0.02 | 0.01 | 0.02 | 0.01 |
| 29 | *n*-C29 | 2897 | 4.26 | 0.36 | 5.73 | 0.97 | 4.41 | 0.55 |
| 30 | Unknown | 2919 | 0.24 | 0.06 | 0.13 | 0.06 | 0.13 | 0.05 |
| 31 | 13-, 11-MeC29 | 2928 | 2.66 | 0.17 | 2.62 | 0.14 | 2.10 | 0.07 |
| 32 | 7-MeC29 | 2937 | 0.23 | 0.03 | 0.83 | 0.13 | 0.60 | 0.09 |
| 33 | 5-MeC29 | 2950 | 11.89 | 0.58 | 13.03 | 0.92 | 10.51 | 0.63 |
| 34 | 3-MeC29 | 2969 | 0.17 | 0.02 | 0.48 | 0.12 | 0.57 | 0.17 |
| 35 | 5,17-diMeC29 | 2977 | 1.16 | 0.11 | 1.79 | 0.36 | 1.31 | 0.10 |
| 36 | Unknown | 2998 | 0.08 | 0.02 | 0.25 | 0.09 | 0.22 | 0.04 |
| 37 | *n*-C30 | 3003 | 0.20 | 0.05 | 0.36 | 0.09 | 0.28 | 0.07 |
| 38 | Unknown | 3013 | 0.02 | 0.00 | 0.04 | 0.02 | 0.02 | 0.00 |
| 39 | 14-, 12-MeC30 | 3025 | 0.09 | 0.01 | 0.08 | 0.01 | 0.08 | 0.01 |
| 40 | 6-MeC30 | 3040 | 0.07 | 0.01 | 0.07 | 0.01 | 0.06 | 0.01 |
| 41 | 2-MeC30 | 3052 | 0.16 | 0.03 | 0.13 | 0.02 | 0.12 | 0.02 |
| 42 | Unknown | 3074 | 0.03 | 0.01 | 0.34 | 0.11 | 0.11 | 0.04 |
| 43 | 15-, 13-, 11-MeC31 | 3126 | 0.66 | 0.09 | 1.22 | 0.31 | 0.57 | 0.09 |
| 44 | 13,17-diMeC31 | 3150 | 1.45 | 0.14 | 1.53 | 0.22 | 1.17 | 0.08 |
| 45 | 5,17-diMeC31 | 3173 | 0.25 | 0.03 | 0.38 | 0.04 | 0.29 | 0.05 |
| 46 | 17-, 15-, 13-MeC33 | 3324 | 0.35 | 0.06 | 0.59 | 0.14 | 0.21 | 0.05 |
| 47 | 17-, 15-, 13-MeC35 | 3524 | 0.19 | 0.04 | 0.23 | 0.09 | 0.09 | 0.03 |
| 48 | 5,17-dimeC35 | 3572 | 0.14 | 0.03 | 0.08 | 0.02 | 0.03 | 0.01 |
| 49 | 5,17-diMeC37 | 3768 | 0.38 | 0.09 | 0.16 | 0.07 | 0.04 | 0.01 |

The relative areas are reported in proportions (mean ± SE) for the field nests on day 0, and for the two treatment groups (solvent and predator odour) on day 56. For each column (field nests, solvent, predator odour) the same five field nests were used. Peaks, peak numbers (IS, internal standard); compounds, identified compound with their names; KI, Kovats retention index (mean).

 

**Table 2.** Compounds identification of the cuticular profiles of *Reticulitermes flavipes*.

| | | | Field nests (day 0) | | Solvent (day 56) | | Predator odour (day 56) | |
|---|---|---|---|---|---|---|---|---|
| Peaks | Compounds | KI | Mean | SE | Mean | SE | Mean | SE |
| IS | *n*-C20 | 2016 | – | – | – | – | – | – |
| 1 | *n*-C22 | 2194 | 0.09 | 0.01 | 0.14 | 0.02 | 0.08 | 0.01 |
| 2 | 2-MeC22 | 2260 | 0.02 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| 3 | 9-C23:1 | 2271 | 0.55 | 0.09 | 0.62 | 0.20 | 0.25 | 0.07 |
| 4 | X-C23:1 | 2277 | 0.06 | 0.01 | 0.05 | 0.01 | 0.02 | 0.01 |
| 5 | X'-C23:1 | 2286 | 0.07 | 0.02 | 0.09 | 0.03 | 0.13 | 0.04 |
| 6 | *n*-C23 | 2301 | 6.81 | 0.44 | 9.40 | 1.04 | 8.78 | 0.91 |
| 7 | 11-, 7-MeC23 | 2334 | 0.91 | 0.07 | 1.17 | 0.18 | 0.83 | 0.13 |
| 8 | 2-MeC23 | 2360 | 1.47 | 0.07 | 1.48 | 0.15 | 1.24 | 0.08 |
| 9 | X-C24:1 + 3-MeC23 | 2370 | 0.69 | 0.07 | 0.63 | 0.06 | 0.41 | 0.05 |
| 10 | *n*-C24 | 2397 | 3.85 | 0.20 | 3.54 | 0.15 | 4.03 | 0.43 |
| 11 | 12-, 11-MeC24 | 2433 | 1.34 | 0.10 | 1.38 | 0.16 | 1.06 | 0.16 |
| 12 | 3-MeC24 | 2452 | 0.22 | 0.03 | 0.18 | 0.03 | 0.05 | 0.02 |
| 13 | 2-MeC24 | 2468 | 11.99 | 0.30 | 12.79 | 0.79 | 12.86 | 0.99 |
| 14 | 9-C25:1 + X,X'-C25:2 | 2482 | 15.86 | 1.19 | 12.67 | 0.92 | 9.43 | 1.19 |
| 15 | *n*-C25 | 2505 | 15.86 | 0.93 | 15.97 | 0.80 | 19.74 | 1.93 |
| 16 | 13-, 11-MeC25 | 2540 | 19.03 | 1.26 | 23.05 | 2.30 | 24.43 | 2.60 |
| 17 | 7,9-C25:2 + 5-MeC25 | 2547 | 4.42 | 0.33 | 3.83 | 0.89 | 2.79 | 0.36 |
| 18 | 2-MeC25 + X,X'-C25:2 | 2565 | 6.40 | 0.28 | 4.89 | 0.38 | 4.48 | 0.46 |
| 19 | 3-MeC25 | 2573 | 6.80 | 0.39 | 6.40 | 0.82 | 8.20 | 0.17 |
| 20 | Unknown | 2593 | 0.44 | 0.13 | 0.11 | 0.04 | 0.23 | 0.10 |
| 21 | *n*-C26 | 2606 | 0.95 | 0.23 | 0.47 | 0.10 | 0.41 | 0.20 |
| 22 | Unknown | 2624 | 0.11 | 0.02 | 0.05 | 0.01 | 0.01 | 0.00 |
| 23 | 12-, 11-MeC26 | 2629 | 0.31 | 0.03 | 0.29 | 0.04 | 0.21 | 0.05 |
| 24 | 2-MeC26 | 2661 | 0.37 | 0.06 | 0.25 | 0.04 | 0.15 | 0.05 |
| 25 | 11-MeC35 | 3526 | 0.16 | 0.03 | 0.13 | 0.01 | 0.06 | 0.03 |
| 26 | 11-MeC37 | 3721 | 0.33 | 0.10 | 0.14 | 0.02 | 0.04 | 0.03 |
| 27 | 11,15-diMeC37 | 3743 | 0.28 | 0.07 | 0.11 | 0.02 | 0.02 | 0.02 |
| 28 | 5,17-diMeC37 | 3768 | 0.27 | 0.06 | 0.11 | 0.01 | 0.03 | 0.02 |
| 29 | 11-MeC39 | 3917 | 0.11 | 0.05 | 0.02 | 0.01 | 0.00 | 0.00 |
| 30 | 11,15-diMeC39 | 3939 | 0.12 | 0.05 | 0.03 | 0.01 | 0.00 | 0.00 |
| 31 | 5,17-diMeC39 | 3965 | 0.12 | 0.05 | 0.02 | 0.01 | 0.00 | 0.00 |

The relative areas are reported in proportions (mean ± SE) for the field nests on day 0, and for the two treatment groups (solvent and predator odour) on day 56. For each column (field nests, solvent, predator odour) the same five field nests were used. Peaks, peak numbers (IS, internal standard); compounds, identified compound with their names; KI, Kovats retention index (mean).

experienced the same treatment (i.e. predator odour or solvent); and (ii) treatment trials, in which individuals that had experienced a treatment (i.e. predator odour or solvent) encountered naive individuals, meaning that they were non-nestmates that had or had not experienced a treatment. Individuals from each subgroup were placed in separate 25-ml glass containers positioned upside down on their own humidified paper disc (47 mm diameter; Whatman 42) in a 100-mm-diameter closed plastic box (Star-pack, 112218); there was no contact between the two paper discs of each subgroup. The boxes were kept at 26 °C and 60% RH for 24 h. Next, a glass divider was inserted into the box so that termites were kept separated as the glass containers were removed. One minute later, the glass divider was also removed, and the termites were able to freely interact for 24 h. After this time period, a mortality survey in a competitive test was conducted to evaluate aggressiveness (Clément, 1986).

Treated individuals were distinguished from naive ones using Nile blue A coloration (Aldrich, 121479) (Su *et al.*, 1991). Only naive workers were stained (a control experiment was done; see later). Nile blue is known to stain lipoid tissues and was successfully used to follow termites over a period of 11 weeks (Evans, 1997). The dye was diluted in acetone at 200 $\mu$g ml$^{-1}$ and stirred for 1 h with a magnetic bar. Paper discs of pure cellulose (90 mm diameter; Whatman 42) were impregnated with 20 ml of the solution. They were placed in the solution twice, for 5 min each time (the discs were flipped before the second soaking). The discs were then dried overnight at 40 °C to remove all traces of the solvent. Two of the dyed discs were then moistened and added to a plastic box (100 mm diameter; Star-pack, 112218) containing 700 naive workers during 7 days. Only coloured naive workers were used in the competitive tests.

A control experiment exanimating the potential effects of the Nile blue dye was performed using the same behavioural setup with 100 naive termites. Workers were taken from five field nests per species (these field nests were different from the one used in previous experiments) and dyed as described earlier. They were divided up equally into two subgroups with 50 termites each. The two subgroups from the same nest were then

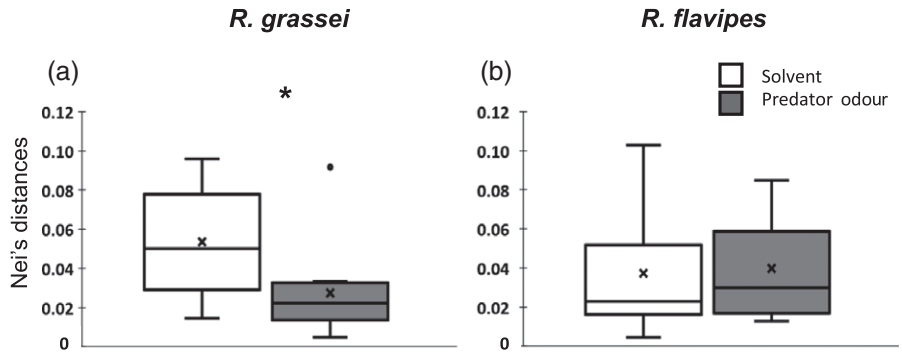


**Fig. 3.** Chemical heterogeneity indexes (Nei's distances) for *Reticulitermes grassei* (a) and *Reticulitermes flavipes* (b). They were calculated using the relative areas of the chemical compounds present on termite cuticles after 2 months of treatments (solvent and predator odour treatments) (*, $P < 0.05$). Ten pairwise comparisons per treatment and per species were analysed, built up from five field nests. Box plots show the median, upper and lower quartiles and range; dots, outliers; crosses, means.

allowed to engage in encounters with each other. No evidence of toxicity or enhanced aggressiveness was observed. Indeed, mortality did not differ from zero (one-sample signed-rank test) for both species (*R. grassei*, first quartile = 0.000, fourth quartile = 0.010, $W = 3$, $P > 0.05$; *R. flavipes*, first quartile = 0.000, fourth quartile = 0.085, $W = 6$, $P > 0.05$).

### *Statistical analyses*

The Nei's distances, mortality rates and the number of neotenic reproductives and soldiers were analysed with treatments and species as explanatory variables. The data were analysed using a non-parametric paired test (Wilcoxon) because the same colonies experienced different treatments and because of sample sizes. Comparison between species for the same treatment was done using a non-parametric non-paired test (Mann–Whitney). Data from the control experiments examining the effects of the Nile blue dye were analysed using a one-sample signed-rank test. All statistics were performed using SIGMASTAT (v. 12.5; Systat Software Inc., San Jose, California).

## Results

### *Chemical profiles*

The chromatograms revealed that the chemical profiles had 49 peaks for *R. grassei* (Table 1) and 31 peaks for *R. flavipes* (Table 2). The identified cuticular compounds of the two-termite species were enriched compared with previous publication (Clément *et al.*, 2001) and no differences were found for *L. niger* (Lenoir *et al.*, 2009). Although quantitative differences appeared over time for the predator odour and solvent groups, there were no qualitative differences (all peaks were present for all treatments for both species). The absence of qualitative differences between treatments (Tables 1 and 2) means that, in the predator odour treatment, cuticular compounds extracted from ants and deposited on the odour disc did not become part of the chemical profiles of the termites, even after 2 months' exposure.

Quantitative differences in chemical profiles were analysed using Nei's distances, which are based on the relative areas of all the peaks for both species and treatments. It was thus possible to quantify chemical profile homogeneity after 2 months (Fig. 3). For *R. grassei*, Nei's distances decreased significantly in the predator odour group compared with the solvent group ($W = -41$, $P = 0.037$; Fig. 3a), meaning that chemical profiles were more homogeneous in the predator odour group than in the solvent group. This was not true for *R. flavipes*, for which no treatment-related differences were observed ($W = -1$, $P > 0.05$; Fig. 3b). Analyses between species within treatments after 2 months (Fig. 3a,b) revealed no significant differences for the solvent treatment ($U = 34.0$, $P > 0.05$) and the predator odour treatment ($U = 22.5$, $P > 0.05$). We further compared the two species directly with other Nei's distances. For this dataset, distances between treatments were calculated for each pair of groups originating from the same field nests on day 56, for each species. The two species did not differ significantly ($U = 8.0$, $P > 0.05$; data not shown).

The shift over the 2-month period was calculated between day 0 and day 56 within treatments for each species, using Nei's distances. No shift overtime of the chemical profiles was found for any treatment for *R. grassei* (solvent group, $W = 33$, $P > 0.05$; predator odour group, $W = -1$, $P > 0.05$) and for *R. flavipes* (solvent group, $W = -15$, $P > 0.05$; predator odour group, $W = -9$, $P > 0.05$) (data not shown).

### *Caste ratios and mortality inside the odour nests*

We tested the effects of the two treatments over the 2-month period inside the odour nests. The treatments did not significantly affect the number of dead workers inside the odour nests in either species (all $P > 0.05$; Figure S1a,b). There was also no significant effect of the treatments on the production of neotenic reproductives (Figure S2) or soldiers (Figure S1c,d) in either species (all $P > 0.05$).

### *Competitive tests*

The control trials showed that the odour treatments did not affect mortality in either species [*R. grassei*, $W = -6$, $P > 0.05$ (Fig. 4a); *R.flavipes*, $W = -6$, $P > 0.05$ (Fig. 4b)].

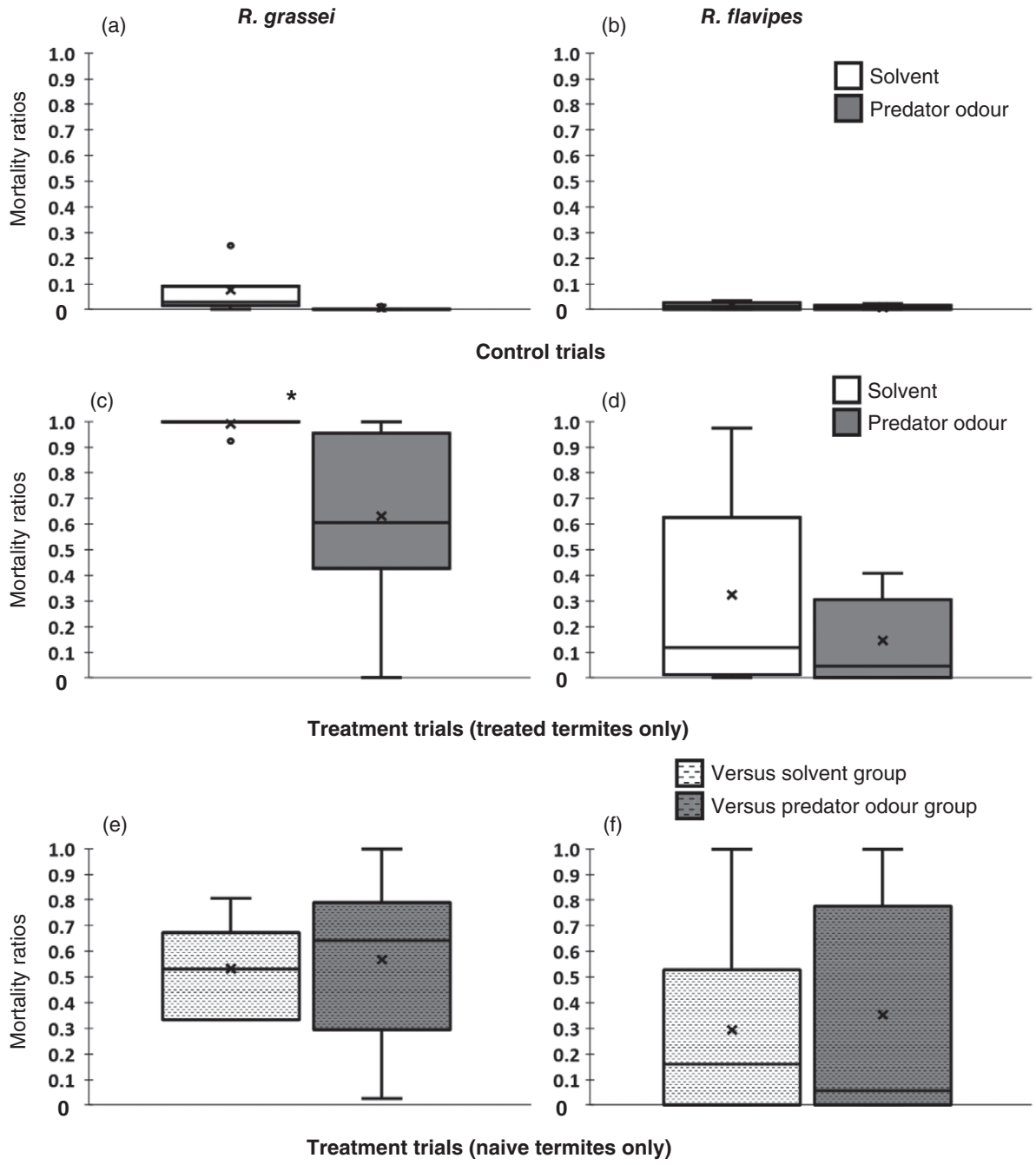


**Fig. 4.** Mortality ratios observed during competitive tests conducted with *Reticulitermes grassei* (a, c, e) and *Reticulitermes flavipes* (b, d, f) (*, $P < 0.05$; a value of 1 indicates that all the individuals died). Two types of trials were performed: control trials in which encounters occurred among termites originated from the same treated box (a, b) and treatment trials in which encounters occurred between treated and naive termites (c, d: mortality of treated termites only; e, f: mortality of naive termites only). For both treatments, five field nests were used (per species). Treated and naive termites were collected from different field nests but were associated by pairs. Box plots show the median, upper and lower quartiles and range; dots, outliers; crosses, means.

Analyses within species revealed that each species displayed different responses in the treatment trials. In *R. grassei*, mortality was lower in the predator odour group than in the solvent group ($W = -28$, $P = 0.016$; Fig. 4c). No such difference was observed in *R. flavipes* ($W = -10$, $P > 0.05$; Fig. 4d). In the case of the naive termites, which were tested against treated individuals, there was no treatment-related effect on mortality in either species [*R. grassei*, $W = 2$, $P > 0.05$ (Fig. 4e); *R. flavipes*, $W = 4$, $P > 0.05$ (Fig. 4f)].

Analyses between species within treatments (Fig. 4c,d) revealed significant differences for the solvent treatment ($U = 1.0, P < 0.001$) and the predator odour treatment ($U = 9.5$, $P = 0.007$).

## Discussion

Our study focused on how termites of two species – *R. grassei* and *R. flavipes* – responded when exposed to the odour of a predator. More specifically, we examined caste ratio, mortality rates, variation of the chemical profiles, and aggressiveness. We found that the two species responded differently to the treatments. First, in *R. grassei*, chemical profile homogeneity increased in the group exposed to predator odour but not in the group exposed to solvent. Secondly, *R. grassei* workers exposed to predator odour had lower mortality rates than those exposed to solvent; there was no difference for *R. flavipes*. The treatments did not affect caste development in either species, as the production of neotenic reproductives and soldiers was unchanged. No chemical shifts (between day 0 and 56) were observed for the same colonies within treatments for both species. Altogether, these results suggest that, at least in *R. grassei*, prolonged exposure to predator odour influenced survival rates and modified chemical profiles. Interestingly, it was not the case for the invasive species *R. flavipes*.

Our analysis of the cuticular compounds showed that, in *R. grassei*, exposure to predator odour increased chemical profile homogeneity. In contrast, chemical profiles remained relatively heterogeneous in the group exposed to solvent. These results match those of previous studies, in which *R. grassei* displayed a high degree of chemical profile variation (Perdereau *et al.*, 2011). In several social insect species, colony odour is kept homogeneous thanks to chemical cues transferred among nestmates via social interactions such as trophallaxis and physical contacts (Lenoir *et al.*, 2001; Soroker *et al.*, 2003; Lucas *et al.*, 2004, 2005; Blomquist & Bagnères, 2010). The presence of predator odour may have induced a stress response in *R. grassei* colonies, resulting in a stronger clustering. These increased physical contacts may have involved more social interactions (with higher chemical transfer), resulting in a stronger social cohesion. This hypothesis could explain the greater homogeneity in chemical profiles that was reflected in the decreased Nei's distances observed in *R. grassei*.

Interestingly, *R. flavipes* did not show a similar response to predator odour. Chemical profile homogeneity was the same in the predator odour group as in the solvent group, suggesting that social interactions in *R. flavipes* are less affected by environmental conditions. Again, this finding matches those of previous studies on *R. flavipes* (Perdereau *et al.*, 2010b). Maintaining a high degree of chemical profile homogeneity across varying environmental conditions could actually be advantageous. *Reticulitermes flavipes* invaded Europe after arriving from North America in lumber shipments during the 18th century (Perdereau *et al.*, 2013). Several authors have argued that reduced chemical variability is a way in which invasive species adapt to the new environments of their invasive range (Errard *et al.*, 2005; Brandt *et al.*, 2009; Perdereau *et al.*, 2011). On the one hand, the profiles of *R. flavipes* appeared to be stable and less reactive to external influences (predator odour effect). On the other hand, our results seem to show that the initial heterogeneity of chemical profiles in *R. flavipes* was not significantly different from that of *R. grassei*.

The chemical profiles showed no qualitative differences in either species, including in the predator odour group, in which termites were exposed to predator odour for 2 months. *Lasius niger*'s profile largely consists of dimethyl- and trimethyl-branched alkanes, which are not at all present in *R. grassei* and *R. flavipes*. As these compounds were absent from the termite profiles in this study, it is quite clear that no chemical transfer occurred (at least in significant quantities). As for compounds that the ant and the termites share, if chemical transfer had occurred, then chemical heterogeneity should have increased in the predator odour groups in both species. In fact, the predator odour treatment did increase chemical homogeneity for *R. grassei* but had no effect on *R. flavipes* profiles. Previous publications showed that external exogenous CHCs were transferred upon first contact but were not incorporated into the cuticular profiles over several days (Meskali *et al.*, 1995; Sevala *et al.*, 2000; Soroker *et al.*, 2003), unless these CHCs were continuously produced and transferred through social interactions (Errard & Jallon, 1987; Vauchot *et al.*, 1996). In the present study, the treatment was 2 months long and was deposited twice on paper.

Social insects have complex caste systems in which individuals are specialised for different tasks (e.g. defence, siblings care, nest maintenance, reproduction). Caste differentiation in the genus *Reticulitermes* has been shown to be influenced by genetic factors (Hayashi *et al.*, 2007; Kitade *et al.*, 2011) and environmental factors (Korb & Hartfelder, 2008; Lo *et al.*, 2009). Thus, social insects can alter caste ratios to deal with environmental pressures. In termites, stress conditions like food quantities or seasonal variations (Korb & Katrantzis, 2004), as well as queen pheromonal control (Matsuura *et al.*, 2010) can induce variability in caste ratios. This caste polyphenism seems to be controlled by juvenile hormone titre in termites (Zhou *et al.*, 2006; Cornette *et al.*, 2008). In the ant *Pheidole pallidula*, Passera *et al.* (1996) found that colonies exposed to intraspecific competitors were able to modify their caste ratios in less than 1 month, increasing the number of soldiers produced. Because the *Reticulitermes* caste system is also influenced by environmental factors, we predicted that caste ratio would vary in response to prolonged exposure to predator odour. Surprisingly, although predator odour seemed to influence social interactions, there was no change in caste ratio, even after 2 months of exposure. It is possible that the treatment period was not long enough to significantly increase the number of neotenic reproductives and/or soldiers. An alternative explanation is that some genetic factors override the potential influence of the environmental factors on the caste differentiation for the two studied species.

Social organisation is also characterised by specific nesting and foraging strategies in social insects (Bignell *et al.*, 2011). Here we have shown that the two study species seemed to display different strategies in the presence of predator cues. The results of the competitive tests confirmed that predator odour had an impact on *R. grassei*. Indeed, mortality was lower in the predator

odour group than in the solvent group. There are two possible explanations for this pattern. First, workers exposed to predator odour may have become more aggressive. Secondly, they may have displayed improved defensive mechanisms. Both would have resulted in greater survivorship during the competitive tests for the predator odour-treated workers. To determine the best explanation, we examined the mortality rates of the facing naive workers. If predator odour-treated workers were indeed more aggressive, they would have killed more naive workers. This was not the case because the mortality rates of naive workers did not differ whether they were facing predator odour-exposed workers or solvent-exposed workers. This result therefore suggests that exposure to predator odour improved defensive mechanisms rather than aggressiveness. Of course, dedicated experiments are required to confirm this hypothesis on the improved defences of workers exposed to predator odour. Nevertheless, this working hypothesis fits with the greater chemical homogeneity observed in the predator odour group. The results for *R. flavipes* were different from those for *R. grassei*. Neither the predator odour nor the solvent treatment affected worker mortality. Moreover, when the two species were compared, mortality rates were lower in *R. flavipes* than in *R. grassei* for both treatment types. It has been suggested that invasive species display a unique combination of reduced intraspecific aggressiveness and chemical homogeneity, which allows colonial fusion events (Brandt *et al.*, 2009; Vásquez *et al.*, 2009; Perdereau *et al.*, 2010b, 2011). *Reticulitermes flavipes* is a well-known invader that often engages in colony fusion, our results fit with this point.

Several specific characteristics of *R. flavipes* and *R. grassei* were confirmed in this study. We have also added to the body of knowledge the influence of environmental predator cues on chemical and behavioural adaptations of these termites. Our results underscore the important role played by local foreign chemical cues on species' social organisation. Thanks to our experimental design, we were able to test hypotheses focusing on how a predator signal affected some biological traits of termites. It could be further extended to study learning ability of termites in response to foreign chemical cues. Hence the next step will be to conduct more detailed studies on specific identified chemicals, such as alarm pheromones from predators, to determine their impact on these two termite species, which are the most widespread termite pests. Indeed, it could be useful to further explore the effect of the environmental factors on these species' colonisation strategies in order to improve the control of their populations.

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The experiment was designed by CL, SD and AGB. SD and CL performed the field work. CL and LL performed experiments with the help of JPC. CL conducted the statistical analyses. CL and LB wrote the paper along with advice from AGB. All the authors read and approved the final version of the manuscript.

## Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12529

**Figure S1.** Number of dead workers inside the odour nests after 2 months of treatment in *R. grassei* (a) and *R. flavipes* (b). For both treatments, five field nests were used (per species). Box plots show the median, upper and lower quartiles and range; dots, outliers; crosses, means.

**Figure S2.** Number of neotenic reproductives and soldiers inside the odour nests for both treatments on day 56 (2 months), for *R. grassei* (a and c, respectively) and *R. flavipes* (b and d, respectively). For both treatments, five field nests were used (per species). Box plots show the median, upper and lower quartiles and range; dots, outliers; crosses, means.

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MINI-REVIEW

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# Job switching in ants
## Role of a kinase

Christophe Lucas,[1,*] Bryon N. Hughson[2] and Marla B. Sokolowski[2]

[1]Department of Ecology and Evolution; University of Lausanne; Lausanne Switzerland;
[2]Department of Biology; University of Toronto; Mississauga, ON Canada

**Key words:** *foraging*, defence, *Pheidole pallidula*, ants, kinase

**Abbreviations:** *for*, *foraging* gene; PKG, cGMP dependent protein kinase; *ppfor*, *Pheidole pallidula foraging* gene

Reproductive division of labor is a defining characteristic of eusociality in insect societies. The task of reproduction is performed by the fertile males and queens of the colony, while the non-fertile female worker caste performs all other tasks related to colony upkeep, foraging and nest defence. Division of labor, or polyethism, within the worker caste is organized such that specific tasks are performed by discrete groups of individuals. Ordinarily, workers of one group will not participate in the tasks of other groups making the groups of workers behaviorally distinct. In some eusocial species, this has led to the evolution of a remarkable diversity of subcaste morphologies within the worker caste, and a division of labor amongst the subcastes. This caste polyethism is best represented in many species of ants where a smaller-bodied minor subcaste typically performs foraging duties while larger individuals of the major subcaste are tasked with nest defence. Recent work suggests that polyethism in the worker caste is influenced by an evolutionarily conserved, yet diversely regulated, gene called *foraging (for),* which encodes a cGMP-dependent protein kinase (PKG). Additionally, flexibility in the activity of this enzyme allows for workers from one task group to assist the workers of other task groups in times of need during the colony's life.

In a recent article, Lucas and Sokolowski[1] report that PKG mediates behavioral flexibility in the minor and major worker subcastes of the ant *Pheidole pallidula*. By changing the task-specific stimulus (a mealworm to induce foraging or alien intruders to induce defensive behavior) or pharmacologically manipulating PKG activity, they are able to alter the behavior of both subcastes. They also show differences in the spatial localization of the FOR protein in minor and major brains. Furthermore, manipulation of *ppfor* activity levels in the brain alters the behavior of both *P. pallidula* subcastes. The *foraging* gene is thus emerging as a major player in regulating the flexibility of responses to environmental change.

*Correspondence to: Christophe Lucas; Email: christophe.lucas@unil.ch
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### Evolutionary Conservation of the *foraging* Gene

To date the *foraging* gene is found in 42 species and in several taxa including bacteria, insects, nematode, fish, birds and mammals.[1-12] In several species *for* is associated with natural behavioral variations linked to foraging activities. This link was first described in *Drosophila melanogaster* where *for* allelic variants (rover, $for^R$, and sitter, $for^s$) produce a natural polymorphism in foraging behavior.[6] Rover larvae have longer foraging trails, higher *for* mRNA and PKG activity levels as compared to sitters. These traits continue into adulthood where adult rovers display greater post-feeding locomotion.[13] Drosophila *for* is implicated in metabolic plasticity permitting the adoption of alternative metabolic strategies in nutritionally stressful environments.[14] In the honey bee *Apis mellifera*, *Amfor* plays a role in the long term behavioral transition from nursing to foraging that occurs as workers mature. In the worm *Caenorhabditis elegans*, mutating *for*'s counterpart *egl-4* alters foraging and olfactory adaptation, in addition to body size and lifespan.[2,3,15] In flies and honey bees, increases in PKG lead to more foraging whereas in *C. elegans* and species of ants investigated to date (*Pheidole pallidula*, *Pogonomyrmex barbatus* and *Solenopsis invicta*) decreases in PKG lead to foraging. These findings suggest that although the regulatory pathways underlying this gene may differ, the behavioral function of *for* may be evolutionarily conserved across species.

### *foraging* Gene and Behavioral Polyethism

In *D. melanogaster* and *C. elegans*, allelic variation of the *for* gene produces distinct behavioral phenotypes. Alternatively, in the eusocial honey bee, members of the worker caste display an age-related transition in behavioral repertoires, known as temporal polyethism, where young workers perform tasks such as nursing inside the colony and move outside the nest to fill the role of foragers only later in life.[10,16] Temporal polyethism in *A. mellifera* is correlated with *for* mRNA expression.[10,17]

Nearly all species of eusocial insects display temporal polyethism, but as mentioned above, in some species the division of labor within the worker caste has led to the evolution of morphologically distinct groups, or subcastes, of workers. The most highly derived form in the evolution of physical castes, complete dimorphism, is displayed by the ant *Pheidole pallidula*, where the

worker caste is subdivided into two morphologically distinct size groups; members of the two subcastes ordinarily display distinct behavioral repertoires in the same environment.[16] The subcaste of major workers (soldiers) have enlarged heads that house hypertrophied mandibular muscles and large mandibles, and are primarily involved in colony defense and patrolling. The subcaste of minor workers (foragers) are smaller and perform foraging tasks. We have shown that the behavioral differences between subcastes are linked to different levels of PKG activity by manipulating foraging and defense stimuli. Interestingly, pharmacological manipulation of PKG activity is enough to stimulate foraging behavior in majors. This implies a causal relationship between PKG activity, behavioral flexibility and the environmental stimulus. Although many genes are involved in the development and regulation of caste polyethism in *P. pallidula*, altering *ppfor* activity alone changes foraging and defense behavior in a predictable manner. Thus, *ppfor* plays a role in behavioral flexibility in ants.

## PKG and Adaptive Foraging

Optimal foraging theory[18,19] predicts that animals weigh the costs and benefits associated with different foraging strategies in order to maximize their fitness. Differences between majors and minors can be seen as an adaptive response of the colony used to moderate the conflict between performing foraging or defensive behavior. Where a solitary animal has evolved to weigh the costs and benefits of performing a given behavior, the worker population of the eusocial colony divides the workforce and thus shares the costs and benefits of the tasks performed by the specialized subcastes. The presence of morphologically distinct worker subcastes in the colony, such as in *P. pallidula* where majors are built for defense and minors for foraging, should confer an advantage upon eusocial species. In natural populations of *P. pallidula* the majors comprise around 10% of the colony population and at this abundance prevent the production of more majors, but in colonies exposed to intraspecific competition the ratio of majors produced by the colony increases.[16,20]

## PKG Activity Levels: Predetermination and Flexibility

Flexibility in caste polyethism also permits rapid responses to changing environmental and social stimuli. In ants, it is inefficient for behaviors such as foraging and defense to be performed simultaneously by an individual. One hypothesis emerging from Lucas and Sokolowski[1] is that PKG acts as a molecular switch between foraging and defense. In stable colonies, majors and minors exhibit PKG activity levels characteristic of their subcaste. This "baseline" PKG activity level is likely established early in life concurrently with subcaste determination, perhaps during larval development when the decision point that determines subcaste development is reached.[16] Lucas and Sokolowski[1] show that the different "baseline" PKG activity levels of majors and minors can increase or decrease in response to colony stress (introduction of intruder ants or a live prey item). In a social insect colony, the recruitment of workers to foraging or defense activities fluctuates according to the colony's needs, and the regulation of these behaviors within a subcaste may be most efficiently accomplished by changing the level of a single molecule like PKG. The need to recruit greater numbers of workers to a task than are present within a single subcaste could produce a selection pressure to exploit the pre-existing behavioral regulatory system and lead to the evolution of a molecular switch.

PKG activity was higher in majors than minors in all test conditions. The finding that majors have more FOR-immunoreactive brain cell clusters than minors suggests a neuroanatomical basis for the reported major-minor behavioral differences. Analysis of PPFOR expression in *P. pallidula* worker brain show several clusters of immunoreactivity, some of which are near the central complex, a region known to be involved in spatial perception and navigation. Given the role of this centre in the integration and coordination of sensory inputs with motor output, it is tempting to investigate the potential of the central complex to act as a switch for moving from one behavioral state to another.[21-23] Whether PKG functions in this manner requires more subtle manipulations of PKG enzyme activities and measures of possible thresholds involved in switches between foraging and defense under stressful conditions. The rapid behavioral response (less than 5 min) of *P. pallidula* workers to the foraging and defense stimuli suggests that the PKG protein acts directly on the nervous system, allowing socially-induced behavioral flexibility. This is in contrast to *Amfor*'s role in the more long term transcriptional changes associated with temporal polyethism in honey bees.[10]

Few genes and molecules have been definitively shown to influence social behavior in eusocial insects. We demonstrate that the activity of the foraging gene which encodes a cGMP-dependent protein kinase (PKG) determines whether a worker ant will adopt the role of forager or defender. Majors have more cells in their brains that express PPFOR and higher PKG activity than minors. Environmental stimulation or pharmacological manipulation of PKG activity alters the tendency of both subcastes to forage and defend, demonstrating the pivotal role played by this enzyme in social interactions.

Our study is one of the first to investigate the molecular basis of ant social behavior and provides a framework for future mechanistic and evolutionary studies. It also provides a means to characterize the role of PKG in the evolution of physical castes, and has identified candidate regions of the CNS to be targeted in future investigations. Localization of PKG in the brain also suggests a role for this molecule in the integration and interpretation of context-specific sensory input that is required for the generation of appropriate behavioral output. PKG is known to mediate flexibility in an array of disparate behaviors, such as feeding, food search behavior, stress tolerance, learning and memory, and now defense.[24] This recurring theme across such a wide variety of organisms suggests that the foraging gene and the PKG molecule that it encodes may have evolved as a general behavioral modifier.

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Article

# THE LOCUST *FORAGING* GENE

C. Lucas
*Department of Biology, University of Toronto, Mississauga, Ontario, Canada; Department of Ecology and Evolution, University of Lausanne, Lausanne, CH-1015, Switzerland*

R. Kornfein
*Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*

M. Chakaborty-Chatterjee and J. Schonfeld
*Department of Biology, University of Toronto, Mississauga, Ontario, Canada*

N. Geva
*Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*

M. B. Sokolowski
*Department of Biology, University of Toronto, Mississauga, Ontario, Canada*

A. Ayali
*Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel*

*Our knowledge of how genes act on the nervous system in response to the environment to generate behavioral plasticity is limited. A number of recent advancements in this area concern food-related behaviors and a specific gene family called foraging (for), which encodes a cGMP-dependent protein kinase (PKG). The desert locust (Schistocerca gregaria) is notorious for its destructive feeding and long-term migratory behavior. Locust phase polyphenism is an extreme example of environmentally induced behavioral plasticity. In response to changes in population density, locusts dramatically alter their behavior, from solitary*

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Correspondence to: A. Ayali, Department of Zoology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, 69978, Israel. E-mail: ayali@post.tau.ac.il

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*and relatively sedentary behavior to active aggregation and swarming. Very little is known about the molecular and genetic basis of this striking behavioral phenomenon. Here we initiated studies into the locust for gene by identifying, cloning, and studying expression of the gene in the locust brain. We determined the phylogenetic relationships between the locust PKG and other known PKG proteins in insects. FOR expression was found to be confined to neurons of the anterior midline of the brain, the pars intercerebralis. Our results suggest that differences in PKG enzyme activity are correlated to well-established phase-related behavioral differences. These results lay the groundwork for functional studies of the locust for gene and its possible relations to locust phase polyphenism.*


**Keywords:** *foraging* gene; locust; swarming behavior; kinase; density-dependent phase polyphenism

## INTRODUCTION

Natural variation in behavior is influenced by interactions between many genes. These genes interact with the environment to generate behavioral plasticity. A remarkable example of environmentally induced behavioral plasticity can be found in locusts. In the desert locust (*Schistocerca gregaria*), solitary-phase locusts are cryptic in physical appearance and behavior (Rogers et al., 2003). They actively avoid contact with other locusts (Roessingh et al., 1993), and are relatively sedentary (Simpson et al., 1999). An increase in population density dramatically alters locust behavior; the gregarious locusts are generally more active, and march in huge bands of hoppers or form flying swarms as adults. The major behavioral characteristic of gregarious-phase locusts is their strong attraction to conspecifics, which translates into active aggregation behavior (Ellis, 1959, 1963; Uvarov, 1966). The phase transformation is thought to be a positive feedback process, with changes in behavior preceding and facilitating other phase-changes including biochemical, physiological, and morphological ones (Pener, 1991; Pener and Yerushalmi, 1998; Applebaum and Heifetz, 1999; Pener and Simpson, 2009). Recent findings have identified the primary sensory inputs inducing a density-dependent phase change in locusts (Rogers et al., 2004). Other neurobiology research has characterized differences in sensory interneurons that mediate behavioral changes between the locust phases (e.g., wind-sensitive interneurons, Fuchs et al., 2003; visual interneurons, Matheson et al., 2004). In marked contrast, little has been achieved in the study of the molecular and genetic contributions to this phenomenon (see recent reviews in Simpson et al., 2005; Simpson and Sword, 2008; Pener and Simpson, 2009). As a result, one is compelled to initiate an investigation into possible candidate genes from other organisms that could be involved in the locust phase-dependent behavioral polymorphism (Fitzpatrick et al., 2005).

In other species, there are a handful of well-studied examples that shed light on the genes and pathways that underlie natural behavioral variations and plasticity. In some cases, natural variation in a single gene can be identified that affects the regulation of complex yet distinct behavior patterns (e.g., de Bono and Bargmann, 1998; Krieger and Ross, 2002; Davies et al., 2004; Hammock, 2007). Interestingly, a number of recent achievements in this area involve food-related behaviors and the

*foraging* gene, which encodes a cGMP-dependent protein kinase (PKG) (Osborne et al., 1997; Ben-Shahar et al., 2002, 2003; Hirose et al., 2003; Fitzpatrick and Sokolowski, 2004; Ingram et al., 2005; Fussnecker and Grozinger, 2008; Garabagi et al., 2008; Heylen et al., 2008; Lucas et al., 2009; Lucas and Sokolowski, 2009).

The fruit fly, *Drosophila melanogaster,* exhibits a behavioral polymorphism in larval and adult-foraging behaviors, which arises from allelic variation in *for*. Larvae and adult flies with a rover allele ($for^R$) move greater distances while feeding than those with the sitter alleles ($for^s$) (Sokolowski, 1980; Pereira and Sokolowski, 1993). The *for* gene encodes a cGMP-dependent protein kinase (PKG) (Reaume and Sokolowski, 2009). Rover heads exhibit higher levels of *for* mRNA and higher PKG activity than sitter heads (Osborne et al., 1997). Interestingly, in flies, crowded populations select for animals with rover alleles whereas uncrowded ones select for those with sitter alleles (Sokolowski et al., 1997). The *for* gene also plays a role in behavioral plasticity in *D. melanogaster* in response to food deprivation (Kaun et al., 2007b). Moreover, the *for* gene was successfully used as a candidate gene to investigate the plasticity of food-related behaviors in other insect species including ants (Ingram et al., 2005; Lucas et al., 2009; Lucas and Sokolowski, 2009) and honey bees (Ben-Shahar et al., 2002).

In the present study, we cloned the locust *for* gene and placed it in an insect phylogeny of cGMP-dependent protein kinases (PKGs). We also examined FOR expression in locust brains and measured the PKG enzyme activities in female and male solitary and gregarious locusts. Our results lay the groundwork for functional studies of the locust *for* gene and its possible relations to locust phase polyphenism.

## MATERIALS AND METHODS

### *Animals*

Desert locusts, *Schistocerca gregaria* (Forskål), were reared for many consecutive generations under heavy crowding conditions, 100–160 animals in 60-liter metal cages. Cages were kept under controlled temperature and humidity conditions (30°C, 35–60%) under a 12:12 light-dark cycle (with lights on at 7 am). Direct radiant heat was supplied during daytime by incandescent electric bulbs to reach a final day temperature of 35–37°C. Locusts were fed daily with fresh grass and dry oats. In order to obtain locusts approaching the solitary phase, hatchlings from eggs laid by crowded-reared locusts were isolated within 4 h post-hatching and kept under isolation, one locust per 1.5-liter metal cage. Care was taken to keep locusts of the different phase groups under similar conditions (except density).

Migratory locusts, *Locusta migratoria*, were generously provided by Angela Lange from a colony housed at the University of Toronto (Mississauga, Canada). The locusts were raised under crowded conditions on a 12:12 light-dark cycle, at a temperature range of 30–34°C, and fed fresh wheat seedlings supplemented with bran and carrots.

### *Cloning the Locust for Gene*

mRNA was extracted from 10 brains of *S. gregaria* (5 males and 5 females) or *L. migratoria* (females) dissected in saline PBS solution. All tissues were then quickly placed in RNA later solution until mRNA extraction using an Amersham Biosciences kit (GE Healthcare). Specific cDNA was amplified using a RACE kit from Clontech (Palo Alto, CA) with degenerate primers designed based on conservative regions of *for*

ortholog alignment of several species (sense primer: TGGGCCATYGANCGACARTG, antisense primer: AAGCCMTCRAACCAYTTGTGYTT). The primers were used for both locust species but with different annealing temperature (56°C for *S. gregaria* and 53°C for *L. migratoria*). Amplified bands were cloned with a TOPO TA Cloning from Invitrogen (Carlsbad, CA), using the pCR 2.1-TOPO vector in electrocompetent *E. coli* cells. Vectors from positive clones were extracted, purified, and sequenced. The cloned locust sequences were examined for the extent of their homology with known *for* genes using the NCBI database. After confirming that the newly cloned locust sequences matched known *for* gene sequences, nested specific primers were designed and used with newly extracted mRNA samples (*S. gregaria* sense primer: CCGGCAGGCAGAGTACACCGATTTC, antisense primer: CAACACGGCCAAAACCACCAACTCC; *L. migratoria* sense primer: TGTCAAACTGGTGGATTTTGGTTTTGC, antisense primer: TCGTTCTTGGGAAGTCAATAGCATCG) in order to extend and confirm the cloned locust *for* sequences.

### *Phylogeny*

PKG protein sequences were obtained from the NCBI database using Blastx and nomenclature searches. As of November 2009, a total of 44 PKG proteins were found for over 30 insect species (Table 1) (variant sequences were discarded). Pairwise protein sequence alignments were made using the default settings of Muscle (SeaView; Galtier et al., 1996). Phylogenetic distances were used to build a neighbor joining tree using Phylowin (Galtier et al., 1996) on the conserved kinase domains and the remaining carboxyl terminal residues (around 300 aa) with 5,000 bootstrap replications. Bootstrapping is a resampling technique used to generate confidence estimates for the placement of nodes in phylogenetic trees (Page and Holmes, 1998). Bootstrap values range from 0 to 100 where higher values indicate low sampling error and, therefore, higher support for those nodes.

### *Immunocytochemistry and Neuronal Staining*

Immunohistochemistry was as in Belay et al. (2007). Briefly, whole brains were dissected in PBS (0.1 M, pH 7.4), fixed in 4% paraformaldehyde, and blocked in 4% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in 0.5% Triton X-100/PBS. The specific guinea pig antibody called anti-FOR, described in Belay et al. (2007), was used at 1:150; the neuropile marker mouse mAb nc82 was used at 1:20 (22, 23). Incubation was for 48 h at 4°C. After incubation, brains were washed several times in 0.5% Triton X-100/PBS before adding a goat Cy2-conjugated anti-mouse and a Cy5-conjugated anti-guinea pig Ig (1:100, Jackson ImmunoResearch) for 24 h at 4°C. For negative controls, brains were incubated in only secondary antibody, in the absence of primary antibody or in pre-absorbed anti-FOR serum. The specificity of the primary antibody, anti-FOR antibody generated in guinea pig, was measured by using Western blot immunodetection.

For neurobiotin staining, stumps of the cut nerves (NCC1) were isolated in Vaseline vessels and exposed to distilled water for 5 min. Incubation was in neurobiotin (5% in distilled water, Vector Laboratories, Burlingame, CA) at 4°C or for 2–3 days. After overnight fixation in paraformaldehyde (4% in distilled water), preparations were rinsed in several changes of PBS containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Subsequently, tissues were incubated overnight in Cy3-conjugated streptavidine (Amersham Biosciences, Piscataway, NJ) in PBS

***Table 1. GenBank Accession Number and Detailed Informations About the Insect Species Used for the Phylogenetic Analysis***

| *Scientific name* | *Common name* | *PKG type* | *GenBank* | *Unigene* |
|---|---|---|---|---|
| *Acyrthosiphon pisum* | Pea aphid | I | XM_001952056.1 | Aps.15600 |
| *Acyrthosiphon pisum* | Pea aphid | II | XM_001947008.1 | |
| *Aedes aegypti* | Yellow fever mosquito | I | XM_001652896.1 | Aae.12121 |
| *Aedes aegypti* | Yellow fever mosquito | II | XM_001651359.1 | Aae.709 |
| *Anopheles gambiae* | Mosquito | I | XM_319605.4 | |
| *Anopheles gambiae* | Mosquito | II | XM_314690.4 | |
| *Apis mellifera* | Honey bee | I | AF469010.1 | |
| *Bombus ignitus* | Bees | I | dbj_AB491725.1 | |
| *Bombus terrestris* | Bees | I | gb_FJ816699.1 | |
| *Bombyx mori* | Silkworm moth | I | AF465601.1 | Bmo.749 |
| *Diabrotica virgifera virgifera* | Western corn rootworm beetle | I | gb_DQ913742.1 | |
| *Drosophila ananassae* | Flies | I | XM_001961954.1 | |
| *Drosophila ananassae* | Flies | II | XM_001961529.1 | |
| *Drosophila erecta* | Flies | I | XM_001968555.1 | |
| *Drosophila erecta* | Flies | II | XM_001968175.1 | |
| *Drosophila grimshawi* | Flies | I | XM_001993194.1 | |
| *Drosophila grimshawi* | Flies | II | XM_001988546.1 | |
| *Drosophila melanogaster* | Fruit fly | I | NM_058139.3 | Dm.6584 |
| *Drosophila melanogaster* | Fruit fly | II | NM_057865.3 | Dm.4323 |
| *Drosophila mojavensis* | Flies | I | XM_002003128.1 | |
| *Drosophila mojavensis* | Flies | II | XM_002003588.1 | |
| *Drosophila persimilis* | Flies | I | XM_002014941.1 | |
| *Drosophila persimilis* | Flies | II | XM_002020546.1 | |
| *Drosophila pseudoobscura pseudoobscura* | Flies | I | XM_001356655.2 | |
| *Drosophila sechellia* | Flies | II | XM_002041587.1 | |
| *Drosophila simulans* | Flies | I | XM_002078022.1 | |
| *Drosophila simulans* | Flies | II | XM_002077639.1 | |
| *Drosophila virilis* | Flies | II | XM_002052831.1 | |
| *Drosophila willistoni* | Flies | I | XM_002065449.1 | |
| *Drosophila willistoni* | Flies | II | XM_002066659.1 | |
| *Drosophila yakuba* | Flies | I | XM_002087818.1 | |
| *Drosophila yakuba* | Flies | II | XM_002087431.1 | |
| *Lobesia botrana* | Moth | I | gb_DQ666642.1 | |
| *Locusta migratoria* | Migratory locust | I | FJ214984 | |
| *Mythimna separata* | Northern armyworm moth | I | gb_GQ844298.1 | |
| *Nasonia vitripennis* | Jewel wasp | I | XM_001603499.1 | |
| *Nasonia vitripennis* | Jewel wasp | II | XM_001599276.1 | Nvi.4830 |
| *Pediculus humanus corporis* | Human body louse | II | XM_002432638.1 | |
| *Pheidole pallidula* | Ant | I | EF999975 | |
| *Pogonomyrmex barbatus* | Red harvester ant | I | AY800387.1 | |
| *Schistocerca gregaria* | Desert locust | I | FJ214985 | |
| *Tribolium castaneum* | Red flour beetle | I | XM_968614.2 | Tca.5335 |
| *Tribolium castaneum* | Red flour beetle | II | XM_963625.1 | Tca.3123 |
| *Vespula vulgaris* | Wasp | I | gb_EF136648.1 | |

containing 0.3% Triton X-100 at room temperature. After rinsing with PBS, preparations were dehydrated in a graded series of ethanol, cleared in 60% glycerol at 4°C overnight and mounted onto polylysine-coated glass slides under Fluorescent Mounting Medium (Golden Bridge Life Science, WA) and cove-slipped.

All labeled preparations were analyzed and photographed using an Olympus inverted system microscope (IX70, Olympus, Tokyo, Japan) equipped with a digital camera or alternatively were examined using a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

### *PKG Assays*

PKG enzyme assays (modified from Kaun et al. 2007b) were performed on dissected brain tissues of *S. gregaria* solitary and gregarious locusts one week after adult emergence. Locusts were briefly anesthetized in $CO_2$, their brain dissected out and stored at −80°C. Individual brains were homogenized on ice, for males and females of both phases separately, in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 0.05% Triton X-100, 5 mM β-mercaptoethanol (Sigma Aldrich), and protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Samples were sonicated 4 times for 5 sec on ice in a Branson Sonifier 250 (Branson, CT) at the lowest setting (duty cycle 20 and output control 4). Samples were then centrifuged at 10,000 RPM for 5 min at 4°C. The supernatant of each sample was then removed and used to determine the total protein amount using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) prior to the cGMP-dependent protein kinase activity assays. The final concentration of the PKG activity mixture contained 40 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM [$\gamma^{32}$P]ATP (500–1,000 cpm/pmol) (Amersham, Pharmacia Biotech, Baie D'Urfe, QC, Canada), 13 μg/ml of a heptapeptide substrate highly specific to the PKG (RKRSRAE) (Promega, Burlington, ON, Canada), 3 μM c-GMP (Promega), and 4.6 nM of a highly specific c-AMP-dependent protein kinase inhibitor (Ki50% = 2.3 nM) (5–24 from Calbiochem, San Diego, CA). As a control, reactions were performed in the presence of 468 nM of a PKG inhibitor K-5823 (Calbiochem). The reaction mixtures were incubated at 30°C for 10 min. The reaction was terminated by spotting 70 μl of the reaction mixture onto Whatman P-81 filters, which were then soaked with 75 mM $H_3PO_4$ for 5 min and washed three times with 75 mM $H_3PO_4$ to remove any unbound [$^{32}$P]ATP. Finally, filters were rinsed with 100% ethanol and air dried before quantification. For quantification of PKG activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter (Perkin Elmer, Woodbridge, ON, Canada) using a universal scintillation cocktail (ICN). The specific PKG activity was expressed as pmol of $^{32}$P incorporated of the PKG substrate per min per mg of protein. Data were analyzed using two-way ANOVA and presented as means ± S.E. In all figures, $P < 0.001$, $P < 0.01$, and $P < 0.05$ are represented as ***, **, and *.

## RESULTS

### *Laboratory-Reared Locusts Approach the Two Extreme Density-Dependent Phases*

The desert locust colony at Tel Aviv University shows clear and very consistent phase differences in response to the distinct rearing conditions used. These included all reported differences in morphometric ratios, coloration and pattern, and development. Further, recently reported neurophysiological differences (Fuchs et al., 2003) and behavioral differences (Geva et al., 2010) are also very robust and consistent.

### *The Locust Foraging Gene*

We isolated a 1,258-bp fragment of the *S. gregaria for* ortholog, which we call *sgfor* (GenBank accession number FJ214985). It had typical PKG domains (Hofmann et al., 2006) including a serine/threonine kinase domain (with a catalytic site) and two cGMP-binding domains. The same degenerate primers and techniques were used to clone the *L. migratoria* for ortholog *lmfor* (GenBank accession number FJ214984). The *lmfor* fragment was 560 bp long and did not differ from a fragment of *sgfor*'s kinase domain, which contained the catalytic site. The *S. gregaria sgfor* DNA sequence has 91% pairwise identity with *L. migratoria*'s *lmfor* sequence. Both locust DNA sequences are around 70% pairwise identical to the *for* gene sequence of *D. melanogaster.* A comparison of the two locusts *for* amino acid sequences and the corresponding region of the fly FOR protein are shown in Figure 1. The kinase domain is well conserved in these species and the catalytic sites are identical. We studied the phylogenetic relationships of the newly-cloned locust *for* sequences as compared to all currently known insect PKG-encoding genes (Fig. 2). Two types of PKG (I and II) are represented on the tree. PKG type I is known to play a role in food-related behaviors and plasticity (reviewed in Lohmann et al., 1997). As shown in Figure 2, all the PKG type I protein sequences cluster together and appear to be derived from PKG type II sequences. Within the type I cluster, we can distinguish a group formed by the dipterans and a group with all the social insects except the wasp *Vespula vulgaris*. The two locust sequences are the locust *foraging* genes and are part of the PKG type I cluster.

### *FOR Expression in the Locust Brain*

The FOR protein was found to be expressed in a very distinct group of neuronal cell bodies of the anterior midline of the brain (Fig. 3A, B). The examples shown in Figure 3C demonstrate a very robust and consistent expression pattern of FOR in the locust

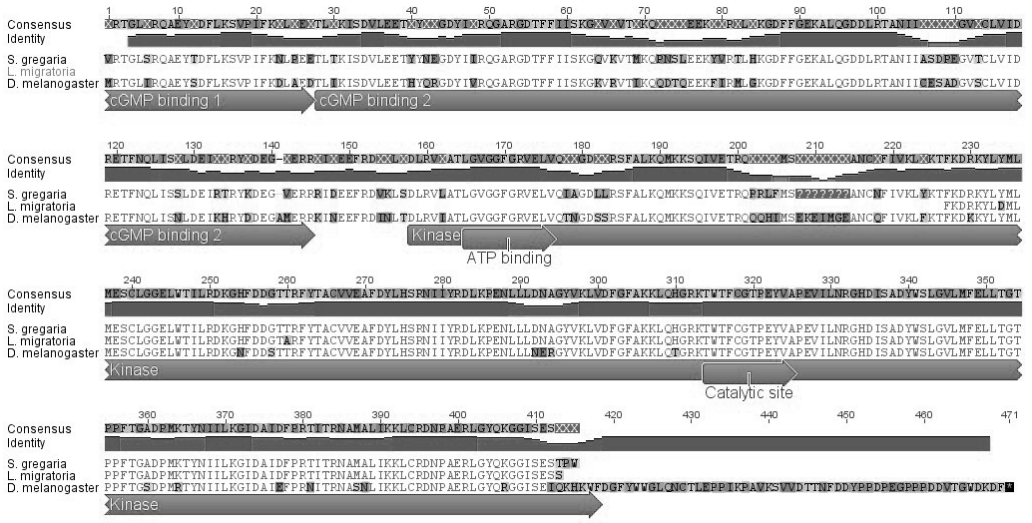


**Figure 1.** Alignments of amino acid sequence of *S. gregaria*, *L. migratoria,* and *D. melanogaster* using Muscle (SeaView, Galtier et al., 1996). Identity and consensus sequence is shown using Geneious software. The different identified domains of the PKG are shown for *D. melanogaster.*

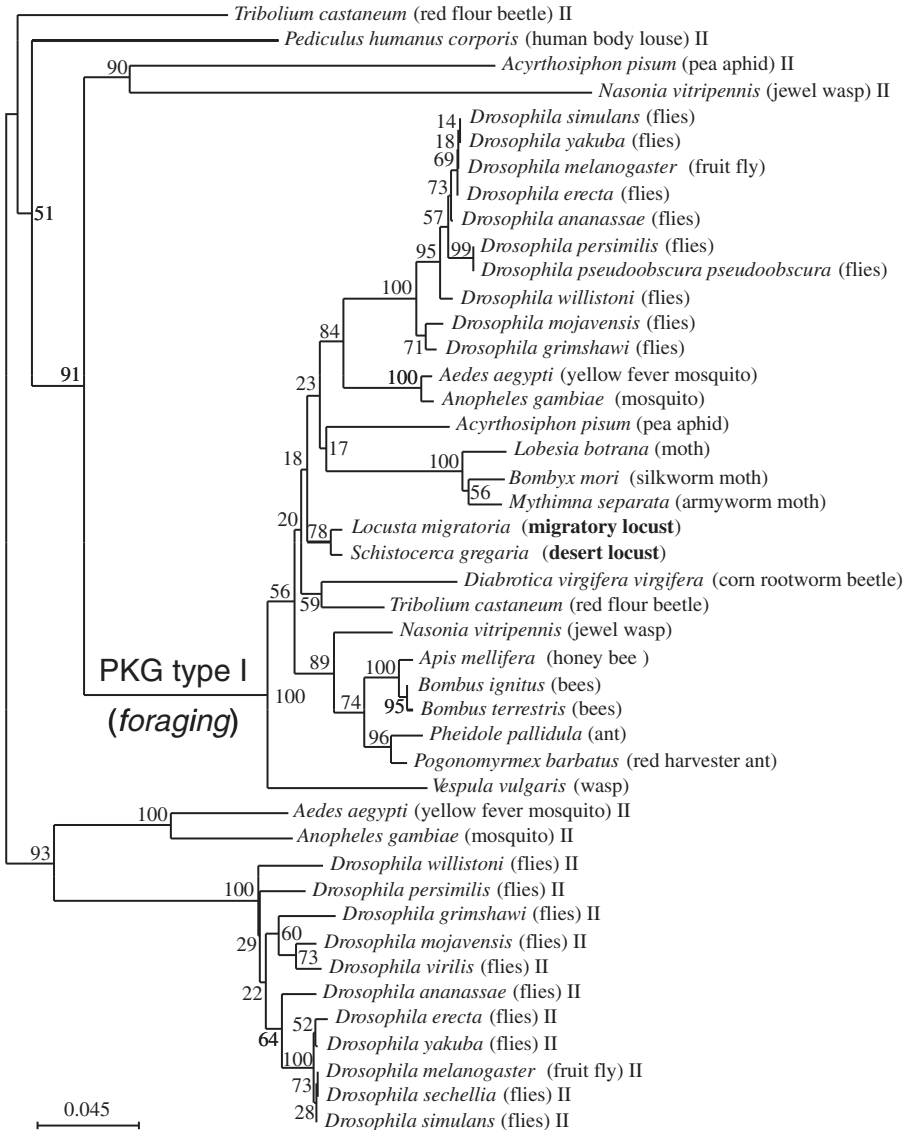


**Figure 2.** Phylogenetic analysis (Neighbour joining method) of the relationship of 44 PKG sequences spanning 30 insect species (variant sequences were discarded). Pairwise comparisons using 5,000 bootstrap replications were used to build the tree. The 2 locust species are in bold. Further detailed information about the insect species used can be found in Table 1.

brain. Similar staining patterns were observed in adult male and female locusts, as well as in gregarious and solitary-reared animals and also in larvae. The brain area stained is known as the pars intercerebralis (PI) of the protocerebrum. It contains the largest collection of neurosecretory cells in the central nervous system of the locust, most of which have fibers that run directly to the corpora cardiaca (CC) via nervus corporis cardiaci I (NCCI, Burrows, 1996). Hence, in order to confirm the identity of the FOR-positive cells, neurons of the PI were back-filled through the NCCI nerve leading from the PI to the CC. As can be seen in Figure 4A, the area of the brain stained by

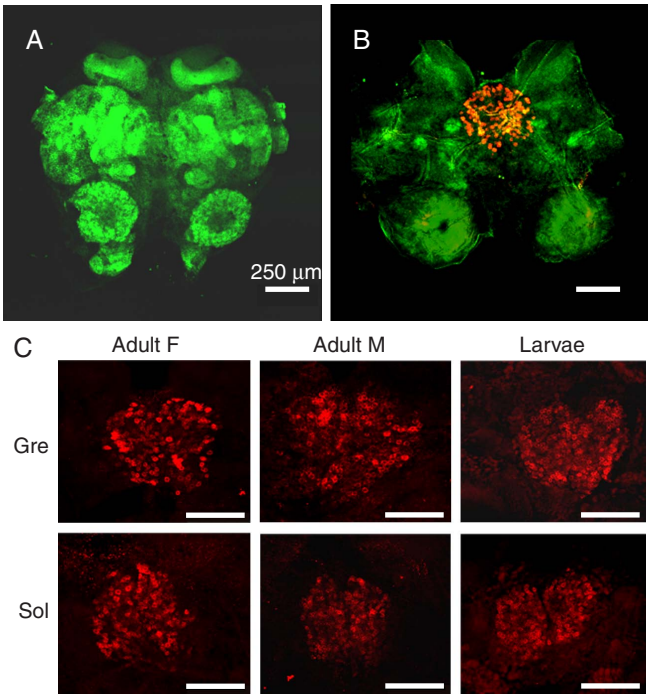


**Figure 3.** FOR expression patterns in the locust brain. **A:** Merged confocal image of frontal 3-µm optical sections of a locust brain showing the major brain neuropils (in green, mouse antibody, nc82). **B**: A distinct PKG-IR region is seen as a cluster of cell bodies in the anterior midline of the brain (double staining with anti-FOR in red and nc82 in green). **C:** Similar FOR expression patterns can be observed in gregarious (Gre) or solitary-reared animals (Sol), in adult male (M), female (F), and larvae. Scale bars = 250 µm.

backfilling NCCI was very similar to the area expressing FOR. Indeed, when we employed double staining of FOR together with neurobiotin backfilling of the NCCI, many cells within this area showed double labeling (Fig. 4B).

### *Phase-Related Differences in PKG Activity*

The PKG activity of gregarious locusts was found to be significantly higher than that of solitary locusts ($F_{(1,28)} = 13.29$, $P < 0.001$) (Fig. 5). Differences in PKG activity are sex specific ($F_{(1,28)} = 23.16$, $P < 0.001$), with higher PKG activity found in males than in females. No interaction was found between sex and phases ($F_{(1,28)} = 0.17$, $P = 0.68$). This sex difference in PKG activity was found for both solitary ($P < 0.01$) and gregarious locusts ($P < 0.001$).

## DISCUSSION

The *foraging* gene functions in plastic responses to environmental change in a variety of animals. In *D. melanogaster*, a previous history of food deprivation changes rovers into sitters and decreases PKG levels (Kaun et al., 2007b, 2008) and *for* plays a role in learning and memory in larval and adult flies (Kaun et al., 2007a; Mery et al., 2007). In *P. pallidula*, the plastic behavior of workers is related to PKG enzyme activity and worker ants who work as guards have five more FOR immunoreactive clusters of cells in their

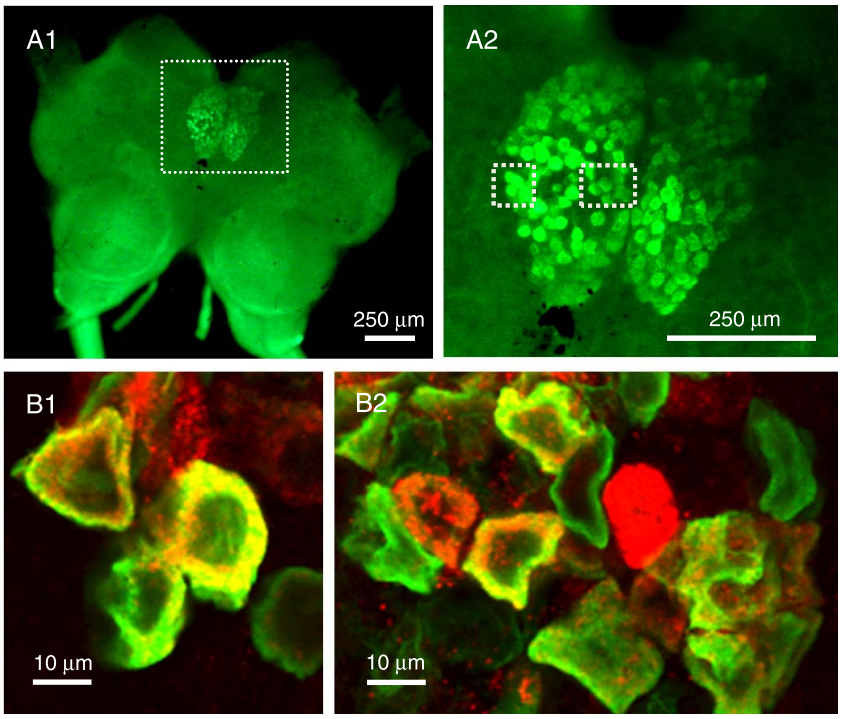


**Figure 4.** **A:** Merged confocal image of frontal 3-µm optical sections of a locust brain following neurobiotin backfilling from both left and right NCC1 nerves. The cell bodies stained are in the brain area known as the pars intercerebralis (PI) of the protocerebrum and largely overlap the area immunostained by anti-FOR (compare to Fig. 3). The area marked in A1 is enlarged in A2. Scale bars = 250 µm. **B:** Many cell bodies in this area were yellow after double staining with anti-FOR (red) and neurobiotin backfill (green). Cells shown in B correspond to the areas marked in A2. Scale bar = 10 µm.

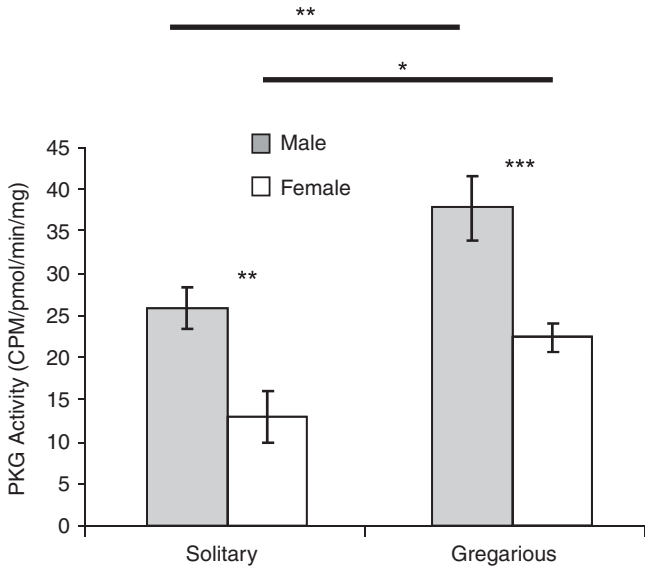


**Figure 5.** PKG activity (mean ± S.E.) of solitary ($n = 24$) and gregarious ($n = 20$), and male ($n = 18$) and female ($n = 26$) *S. gregaria*. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

brains than do their smaller-sized sister worker ants who work as foragers (Lucas and Sokolowski, 2009). Ants become either defenders or foragers depending on the food and social cues they receive as larvae (Passera and Suzzoni, 1991; Bloch et al., 2002). Defenders have higher PKG enzyme activity than foragers; however, defenders can exhibit flexibility in behavior and are able to engage in foraging activities, depending on the needs of the colony (Lucas et al., 2009). This plastic behavior is correlated to PKG enzyme activity and pharmacological manipulations of PKG activity change this behavioral plasticity (Lucas and Sokolowski, 2009). In *C. elegans*, PKG plays a role in olfactory adaptation as well as other plastic phenotypes (Fujiwara et al., 2002; L'Etoile et al., 2002; Hirose et al., 2003; Raizen et al., 2008). In the honey bee, *for* plays a role in the long-term plasticity changes involved in the switch from nursing to foraging (Ben-Shahar et al., 2002, 2003). No differences in spatial localization of the *for* transcripts were found in nurse and forager honey bees (Ben-Shahar et al., 2002) or in rover and sitter flies (Belay et al., 2007). Finally, PKG is known to function in learning and memory in mammals (Reaume and Sokolowski, 2009).

Density-dependent phase polyphenism in the desert locust is another example of plastic changes in response to environmental change. Previous pioneering work has provided some evidence for molecularly based phase differences (e.g., differences in the number and amount of peptides present in the corpora cardiaca and the hemolymph, Clynen et al., 2002; An unidentified solitary-specific gene and a SPARC-like gregarious-specific gene, Rahman et al., 2003). However, a full understanding of the molecular basis of this phenomenon is still lacking.

How might PKG act to affect locust behavior? Our findings of FOR expression in the brain PI cells, in close association with the major locust neurosecretory centers, opens multiple routes for PKG to affect locust physiology and behavior. In respect to locust phases, gregarious locusts are generally more active than solitary ones and show increased propensity for long-range marching or migratory flight (Pener and Simpson, 2009). This could be correlated to the higher PKG activity in gregarious locusts, similar to rover flies and forager honey bees. Rovers and sitters differ in levels of adipokinetic hormone and in acquisition, allocation, and storage of energy (Kaun et al., 2008). In locusts, in addition to the high PKG activity reported here, the gregarious phase is characterized by high levels of lipid reserves, higher hiperlipaemic response to flight, and increased adipokinetic response (mediated by CC neurohormones; Ayali and Pener, 1992, 1995; Ayali et al., 1996; Pener et al., 1997). PKG signaling also plays a role in modulating environmental stresses, such as thermal stress (in *D. melanogaster*, Dawson-Scully et al., 2007). Phase differences are also related to thermotolerance in locusts, including, for example, expression of heat shock proteins (Wang et al., 2007) and response to pathogens (Elliot et al., 2003, 2005). PKG is also involved in phototaxis behavior in bees (Ben-Shahar et al., 2003) and in circadian clock-related behaviors such as quiescence or sleep in both *C. elegans* and *D. melanogaster* (e.g., Raizen et al., 2008). In locusts, gregarious animals are characterized by diurnal flight behavior, while solitary locusts fly at night. Fuchs et al. (2003) describe an identified flight-related inhibitory interneuron, which is sensitive to illumination level, and show a remarkable and highly significant increase in activity during the dark but only when locusts are crowded locusts. Lastly, serotonin (5-hydroxtryptamine, 5HT) is both necessary and sufficient for locust phase transformation, with increasing levels of 5HT accompanying (and inducing) gregariousness (Anstey et al., 2009). A connection between the 5HT transporter (SERT), present in all animals from flatworm to human (Caveney et al., 2006) and

cGMP/PKG signaling is also known; activation of cGMP/PKG-linked pathways increases SERT activity and rapidly alters both 5HT uptake and clearance rates (e.g., Miller and Hoffman, 1994; Zhu et al., 2004a,b). Specifically, Zhang et al. (2007) reported that PKG phosphorylates human SERT (at Thr-276) and thus increases its activity. These reports are not fully consistent with our own results, as higher PKG activity in gregarious locusts compared to solitary ones, implies a down-regulation of 5HT in the gregarious phase (in contrast to Anstey et al., 2009). Reported differences in 5HT action and its regulation in vertebrates as compared to invertebrates (e.g., 5HT and aggression; Edwards and Kravitz, 1997), and even within the same animal depending on the social context, suggest that the relationships between PKG and 5HT require further study.

Our study lays a foundation for investigations into the functional role of the *for* gene in locust behavior, and specifically density-dependent phase polyphenism. A comparison of studies across species confirms a general role for the *foraging* gene and the PKG molecule in behavioral plasticity with an emphasis on food-related behaviors. Interestingly, the phylogeny suggests that species within a cluster with their closely related protein sequences can have an inverse relationship between *foraging* gene levels and behavior. Further studies are needed to understand the species-specific mechanisms underlying the *foraging* gene's function in suites of plastic behaviours.

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# Expression of *foraging* and *Gp-9* are associated with social organization in the fire ant *Solenopsis invicta*

**C. Lucas*†, M. Nicolas*‡ and L. Keller***

**Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland; †Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS, University of Tours, Tours, France; and ‡Department of Plants Molecular Biology, Centro Nacional de Biotecnologia, Madrid, Spain*

## Abstract

**The aim of this study was to investigate levels of expression of two major genes, the odorant binding protein *Gp-9* (*general protein-9*) and *foraging*, that have been shown to be associated with behavioural polymorphisms in ants. We analysed workers and young nonreproductive queens collected from nests of the monogyne (single reproductive queen per nest) and polygyne (multiple reproductive queens) social forms of *Solenopsis invicta*. In workers but not young queens, the level of *foraging* expression was significantly associated with social form and the task performed (ie localization in the nest or foraging area). The level of expression of *Gp-9* was also associated with social form and worker localization. In addition there was a higher level of expression of the $Gp\text{-}9^{b}$ allele compared with the $Gp\text{-}9^{B}$ allele in the heterozygote workers and the young nonreproductive queens. Finally, in the polygyne colonies the level of expression of *foraging* was not significantly associated with the *Gp-9* genotype for either workers or young nonreproductive queens, suggesting that both genes have independent non-epistatic effects on behaviour in *S. invicta*.**

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Correspondence: Christophe Lucas, Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS, University of Tours, Parc de Grandmont, 37200 Tours, France. Tel.: + 33 247 367 372; fax: + 33 247 366 966; e-mail: christophe.lucas@univ-tours.fr
Laurent Keller, Department of Ecology and Evolution, University of Lausanne, Biophore, Unil-Sorge, 1015 Lausanne, Switzerland. E-mail: laurent.keller@unil.ch

**Keywords: social organization, gene expression, *foraging*, *Gp-9*, *Solenopsis invicta*, behaviour.**

## Introduction

Explaining how interactions between genes and environment influence social behaviour is a fundamental research goal, yet there is still only limited information for species exhibiting complex social behaviour (Linksvayer & Wade, 2005; Robinson *et al.*, 2005; Linksvayer, 2006; Nedelcu & Michod, 2006). For example, in ants, which have played a central role in the study of the mechanisms regulating social behaviour, two major genes have been shown to be associated with behavioural polymorphisms.

The first example was discovered in the fire ant *Solenopsis invicta*, in which a genomic region containing the odorant-binding protein (OBP) *Gp-9* (*general protein-9*) determines whether colonies contain one queen (monogyne social form) or several queens (polygyne social form) (Ross, 1997; Ross & Keller, 1998; Krieger & Ross, 2002; Gotzek & Ross, 2007). Recently, *Gp-9* was found to be located on a large (13.8 Mb, 55% of the chromosome) genomic region that does not recombine between the two variants [ie the social b (Sb) chromosome contains the $Gp\text{-}9^{b}$ allele and the social B (SB) chromosome contains the $Gp\text{-}9^{B}$ allele (Wang *et al.*, 2013)]. Monogyne colonies invariably contain one SB/SB queen and only SB/SB workers. By contrast, polygyne colonies contain several SB/Sb queens and both SB/SB and SB/Sb workers. This very unusual genotypic distribution stems from the Sb chromosome containing one or more lethal recessive genes, leading to the death of all females (ie queens and workers) with two copies of this chromosome soon after they hatch from the pupae (Ross, 1997; Hallar *et al.*, 2007). Moreover, there is a complete lack of SB/SB queens in polygyne colonies because the Sb chromosome behaves as a selfish genetic element by inducing workers with one copy of this chromosome to eliminate all the reproductive queens lacking a copy of

this chromosome (ie SB/SB queens) when they initiate reproduction (Keller & Ross, 1998). The nonrecombining region on the pair of social chromosomes contains slightly more than 600 genes. It is likely that several of these genes in the nonrecombining region are implicated in the many other traits [eg queen fecundity, queen tendency to accumulate fat during sexual maturation, the odour of mature queens, the quantity of sperm produced by males and worker size (Keller & Ross, 1998; Goodisman *et al*., 1999; DeHeer, 2002; Ross & Keller, 2002; Vander Meer & Alonso, 2002; Krieger, 2005; Lawson *et al*., 2012)] that differentiate the two social forms.

The other identified genetic component influencing behaviour in ants is the *foraging* gene (*for*). This gene was first described in *Drosophila melanogaster*, in which allelic variations result in differences in the level of foraging behaviour of larvae (Sokolowski, 1980; de Belle & Sokolowski, 1987; de Belle *et al*., 1989). The *for* gene, also known as *dg2*, encodes a cyclic guanosine monophosphate (cGMP)-dependent kinase (PKG) expressed from the *dg2* locus (Osborne *et al*., 1997) and affects hundreds of downstream genes (Kent *et al*., 2009). Genetic manipulations have shown that behavioural polymorphism is related to allelic variation at the *for* locus in *Drosophila melanogaster* (Osborne *et al*., 1997). The *for* gene is also associated with behavioural polymorphisms in food related behaviours in the worm *Caenorhabditis elegans* (Fujiwara *et al*., 2002) and swarming polymorphism in the locust *Schistocerca gregaria* (Lucas *et al*., 2010b). In social insects, variation in the level of expression of *for* regulates temporal division of labour in the honey bee *Apis mellifera* and the ant *Pogonomyrmex barbatus* (Ben-Shahar *et al*., 2002; Ingram *et al*., 2005), as well as caste polyethism and aggressive behaviour in the ant *Pheidole pallidula* (Lucas & Sokolowski, 2009). Thus, the *for*-PKG molecule is emerging as a major player in regulating behavioural polyethism and the observed effect in a wide variety of organisms suggests the possibility that the *for* gene is a general behavioural modifier in the animal kingdom (Sokolowski, 2010; Székely *et al*., 2010; Lucas *et al*., 2010a).

The aim of this study was to investigate the interplay between the level of expression of the *for* gene and the presence or absence of the Sb chromosome in queens and workers of the fire ant *S. invicta*. To this end we collected young winged queens that had not yet departed on a mating flight as well as workers both within the nest and in the foraging area. Monogyne and polygyne colonies were both used and reared under laboratory competition to address four main questions.

First, we compared the level of expression of *for* between SB/SB and SB/Sb young winged queens that had not yet initiated reproduction (gynes). Previous work has shown that SB/SB gynes produced by monogyne colonies disperse on the wing to initiate a new colony by feeding the first brood produced from their body reserves (DeHeer, 2002). By contrast, SB/Sb gynes from polygyne colonies disperse much shorter distances and usually infiltrate an existing polygyne colony immediately after the mating flight (Porter *et al*., 1991; Gotzek & Ross, 2007). Given that the level of *for* expression and *for*-PKG activity have been shown to differ in the brains of singly vs. multiply mated queens (Richard *et al*., 2007) and affect search trajectories, foraging strategies, energy homeostasis and lipid storage in *Drosophila* (Sokolowski, 2001; Kaun & Sokolowski, 2009), as well as behavioural polymorphisms in ants (Lucas & Sokolowski, 2009; Lucas *et al*., 2010a), we tested whether the differential tendency of SB/SB and SB/Sb queens to disperse was associated with differences in the level of expression of *for*.

Second, we compared the level of expression of *for* between SB/SB and SB/Sb workers from the nest and the foraging area. In the honey bee *Apis mellifera* and in the ant *P. barbatus*, *for* gene function is not only linked to food search behaviour but also to temporal polyethism (Ben-Shahar *et al*., 2002; Ingram *et al*., 2005). In these species, inside-nest workers involved in tasks such as nest cleaning and brood care have different *for* gene expression levels than workers involved in foraging. We therefore hypothesized that there might be a difference in *for* expression between *S. invicta* workers collected inside and outside the nest as they exhibit marked differences in behaviour. To test this we compared the *for* expression of SB/SB and SB/Sb workers collected in the nest and from the foraging area.

Third, we tested whether there was an association between the level of expression of *Gp-9* and the *Gp-9* genotype. *Gp-9* is not expressed in the larval or pupae stages and is undetectable in newly emerged workers and queens. It becomes abundant in females once they are more than 8–14 days old (Ross, 1997; Liu & Zhang, 2004). Despite the proposed role of *Gp-9* in social organization, there is only limited information on the level of expression of this gene in monogyne and polygyne queens and workers (Gotzek & Ross, 2007). Two microarray studies failed to detect variations in expression between SB/SB and SB/Sb workers (Wang *et al*., 2008; Nipitwattanaphon *et al*., 2013), but these studies were not specifically designed for this purpose and there is currently no information for gynes of alternate genotypes. We therefore compared the level of expression of *Gp-9* between SB/SB and SB/Sb queens as well as SB/SB and SB/Sb workers. Moreover, we compared the allelic-specific gene expression of $Gp\text{-}9^{B}$ and $Gp\text{-}9^{b}$ in both SB/Sb queens and workers.

Finally, we investigated whether there were differences in the proportion of SB/SB and SB/Sb workers and gynes

in the nest and in the foraging area in polygyne colonies. Previous studies have shown differences between the expected and observed proportions of SB/SB and SB/Sb adults (Ross, 1997; Goodisman *et al.*, 2000; Fritz *et al.*, 2006). For example, in colonies headed by a SB/Sb queen fertilized by a single SB male, there were significant deviations from the expected proportion of 50% SB/SB and 50% SB/Sb workers. However, these studies sampled individuals only from inside the nest, raising the possibility that discrepancies between the expected and observed proportions arose from a nonrandom distribution of workers for each genotype between the nest and foraging area. We therefore compared, for both workers and gynes, the *Gp-9* genotype frequencies between individuals collected in the nest and individuals collected in the foraging area.

## Results

### foraging *gene expression*

In polygyne colonies, the level of expression of *for* was not significantly associated with the *Gp-9* genotype for workers ($F_{1,126} = 1.19$, $P = 0.28$) and gynes ($F_{1,98} = 0.82$, $P = 0.37$; Fig. 1). Gene expression data from polygyne SB/SB and SB/Sb individuals were therefore pooled together in later analyses. By contrast, the level of expression of *for* was associated with social form, the level of expression being higher in monogyne than polygyne workers ($F_{1,12} = 5.16$, $P < 0.05$) and lower in monogyne than polygyne gynes ($F_{1,9} = 6.32$, $P < 0.05$; Fig. 1).

In colonies of both social forms, there was a large difference in the level of *for* expression between workers

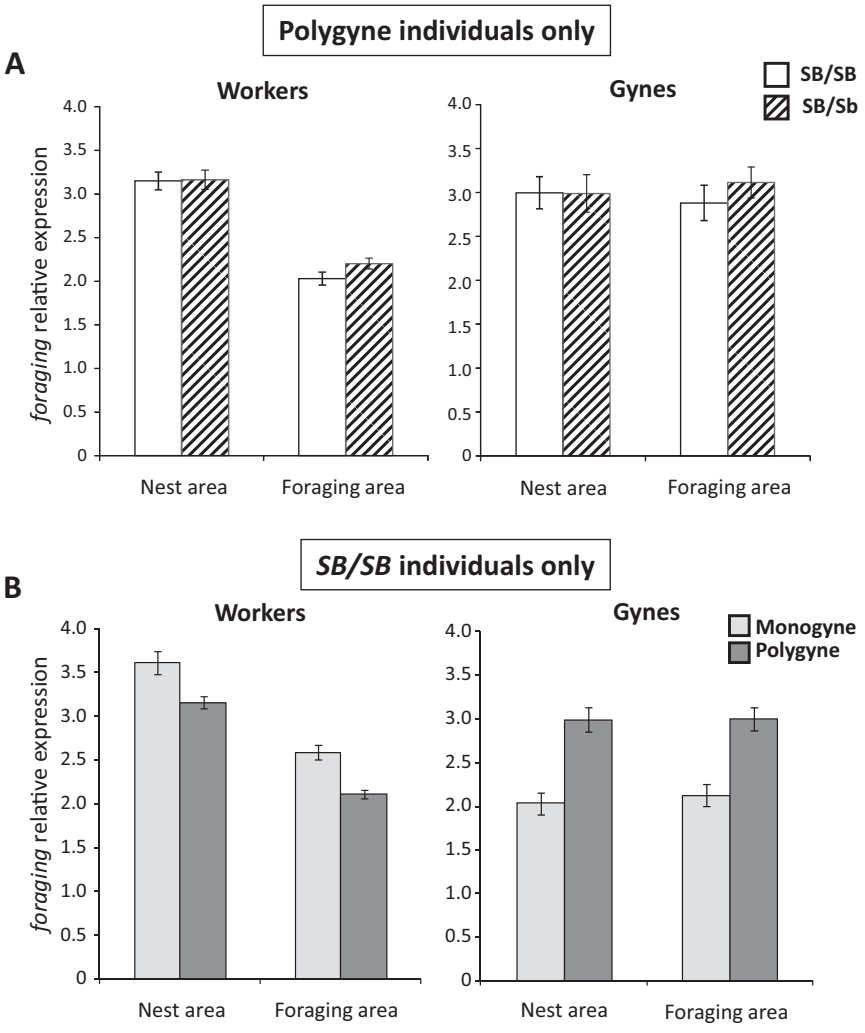


**Figure 1.** Relative expression of the *foraging* gene. (A) Polygyne individuals only [statistics for workers (social form: NS; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: NS; location: NS; social form × location: NS)]. (B) SB/SB individuals only [statistics for workers (social form: $P < 0.05$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: $P < 0.05$; location: NS; social form × location: NS)]. SB, social B chromosome; Sb, social b chromosome.

collected from the nest and workers collected from the foraging area ($F_{1,175} = 216.21$, $P < 0.001$) but no significant interaction between location and social form ($F_{1,175} = 0.001$, $P = 0.97$). The level of expression was always higher in workers from the nest than those from the foraging area. By contrast, there was no significant association between the location where gynes were collected and the level of expression of *for* ($F_{1,133} = 0.13$, $P = 0.72$).

### Gp-9 *gene expression*

In polygyne colonies, there was a significant association between the *Gp-9* genotype and level of expression of *Gp-9*. The level of expression was higher for *Gp-9$^{Bb}$* than *Gp-9$^{BB}$* individuals for both workers ($F_{1,126} = 33.17$, $P < 0.001$) and gynes ($F_{1,98} = 12.90$, $P < 0.001$; Fig. 2A). There was also an effect of the location of workers ($F_{1,126} = 50.85$, $P < 0.001$). For workers of both genotypes and both social forms the level of expression was greater in individuals from the nest compared with individuals outside the nest (Fig. 2A, B). When considering SB/SB workers only (Fig. 2B), the level of expression was higher in monogyne than polygyne individuals ($F_{1,12} = 8.40$, $P = 0.013$). An analysis of SB/Sb individuals (Fig. 2C) revealed a higher level of expression of the *Gp-9$^{b}$* than the *Gp-9$^{B}$* allele in both workers ($F_{1,121} = 9.59$, $P = 0.002$) and gynes ($F_{1,95} = 12.82$, $P < 0.001$).

### *Area repartitions of polygyne individuals*

The meta-analyses of the *P*-values (Pearson's Chi$^2$ tests) revealed that the spatial distribution of workers was different from random with SB/SB individuals being more common in the foraging area than inside the nest ($P = 0.002$; Fig. 3, Table 1). By contrast, there was no such effect for gynes ($P = 0.106$; Table 1).

## Discussion

### foraging *gene expression in workers and gynes*

This study revealed that the level of expression of *for* is lower in fire ant workers collected from the foraging area than individuals collected inside the nest. This finding is in line with the finding that *for* expression is lower in foragers than nurses in the ant *P. barbatus* (Ingram *et al*., 2005) and that pharmacological activation of the cGMP-dependent protein kinase encoded by *for* reduces foraging behaviour in the ant *P. pallidula* (Lucas & Sokolowski, 2009). Interestingly, these results in ants differ from the pattern observed in honey bees (Ben-Shahar *et al*., 2002) and bumble bees (Tobback *et al*., 2011), in which foragers have a higher expression of *for* than nurses. In nonsocial insects, contrasting results have also been reported, with *for* expression being up-regulated with foraging behaviour in *D. melanogaster* or *Schistocerca gregaria*, but down-regulated in *C. elegans* (Osborne *et al*., 1997; Fujiwara *et al*., 2002; Lucas *et al*., 2010b). In *D. melanogaster*, the precise DNA polymorphism responsible for rover/sitter behavioural polymorphism is still not known (Reaume *et al*., 2011). In social insects, in all seven ant species sequenced as well as *A. mellifera*, there is only one-to-one orthologue for the *for* gene (unpublished part of the fourmidable database, Wurm *et al*., 2009). In *S. invicta*, the *for* gene is located on the linkage group LG1, which is not on the social chromosome (Wurm *et al*., 2011) and no information is available on sequence polymorphisms.

Our data also revealed that the expression of *for* is lower in workers from polygyne colonies than those from monogyne colonies. This finding is interesting with regards to the difference in the level of aggression between the two types of workers. Several experiments have shown that monogyne workers are more aggressive than polygyne workers (Chirino *et al*., 2012), whereas studies in the ant *P. pallidula* found that the soldiers, which are involved in colony defence, have higher *for*-PKG activity than workers less involved in colony defence. Moreover, pharmacological treatments using a specific activator of *for*-PKG did increase aggressive behaviour (Lucas & Sokolowski, 2009), raising the possibility that differences in the expression of *for* may mediate the differences in aggressiveness between monogyne and polygyne fire ant workers. By contrast, the opposite pattern was observed in gynes in which *for* expression was lower for monogyne individuals than polygyne individuals. This social form effect on *for* expression may be correlated to the higher capacity to disperse of the monogyne gynes (DeHeer, 2002). However, because there is no genotypic effect, more experiments are needed to determine whether the level of *for* expression has effects on patterns of dispersal. Importantly, the difference in expression of *for* in gynes and workers of the two social forms is unlikely to be explained by genotypic differences because, in polygyne colonies, there was no significant difference in the level of expression of *for* between SB/SB and SB/Sb individuals (both for workers and gynes).

### Gp-9 *gene expression in workers and gynes*

Our analyses also revealed that the expression of *Gp-9* is differentially regulated between monogyne and polygyne workers. When considering only SB/SB workers, there was a higher level of expression in monogyne than polygyne individuals. By contrast, a previous study performed on whole bodies showed no significant difference between the two social forms (Wang *et al*., 2008). A possible explanation for this discrepancy lies in the type of tissues analysed, as we used only brains in this study. Nevertheless, consistent with Wang *et al*. (2008), we

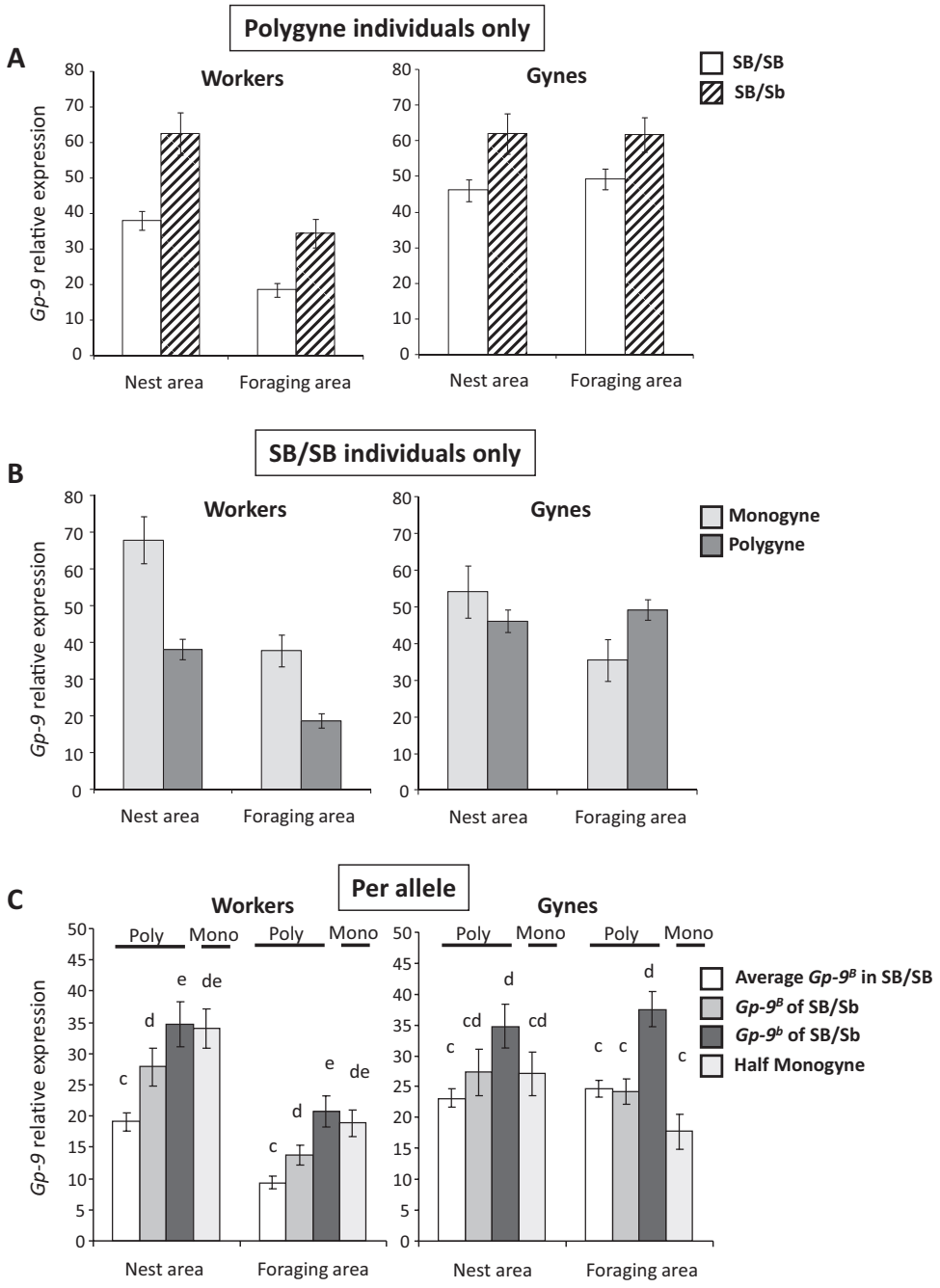


**Figure 2.** Relative expression of *Gp-9*. (A) Polygyne individuals only [statistics for workers (social form: $P < 0.001$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: $P < 0.001$; location: NS; social form × location: NS)]. (B) S*B/SB* individuals only [statistics for workers (social form: $P < 0.05$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: NS; location: NS; social form × location: $P < 0.01$)]. (C) Per allele [significant differences between pairwise tests ($P < 0.05$) within the same location are represented with different letters]. Statistical comparisons between locations are not shown but each genotype comparison was significant for workers ($P < 0.05$) and only one was significant for gynes (half monogyne vs. *Gp-9*$^{B}$ allele, $P < 0.01$). SB, social B; Sb, social b.

found that the level of *Gp-9* expression was associated with the genotype, with higher expression in SB/Sb than SB/SB workers. For gynes, the expression of *Gp-9* was also higher in SB/Sb than SB/SB individuals. This finding also contrasts with a previous study that showed no differences in the level of expression of *Gp-9* between SB/Sb and SB/SB queens within the age classes investigated (1-day-old, 11-day-old and reproductive queens; Nipitwattanaphon *et al.*, 2013). There are two possible explanations for this discrepancy. The first again relates to

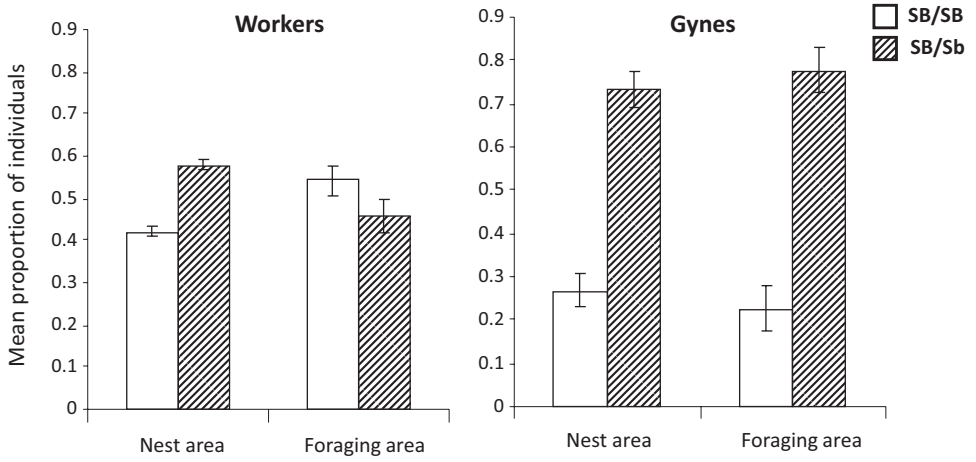


**Figure 3.** Mean proportion (± SE) of polygyne individuals in each location according to their *Gp-9* genotypes. Worker values were calculated from eight colonies with 166 to 189 individuals per colony; gyne values were calculated from six colonies with 107 to 154 individuals per colony. See detailed statistical results in Table 1. SB, social B; Sb, social b.

the type of tissue analysed (brains for this study vs. whole bodies for previous studies). Alternatively, it could also be that the difference observed in our study reflects age differences between the gynes analysed. The level of expression of *Gp-9* seems to be age-related in queens, as suggested by microarray data showing that the level of expression is highest in reproductive queens, intermediate in 11-day-old gynes and the lowest in 1-day-old gynes (Nipitwattanaphon *et al*., 2013). Thus, the difference between genotypes in our study could possibly have arisen if the SB/Sb gynes were older than the SB/SB gynes (although there is no reason for such a bias to occur in our design). Finally, it is important to note that the difference in Gp-9 expression observed in this study was quite small and less likely to be detected with a cDNA-probes microarray approach [as used in the studies of Wang *et al*. (2008) and Nipitwattanaphon *et al*. (2013)] than a quantitative PCR (qPCR) approach (used in this study), which is more sensitive (see review, Chuaqui *et al*., 2002).

*Gp-9* has been shown to encode for an OBP (Krieger & Ross, 2002; Gotzek & Ross, 2007). OBPs play a role in insect chemoreception by transporting hydrophobic odorant molecules to the odorant receptors of the sensory neurones (Blomquist & Vogt, 2003). Thereby, variations in OBP expression may interfere with pheromone

**Table 1.** Total number of polygyne individuals in each location according to their *Gp-9* genotypes. The column 'SB/SB proportions' represents the proportions of individuals in the nest or foraging area. Positive correlation values are in bold as well as significant *P*-values. The *P*-values were calculated with Pearson's Chi$^2$ test (meta-analysis *P*-values: workers $P = 0.002$; gynes $P = 0.106$)

| | | Nest area | | | Foraging area | | | SB/SB proportions | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Caste | Colony | SB/SB | SB/Sb | Total | SB/SB | SB/Sb | Total | Nest | Foraging | Chi$^2$ | *P*-value | r |
| Workers | 191Pg8 | 36 | 50 | 86 | 51 | 40 | 91 | 0.42 | 0.56 | 3.559 | 0.059 | **0.142** |
| Workers | 208Pg8 | 43 | 51 | 94 | 61 | 34 | 95 | 0.46 | 0.64 | 6.510 | **0.011** | **0.186** |
| Workers | 210Pg8 | 43 | 46 | 89 | 63 | 30 | 93 | 0.48 | 0.68 | 7.058 | **0.008** | **0.197** |
| Workers | 212Pg8 | 38 | 53 | 91 | 36 | 56 | 92 | 0.42 | 0.39 | 0.131 | 0.717 | –0.027 |
| Workers | 213Pg8 | 36 | 54 | 90 | 38 | 49 | 87 | 0.40 | 0.44 | 0.246 | 0.620 | **0.037** |
| Workers | 216Pg8 | 36 | 53 | 89 | 37 | 49 | 86 | 0.40 | 0.43 | 0.119 | 0.730 | **0.026** |
| Workers | 241Pg8 | 34 | 48 | 82 | 50 | 34 | 84 | 0.41 | 0.60 | 5.415 | **0.020** | **0.181** |
| Workers | 244Pg8 | 34 | 57 | 91 | 54 | 37 | 91 | 0.37 | 0.59 | 8.801 | **0.003** | **0.220** |
| Gynes | 197Pg8 | 25 | 63 | 88 | 15 | 51 | 66 | 0.28 | 0.23 | 0.633 | 0.426 | –0.064 |
| Gynes | 215Pg8 | 11 | 48 | 59 | 4 | 44 | 48 | 0.19 | 0.08 | 2.334 | 0.127 | –0.148 |
| Gynes | 218Pg8 | 23 | 63 | 86 | 14 | 70 | 84 | 0.27 | 0.17 | 2.534 | 0.111 | –0.122 |
| Gynes | 219Pg8 | 16 | 40 | 56 | 32 | 43 | 75 | 0.29 | 0.43 | 2.744 | 0.098 | **0.145** |
| Gynes | 222Pg8 | 14 | 81 | 95 | 16 | 34 | 50 | 0.15 | 0.32 | 5.950 | **0.015** | **0.203** |
| Gynes | 244Pg8 | 41 | 53 | 94 | 7 | 49 | 56 | 0.44 | 0.13 | 15.616 | **0.000** | –0.323 |

SB, social B chromosome; Sb, social b chromosome.

transduction but there is a complex functional mosaic of combinatorial recognition patterns between OBPs and odorant receptors, making straightforward interpretations difficult (Swarup *et al*., 2011). In insects, some OBPs are also expressed in nonchemosensory tissues such as the male accessory gland (Paesen & Happ, 1995), the abdomen (He *et al*., 2011), and the haemolymph (Graham *et al*., 2001; Paskewitz & Shi, 2005) raising question about what their function is (Vogt, 2005; Forêt & Maleszka, 2006; Gotzek & Ross, 2007). In female ants, OBPs are only present in small quantities in the antennae (Ishida *et al*., 2002; Ozaki *et al*., 2005) and it has been suggested that *Gp-9* might not be involved in pheromone detection within chemosensilla but may act as a transporter of pheromone into the haemolymph (Calvello *et al*., 2003; Pelosi *et al*., 2005; Gotzek & Ross, 2007). Regardless of the specificity of *Gp-9* compared with 'standard' OBPs, the regulation of colony queen number is clearly linked to specific chemical signals emanating from the queens (Keller & Ross, 1998; Ross & Keller, 1998). There has been considerable controversy over the role of *Gp-9* in mediating odour differences amongst individuals (Leal & Ishida, 2008; Gotzek & Ross, 2009). A recent study showed that *Gp-9* is in a large (*c*. 13.8 Mb), nonrecombining region, raising the possibility that one or several of the *c*. 600 linked genes may be involved in odour differences amongst individuals with alternate genotypes (Wang *et al*., 2013). Further support for the view that other genes may be involved in odour differences comes from microarray analyses showing that 18 genes involved in chemical signalling are differentially expressed between $Gp\text{-}9^{BB}$ and $Gp\text{-}9^{Bb}$ queens (Wang *et al*., 2008; Nipitwattanaphon *et al*., 2013). Thus, variations in *Gp-9* expression such as those observed in this study call for future experiments to disentangle the causes and consequences of the observed variations of *Gp-9* expression and their potential role in the regulation of colony queen numbers.

Interestingly, our study revealed higher expression of the $Gp\text{-}9^{b}$ allele than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes. The significance of the higher expression of the $Gp\text{-}9^{b}$ than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes remains unclear but may also be associated with the selective elimination of $Gp\text{-}9^{BB}$ queens by $Gp\text{-}9^{Bb}$ workers (Keller & Ross, 1998). The finding of higher expression of the $Gp\text{-}9^{b}$ than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes is interesting because previous work on sex chromosomes has revealed that genes in the Y or Z nonrecombining chromosome usually have lower levels of expression than their homologues on the X or W recombining chromosomes (Hahn & Lanzaro, 2005; Xiong *et al*., 2010; Deng *et al*., 2011; Larschan *et al*., 2011). An analysis of the level of expression of 288 genes in the nonrecombining region of SB/Sb queens revealed significant allele-specific expression differences for about 11% of the genes but there was no evidence for a pattern of higher expression of alleles on the SB haplotype compared with the Sb haplotype (Wang *et al*., 2013). The lack of a consistent difference in the level of expression of genes located on the SB and Sb chromosomes may reflect the fact that there has been only limited degeneration of the Sb chromosome, possibly because it recently evolved and also because there is selection at the haploid stage on Sb males (Correns, 1908; Bachtrog *et al*., 2011; Bergero & Charlesworth, 2011; Wang *et al*., 2013). It remains to be investigated whether the higher expression of the $Gp\text{-}9^{b}$ than $Gp\text{-}9^{B}$ allele is because of selection or just random changes in the level of expression as has been documented for the neo-Y chromosome in *Drosophila miranda* (Bachtrog, 2006). It may also imply differences in the underlying regulatory machinery amongst individuals of alternative genotypes or possible some genomic rearrangement in the nonrecombining region.

### *Area repartitions of polygyne individuals*

The *Gp-9* genotypic distribution of workers was found to be associated with their location in the colony. Although significant, the effect was small and not found in all colonies. In polygyne colonies, there was an overrepresentation of SB/SB workers in the foraging area compared with the nest. Given that $Gp\text{-}9^{b}$ is linked to more than 600 other genes in the nonrecombining region (Wang *et al*., 2013), it is currently not possible to determine why there is an association between genotype and spatial distribution. Previous work has shown that complex interactions between genetic backgrounds can affect division of labour through task efficiency (Oldroyd & Fewell, 2007; Libbrecht & Keller, 2013). It is thus possible that one or several genes in the nonrecombining region may affect worker propensity to move from one within-nest task to foraging. Those genes may also affect lifespan thus leading to different genotypic representation in the nest and outside. Whatever the mechanism, the finding of differential genotypic representation inside and outside the nest calls for special care when collecting individuals in studies aimed at determining behavioural and other phenotypic differences associated with genotype. In that respect it is notable that several previous studies showed discrepancies between the expected and observed $Gp\text{-}9^{BB}$ genotype proportions (Ross, 1997; Goodisman *et al*., 2000; Fritz *et al*., 2006). Detailed studies on the differential distribution of workers of alternative genotypes remain to be conducted to elucidate the reasons underlying these discrepancies between the expected and observed differences in genotypic distribution.

In contrast to workers, there was no significant association between genotype and location in the nest for gynes.

However, there was a low proportion of SB/SB gynes ($0.25 \pm 0.03$ mean proportion $\pm$ SE) amongst the gynes collected, similarly to previous studies (Ross, 1997; Goodisman *et al*., 2000). This low proportion reflects the fact that they are selectively eliminated by SB/Sb workers when they initiate reproduction in polygyne colonies (Keller & Ross, 1998, 1999).

### *Conclusions*

This study revealed that two genes associated with behavioural polymorphisms, *for* and *Gp-9*, are related to social organization in the fire ant *S. invicta*. The level of *for* and *Gp-9* expression was associated with social form and there was a large difference in the level of expression between workers collected from the nest and workers collected from the foraging area. It is likely that other factors such as worker size and age (foragers tend to be older than nurses) could also have an impact on expression of these genes and it would be interesting to investigate the exact relationship amongst all of these variables. In addition, there was also a higher level of expression of the $Gp\text{-}9^{b}$ allele compared with the $Gp\text{-}9^{B}$ allele for both workers and gynes. Finally, the level of expression of *for* was not significantly associated with the *Gp-9* genotype for either workers or gynes, suggesting that both genes have independent non-epistatic effects on behaviour in *S. invicta*.

## Experimental procedures

### *Colonies collection and rearing conditions*

Monogyne and polygyne colonies of *S. invicta* were collected near Athens (Georgia, USA) in April 2008 and reared under standard laboratory conditions to minimize environmental effects (Jouvenaz *et al*., 1977). Social forms were determined after collection by genotyping several individuals per nest and queens were morphologically checked (Ross & Keller, 1995; Ross, 1997). Colonies were acclimatized for 3 months in the laboratory before sampling. Ants were reared into a plaster nest localized in a plastic box covered with Teflon (Whitford LTD., Runcorn Cheshire, UK) on the top. Feeding occurred on the same days twice a week. Food and water were deposited only into a second plastic box connected with the first box by a 1-m plastic tube (outside surface was covered with Teflon). To get access to the food, ants had to get out of the plaster nest (nest area) and move through the tube from the first to the second box and reach the food in a cup (foraging area).

### *Sampling of individuals*

Individuals were sampled with a vacuum aspirator 2 days after feeding between 13:30 and 17:00 h in order to minimize variations in gene expression. To avoid DNA/RNA sample bias owing to size or age, we collected only winged queens and medium-sized workers with dark cuticles to avoid very young individuals, which are yellowish. The first group of individuals was collected in the plaster nest near the brood and another group in the foraging area near the food cup. All individuals collected in the foraging arena were foragers whereas the vast majority of individuals collected near the brood were nurses [as for other ants (Hölldobler & Wilson, 1990)]. There is a clear spatial separation between nurses and foragers in *S. invicta* and, like other social insects that have been studied (Mersch *et al*., 2013), foragers tend to be older than nurses (Mirenda & Vinson, 1981). All collected individuals were immediately anaesthetized with $CO_2$ and flash-frozen in liquid nitrogen. Ants were genotyped using DNA from the body (thorax and abdomen) and qPCR analyses were carried out using heads only. To do so, heads were removed from the body using a scalpel blade on a cooled sterile pod. To avoid DNA and RNA contamination, blades and forceps were cleaned in ethanol then flamed and cooled down in liquid nitrogen for the dissection of each individual. Bodies and heads were then flash-frozen in liquid nitrogen and stored at –80 °C until extraction.

**Table 2.** Primer sequences [final concentrations: 500 nM for genotyping and pyrosequencing; 200 nM for quantitative PCR (qPCR)]

| Name | Sequence 5′–3′ | Experiment |
|---|---|---|
| GP9CLF2 | TCT CGA TTG GTG AAG TAT CAA GT | Genotyping |
| GP9CLR2 | CAT GTC AAT ACA AAA GAA AGC TG | Genotyping |
| GP9CLF1 | GAT ACC GAA CTA CAC AAA AAT GGT TGC | Genotyping |
| GP9CLR1 | TTA GAA TCG GCG AGC ACA GCT T | Genotyping |
| Eef1a1_F2 | CAG CCG ATG TAG CTC ACC CT | qPCR |
| Eef1a1_R2 | TGT CAA ATT CGT CTC CCG TG | qPCR |
| qGAPDHP1_F1 | GCT ACA CCG AGG ACG AAG TTG | qPCR |
| qGAPDHP1_R1 | CCA CGA GAT CAG TTT CAC GAA G | qPCR |
| RPS-3_F3 | GTG ACG ATC TTT CGG CAT GG | qPCR |
| RPS-3_R3 | TTG GAG AAG ACC GAC GGA AT | qPCR |
| qforP1_F1 | CTG CCC GAG GAA ACT CTA ATC | qPCR |
| qforP1_R1 | CAC CTC TCG CTC CTT GTC TTA T | qPCR |
| GP9-41F | TGG CTT TCG CTT CTG CAT C | qPCR |
| GP9-141R | GTC ATC CTC TGT TAG ACT ATG TTC GG | qPCR |
| GP9SNIP_F1 | AGG ATC CCA ATA TGA CAA TTA CGC | Pyrosequencing |
| GP9SNIP_R1 | Biotinilated-TTT TGT GCT GGC CAC TTG ATA CT | Pyrosequencing |
| GP9SNIP_S1 | TAC GCG ACT TGC TTA | Pyrosequencing |

### Gp-9 *genotyping*

Bodies (thorax and abdomen) were ground by using 3 mm Tungsten Carbide Beads (Qiagen, Hombrechtikon, Switzerland) in a TissueLyser (Qiagen). DNA was extracted using a DNeasy 96 Blood & Tissue Kit with the Biosprint 96 extraction robot following the manufacturer's protocol (extraction of animal tissues, Qiagen). DNA was suspended in 150 µl Milli-Q water (Millipore, Billerica, MA, USA) after extraction and then concentrated to 10 µl with a Vacufuge speedvac (Eppendorf, Montesson, France). The *Gp-9* gene was amplified using a nested PCR amplification method. Two µl of DNA were amplified by a first PCR reaction with primers GP9CLF2 and R2 (Table 2) with 0.5 units of Taq DNA polymerase (Qiagen, 201203) in 20 µl PCR mix [94 °C, 5 min; 30× (94 °C, 30 s; 57 °C, 30 s; 72 °C, 45 s); 72 °C, 10 min; 4 °C until stored). Two µl of the first PCR were used for the second nested PCR amplification using primers GP9CLF1 and R1 with 0.5 units of Taq polymerase in 20 µl mix [94 °C, 5 min; 35× (94 °C, 30 s; 64 °C, 30 s; 72 °C, 45 s); 72 °C, 10 min; 4 °C until stored].

The *Gp-9* genotype was determined by performing two independent allele-specific restriction fragment length polymorphism

analyses using two different enzymes specific to either *GP-9$^B$* and *GP-9$^b$* allele (on amino acid position 117). First the *Gp-9$^B$* allele band was cut by the *Bsm*I restriction enzyme (New England BioLabs, Beverly, MA, USA; R0134L), which gave rise to a 470-bp and a 298-bp product (this enzyme does not cut the *b* allele). Three μl of PCR products were used in a total mix of 15 μl using 10 units of *Bsm*I following the conditions given by the manufacturer. For additional verification, we used the *Bve*I (*Bsp*MI) restriction enzyme (New England Biolabs, R0502L). This enzyme only cuts the *Gp-9$^b$* allele, giving rise to a 481-bp and a 289-bp product. Three μl of PCR products were used in a total mix of 15 μl using 1 unit of *Bve*I. Digestion products were then electrophoresed in a 2% TAE (Tris-acetate-EDTA buffer) agarose gel and analysed for band patterns.

### *RNA extraction*

Pools of five worker heads with the same genotype, or individual head for gynes, were ground using 1.4 mm zirconium silicate beads (QuackenBush, Crystal Lake, IL, USA) in a FastPrep24 tissue lyser (MP, 116004500) at 4 °C for two periods of 60 s with a 30-s gap. Between three and five samples were processed per colony. *Gp-9$^{bb}$* individuals were too rare to permit meaningful analyses and were thus discarded from the study. RNAs were extracted using the RNeasy Mini kit (Qiagen, 74106) with DNase treatment then eluted in 50 μl Milli-Q water (Millipore). Samples were concentrated with a Vacufuge speedvac (Eppendorf), resuspended in 11 μl of Milli-Q water and stored at −80 °C. Owing to the low RNA concentration, a Quant-iT RiboGreen kit (Invitrogen, Carlsbad, CA, USA; R11490) was used for quantification using 1 μl of each sample. Three technical replicates were measured using a black-coloured F96 MicroWell plate (Nunc, Thermo Fisher Scientific, Villebon sur Yevette, France; 137103) in a SpectraFluor Plus fluorometer (Tecan). RNA quality was controlled using an Agilent RNA 6000 Pico Kit (Agilent, Palo Alto, CA, USA; 5067-1513) with a Pico Chip in a 2100 Bioanalyser (Agilent).

### *cDNA synthesis and qPCR*

The same cDNA samples were used for qPCR analyses of *Gp-9* and of *for*. Worker and gyne samples were treated separately in two independent experiments but under the same conditions. Six monogyne and eight polygyne colonies were used for worker analyses; four monogyne and six polygyne colonies for gyne analyses. cDNA synthesis was performed using 56.5 ng of total RNA for all samples with DNA polymerase Superscript III (Invitrogen, 12574-018), random hexamers (Invitrogen 48190-011) and RNasin Plus RNase Inhibitor (Promega, Madison, WI, USA; N2611). Reverse tanscriptase reactions were diluted 10 times before performing quantitative expression analyses (qPCR) with an ABI 7900 thermocycler (Applied Biosystems, Foster City, CA, USA) at the DNA array facility of Lausanne (DAFL). Pipetting was carried out with a robot (Tecan) on a 384-well plate. Amplification was monitored by Power SYBR Green detection (Applied Biosystems, 4309155). Three technical replicates were performed per sample and a unique reference sample was used for all plates to ensure direct normalization and comparison between plates. cDNA of the reference sample was synthesized from 950 ng of RNA, extracted from 100 whole body monogyne workers. It was then conserved in aliquots at −80 °C and the stability (relative abundance) was validated by qPCR. Specific primers for both genes of interest and for the reference genes were designed with PRIMER3 software (Rozen & Skaletsky, 2000) using the fourmidable database (Wurm *et al*., 2009; Table 2). *Gp-9* primers were designed from a consensus sequence of all *GP-9* alleles (*B* or *b*). The *for* gene sequence is available with the accession number KJ874960 (GenBank). The three reference genes used to normalize expression [eukaryotic translation elongation factor 1A (EeF1A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S3 (RPS-3)] underwent preliminary validation by checking their dissociation curves and using sample dilutions. We also tested the efficiency and specificity of the primers (efficiency tests showed a dissociation curve with only one peak).

### *Pyrosequencing*

Because of the genotype effects within polygyne individuals we sequenced *Gp-9$^{Bb}$* individuals using pyrosequencing with the samples used for qPCR and estimated the relative expression of the *Gp-9$^B$* or *Gp-9$^b$* alleles. Single nucleotide polymorphism (SNP) analyses were performed on a PSQ96 machine (Qiagen) with specific primers designed with PSQ ASSAY DESIGN software from Qiagen (Table 2). Primers amplified two SNPs (amino acid positions 39 and 42) specific for each *Gp-9* allele. The use of two SNPs allow to perform a double control. The primer GP9SNIP_S1 was used for sequencing purposes. Two μl of DNA was amplified by PCR reaction with the primers GP9SNIP_F1 and R1 [94 °C, 5 min; 45× (94 °C, 15 s; 57 °C, 30 s; 72 °C, 15 s); 72 °C, 5 min; 4 °C until stored] with 1.5 units of Taq DNA polymerase (Qiagen) in 50 μl PCR mix.

### *Statistical analyses*

Relative expression of target genes were calculated using ΔΔCt with QBASE software v. 1.3.5 (Hellemans *et al*., 2007). Normalized gene expression means were tested using generalized linear mixed-effect models (GLMMs) within workers and gynes with JMP 7 software (SAS Institute, Cary, NC, USA). Social forms and locations were processed as fixed factors and colony of origin as a random factor. Pairwise comparisons were carried out with GLMMs with colony as a random effect and the normality of residuals was checked (Bolker *et al*., 2009).

We used a meta-analysis method to test whether there was an effect of genotype on the repartition of individuals in the nest vs. foraging area in polygyne colonies. The number of individuals of each genotype in each area was counted, transformed into proportions and used as a response variable (repartition) in a global analysis (Rosenthal, 1991; Rosenberg *et al*., 2000). Repartition effect is a correlation coefficient (r) that gives an estimate of how individuals are distributed between the two areas depending on their genotypes. We defined a positive effect when SB/SB individuals were more present in the foraging area than the nest area and a negative effect when they were more present in the nest area than the foraging area. The *P*-values were calculated with Pearson's Chi$^2$ test using R 2.7 software (http://www.r-project.org/).

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# Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*

C. Lucas [a,*], D.B. Pho [a], D. Fresneau [b], J.M. Jallon [a]

[a] *Mécanismes de communication, Université Paris Sud, CNRS-UMR 8620, 91405 Orsay Cedex, France*
[b] *LEEC, Université Paris Nord, CNRS-UPRESA n° 7025, F-93430 Villetaneuse, France*

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**Abstract**

In ants, both cuticular and postpharyngeal gland (PPG) hydrocarbons (HCs) have been involved in nestmate recognition. However, no detailed comparison is available. A comparative study including also high density lipophorin (HDLp), an internal HC carrier, was therefore undertaken on *Pachycondyla villosa*. Purified HDLp is an 820 kDa lipoprotein with a density of 1.114 g/ml and two 245 and 80 kDa apo-proteins. Its hydrocarbon profile is very similar with the cuticular one, in agreement with its hydrocarbon carrier function. Conversely, *n*-alkanes and externally branched monomethylalkanes are markedly decreased in the PPG. According to their physical properties, this suggests that they are involved in waterproofing on the cuticle. The PPG actually contains only internally branched mono-, dimethylalkanes or monomethylalkenes; their greater fluidity is more adequate for chemical communication. The percentages of some of them are statistically not different between the cuticle and PPG. Their mixtures vary with colonies and they may thus be involved in colonial signature. A scheme for hydrocarbon circulation is discussed, involving lipophorin, cuticle, PPG and self-grooming in one individual, a pathway complementary or alternative to the selective delivery by lipophorin in some other insects. HCs are then distributed between nestmates' cuticles through allo-grooming and physical contacts.


*Keywords:* Cuticle; Lipophorin; Postpharyngeal gland; Hydrocarbons; Ants' nestmate recognition

## 1. Introduction

Insect hydrocarbons (HCs) play important roles in waterproofing and chemical communication. They make up an effective barrier against desiccation due to their hydrophobicity and organization on the cuticle (Noble-Nesbitt, 1991; Gibbs, 1998; Rouault et al., 2000; Young et al., 2000). Interestingly, their involvement in chemical communication induces various behaviours in solitary as well as social insects: male–female mating (Antony et al., 1985; Singer, 1998), reproductive isolation (Blomquist et al., 1987; Cobb and Jallon, 1990; Chase et al., 1992), chemical mimicry (Dettner and Liepert, 1994), species-/colony-/caste-recognition (Vander Meer and Morel, 1998; Lenoir et al., 1999) and the signalling of fertility status (Monnin et al., 1998; Cuvillier-Hot et al., 2002; Heinze et al., 2002).

The site of biosynthesis of insect hydrocarbons has been shown to be abdominal, internal but close to the integument (Dillwith and Blomquist, 1982; Gu et al., 1995; Ferveur et al., 1997). Since the studies of Diehl (1975) on *Schistocerca gregaria* and Romer (1980) on *Tenebrio molitor* larvae, oenocytes are thought to synthesize hydrocarbons in adult insects also and this has been recently demonstrated by Fan et al. (2003) with an 'oenocyte-enriched fraction' of the abdominal integument of *Blatella germanica*.

Transfer of internally synthesized hydrocarbons to the cuticle of several insect species is performed by lipophorin (Schal et al., 2001 and references therein). This ubiquitous lipoprotein shuttles different classes of

* Corresponding author. Present address: Department of Zoology, University of Toronto at Mississauga, Room A3069 South Building, 3359 Mississauga Road, Mississauga, Ont., Canada L5L 1C6. Tel.: +1-905-569-4707; fax: +1-905-828-3792.
*E-mail address:* clucas@utm.utoronto.ca (C. Lucas).

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lipids between their sites of synthesis or absorption and storage or utilization in various target organs or tissues through the aqueous environment of hemolymph. Hydrocarbons are transported in the hydrophobic core of high density lipophorin (HDLp), as has been shown in different structural studies (Van der Horst, 1990), and delivered to the cuticle.

In social insects, cuticular long chain HCs have been often involved in nestmate recognition. Typically, ants from other colonies that do not bear the colony-specific blend of these long chain hydrocarbons ('gestalt') are rejected and even killed. Similar long chain HCs are also found in abundance in the post-pharyngeal gland (PPG), an exocrine gland that is unique to ants (Jackson and Morgan, 1993) and occupies a situation very propitious to secretion/exchanges through oral contact and grooming. These hydrocarbons originate behaviours similar to those elicited by cuticular HCs. Thus, in a colony, an individual coated with alien PPG HCs undergoes aggressive behaviour from its nestmates, whereas an alien ant treated with PPG HCs from this colony is accepted as a nestmate (Soroker et al., 1994; Lahav et al., 1999).

Despite this interesting observation, detailed chemical comparisons of HCs from cuticle and PPG have been rarely done. A question of central interest in the present study is what the origin of these HCs may be. Are they synthesized by the PPG, brought in internally by lipophorin through the hemolymph or transferred from the cuticle by self-grooming (Hefetz et al., 2001)? The PPG of an individual, however, has also been shown to contain HCs of other nestmates brought through physical contacts, allo-grooming and trophallaxis (Soroker et al., 1994, 1995, 2003; Dahbi et al., 1998; Hefetz et al., 2001). Together, these considerations led to the hypothesis that HDLp, PPG and cuticle are involved in the HC transport pathway within and between individuals.

The complete description of the composition of cuticular hydrocarbons has already been performed on three *Pachycondyla* close species (Lucas et al., 2002). *Pachycondyla villosa* exhibits the broadest pattern of hydrocarbons. We have purified the HDLp of this primitive ant. This enabled us to compare HCs associated to HDLp to those of the cuticle and the PPG and investigate their roles in these social insects. Our data shed new light on the potential roles of different types of HCs found on the cuticle and in the PPG.

## 2. Material and methods

### 2.1. Ants

Queen-right colonies of the species *P. villosa* (Lucas et al., 2002) were collected in the experimental fields of the Cocoa Research Center at Ilhéus, Bahia, Brazil. All the colonies were reared in the laboratory in artificial nests at least 6 months before being analysed. The nests were maintained at $27 \pm 1\ ^{\circ}C$, with about 60–80% relative humidity, and a 12L:12D photoperiod. All the colonies were provided with an identical diet (honey/apple mixture, *Calliphora* sp. larvae) twice weekly.

### 2.2. Extraction procedure and characterization of hydrocarbons

Three colonies were used (6–9 foragers per colony) for chemical analysis. Each individual was picked using clean forceps and placed into chilled vials for 10 min before analysis. Secretion of the Dufour gland compounds—the only contaminants otherwise observed—was thus inhibited on subsequent solvent addition. Cuticular hydrocarbons (HCs) were extracted from each entire individual by 5 min immersion in 200 μl of heptane with agitation.

Workers were then dissected and the PPGs were individually collected. The PPG is glove-shaped, with two symmetrical halves terminating in a varying number of finger-shaped projections (Fig. 1). PPG's hydrocarbons were extracted under the same conditions.

Samples were dried under nitrogen and dissolved in 50 μl of heptane. Each sample was analysed using gas chromatography–mass spectrometry (GC–MS) carried out with a Fisons mass spectrometer MD 800 (electron

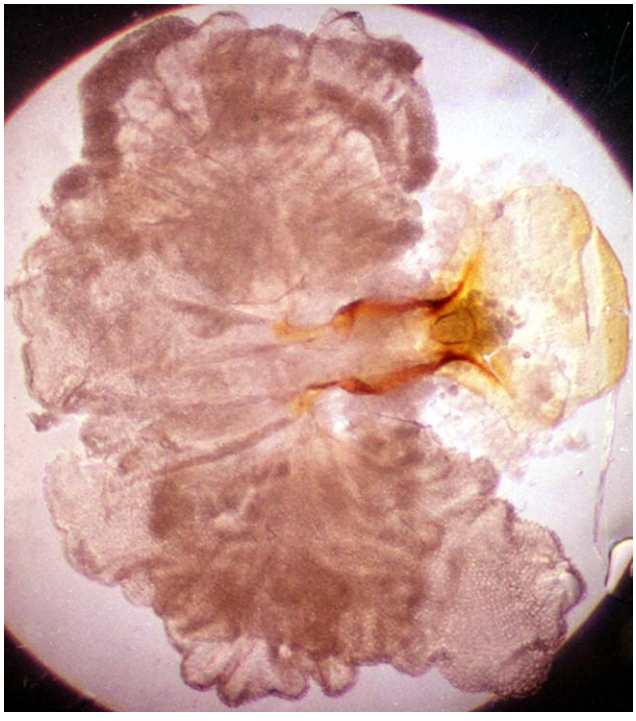

Fig. 1. *Pachycondyla villosa* postpharyngeal gland.

impact at 70 eV) coupled directly with a Carlo Erba gas chromatograph GC 8065MS. The GC–MS was fitted with a 25QC2 BP1 methylsilicone capillary column (25 m × 0.22 mm ID × 0.1 μm). Injections of 5 μl of solution were done at 60 °C. The oven temperature was increased 1 min after injection from 60 to 225 °C at 20 °C/min and from 225 to 320 °C at 3 °C/min (isotherm 10 min). Data were integrated using an IBM-PC with MassLab 1.27 data acquisition, plotting and analysis software. Masses were scanned between 40 and 700 amu at 0.45 scan/s. The mass spectra of HCs were interpreted according to published criteria (McCarthy et al., 1968; Nelson et al., 1972; Nelson, 1978; Pomonis et al., 1978, 1980).

### 2.3. Collection of hemolymph proteins

Forty workers (only foragers) were picked from one colony using clean forceps and put into chilled vials. Cuticular HCs were extracted from this pool by immersion for 5 min in 40 ml of heptane with three-dimensional agitation. The ants were put at 5 °C into an extraction buffer containing protease inhibitors (1 ml/worker). This buffer consisted of 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 2 mM EDTA, 5 mM benzamidin, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin and aprotinin at 20 μg/ml, glutathione at 2 mg/ml, soybean trypsin inhibitor at 2 mg/ml and 0.01% sodium azide. Heads and legs were removed to facilitate the collection of hemolymph. Individual heads were stored separately for later dissection of PPGs and extraction of the HCs as described above. Hemolymph was obtained by centrifuging the remaining parts of the ants with 10 μm membrane tubes (VectaSpin 20, Whatman, Kent, UK) at 2000 g/4 °C for 2 min (Sigma centrifuge). The collected hemolymph and extraction buffer were centrifuged at 8 °C in a Kontron T 124 centrifuge (Kontron Instruments, Milano, Italy) with an A824 rotor at 39,120 $g^{max}$ for 35 min. The protein content of the supernatant was precipitated by adding 0.533 g/ml ammonium sulphate at 4 °C, and the precipitate was collected by centrifugation under the conditions described above (39,120 $g^{max}$ for 35 min). The pellet was redissolved in extraction buffer and recentrifuged to get a clear supernatant containing the dissolved proteins.

### 2.4. Lipophorin purification

HDLp was isolated by density gradient ultracentrifugation (Shapiro et al., 1984). The last supernatant was stained with 400 μl of Sudan Black (5 mg/ml in ethylene glycol), mixed with 2.3 g of solid sodium bromide and adjusted to a final volume of 5 ml. The solution was transferred to a 11.5 ml ultracentrifuge tube and overlaid with a solution of 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 2 mM EDTA and 0.01% sodium azide. Tubes were sealed and centrifuged with a slow acceleration mode at 235,340 $g^{max}$ for 60 min at 4 °C in a Kontron TVF 65.13 vertical rotor. The stained Lp band was withdrawn and its concentration and salt removal were performed by ultrafiltration through Biomax-100 membrane (100 kDa cut-off, Ultrafree-4 centrifugal filter unit, Millipore, Bedford, MA, USA). For further purification, this stained Lp material was diluted in a final volume of 5 ml extraction buffer without protease inhibitors and containing 2.3 g solid sodium bromide, and subjected to ultracentrifugation again. The purity of HDLp was checked by native and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoreses (PAGE) (Laëmmli, 1970) using a concave exponential gradient of acrylamide (5–25%) for the resolving gel in both types of electrophoresis. Silver staining was performed according to Blum et al. (1987). The molecular mass of Lp and its subunits were determined with high and low molecular standard kits from Pharmacia (Saint-Quentin-en-Yvelines, France). Platelet myosin was added to the latter kit.

The density of isolated HDLp was determined by comparative centrifugation with a density gradient reference tube. Fractions (400 μl) were collected from the top of the tubes. Each fraction was measured for absorbance at 280 nm (Lp tube) and for refractive index (blank tube) to calculate the density.

### 2.5. Lipid extraction from lipophorin

Isolated HDLp was submitted to lipid extraction (Folch et al., 1957). The chloroform extract was dried under nitrogen and redissolved in heptane. The HCs were separated from other lipids by thin layer chromatography (TLC) on Silica Gel 60 F-254 in heptane/diethyl ether/acetic acid solvent (80/20/2, v/v). The HC band was located by reference to control lipid standards subjected to the same run and stained with $I_2$, scraped from the plates and extracted with heptane, before analysis by GC–MS.

### 2.6. Statistical analysis

Mann–Whitney *U*-tests were performed using Statistica 5.5 software (Statsoft Inc., Tulsa, OK, USA) with the sequential Bonferroni correction of the *P*-values. Principal component analysis (PCA), on normalized variables, used SPAD 3.0 software (Decisia, Paris, France).

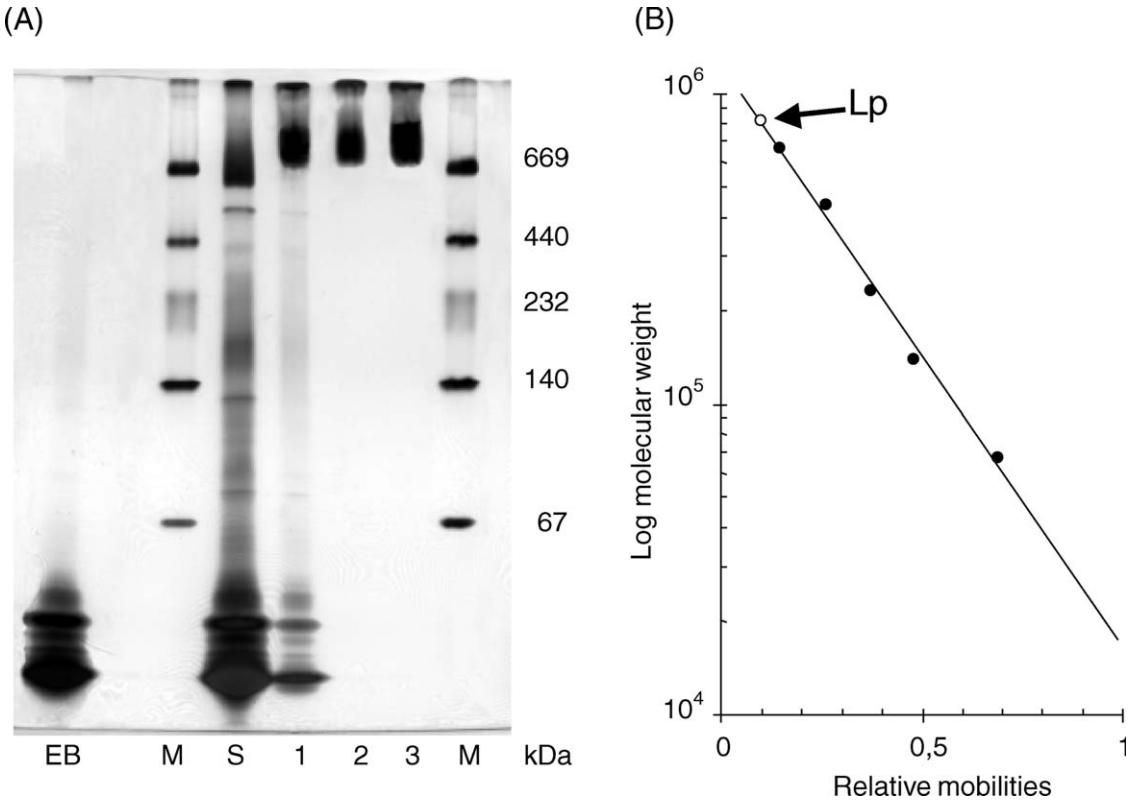


Fig. 2. (A) Electrophoretic analysis of NaBr gradient fractions by native slab gel PAGE. EB: extraction buffer with antiproteases; M: molecular weight markers (in kDa) were thyroglobulin (669), ferritin (440), catalase (232), lactate dehydrogenase (140) and bovine serum albumin (67); S: starting $(NH_4)_2SO_4$ precipitated material redissolved in EB; 1: lipophorin after ultracentrifugation with EB; 2–3: lipophorin after ultracentrifugations without antiproteases; (B) Molecular mass determination. Open point: native lipophorin (Lp), 820 kDa.

## 3. Results

Comparison of the HC profiles of the cuticle, HDLp and PPG implies two prerequisites: (1) HDLp be pure and (2) the cuticle, HDLp and PPG originate from the same individuals because in social insects, like ants, differences do exist between HC profiles of workers from different colonies and even within colonies (Tentschert et al., 2002; Greene and Gordon, 2003).

### 3.1. Lipophorin purification

The HDLp isolated using density gradient ultracentrifugation was pure as shown by slab gel native (Fig. 2, lanes 2–3) and SDS PAGE (Fig. 3, lanes 2–3) with a concave exponential gradient of polyacrylamide (5–25%). On the top of each slab gel shown (Figs. 2 and Fig. 3), which corresponds to the interface between the concentrating and resolving gels, a stained band is visible in all lanes. This is due to the formation of aggregates, which cannot enter the resolving gel. Otherwise, the lane with the starting precipitated material (Figs. 2 and 3, lane S) shows a weak band of HDLp and a multitude of proteins whereas lanes 2 and 3, show a single Sudan Black-stainable native protein (Fig. 2) or two polypeptides characteristic of HDLp (Fig. 3) when ultracentrifugations were performed without antiproteases. The native HDLp has a molecular weight of approximately 820 kDa (Fig. 2B). Its band is broad, due to the high concentration which was used in order to reveal any kind of contaminant. As shown in Fig. 4, there is only one peak of absorption ($\lambda = 280$ nm) for purified HDLp. A density gradient reference tube permits to locate it at a density of 1.114 g/ml (Fig. 4). The molecular mass of the two subunits, apoLp-I and apoLp-II, are approximately 245 and 80 kDa, respectively (Fig. 3B).

Before this purification procedure was set up, direct puncture collection of hemolymph had been tried. Electrophoresis of this hemolymph showed the same Sudan black-stained native protein and the same predominant subunits, with less protein or polypeptide contaminants (not shown). However, as clotting rapidly occurred, insufficient volumes of hemolymph were available for purification. Nevertheless, these data show that the different steps of our present procedure did not alter the purified HDLp.

### 3.2. Lipophorin and cuticle hydrocarbons

In several insect species, HDLp has been shown to carry HCs from the site of biosynthesis, probably

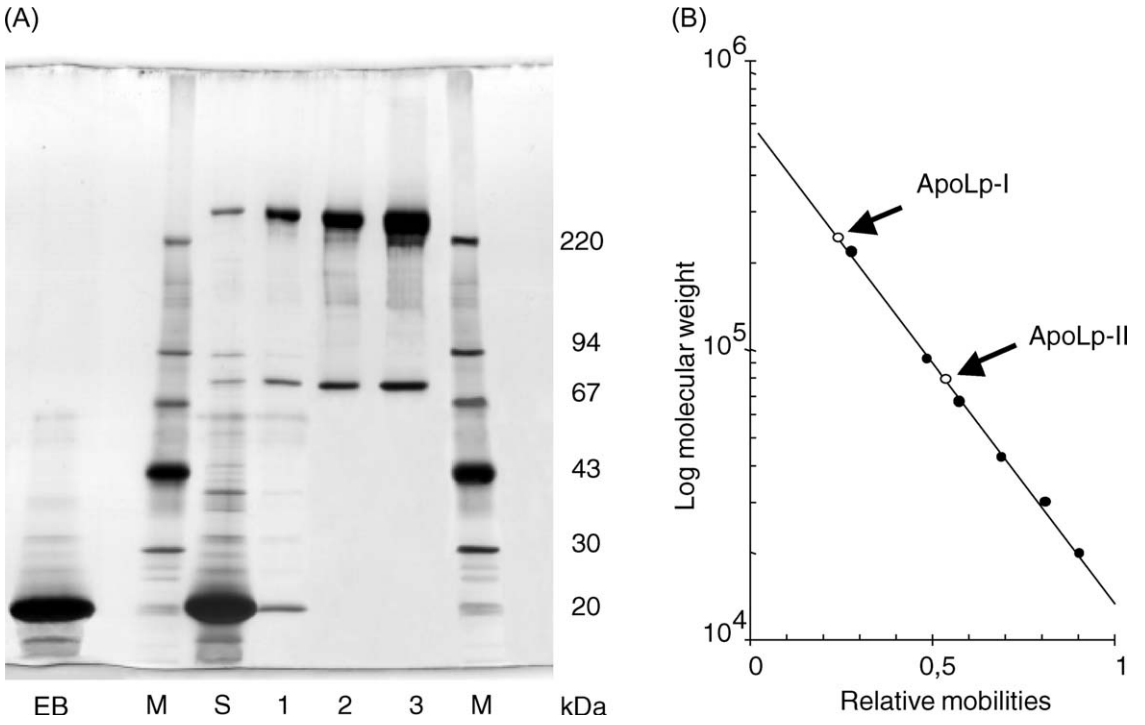


Fig. 3. (A) Electrophoretic analysis of NaBr gradient fractions by SDS slab gel PAGE. M: molecular weight markers (in kDa) were platelet myosin heavy chain (220), phosphorylase B (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and soybean trypsin inhibitor (20). EB, S, 1 and 2–3: as in Fig. 2; (B) Molecular mass determination. Open points: apoLp-I: 245 kDa, apoLp-II: 80 kDa.

oenocytes as in *B. germanica* (Fan et al., 2003), to the cuticle. This has never been studied in Hymenoptera where it is known that HCs are especially important for chemical communication. Therefore, lipid extraction was performed on the purified HDLp and its HCs isolated by TLC and analysed by GC–MS. The HCs associated with *P. villosa* HDLp are qualitatively and quantitatively similar to those of the cuticle (Table 1 and Fig. 5, cuticle pool and lipophorin). As for the cuticle (Lucas et al., 2002), HDLp HCs cover a large range of chain lengths (C26–47). They have even- or odd-numbered chain lengths, are saturated or unsaturated, linear or branched with branching occurring on even- or odd-numbered carbon atoms. Internally branched monomethylalkanes (on carbons inner than $C_6$) appear as mixtures of unresolved isomers among which the 11- to 13-isomers are dominant. Dimethylalkanes are also observed as mixtures, but there is more diversity in branchings. Trimethylalkanes are present in very small quantities whereas *n*-alkadienes—which are more prevalent HCs in another species, *Pachycondyla apicalis* (Hefetz et al., 2001), are completely absent.

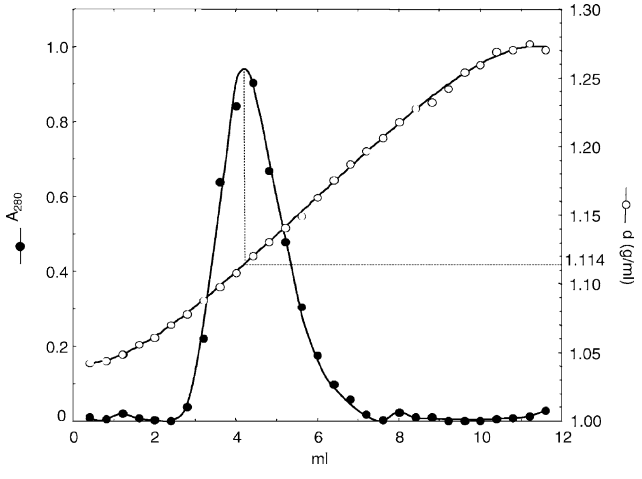


Fig. 4. Density determination of isolated HDLp.

### 3.3. Lipophorin and postpharyngeal gland hydrocarbons

Detailed comparison of hydrocarbons associated with HDLp or extracted from PPGs of the same set of individuals showed clear quantitative differences, even if we do not take into account the unresolved isomers (Fig. 5, PPG pool and lipophorin). Both HDLp and cuticle HC profiles are divided into two subgroups (Fig. 5). The first eluting subgroup (peaks 1–27, Table 1) represents, in HDLp for instance, 20.6% of all HCs, *n*-alkanes and externally branched monomethylalkanes (ext-MeA) being its main components (0.7% and 16.2%, respectively). The later eluting subgroup (peaks 28–63) represents 79.4% and is essentially

Table 1
Percentages of HCs from cuticle, PPG (mean values ± s.d., $n = 18$) and HDLp in order of increasing retention times

| Peak | Compound | Cuticle | | PPG | | Cuticle pool | PPG pool | HDLp | Diagnostic ions |
|---|---|---|---|---|---|---|---|---|---|
| 1 | nC26 | tr | | tr | | tr | tr | tr | 366 |
| 2 | nC27 | tr | | tr | | 0.11 | tr | tr | 380 |
| 3 | 13-, 11-MeC27 | tr | | tr | | tr | tr | tr | 196/7, 224/5; 168/9, 252/3; 379 (M − 15) |
| 4 | 9-MeC27 | tr | | tr | | tr | tr | tr | 140/1, 280/1, 379 (M − 15) |
| 5 | 7-MeC27 | tr | | tr | | tr | tr | tr | 112/3, 308/9, 379 (M − 15) |
| 6 | 5-MeC27 | tr | | tr | | tr | tr | tr | 84/5, 336/7, 379 (M − 15) |
| 7 | nC28 | tr | | tr | | 0.17 | tr | tr | 394 |
| **8** | **2-MeC28** | **2.60** | (**0.37**) | tr | | **2.91** | tr | tr | 365 (M − 43), 393 (M − 15), 408 |
| 9 | X-nC29:1 | tr | | tr | | tr | tr | tr | 406 |
| 10 | X′-nC29:1 | tr | | tr | | tr | tr | tr | 406 |
| 11 | 2,16-; 2,14-diMeC28 | tr | | tr | | tr | tr | tr | 196/7, 252/3; 224/5; 407 (M − 15) |
| **12** | **nC29** | 1.90 | (0.35) | tr | | **2.09** | tr | 0.44 | 408 |
| 13 | 15-, 13-, 11-, 7-MeC29 | 0.35 | (0.08) | 0.14 | (0.03) | 0.35 | tr | 0.97 | 224/5; 196/7, 252/3; 168/9, 280/1; 407 (M − 15) |
| 14 | 13,17-diMeC29 | tr | | tr | | 0.13 | tr | 0.34 | 196/7, 267, 421 (M − 15) |
| 15 | 11,19-diMeC29 | tr | | tr | | tr | tr | tr | 168/9, 295, 421 (M − 15) |
| 16 | 2-MeC29 | tr | | tr | | tr | tr | tr | 379 (M − 43), 407 (M − 15), 422 |
| 17 | 9,17-diMeC29 | tr | | tr | | tr | tr | tr | 140/1, 196/7, 267, 323, 421 (M − 15) |
| **18** | **3-MeC29** | **8.43** | (**0.85**) | 0.15 | (0.02) | **6.70** | tr | **9.25** | 364/5 (M − 57), 393 (M − 29), 422 |
| 19 | nC30 | 0.82 | (0.14) | tr | | 0.58 | tr | 0.25 | 422 |
| 20 | 14-, 13-, 11-MeC30 | tr | | tr | | tr | tr | 0.47 | 210/1, 252/3; 196/7, 266/7; 168/9, 294/5, 421 (M − 15) |
| 21 | 8-, 9-MeC30 | tr | | tr | | tr | tr | 0.43 | 126/7, 336/7; 140/1, 322/3; 421 (M − 15) |
| **22** | **2-MeC30** | **4.53** | (**0.50**) | 0.16 | (0.02) | **5.45** | 0.10 | **5.09** | 393 (M − 43), 421 (M − 15), 436 |
| 23 | 2,20-; 2,18-; 2,16-diMeC30 | tr | | tr | | 0.40 | tr | 1.02 | 168/9, 308/9; 196/7, 280/1; 224/5, 252/3, 435 (M − 15) |
| 24 | nC31 | 0.41 | (0.08) | tr | | tr | tr | tr | 436 |
| 25 | 15-, 13-, 11-MeC31 | 0.20 | (0.05) | 0.17 | (0.03) | 0.23 | tr | 0.52 | 224/5, 252/3; 196/7, 280/1; 168/9, 308/9; 435 (M − 15) |
| 26 | 13,19-; 11,19-diMeC31 | tr | | 0.13 | (0.02) | tr | tr | tr | 196/7, 295; 168/9, 196/7, 295, 323, 449 (M − 15) |
| 27 | 3-MeC31 | 1.56 | (0.21) | tr | | 1.91 | tr | 1.84 | 392/3 (M − 57), 421 (M − 29), 450 |
| 28 | Xi-MeC33:1 | tr | | tr | | tr | tr | tr | 476 |
| 29 | 17-, 15-, 13-, 11-MeC33 | tr | | 0.18 | (0.02) | tr | tr | tr | 252/253; 224/5, 280/1; 196/7, 308/9; 168/169, 336/7, 463 (M − 15) |
| 30 | 13,17-diMeC33 | tr | | 0.37 | (0.04) | tr | tr | tr | 196/7, 252/3, 267, 323, 477 (M − 15) |
| 31 | 17-, 15-, 14-, 13-, 12-MeC34 | tr | | tr | | tr | tr | tr | 252/3, 266/7; 224/5, 294/5; 210/1, 308/9; 196/7, 322/3; 182/3, 336/7, 477 (M − 15) |
| 32 | 15,19-; 13,17-diMeC34 | tr | | 0.22 | (0.02) | tr | 0.11 | tr | 224/5, 238/9, 295, 309; 196/7, 266/7, 337, 491 (M − 15) |
| 33 | Xi-MeC35:1 | 0.20 | (0.05) | 0.97 | (0.13) | 0.42 | 0.31 | 0.60 | 504 |
| **34** | **17-, 15-, 13-MeC35** | 0.59 | (0.12) | **2.09** | (**0.15**) | 0.81 | 0.95 | 1.24 | 252/3, 280/1; 224/5, 308/9; 196/7, 336/7, 491 (M − 15) |
| 35 | Xi-diMeC35:1 | tr | | tr | | tr | tr | tr | 518 |
| **36** | **13,19-; 13,17-diMeC35** | **6.65** | (**0.69**) | **15.44** | (**0.94**) | **3.99** | **6.41** | **4.09** | 196/7, 252/3, 295, 351; 196/7, 267, 280/1, 351, 505 (M − 15) |
| 37 | Xi-MeC36:1 | tr | | 0.16 | (0.02) | tr | tr | 0.14 | 518 |
| 38 | 14-MeC36 | tr | | 0.40 | (0.03) | 0.25 | 0.26 | 0.29 | 210/1, 336/7, 505 (M − 15) |
| **39** | **14,18-; 12,16-diMeC36** | 0.66 | (0.14) | **2.65** | (**0.11**) | 1.12 | 1.66 | 1.63 | 210/1, 280/1, 351; 182/3, 253, 308/9, 379, 519 (M − 15) |
| **40** | **Xi-MeC37:1** | 1.39 | (0.39) | **2.83** | (**0.24**) | **3.30** | 1.72 | **2.96** | 532 |
| **41** | **19-, 17-, 15-, 13-MeC37** | **5.73** | (**0.31**) | **4.50** | (**0.43**) | **7.69** | **6.45** | **7.69** | 280/1; 252/3, 308/9; 224/5, 336/7; 196/7, 364/5, 519 (M − 15), |
| **42** | **15,19-; 13,17-diMeC37** | **50.23** | (**3.26**) | **50.10** | (**1.99**) | **35.95** | **61.78** | **42.44** | 224/5, 280/1, 295, 351; 196/7, 267, 308/9, 379, 533 (M − 15) |
| 43 | 13,17,21-triMeC37 | tr | | 0.69 | (0.09) | 0.75 | 0.53 | 0.64 | 196/7, 252/3, 267, 323, 337, 393, 547 (M − 15) |
| 44 | Xi-MeC38:1 | tr | | 0.24 | (0.03) | 0.52 | 0.22 | 0.37 | 546 |

Table 1 (*continued*)

| Peak | Compound | Cuticle | | PPG | | Cuticle pool | PPG pool | HDLp | Diagnostic ions |
|---|---|---|---|---|---|---|---|---|---|
| 45 | 15-, 14-, 13-, 12-, 11-, 7-MeC38 | tr | | 0.34 | (0.03) | 0.60 | 0.37 | 0.49 | 224/5, 350/1; 210/1, 364/5; 196/7, 378/9; 182/3, 392/3; 168/9, 406/7; 112/3, 462/3, 533 (M – 15) |
| 46 | 14,18-; 13,17-diMeC38 | 0.50 | (0.14) | 1.16 | (0.08) | 1.19 | 1.14 | 1.11 | 210/1, 281, 308/9, 379; 196/7, 267, 322/3, 393, 547 (M – 15) |
| 47 | 12,16,20-triMeC38 | tr | | 0.10 | (0.01) | tr | tr | tr | 182/3, 253, 280/1, 323, 351, 421, 561 (M – 15) |
| **48** | **Xi-MeC39:1** | 1.08 | (0.36) | 1.33 | (0.13) | **3.24** | 1.40 | 1.84 | 560 |
| **49** | **19-, 17-, 15-, 14-, 13-MeC39** | **3.33** | **(0.48)** | **2.74** | **(0.20)** | **5.53** | **2.95** | **3.59** | 280/1, 308/9; 252/3, 336/7; 224/5, 364/5; 210/1, 378/9; 196/7, 392/3, 547 (M – 15) |
| **50** | **13,19-diMeC39** | **8.11** | **(0.84)** | **8.08** | **(0.28)** | **8.70** | **8.87** | **7.00** | 196/7, 295, 308/9, 407, 561 (M – 15) |
| 51 | 13,17,21-triMeC39 | tr | | 0.53 | (0.05) | 0.69 | 0.57 | 0.63 | 196/7, 267, 280/1, 337, 351, 421, 575 (M – 15) |
| 52 | 20-, 14-MeC40 | tr | | 0.11 | (0.01) | 0.17 | tr | 0.13 | 294/5, 308/9; 210/1, 392/3, 561 (M – 15) |
| 53 | 14,20-diMeC40 | tr | | 0.23 | (0.02) | 0.23 | 0.22 | 0.18 | 210/1, 308/9, 407, 575 (M – 15) |
| 54 | Xi-MeC41:1 | tr | | 0.18 | (0.03) | 0.28 | 0.13 | 0.16 | 588 |
| 55 | X′i-MeC41:1 | tr | | tr | | 0.30 | 0.11 | 0.17 | 588 |
| 56 | 21-, 19-, 17-, 15-, 13-MeC41 | 0.16 | (0.07) | 0.48 | (0.05) | 0.81 | 0.46 | 0.55 | 308/9; 280/1, 336/7; 252/3, 364/5; 224/5, 392/3; 196/7, 420/1, 575 (M – 15) |
| 57 | 15,21-; 13,21-; 13,19-diMeC41 | 0.25 | (0.11) | 0.99 | (0.09) | 0.98 | 0.98 | 0.76 | 224/5, 308/9, 323, 407; 196/7, 308/9, 323, 435; 196/7, 295, 336/7, 435, 589 (M – 15) |
| 58 | 13,17,21-triMeC41 | tr | | 0.17 | (0.02) | 0.14 | 0.17 | tr | 196/7, 267, 308/9, 337, 379, 449, 603 (M – 15) |
| 59 | 14,20-diMeC42 | tr | | tr | | tr | tr | tr | 210/1, 309, 336/7, 435, 603 (M – 15) |
| 60 | 13-MeC43 | tr | | 0.15 | (0.02) | 0.20 | 0.25 | tr | 196/7, 448/9, 603 (M – 15) |
| 61 | 13,19-; 13,21-diMeC43 | 0.12 | (0.06) | 0.65 | (0.11) | 0.47 | 0.56 | 0.30 | 196/7, 295, 364/5, 463; 196/7, 323, 336/7, 463, 617 (M – 15) |
| 62 | 13,17,21-triMeC43 | tr | | tr | | tr | tr | tr | 196/7, 267, 336/7, 407, 477, 631 (M – 15) |
| 63 | 13,21-diMeC45 | tr | | 0.52 | (0.07) | 0.26 | 0.41 | 0.18 | 196/7, 323, 364/5, 491, 645 (M – 15) |

The 'cuticle pool', 'PPG pool' and the HDLp extract came from the same 40 workers. Compounds in bold type: >2%; trace (tr): <0.1%; X: unknown position of double bond; Xi: unknown position of internal methyl branch.

composed of successive associations of small amounts of Xi-methylalkenes (methylalkenes with undetermined double bond and internal methyl branching positions, peaks 33, 40 and 48), monomethylalkanes (mainly the 13-isomer) (34, 41 and 49) and 13,17- and/or 13,19-dimethylalkanes (36, 42 and 50), in order of increasing abundance (5.4%, 12.5% and 53.5%). In the PPG, the first subgroup of *n*-alkanes and ext-MeA is almost completely absent and represents only 1.1% of the total. The second subgroup represents 98.9% and conserves the same organization (same peak number) with similar increase order (5.1%, 9.3% and 73.6%, respectively). Representative mass spectra are shown for externally branched monomethylalkanes (Fig. 6A), internally branched monomethylalkanes (Fig. 6B) Xi-monomethylalkenes (Fig. 6C), and internally branched dimethylalkanes (Fig. 6D).

The results of PCA of the percentages of the prevalent HCs (mean percentage >2%) from PPG and cuticle are shown in Fig. 7. The first two factors, F1 and F2, represent 44% and 26% of the total variance, respectively. The 'cuticle pool' and 'PPG pool' data arose from the total HC extracts of cuticles and PPGs from the same 40 workers used to isolate the HDLp. The PPG and the cuticle points cluster at opposite sides along the F1 axis. The HDLp point is also found within the cuticle cluster whereas the 'PPG pool' point is close to the PPG cluster.

Plotting the percentages of HCs by structural classes confirms these observations (Fig. 8). The *n*-alkanes and externally branched monomethylalkanes are mostly absent from the PPG compared to HDLp or the cuticle ($P < 0.001$). The internally branched monomethylalkanes are not statistically significantly different ($P > 0.05$) between PPG and cuticle. Conversely, the dimethylalkanes (and also trimethylalkanes which are in small quantities) are statistically significantly ($P < 0.001$) lower in cuticle than in PPG. The percentages of this class of HCs from HDLp and 'cuticle pool' are also lower than that of the 'PPG pool'. Concerning alkenes, the cuticle presents lower quantities than the PPG ($P < 0.01$). Unfortunately, the

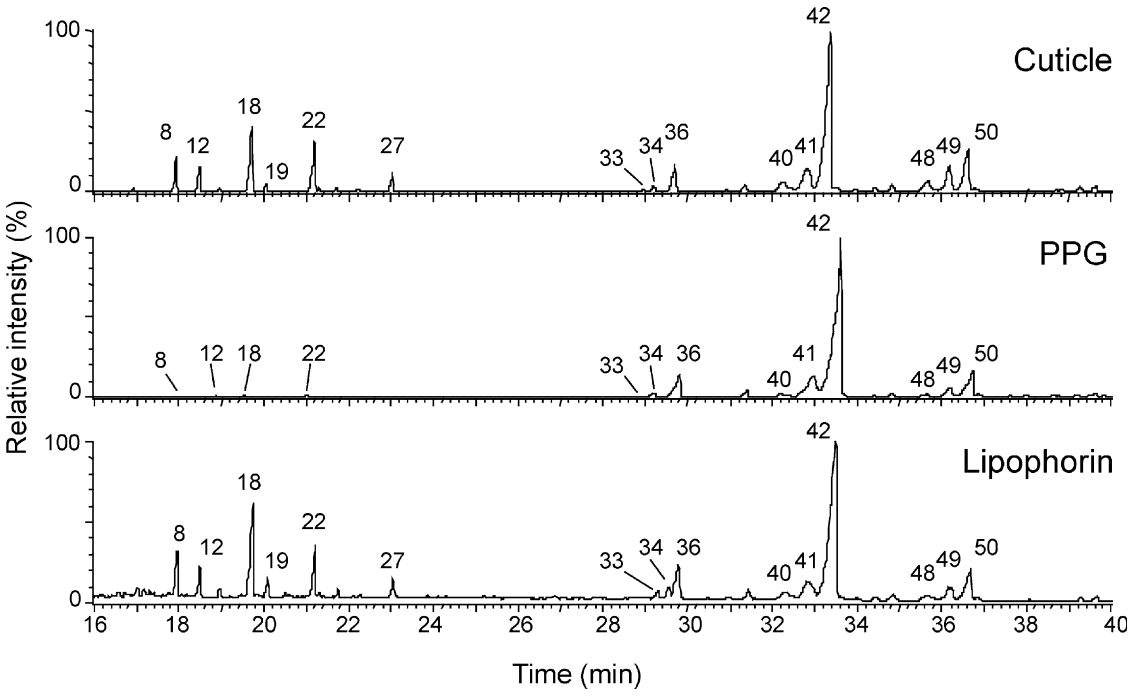


Fig. 5. Total ion GC–MS chromatograms of the HCs from cuticle, PPG and HDLp of the same 40 workers. Numbers refer to the peaks listed in Table 1.

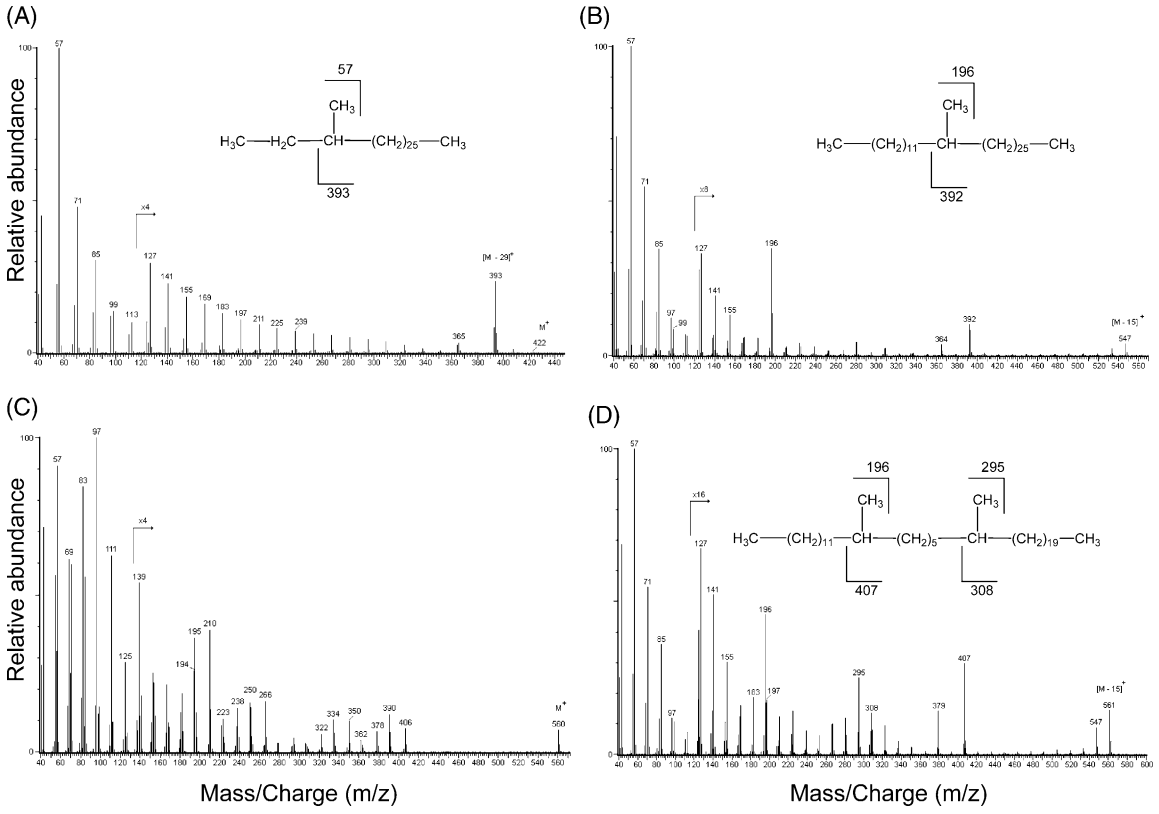


Fig. 6. (A) Mass spectrum of 3-MeC29 (peak 18); (B) mass spectrum of 13-Me C39 (peak 49); (C) mass spectrum of Xi-MeC39:1 (peak 48), probably a mixture of isomers of monounsaturated homologs of 13-MeC39 (peak 49): the $m/z$ values of M+ and fragment ions are those of monounsaturated species, and the diagnostic fragments, arising from cleavages α and β of the branching points, are in clusters 1–3 amu lower than the corresponding doublets of the related methylalkanes ($m/z$ range 139–560). Furthermore, the peak is at about −0.3 equivalent chain length from peak 49. Nevertheless, precise structural determinations (methyl branching and double bond position) would need isolation in sufficient amounts and chemical modifications for further spectral analyses; (D) mass spectrum of 13,19-diMeC39 (peak50).

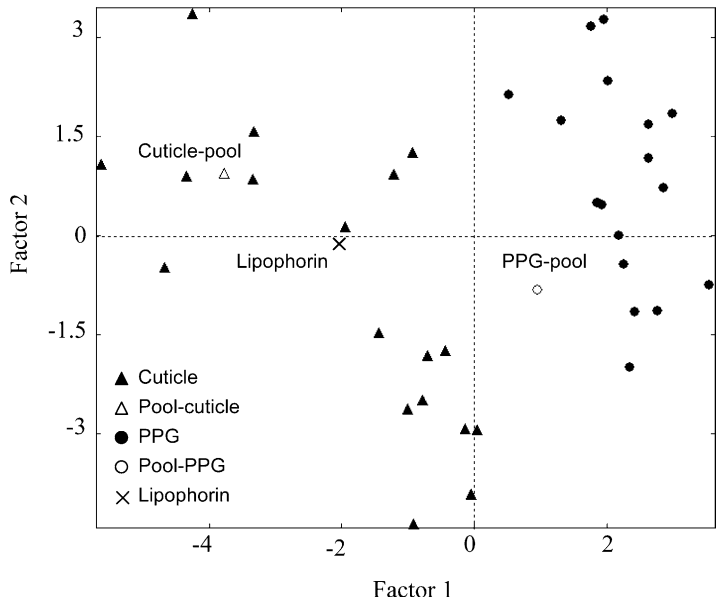


Fig. 7. PCA of HC percentages of cuticle, PPG and lipophorin. The first two factors F1 and F2 represent respectively 44% and 26% of the total variance. The variables were normalized during the analysis in order to obtain a better comparison. The 'cuticle pool' and 'PPG pool' points on the PCA represent the total HC extracts from the same 40 workers that have been used to isolate the lipophorin.

quantities of alkenes are too small to be sorted by class like alkanes, but they are mainly internally branched methylalkenes. *n*-Alkanes and ext-MeA being discarded, the mean percentages of the other HCs have been recalculated: they clearly illustrate the structural similarities between the HCs common to both HDLp and PPG.

Comparison of the HC profiles of the cuticle and the PPG have also been performed, on the two close species *Pachycondyla* cf. *inversa* and *Pachycondyla subversa* (Lucas, 2002), and led to the same conclusions: qualitative similarity but marked decrease of *n*-alkanes and MeA-ext and opposite clustering of cuticle and PPG points in PCA analyses.

### 3.4. Variations between colonies

Statistical comparison of the recalculated data between cuticular and PPG HCs of *P. villosa* shows that several HCs are not significantly different ($P > 0.05$) and are therefore considered as 'stable'. Such is the case, for instance, in Pv3 colony, for 11 HCs listed underlined in Table 2. All are internally branched (on carbons inner than $C_6$) mono-, dimethylakanes or monomethylalkenes. Four of them are prevalent (>2%) and none of them are mixed externally/internally branched dimethylalkanes.

Furthermore, this table also shows that such stable HCs vary between colonies (Table 2), in comparisons

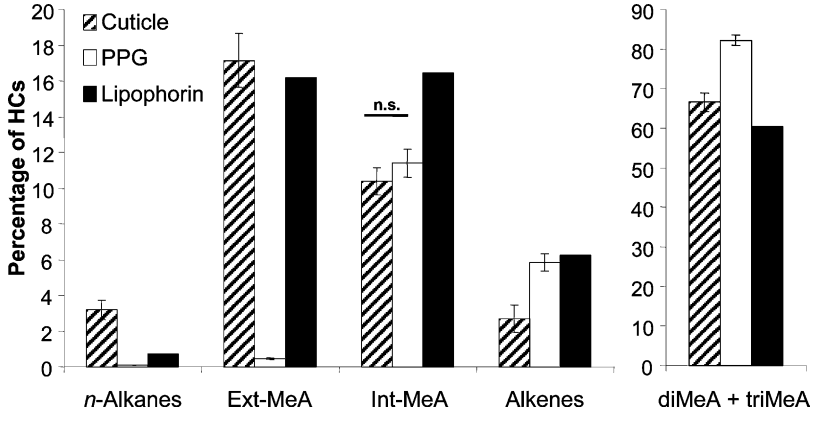


Fig. 8. HC percentages of cuticle, PPG and lipophorin (mean ± s.d.). There are significant differences ($P < 0.05$) between cuticle and PPG values ($n = 18$) except for the group marked n.s. The 'cuticle pool' and 'PPG pool': as in Fig. 7. Ext-MeA: externally branched monomethylalkanes; Int-MeA: internally branched monomethylalkanes; diMeA: dimethylalkanes; triMeA: trimethylalkanes.

Table 2
Recalculated percentages of HCs from cuticle, PPG of three *P. villosa* colonies (mean values ± s.d., $n = 6$, 5 and 7) in order of increasing retention times

| Peak | Compound | Cuticle Pv3 | | PPG Pv3 | | PPG Pv6 | | PPG Pv21 | |
|---|---|---|---|---|---|---|---|---|---|
| 13 | 15-, 13-, 11-, 7-MeC29 | 0.77 | (0.19) | 0.19 | (0.08) | 0.20 | (0.02) | tr | |
| 25 | 15-, 13-, 11-MeC31 | 0.48 | (0.13) | 0.24 | (0.07) | 0.19 | (0.03) | *0.10* | (*0.01*) |
| 26 | 13,19-; 11,19-diMeC31 | tr | | 0.19 | (0.04) | 0.10 | (0.01) | tr | |
| 29 | 17-, 15-, 13-, 11-MeC33 | 0.18 | (0.03) | 0.22 | (0.03) | 0.22 | (0.02) | *0.11* | (*0.01*) |
| 30 | 13, 17-diMeC33 | 0.23 | (0.03) | 0.41 | (0.10) | 0.45 | (0.03) | 0.27 | (0.05) |
| 32 | 15,19-; 13,17-diMeC34 | tr | | 0.20 | (0.04) | 0.31 | (0.02) | 0.17 | (0.03) |
| 33 | Xi-MeC35 : 1 | 0.50 | (0.13) | 0.83 | (0.21) | *1.64* | (*0.09*) | 0.64 | (0.09) |
| **34** | **17-, 15-, 13-MeC35** | 1.31 | (0.20) | **2.05** | (**0.18**) | ***2.88*** | (***0.11***) | 1.58 | (0.16) |
| 35 | Xi-diMeC35:1 | 0.14 | (0.04) | tr | | tr | | tr | |
| **36** | **13, 19-; 13, 17-diMeC35** | **10.16** | (**1.65**) | **15.90** | (**2.49**) | **17.74** | (**0.72**) | **13.62** | (**0.79**) |
| 37 | Xi-MeC36:1 | tr | | 0.11 | (0.02) | *0.29* | (*0.02*) | 0.11 | (0.01) |
| 38 | 14-MeC36 | tr | | 0.33 | (0.02) | *0.57* | (*0.02*) | 0.35 | (0.02) |
| **39** | **14,18-; 12,16-diMeC36** | 1.55 | (0.12) | **2.28** | (**0.12**) | ***3.24*** | (***0.06***) | **2.58** | (**0.1**) |
| **40** | **Xi-MeC37:1** | **4.43** | (**0.29**) | **2.54** | (**0.46**) | ***3.82*** | (***0.22***) | **2.42** | (**0.31**) |
| **41** | **19-, 17-, 15-, 13-MeC37** | **7.40** | (**0.30**) | **4.09** | (**0.61**) | **6.20** | (**0.82**) | **3.71** | (**0.54**) |
| **42** | **15, 19-; 13, 17-diMeC37** | **47.89** | (**4.74**) | **50.33** | (**2.98**) | ***42.31*** | (***1.61***) | **56.12** | (**2.82**) |
| 43 | 13,17,21-triMeC37 | tr | | 0.44 | (0.14) | *1.08* | (*0.11*) | 0.64 | (0.13) |
| 44 | Xi-MeC38:1 | tr | | 0.18 | (0.04) | *0.35* | (*0.04*) | 0.21 | (0.05) |
| 45 | 15-, 14-, 13-, 12-, 11-, 7-MeC38 | tr | | 0.27 | (0.05) | *0.47* | (*0.05*) | 0.32 | (0.05) |
| 46 | 14, 18-; 13, 17-diMeC38 | 1.29 | (0.38) | 1.06 | (0.15) | 1.40 | (0.11) | 1.09 | (0.14) |
| **48** | **Xi-MeC39:1** | **3.42** | (**0.90**) | 1.18 | (0.26) | 1.76 | (0.16) | 1.18 | (0.17) |
| **49** | **19-, 17-, 15-, 14-, 13-MeC39** | **5.78** | (**1.15**) | **2.84** | (**0.48**) | **3.17** | (**0.16**) | **2.39** | (**0.28**) |
| **50** | **13, 19-diMeC39** | **12.45** | (**1.96**) | **8.21** | (**0.73**) | **7.53** | (**0.25**) | **8.46** | (**0.30**) |
| 51 | 13,17,21-triMeC39 | tr | | 0.42 | (0.09) | *0.76* | (*0.06*) | 0.47 | (0.08) |
| 52 | 20-, 14-MeC40 | tr | | 0.11 | (0.02) | 0.12 | (0.01) | 0.10 | (0.02) |
| 53 | 14,20-diMeC40 | tr | | 0.22 | (0.03) | 0.21 | (0.02) | 0.25 | (0.03) |
| 54 | Xi-MeC41:1 | tr | | 0.16 | (0.04) | 0.10 | (0.01) | 0.26 | (0.05) |
| 55 | X′i-MeC41:1 | tr | | 0.12 | (0.03) | tr | | tr | |
| 56 | 21-, 19-, 17-, 15-, 13-MeC41 | 0.62 | (0.18) | 0.61 | (0.10) | *0.38* | (*0.04*) | 0.45 | (0.07) |
| 57 | 15, 21-; 13, 21-; 13, 19-diMeC41 | 0.96 | (0.32) | 1.32 | (0.13) | *0.65* | (*0.07*) | *0.97* | (*0.13*) |
| 58 | 13,17,21-triMeC41 | tr | | 0.22 | (0.05) | 0.16 | (0.02) | 0.14 | (0.03) |
| 60 | 13-MeC43 | tr | | 0.22 | (0.04) | *0.11* | (*0.02*) | *0.11* | (*0.03*) |
| 61 | 13,19-; 13,21-diMeC43 | 0.46 | (0.21) | 1.11 | (0.21) | *0.45* | (*0.08*) | *0.41* | (*0.09*) |
| 62 | 13,17,21-triMeC43 | tr | | 0.14 | (0.02) | 0.11 | (0.02) | tr | |
| 63 | 13,21-diMeC45 | tr | | 0.88 | (0.09) | *0.41* | (*0.07*) | *0.28* | (*0.05*) |

Statistically not different values between cuticle and PPG of Pv3 are underlined.
Significantly different values between Pv3 PPG with either Pv6 or Pv21 PPG are in italics.
Bold type, tr and Xi: as in Table 1.

by pairs (Pv3–Pv6, Pv3–Pv21). Similarly, the same kind of comparison of Pv6 with either Pv3 or Pv21, or Pv21 with either Pv3 or Pv6 leads to similar conclusions (data not shown).

## 4. Discussion

Over the past decades, numerous studies have emphasized the importance of hydrocarbons in insects. Due to their large surface/volume ratio, protection against desiccation is vital, and their hydrophobicity as well as their alignment on the cuticle provide a passive but effective barrier to water loss. Moreover, many insect species utilize hydrocarbons in chemical communication. In social insects particularly, long chain hydrocarbons are involved in species and nestmate recognition. The variety of their structures (chain lengths, number and positions of methyl branching or double bonds) as well as their combinations allow multiple information processing. All these hydrocarbons are deposited on—and not synthesized in—the cuticle. They come from internally synthesized hydrocarbons and are carried by HDLp. In ants, hydrocarbons are also found in the PPG and induce behavioural effects as do the cuticular hydrocarbons. To further investigate the circulation and eventually differentiate the roles of the components of the mixtures of hydrocarbons in each case, we have undertaken a comparative study of cuticular, lipophorin and PPG hydrocarbons.

This comparison implied prior purification of the lipoprotein. We report here the first purification and

characterization of an ant HDLp. The native HDLp has a molecular weight of approximately 820 kDa. This molecular mass is higher than published data on lipophorins of other species which ranged from 580 (*Locusta migratoria*, Van der Horst, 1990) to 730 kDa (*Bombyx mori*, Kim and Kim, 1994). The unique peak of absorbance at 280 nm obtained on recentrifugation, the density of 1.114, the composition in subunits and their molecular masses showed that HDLp had been purified. This purification has allowed a detailed analysis of the HCs associated with this lipoprotein.

Few quantitative differences between HDLp and cuticular extracts were observed as confirmed by the bivariate plot of all the extracts. This supports a possible role of HDLp as an HC carrier from the internal sites of biosynthesis, probably oenocytes (Fan et al., 2003), to the cuticle as already shown by studies on a number of insect species. However, the mechanism of HC transfer from this hemolymph lipoprotein to the epicuticle is not yet clear, in any insect species. The existence of potential specialized structures has been suggested (Noirot and Quennedey, 1991; Quennedey, 1998), but clear-cut evidence remains to be found.

Interestingly, clear differences were observed between PPG and HDLp hydrocarbons: whereas long chain internally branched monomethyl-, dimethylalkanes or alkenes were present in comparable percentages, shorter chain normal and externally branched methylalkanes were almost completely absent from PPG. Do these differences come from the ability of the gland to synthesize hydrocarbons on its own? In the closely related species, *Pachycondyla inversa* (Lucas, 2002) as well as in the more advanced ant *Cataglyphis niger* (Soroker and Hefetz, 2000), when PPGs were incubated with radioactive acetate as a precursor, no labelled HCs were found whereas other radioactive lipids were produced. PPGs thus appear unable to synthesize HCs. Nevertheless, after abdominal injection of the same precursor to ants of the related species *Pachycondyla apicalis*, an appreciable amount of labelled HCs was found in the PPGs (Soroker et al., 1998, 2003).

These HCs may be transferred from hemolymph HDLp but the differences observed in HC compositions between HDLp and PPG would then require some selectivity in this transfer to exclude normal and externally branched methylalkanes. Such selectivity has been observed in the tiger moth *Holomelina aurantiaca* (Schal et al., 1998a, b), and the house fly *Musca domestica* (Schal et al., 2001).

In *Pachycondyla apicalis*, an external mechanism for the transfer of cuticular HCs to the PPG has been suggested to involve self-grooming (Soroker et al., 1998; Hefetz et al., 2001). During this behaviour, ants pass the tarsal brushes of their front legs through their mouths into which the PPG opens. And, indeed, after injection of radioactive acetate precursor, the amounts of labelled HCs in PPG markedly decreased when ants' mouthparts were blocked or front legs immobilized. Such a mechanism may be efficient as these brushes represent a large surface of the cuticle which does bear a large proportion of cuticular HCs (more than one fourth). Such brushes are also present on front legs in *P. villosa* and involved in self-grooming. They may thus be favoured unloading sites for HCs towards the PPG.

This transfer does not occur solely within an individual; labelled HC exchanges were observed between nestmates in several ant species (Soroker et al., 1994, 1995, 2003; Meskali et al., 1995). In those studies, an HC probe first present on only one worker was rapidly transmitted to the cuticles of all other nestmates encountered as well as to their PPGs. In *Pachycondyla apicalis*, this interindividual HC transfer occurred mainly through physical contact and less so through allo-grooming (Soroker et al., 2003).

Taken together, published data and those presented here agree with and complement the general hydrocarbons circulation scheme, for *Pachycondyla* ants, hypothesized by Lenoir et al. (1999). HCs internally synthesized in oenocytes are transported by HDLp through the hemolymph to the cuticle, especially to the basitarsal brushes of the front legs. These are licked during self-grooming and individual cuticular HCs transferred to the PPG. A complementary direct and selective transfer of internally branched methylakanes and alkenes from lipophorin to PPG is possible, but its mechanism has to be clarified (Schal et al., 2003). PPG hydrocarbons are dispatched again on the other parts of the same individual's cuticle or on its nesmates' cuticles during physical contacts and allo-grooming. This recurrent and mutual redistribution of HCs on the cuticle of every member of a colony might allow the formation of a colonial signature.

The chemical properties of the different HC classes present or not in the PPG may be related to different roles. Cuticular *n*-alkanes and externally branched monomethylalkanes have high melting temperatures (Tms) and are more efficiently organized to protect insects from water loss (Gibbs and Pomonis, 1995; Gibbs, 1998 and references in these reviews; Wagner et al., 1998, 2001; Kaib et al., 2000; Young et al., 2000). For the same reason, they are also less easily collected into PPG during grooming. On the contrary, it is well known that internal methyl branching as well as unsaturation markedly lower the Tms of HCs (Gibbs, 1998) and hence increase their fluidity. This might explain why internally branched monomethyl- and dimethylalkanes and alkenes are easily collected into PPG and redistributed during self- or allo-grooming and physical contacts, which supports their possible role in chemical communication. Indeed, despite the

absence of normal and externally branched methylalkanes, PPG HCs keep a behavioural role in nestmate recognition (Soroker et al., 1994; Lahav et al., 1999). Which of the remaining HCs common to cuticle and PPG could have a role in nestmate recognition? Those which are in the same proportion in both of them may be good candidates. *n*-Alkanes and ext-MeA being discarded, we have recalculated the mean percentages of the other HCs. Statistical comparison between cuticle and PPG HCs then showed that several compounds were not significantly different. These 'stable' HCs might be important in chemical communication. Moreover, comparison between different colonies shows that these HCs vary from colony to colony. They might thus be involved in colonial signature. It remains to compare these statistical data with behavioural studies using different hydrocarbon structural classes or components isolated from the extracts.

On the cuticle, the two groups of HCs (waterproofing and insect communication) are precariously balanced. Depending on the environment or life cycle, the use of HCs for waterproofing or chemical communication is favoured. PPG could be considered as an evolutionary answer to the problem raised by the coexistence of the two different types of cuticular HCs. While resistance against high temperatures may be a relatively slow process, colonial signature must be rapidly restored for nestmate recognition to avoid aggressive and even fatal reactions. From this point of view, the PPG constitutes a reservoir for a great quantity of rapidly accessible recognition cues.

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# Role of cuticular hydrocarbons in the chemical recognition between ant species in the *Pachycondyla villosa* species complex

C. Lucas[a,*], D.B. Pho[a], J.M. Jallon[a], D. Fresneau[b]

[a] *Mécanismes de communication, Université Paris Sud, CNRS-UMR 8620, 91405 Orsay Cedex, France*
[b] *LEEC, Université Paris Nord, CNRS-UPRESA 7025, 93430 Villetaneuse, France*

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**Abstract**

Cuticular hydrocarbons (HCs) play important roles in insect communication but few studies clearly demonstrate the direct link between HCs and nestmate recognition. Therefore, cuticular lipids were extracted from ants, their HC and *non*-HC fractions as well as the three principal classes of HCs (*n*-alkanes, branched alkanes and alkenes) were purified and tested using an immobilizing "joust" device which allowed quantification of early pairwise behavioural responses, mandibular opening and antennal retraction, without occurrence of subsequent damages as in classic dyadic encounters.

Chemical recognition of ants was studied at three levels of interactions (intra-colonial, intra-specific and inter-specific). Three closely related species already chemically characterized were used: *Pachycondyla villosa* (Pv), *P. inversa* (Pi) and *P. subversa* (Ps). Each species had its own behavioural responses. Moreover, responses of Pi and Ps towards Pv were significantly longer, than they were between themselves whereas Pv ants were equally aggressive towards Pi and Ps. These differences are in agreement with the results of the cluster analysis of the cuticular HCs profiles that place Pi closer to Ps. In support of the idea that components of cuticular lipids profiles are important for recognition, we found that only the HC fraction and its branched subfraction elicited a behavioural response of Ps workers. It is suggested that internally branched methyl- and dimethylalkanes are involved in recognition behaviour.


*Keywords:* Aggressive behaviour; Chemical recognition; Pachycondyla; Cuticular hydrocarbons; Ethological test

## 1. Introduction

Identification and discrimination are major processes in biological systems, from embryogenesis and immune responses to community dynamics. In social insects, kin, nestmate and species recognition is an important factor for maintaining the cohesion and survival of a colony (Hölldobler and Wilson, 1990) by rejection of alien con- and heterospecific intruders. Elucidation of the mechanisms underlying these different types of recognition is crucial to our understanding of communication between social insects and the evolutionary advantages they may confer.

Cuticular lipids—particularly hydrocarbons (HCs)—are thought to be involved in kin and nestmate recognition as they have been observed as sex-, caste-, reproductive status-, colony- or species-specific in solitary or social insects and linked to courtship, colonial closure, regulation of reproduction and division of labour (Jutsum et al., 1979; Morel et al., 1988; Howard, 1993; Dahbi et al., 1996; Monnin et al., 1998; Singer, 1998; Wagner et al., 1998; Cuvillier-Hot et al., 2002; Heinze et al., 2002; Dietemann et al., 2003; D'Ettore et al., 2004a, b; Endler et al., 2004; Ejima et al.,

*Corresponding author. Temporary new address: Department of Zoology, University of Toronto at Mississauga, Rm. A3069 South Building, Mississauga, Ont., Canada L5L 1C6. Tel.: +1 905 569 4707; fax: +1 905 828 3792.
*E-mail address:* clucas@utm.utoronto.ca (C. Lucas).

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2005). Particularly in social insects, colony- and species-specificity of cuticular HCs composition in a great variety of species suggested that they might constitute the recognition labels. Nevertheless, except in some recent papers discussed below bioassays are generally still needed to directly link chemistry and behaviours (Vander Meer and Morel, 1998).

A great variety of experimental procedures have been used in the study of recognition abilities in ants. Stuart (1987, 1993) introduced foreign conspecific workers into recipient *Leptothorax* colonies and determined whether the intruders were adopted or rejected by the residents' aggressive behaviours. Counting the survival of intruders is another means of describing the discrimination abilities of workers (Sundström, 1997). In double-blind experiments, Heinze et al., 1996 placed in a neutral arena two groups of alien workers of *Leptothorax nylanderi* and observed strong aggression related to the distance between two colonies with an apparent "dear–enemy" phenomenon. A similar procedure was employed by Hefetz et al. (1996) in a circular arena. The authors measured individual distances between a group of resident workers and a foreign individual placed in the center of the arena and the "agitation" of the residents towards the "test" individual. These experiments based on collective responses and an amplification of aggressive behaviours make it difficult to precisely analyse the various responses involved during these encounters. Furthermore, they require large colonies due to their costs in terms of high individual mortality. Dyadic encounter tests are also commonly used for the study of ant recognition; unfortunately, even this kind of test is not entirely satisfactory (Fénéron, 1996; Hefetz et al., 1996). Indeed, the tested ants may not encounter each other for a long time, but when they finally do so, rapid killing or avoidance occurs. Therefore, even if those dyadic tests are informative on the degree of aggressiveness and the social closure of species, they do not provide enough detailed behavioural data on the early decisive steps of recognition. This is the reason why we have set up an immobilizing system that allows a precise investigation of the decision-making period. Indeed, as immobilization impedes injuries, analyses can be performed on colonies that have few individuals or aggressive species that have a complete colonial closure.

Such a device is also useful to test chemicals that might be the discriminating chemical cues. Numerous investigations have emphasized the increasing importance of cuticular HCs in the biology of insects. Indeed, they play various roles in insects, from water proofing, thermal insulation, protection from predators to chemical communication, reproduction isolation and kin and species recognition (Blomquist and Jackson, 1979; Jallon, 1984; Cobb and Jallon, 1990; Lockey, 1991; Blomquist et al., 1998; Carlson et al., 1998; Rouault et al., 2004; Ejima et al., 2005; Howard and Blomquist, 2005). These different roles are directly linked to their great diversity in structure: number of carbon atoms, number and positions of unsaturation, number and positions of methyl branches, as well as combinations of these features, which influence their physical properties (Gibbs and Mousseau, 1994; Gibbs and Pomonis, 1995; Gibbs, 1998a, b). To ascertain the involvement of HCs and identify the class(es) implicated in social communication it would be necessary to isolate them and test their activity.

Studies on *Cataglyphis niger* (Lahav et al., 1999) showed that only the addition to live ants of the purified HC fraction from the cuticular extract was able to modify their aggressive behaviour as predicted from nestmate recognition cues while the non-hydrocarbon did not. In the social wasp *Polistes dominulus*, Dani et al. (2001) also found that treating an individual with some synthetic HCs of alien conspecific workers modified its acceptance by its nestmates. The same kind of analyses was made on *Apis mellifera* with various compounds (Bowden et al., 1998; Breed, 1998a, b; Breed et al., 1998). However, in these studies the HC fraction or synthetic HCs were deposited on living animals and the behavioural tests were thus based on individuals with *modified* cuticular HC profiles and not on the purified HC fraction only. Dead animals previously washed with solvent on which HCs were subsequently applied have also been used instead of living ones, but might not be neutral supports without any visual or non-characterized chemical information (Gamboa et al., 1991). Nevertheless, the interpretation of such data is not unequivocal, as other experimental studies have suggested that nestmate recognition might be related to HC composition and hence to the modifications of the relative concentrations of their constituents (Blomquist et al., 1998; Breed, 1998a, b; Singer, 1998). In a more direct way, Wagner et al. (2000) have shown that the total HC fraction of *Pogonomyrmex barbatus* deposited on small glass blocks elicited behavioural responses in agreement with expected nestmate recognition cues (Wagner et al., 2000), but the *non*-HC fraction was not tested.

Here we report the behavioural tests performed in an immobilizing "joust" device on three closely related ant species (*Pachycondyla villosa* (Pv), *P. inversa* (Pi) and *P. subversa* (Ps)) which have already been chemically characterized (Lucas, 2002; Lucas et al., 2002). Quantification of two early and well characterized behaviours that will be described below, "mandible opening" and "antennal retraction", in different types of social interactions showed discrimination at least at the species level. Using this paradigm with Ps workers and papers perfumed with the purified HC and *non*-HC fractions as well as the three main classes of HCs (*n*-alkanes, branched alkanes and alkenes) isolated from workers'

cuticle, we have shown that cuticular branched HCs are cues of species recognition.

## 2. Methods

### 2.1. Studied species

Three closely related species: Pv, Pi and Ps (Lucas et al., 2002) were collected from sympatric populations in the experimental fields of the Cocoa Research Center at Ilhéus, Bahia, Brazil. These species were chosen in order to better grasp the diversity and the specificity of behavioural responses in closely related biological systems. Moreover, the large size of the individuals facilitates the behavioural observations and allows for obtaining a large quantity of HCs. They were reared in the laboratory in artificial nests at least for 6 months before being studied, at 27±2 °C, with about 60–80% relative humidity, and a 12L:12D photoperiod. All the colonies were provided with an identical diet (honey/apple mixture, *Calliphora sp*. larvae) twice a week.

### 2.2. Immobilizing "joust" device

Confrontations between ants were staged using an immobilizing "joust" device consisting of a Petri dish with a V-shaped opening in its rim (Fig. 1a) which let the head of an ant slip through it, whatever its size. A piece of adhesive tape was then put on the rim just behind the head to block the ant by the "V". A clay cylinder was put under the head to maintain it parallel to the camera objective. The body of the animal was free inside the box (Fig. 1b).

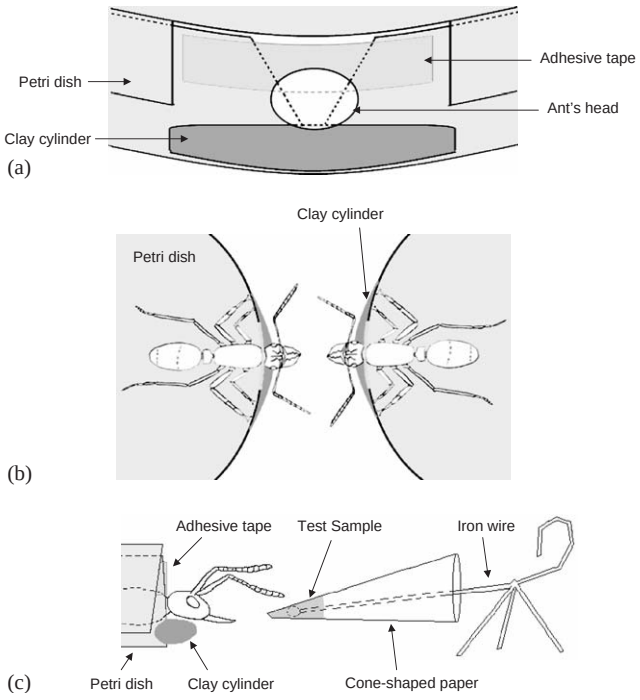


Fig. 1. (a) "Front" view of the immobilizing "joust" device. (b) Same device seen from above without the upper lid, in a pairwise encounter. (c) Side view of the same device, ant confronted to a cone-shaped paper.

Workers were picked up with clean forceps and put into chilled vials before immobilization. They all were foragers. All were tested only once to avoid any habituation. Naïve workers were introduced into their respective disposable Petri dishes for 2 h at 27±2 °C and with 60–80% relative humidity. Every test started 2 h after the beginning of the light phase of the daily cycle and finished 2 h before its end. Two immobilizing devices, containing one worker each, were positioned so that the workers faced each other. An opaque slide previously cleaned with ethanol physically divided the two ants prior to starting the experiment. Distances between workers were the same for all experiments. Five minutes later, the slide was taken off and the encounter was recorded on videotape. Each Petri dish was used only once. Behavioural responses were measured during 1 min starting 20 s after removing the slide.

### 2.3. Statistical analyses

Statistical analyses were performed with SigmaStat 3.0 software (SPSS Inc., Chicago, IL, USA). Normality was assessed by the Kolmogorov–Smirnov distance and homogeneity of variance computed with a Levene median test. Pairwise multiple comparison procedures were done using the Holm–Sidak test (Westfall et al., 1999). Mann–Whitney test was used for non-parametric statistics. Cluster analysis based on relative peak areas was performed using Ward's linkage method (Euclidean distance) (Ward 1963) with Statistica 6.0 software (Statsoft Inc., Tulsa, OK, USA).

### 2.4. Procedures

#### 2.4.1. Pairwise encounters of immobilized ants

The behavioural responses of Pv, Pi and Ps were studied on 116, 67 and 95 naïve workers, respectively from several queen-right colonies for each species. Behavioural analyses were carried out blind to avoid any experimentation effects (Gamboa et al., 1991). Every possible combination was tested between colonies or species. Three levels of interactions were considered: (1) the intra-colonial level, which is expected to lead to the lowest degree of response, (2) the intra-specific level and (3) the inter-specific level where each species encounters successively the two others.

#### 2.4.2. Behavioural responses to a chemical mixture

Ps workers, 161 from 4 queen-right colonies, were presented with a cone-shaped filter paper (Fisherbrand

A7070001) bearing the total lipid fraction, the subfractions of its different HC classes, or the *non*-HC fraction of the extracts from either Pv or Pi (Fig. 1c). Ps workers were tested using cuticular extracts from Pv or Pi workers (queen-right colonies). The cuticular lipids were extracted and separated into different classes as described later on. The total cuticular extracts as well as the extracts of the different lipids classes were concentrated to the same final volume (10 µl/worker). Amounts equivalent to three workers' cuticular extract (or each of its lipids fractions)—i.e. 30 µl—were deposited on a cone-shaped paper 5 min before each test. This paper had been previously washed with hexane 3 times, each time overnight. The control test consisted in deposition of hexane only.

### 2.5. Extraction and purification of classes of HCs

Workers—only foragers—picked up with clean forceps were placed altogether into chilled vials before extraction. Cuticular HCs of entire individuals were extracted (70 Pv or 140 Pi at once) by immersion for 5 min in 300 µl of hexane per worker with agitation. The samples were dried under nitrogen and dissolved in 10 µl of hexane per worker.

Cuticular extracts were first fractionated into different lipid classes by thin layer chromatography (TLC) on Silica Gel 60 F-254 in hexane/diethylether/acetic acid solvent (80/20/2, v/v). Control lipids and the cuticular extract of one worker were chromatographed simultaneously and stained with $I_2$. The HCs of the sample were thus easily located according to these controls, scraped from the plates and extracted with hexane. The rest of the plate, in which several lipid classes could be visualized with primulin (direct yellow 59) but were not identified, was scraped and extracted likewise as the *non*-HC fraction. Then, the total HC fraction (60 µg for Pv and 30 µg for Pi) was fractionated into alkanes and alkenes using a TLC plate impregnated with 10% of $AgNO_3$ (w/w) in hexane/toluene (80/20, v/v). Finally, the methyl-branched components were further separated from the straight-chain components by shaking the alkanes fraction with crushed 5 Å molecular sieve (0.16 cm pellets, Alltech, IL, USA) in 2,2,4-trimethylpentane (iso-octane) (Warthen and Uebel, 1980). The iso-octane extract was dried under nitrogen and dissolved in 10 µl of hexane per worker used for the extraction. The straight-chain components trapped by the molecular sieve may be freed by shaking it during 48 h in pentane. Nevertheless, as the rate of desorption is slow for a total recovery (O'Connor et al., 1962), commercially available *n*-alkanes (Fluka and Sigma-Aldrich, St Quentin Fallavier, France) were used instead. The quantities used were the same as for the cuticular extracts. Altogether, 5 different lipids classes were tested.

The purity of each tested sample was ascertained beforehand by gas chromatography-mass spectrometry (GC-MS) carried out with a Fisons mass spectrometer MD 800 (electron impact at 70 eV) directly coupled with a Carlo Erba gas chromatograph GC 8065MS (Lucas et al., 2002).

## 3. Results

### 3.1. Behavioural discrimination between species

Two behaviours were observed and their durations quantified (as cumulative times, in seconds): (1) mandible opening: when mandibles were opened wide ($\geqslant 120^{\circ}$) and remained in this position without labium extension (Fig. 2a); (2) antennal retraction: when antennae are brought backwards such that at least the basal segments are behind the axis formed by the front ends of the clypeus (Fig. 2b).

These two behaviours are characteristic of a recognition step in two *Pachycondyla apicalis* alien ants' encounter prior to fighting (Fresneau, 1980, 1994) or parts of more complex aggressive behaviours described for *Camponotus* species (Carlin and Hölldobler, 1986; Hölldobler and Wilson, 1990). Our "joust" device allowed individualizing them clearly.

They were then quantified to compare the three levels of interaction (intra-colonial, intra-specific and inter-specific) for the three species. Two-way ANOVA analyses showed a significant effect of the level of interaction (mandible openings: $F_{3,242} = 35.826$, $p < 0.001$; antennal retractions: $F_{3,242} = 23.210$, $p < 0.001$), a significant effect of species (mandible openings: $F_{2,242} = 6.234$, $p = 0.002$; antennal retractions: $F_{2,242} = 3.120$, $p = 0.046$) and an interaction between species and level of interaction (mandible openings: $F_{6,242} = 6.502$, $p < 0.001$; antennal retractions: $F_{6,242} = 7.192$, $p < 0.001$).

At the intra-colonial level of interaction, no differences were found between species for the two behaviours (mandible opening: $t < 1.693$, $p > 0.05$; antennal retraction: $t < 1.799$, $p > 0.05$). Otherwise, each species had its own set of responses.

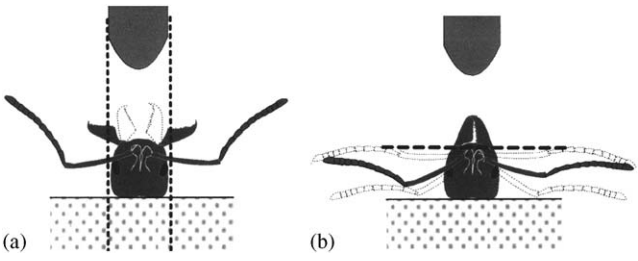


Fig. 2. (a) Mandible opening, (b) antennal retraction.

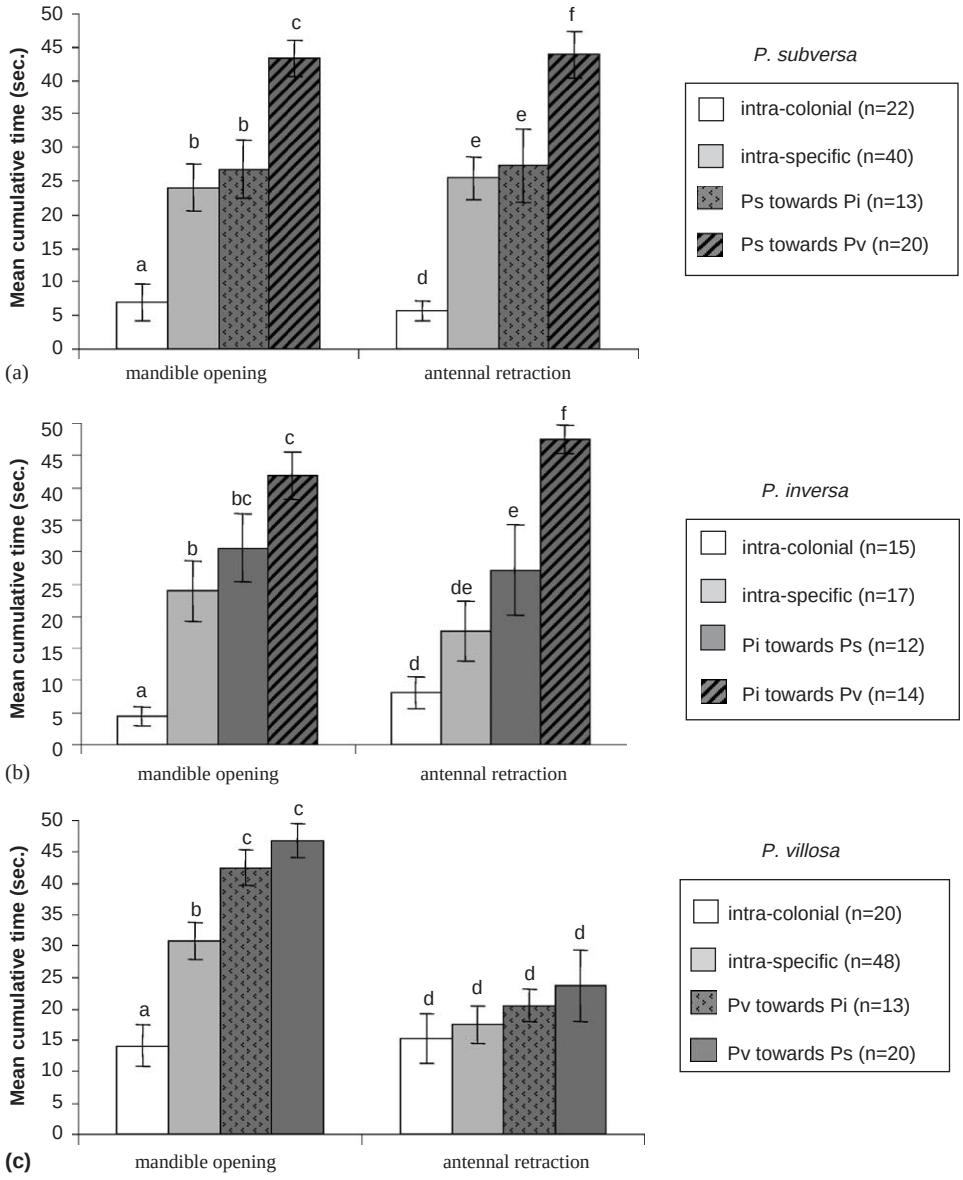


Fig. 3. Behavioural responses of *Pachycondyla subversa* (a), *P. inversa* (b) and *P.villosa* (c) in each type of encounters. Mean ± SEM (in s), bars with different letters are significantly different (two-way ANOVA with Holm–Sidak pairwise multiple comparison). The two behaviours were analysed separately.

Both types of responses of Ps lasted longer towards Pv than towards Pi (mandible opening: $t = 2.764$, $p = 0.006$; antennal retraction: $t = 2.643$, $p = 0.009$), suggesting a stronger discrimination of Pv (Fig. 3a). On the other hand, Ps response towards Pi was not different from its response towards other Ps colonies (mandible opening: $t = 0.527$, $p = 0.598$; antennal retraction: $t = 0.352$, $p = 0.725$; Fig. 3a).

The pattern of responses of Pi was the same as that of Ps. Indeed, the responses of Pi towards Ps were not different from its responses towards other Pi colonies (mandible opening: $t = 1.076$, $p = 0.283$; antennal retraction: $t = 1.439$, $p = 0.151$; Fig. 3b). However, only the antennal retractions responses were significantly longer towards Pv than towards Ps ($t = 3.848$, $p < 0.001$). This was not the case for the mandible opening response ($t = 1.700$, $p = 0.090$). Nevertheless, analyses using the species as a factor of comparison showed no difference between Pi and Ps towards Pv (mandible opening: $t = 0.0246$, $p = 0.980$; antennal retraction: $t = 0.146$, $p = 0.884$).

The mandible opening responses of Pv were shorter when the antagonists were from the same colony than when they belonged to different colonies ($t = 3.716$, $p < 0.001$). These responses were longer when the antagonists were from different colonies, but were longest when the antagonists were from Ps ($t = 3.060$, $p = 0.002$) or Pi ($t = 2.616$, $p = 0.009$). But the

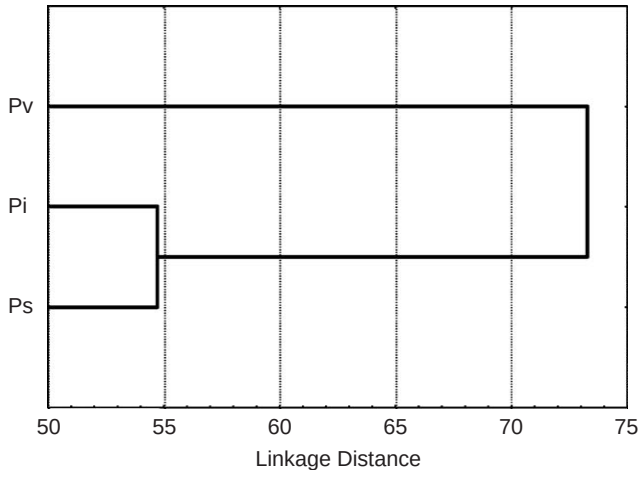


Fig. 4. Cluster analysis of cuticular hydrocarbon compositions of workers of *Pachycondyla villosa* (Pv), *P. inversa* (Pi) and *P.subversa* (Ps) ($n = 22$, 24 and 19, respectively), using Ward's linkage method (Euclidean distance) (Ward, 1963).

durations of antennal retractions did not change regardless of whether a homospecific or heterospecific worker was encountered ($t < 1.351$, $p > 0.05$; Fig. 3c). No statistical difference was found towards Pi or Ps (Fig. 3c) (mandible opening: $t = 0.731$, $p = 0.466$; antennal retraction: $t = 0.516$, $p = 0.606$).

As these results suggest some hierarchy in the behavioural discrimination by these species, a cluster analysis of all the cuticular hydrocarbon components was made (Fig. 4) which confirmed that Pi and Ps were closer to each other than to Pv ($n = 24$, 19 and 22, respectively).

### 3.2. Impact of the chemical extracts on Ps behaviour

As inter-specific interactions elicited the most important effects on Ps, an attempt was made to relate the discriminative behavior in these interactions to specific

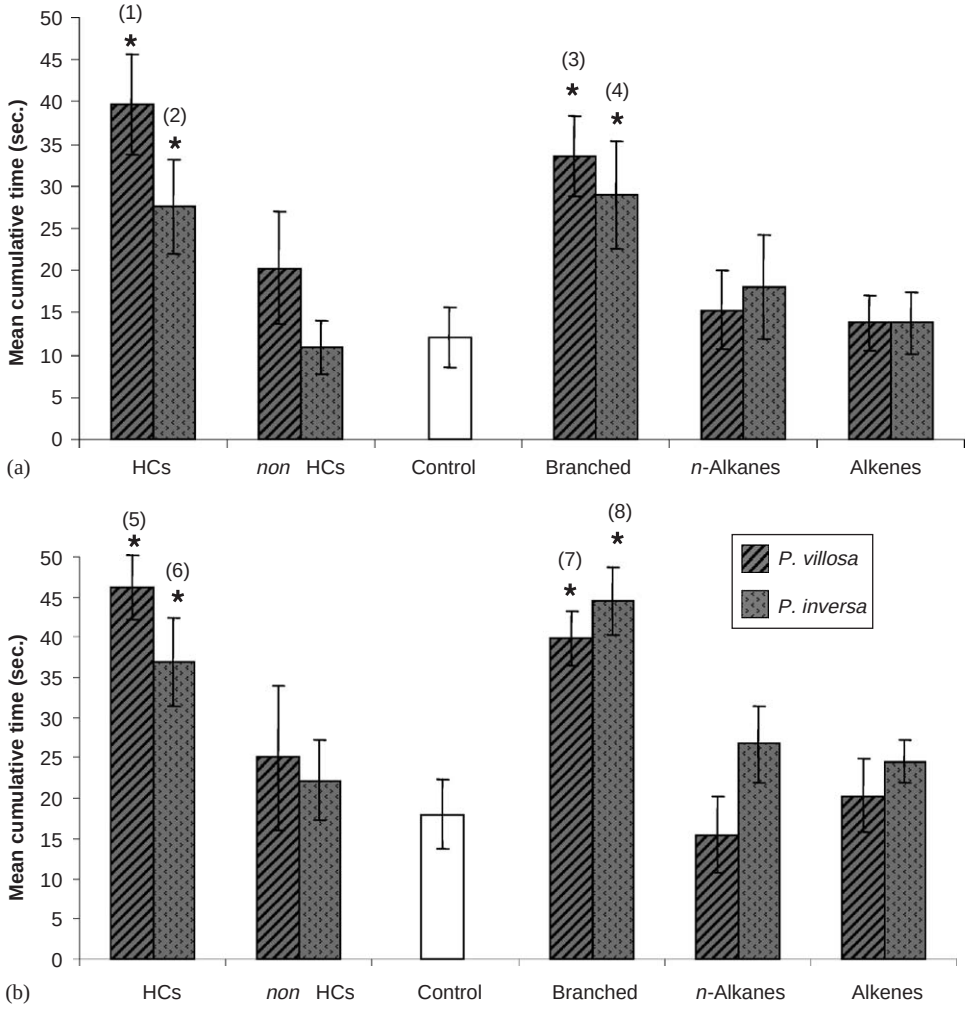


Fig. 5. Behavioural responses of *Pachycondyla subversa* towards chemical compounds tested for (a) mandible opening and (b) antennal retraction. Mean ± SEM (in s), star marks indicate values statistically different from control (Holm–Sidak pairwise comparison: (1) $t = 4.339$, $p < 0.001$; (2) $t = 2.400$, $p = 0.019$; (3) $t = 2.644$, $p = 0.010$; (4) $t = 2.325$, $p = 0.023$; (5) $t = 4.593$, $p < 0.001$; (6) $t = 3.297$, $p = 0.002$; (7) $t = 4.080$, $p < 0.001$; (8) $t = 3.030$, $p = 0.004$).

groups of cuticular HCs using Ps workers. These were confronted with one of the five different chemical classes of cuticular extracts from Pv and Pi. Out of 161 individuals 147, i.e. 91.30% of the encounters, showed clear antennal contacts with the cone-shaped paper regardless of the chemical class present. The mean duration of the first antennal contact with the sample was $12.1 \pm 3.1$ s (SEM) and the mean number of antennal contacts was $40.0 \pm 3.7$ for all the tests. Individuals that showed no contact with the sample were eliminated without further analysis.

As for the preceding experiment, the durations of mandible openings and of antennal retractions were investigated to discriminate the effects of different classes of lipids. Two-way ANOVA analyses showed a significant effect of lipid classes (mandible openings: $F_{10,65} = 4.617$, $p < 0.001$; antennal retractions: $F_{10,65} = 5.971$, $p < 0.001$), no significant effect of colonies (mandible openings: $F_{3,65} = 1.350$, $p > 0.05$; antennal retractions: $F_{3,65} = 0.278$, $p > 0.05$) and no significant interactions between lipid classes and colonies (mandible openings: $F_{30,65} = 1.586$, $p > 0.05$; antennal retractions: $F_{30,65} = 1.275$, $p > 0.05$). Mean values of both types of responses of Ps workers towards HC fractions (''HC response'') showed significant differences with the responses towards the solvent alone (controls) (Fig. 5a and b) while no difference was observed with the *non*-HC fraction. Moreover, the ''HC response'' for mandible openings and antennal retractions was not different from the ''inter-specific response'' of Ps shown in Fig. 3a (Mann–Whitney test, $p > 0.05$; $39.6 \pm 6.0$ s (SEM) and $43.3 \pm 2.7$, respectively for mandible opening towards Pv and $27.5. \pm 5.5$ and $26.8 \pm 4.3$ towards Pi; $46.1 \pm 4$ and $43.9 \pm 3.5$, respectively for antennal retraction towards Pv and $36.8 \pm 5.5$ and $27.4 \pm 5.5$ towards Pi). These two results clearly demonstrate that nestmate recognition cues for Ps are contained in the HC fraction.

Among HCs, the only fraction which induced significant behavioural responses compared to the control was that of branched alkanes (mandible openings: $t = 2.644$, $p = 0.010$ towards Pv and $t = 2.325$, $p = 0.023$ towards Pi as shown in Fig. 5a; antennal retractions: $t = 4.080$, $p < 0.001$ towards Pv and $t = 3.030$, $p = 0.004$ towards Pi as shown in Fig. 5b). The pairwise comparison between the ''HC response'' and the ''branched response'' showed no differences ($p > 0.05$) for all comparisons between species and behaviours. The purity of all tested fractions had been checked by CG-MS, as indicated in the methods.

The behavioural responses of Ps workers confronted to HC classes from Pv or from Pi were nearly significant for mandible openings ($t = 1.909$, $p = 0.061$) but not significant for antennal retractions ($t = 1.224$, $p = 0.225$). Nevertheless, mean response durations for antennal retraction were also lower towards the Pi HC fraction than in front of the Pv HC fraction (Fig. 5a and b) as it was the case in the inter-specific encounters shown in Fig. 3a.

## 4. Discussion

To study recognition abilities between ants a ''joust'' device has been set up in which an immobilized ant can be confronted either to another immobilized ant or to a perfumed paper. This device prevents injuries that may happen in classical dyadic tests but allows the precise quantification of two well-characterized behaviours usually observed in pairwise encounters. These ''mandible opening'' and ''antennal retraction'' take place quickly after antennal contacts. Workers of three closely related Pachycondyla species displayed both behaviours with durations that tended to increase from the intra-colonial to the intra- and inter-specific levels.

Each species displayed its own behavioural set of responses with characteristic durations. Both responses of Ps towards Pv were longer than towards members of other homospecific colonies, but did not differ from those towards Pi. A similar discrimination of Pv by Pi was observed but only in the ''antennal retraction''. Finally, Pv ants display long ''mandible opening'' towards con- and heterospecific ants of both other species—longer towards heterospecifics than towards homospecifics; they do not seem to discriminate Ps from Pi.

Altogether, responses of Pi and Ps towards Pv were statistically longer than between themselves. Conversely, Pv reactions to either Ps or Pi were not significantly different. This suggests that Pv might not discriminate Pi from Ps owing to their relative chemical similarity, whereas Pi and Ps would be more responsive to Pv than between themselves. This is correlated with the result of the cluster analysis of their HCs which shows those of Ps and Pi closer to each other than to those of Pv. Our behavioural data thus support the current recognition hypothesis in social insects which relates behaviours to cuticular hydrocarbon compositions (Howard and Blomquist, 2005).

Our behavioural ''joust'' device also allowed testing of perfumed papers with cuticular extracts and their fractions. The observed behaviours were induced in an artificial context, but this assay turned out to be helpful in the study of early behavioural responses that might not be observed or quantified easily in a classic dyadic context. Ps were selected as testers as they displayed variations of both behaviours and with longer variations towards heterospecifics. Significant differences in the durations of both behavioural responses, ''mandible opening'' and ''antennal retraction'', were induced in Ps workers by the HC total fraction of Pv compared to controls (Fig. 5). Moreover these quantitative responses were not

different from the inter-specific interactions reported in Fig. 3. Among these HCs, only the "branched alkanes" fraction yielded quantitative responses significantly different from controls for both behaviours. Neither *n*-alkanes nor alkenes had a significant effect in Ps, although this species completely lacked the latter class of HCs. Responses induced by total HCs of Pi also induced Ps responses significantly different from those of controls but these look to be shorter than those induced by HCs of Pv. It is noteworthy that Pi and Pv cuticular HC compositions differed in both chain lengths—shorter in Pi—and the abundance of internally branched dimethylalkanes in Pv (Lucas et al., 2002, 2004).

In ants, HCs are present on the surface of the cuticle and in a gland specific for ants, the postpharyngeal gland (PPG). Some experiments have shown that PPG HCs possess a behaviour role in nestmate recognition. For example, in *Cataglyphis niger*, the application of nestmate PPG HCs on alien ants made them acceptable in the colony (Soroker et al., 1994; Lahav et al., 1999). We have shown in Pv (Lucas et al., 2004) that the PPG HC composition differed from that of the cuticle by a marked decrease of *n*-alkanes and externally branched monomethylalkanes (with a methyl-branching on carbon 2–6). It was thus hypothesised that these two classes of HCs were to be excluded from the recognition cues of this species.

This leaves internally branched monomethylalkanes and dimethylalkanes of Pv as candidates for inducing behavioural responses of Ps. The internally branched monomethylalkanes of Pi and Pv differ in their chain-lengths with longer chains in Pv while they are rare in Ps. Conversely, the latest species is rich in externally-internally branched dimethylalkanes (Lucas et al., 2004). This might be one criterion of behavioural reactivity. Besides, internally-internally branched dimethylalkanes are present in markedly different abundances on the cuticles of Pv and Pi (76% and 11%, respectively, as calculated from Lucas et al., 2004). In this latter species there are also abundant internally branched monomethylalkanes. The importance of methylated HCs has also been demonstrated by Gamboa et al. (1996) in kin recognition of social wasps. It will be interesting to further separate the different classes of methylalkanes in order to test them. This would require preparative gas chromatography with larger but not yet available amounts of HCs or synthesis of the different components and their isomers. Indeed, up to now a number of position isomers for methyl branching cannot be separated and their proportions in their mixtures cannot be compared within homospecific colonies yet (Lucas et al., 2004). This would be necessary to elucidate the mechanism of recognition between homospecific colonies but the "joust" device introduced here could be used.

The graduated aggressive responses linked to the differences of HC profiles amongst the three sympatric species could then depend on the capacities of their decoding systems that enable discrimination between these species. Further interpretation of these results is uneasy due to the currently poor understanding of how antennal receptors function and integrate their signals. However, evidence that antennal receptors of Pi workers respond to the queen's fertility signal-3,11-di Me $C_{27}$ , identified by Heinze et al. (2002)—was first reported only recently by D'ettore et al. (2004b). Use of new techniques and tests would thus be helpful in further studies of social communication in ants.

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# Molecular basis for changes in behavioral state in ant social behaviors

Christophe Lucas[a,b] and Marla B. Sokolowski[a,1]

[a]Department of Biology, University of Toronto, Ontario, Canada L5L 1C6; and [b]Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland

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**A hallmark of behavior is that animals respond to environmental change by switching from one behavioral state to another. However, information on the molecular underpinnings of these behavioral shifts and how they are mediated by the environment is lacking. The ant *Pheidole pallidula* with its morphologically and behaviorally distinct major and minor workers is an ideal system to investigate behavioral shifts. The physically larger majors are predisposed to defend the ant nest, whereas the smaller minors are the foragers. Despite this predisposition, majors are able to shift to foraging according to the needs of the colony. We show that the ant foraging (*ppfor*) gene, which encodes a cGMP-dependent protein kinase (PKG), mediates this shift. Majors have higher brain PKG activities than minors, and the spatial distribution of the PPFOR protein differs in these workers. Specifically, majors express the PPFOR protein in 5 cells in the anterior face of the ant brain, whereas minors do not. Environmental manipulations show that PKG is lower in the presence of a foraging stimulus and higher when defense is required. Finally, pharmacological activation of PKG increases defense and reduces foraging behavior. Thus, PKG signaling plays a critical role in *P. pallidula* behavioral shifts.**

cGMP-dependent protein kinase | defense | foraging

Major genes for normal individual differences in behavior are now familiar in a variety of species (1). However, an important feature of behavioral traits is that they are responsive to environmental change. Animals are able to switch from one behavioral state to another, yet little is known about the molecular basis of these behavioral shifts and how they are mediated by the environment. Eusocial insects are excellent models for studying these gene–environment interdependencies because their social organization relies on individuals who belong to behaviorally specialized castes. Wilson (2) showed that, despite this behavioral specialization, individuals of one caste can rapidly modify their behavior, depending on colony requirements; he called this the flexibility of behavioral castes. These quick changes in caste behavioral repertoires provide enough flexibility in colony response to maintain the colony when the environment goes through rapid changes. For example, ants whose role is to defend the nest are able to switch to foraging, depending on the needs of the colony. How might this switch from one behavioral state to another be accomplished?

The ant *Pheidole pallidula* provides us with an excellent system to investigate the molecular underpinnings of flexibilities in behavioral predispositions. This species has 2 morphologically distinct worker castes, called majors and minors (3), that we will refer to here as subcastes. Both reach the same stage of maturity and work outside the nest. Majors have large heads and mandibles and specialize in colony defense; they guard the nest, patrol outside the nest entrance, and kill intruders. Minors are smaller and perform foraging behaviors, including food-search and prey retrieval. However, majors who are built for defense can switch their behavioral state from defense to foraging, depending on the needs of the colony, thereby demonstrating flexibility in their behavioral repertoires (2, 4). Physical differences between majors and minors initiate in developmental pathways that diverge during the larval stage of development. Cues originating from nutrition fed to the larvae and from the social environment are integrated by the endocrine system to initiate the relevant developmental pathway (2, 5, 6).

Here, we investigate the molecular underpinnings of behavioral flexibility in *P. pallidula* workers ants, which are highly specialized both morphologically and behaviorally. We show that the activity of the enzyme cGMP-dependent protein kinase (PKG) encoded by the ant foraging (*ppfor*) gene functions in the change from one behavioral state to another and that this is correlated with differences in the spatial expression of the PPFOR protein in the brains of the 2 worker subcastes. Our study also provides evidence that PKG plays a role in defense.

## Results

**PKG Activity Is Subcaste Specific.** We cloned the *for* ortholog, *ppfor*, from *P. pallidula*. To investigate subcaste-specific differences in PKG enzyme activity, PKG assays were performed on adult brains (7). Majors had significantly higher PKG enzyme activity than minors in all colonies (Fig. 1*A*). This suggested a correlation between brain PKG activity and these physical and behavioral castes.

**PPFOR Differs Spatially in Subcaste Brains.** We were successful in visualizing PPFOR expression in ant brains. We used an antibody made to regions of the *Drosophila melanogaster* FOR protein that had high homology to the PPFOR protein isoform encoded by the longer *ppfor* transcript T1 (8) (see *SI Text* on brain biometry; controls for the FOR antibody are in Fig. 3 and in Fig. S2). We found that *P. pallidula* has 3 main PKG-immunoreactive regions: one cluster dorsal and on the internal face of the lobula, another posterior and ventral to the mushroom body peduncles, and a third cluster on the anterior face of the subesophageal ganglion (Fig. 2). Notably, we observed expression in several regions of the anterior brain in majors but not minors. Specifically, brains of majors, but not minors, display strong immunoreactivity in approximately 5 cells clustered on the anterior face external to the mushroom body peduncles (Fig. 2*E*). An intriguing possibility is that this difference in subcaste PPFOR protein expression may be related to their different behavioral profiles. Such a link would reflect a similar case where social behaviors exhibited by montane and prairie voles are linked to variation in the distributions of vasopressin receptors in their brains (9).

**PKG Activity and Environmental Manipulations of the Colony Environment.** We investigated the flexibility of the behavioral predispositions in majors and minors by manipulating the colony envi-

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[1]To whom correspondence should be addressed. E-mail: marla.sokolowski@utoronto.ca.

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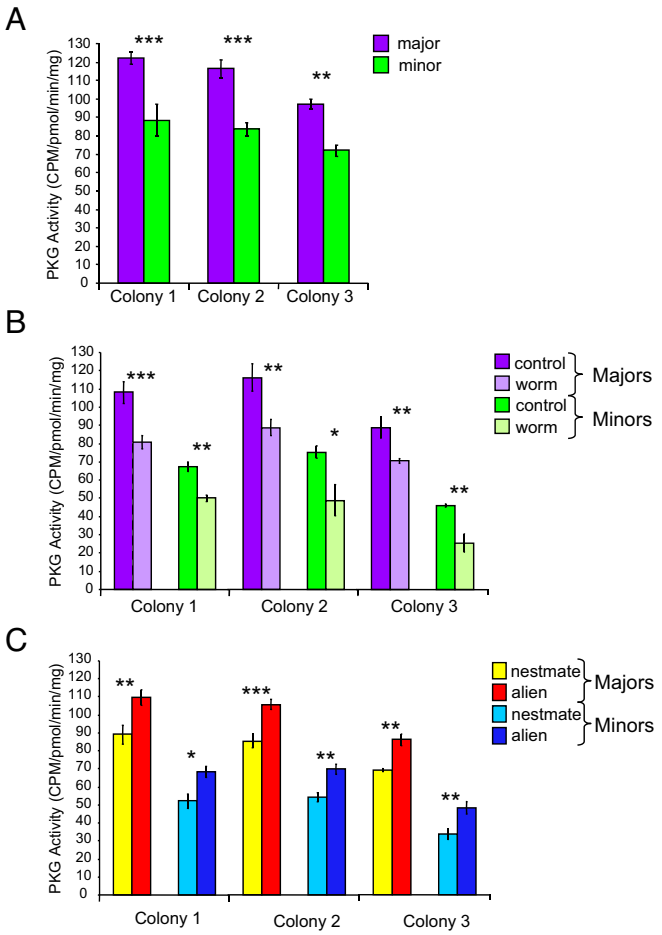


**Fig. 1.** PKG activity of major and minor *P. pallidula* worker ants (mean ± SE). (*A*) Brain PKG activities of majors and minors differ (nested ANOVA, $F_{(3,29)} = 19.04$, $P < 0.001$). (*B*) PKG activity of majors and minors was lower in the presence of the mealworm (foraging stimulus) compared with the control ($F_{(3,45)} = 24.13$, $P < 0.001$); majors had higher PKG activity than minors ($F_{(3,45)} = 73.41$, $P < 0.001$). (*C*) Majors and minors had higher PKG activity in the presence of aliens (defense stimulus) compared with nestmate intruders ($F_{(3,41)} = 23.07$, $P < 0.001$); majors had higher PKG activity than minors ($F_{(3,41)} = 103.19$, $P < 0.001$). For *B* and *C*, the significance of all within-colony comparisons not indicated on the figure was $P < 0.001$. In all figures, *P* levels of $P < 0.001$, $P < 0.01$, and $P < 0.05$ are represented as ***, **, and *, respectively.

ronment. We first introduced either a live mealworm or a mealworm-shaped plastic model (control). In the presence of the mealworm prey, foraging minors rapidly (<5 min) recruit majors to help them cut up the prey and transport it to the nest (4). This successful recruitment of majors for food-related activities confirms that majors are behaviorally flexible. Under certain circumstances and notwithstanding their disposition for defense behaviors, majors will perform foraging tasks. Because minors, which are specialized foragers, have lower PKG activity than majors, we predicted that PKG activities would be lower in majors performing foraging behaviors compared with those in control experiments. Consistent with this prediction, brain PKG activities of both majors and minors, collected from 3 independent colonies, were significantly lower than those of controls when exposed to a foraging stimulus: the mealworm prey (Fig. 1*B*). Another way to stimulate foraging behavior is food deprivation (3). We food-deprived the colonies for 3 weeks, and as expected, PKG activity was lower than that in well-fed controls (Fig. S1) as was found in the mealworm prey experiment (Fig. 1*B*). Together, these results suggest that the mealworm prey is acting as a foraging stimulus for the majors and minors.

Majors have higher PKG activity than minors and are predisposed to act in colony defense. Therefore, second, we asked whether the link between PKG activity and behavioral flexibility extends to colony defense. Methods developed for nestmate recognition experiments were used to assay colony defense (10). We introduced intruder ants who were either alien (from another colony) or nestmates (from the home colony) into an arena placed into a test colony. Ants contact the intruders through small openings in the side of the arena. In all colonies, brain PKG activity was elevated in both subcastes when exposed to alien intruders compared with nestmate controls (Fig. 1*C*), suggesting a possible role for PKG in defense.

**Pharmacological Manipulation of PKG Activity Changes Subcaste Behaviors.** Data presented thus far demonstrate a correlation between PKG enzyme activity and subcaste-specific behaviors, foraging and defense, and suggest that PKG may modulate the probability of performing these behaviors. To initiate testing a causal link, we pharmacologically manipulated PKG activity and measured foraging and defense behavior in majors and minors. We treated ants with a specific PKG activator, 8Br-cGMP, as in Ben-Shahar et al. (11). As expected, the mean specific brain PKG activity of majors and minors was higher after 8Br-cGMP treatment ($F_{(2,20)} = 16.76$, $P < 0.001$): majors, 84.85 ± 3.21 for control and 101.25 ± 4.55 for treatment; minors, 36.64 ± 2.27 for control and 50.51 ± 2.57 for treatment [units are pmol of $^{32}$P-labeled PKG substrate/(min/mg of protein)]. Thus, PKG activities were elevated by the treatment, and this increase was within the normal range (Fig. 1).

To assess the behavioral effect of 8Br-cGMP treatment on foraging behaviors, we quantified the percentage of workers that interacted with a mealworm when treated with the PKG activator as compared with the control. As expected, the pharmacological treatment resulted in fewer majors and minors responding to the presence of the mealworm (Fig. 4*A*), suggesting that this increase in PKG was sufficient to change the probability of responding to the foraging stimulus. The relative decrease in the foraging response of majors was greater than that in minors ($F_{(3,27)} = 69.22$, $P < 0.001$), suggesting that majors may be more flexible than minors. We next tested the effects of the pharmacological treatment on defense behaviors. Consistently, majors showed a significantly higher defense response to the aliens in response to the pharmacological treatment relative to the control (Fig. 4*B*), showing that the treatment was sufficient to increase the probability of responding to the defense stimulus. The pharmacological treatment, however, did not affect the response of the minors to the alien intruders (Fig. 4*B*). Because the pharmacological treatment was administered for a number of days, we cannot yet distinguish the effects of chronic or acute changes in PKG activity on the behaviors of majors and minors.

## Discussion

Physical castes provide a great model for understanding the molecular basis of individual differences in behavior because their discrete alternative phenotypes result in striking behavioral flexibility. One interpretation of our data is that the behavioral differences between majors and minors are due to shifts in levels of response for task-related stimuli (12) and that this occurs in part by tuning the PKG system. In this model, major and minor PKG activity in undisturbed colonies reflects developmentally established predispositions to perform defense and foraging behaviors, respectively. The mealworm and alien intruder experiments suggest that the PKG system can tweak the response, eliciting flexible behavioral responses especially in majors. Shifts in behavioral responses are further shown by using the PKG activator, which was fed to these ants for several days before the behavioral assay. This pharmacological treatment implies a causal relationship among PKG, behavioral flexibility, and the

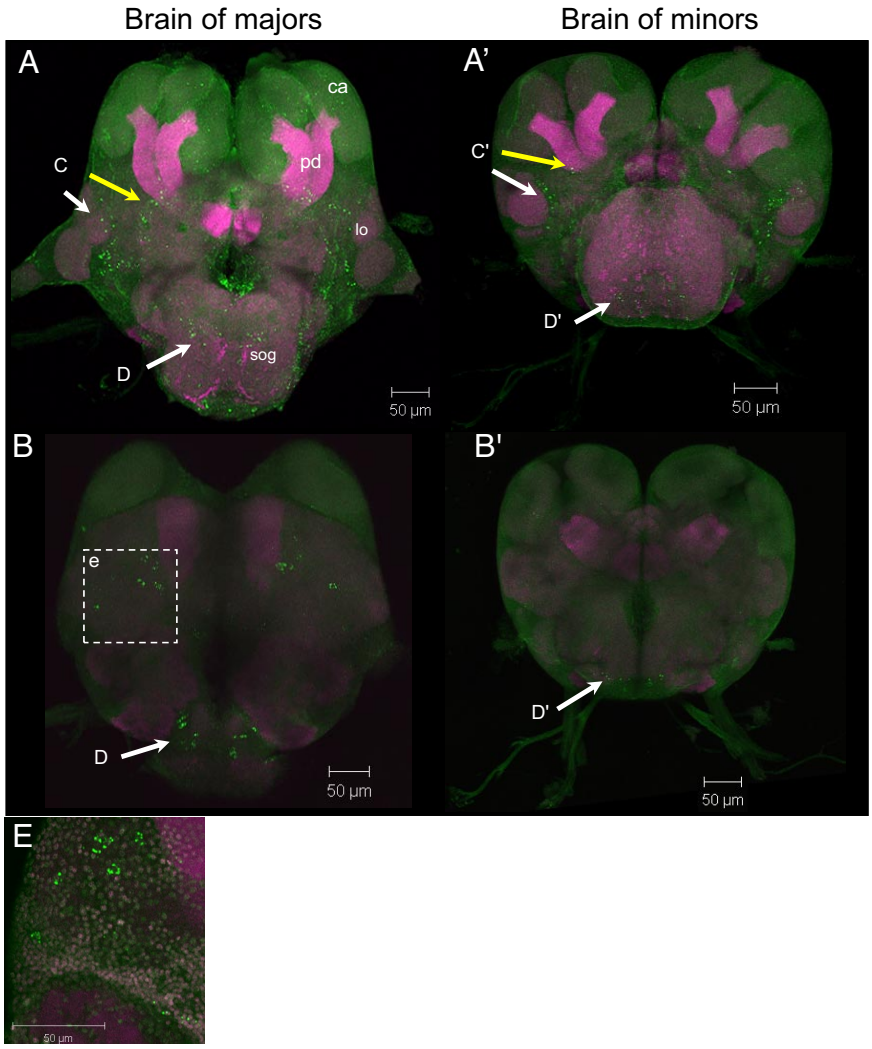


**Fig. 2.** FOR expression patterns differ in the brains of major and minor *P. pallidula* worker ants. All brain images are 3D reconstructions of 1-$\mu$m optical sections shown in a posterior view with dorsal part at the top. PKG immunoreactivity (PKG-IR) is indicated in green, and magenta indicates brain neuropiles (mouse antibody, nc82). (*A* and *A′*) Whole brain reconstruction of majors (*A*) and minors (*A′*) shows 3 main PKG-IR regions: a cluster dorsal and on the internal face of the lobula (*C* and *C′*, white arrows), a cluster posterior and ventral to the peduncles of the mushroom bodies (*C* and *C′*, yellow arrows), and a cluster on the anterior face of the subesophageal ganglion (*D* and *D′*) (enlargement of details is shown in Fig. 3). (*B* and *B′*) Three-dimensional reconstruction of subsections (40 sections) of the whole brains (*A* and *A′*), spanning the posterior part of the brain of majors (*B*) and minors (*B′*), respectively, shows a cluster of PKG-IR cells found in the brain of majors (*E*, 30 sections) but not in minors. This specific cluster is localized on the anterior face external to the peduncles of the mushroom bodies; negative controls using a preabsorption step to abolish PKG-IR and blank controls using only the secondary antibody are in Fig. 3. ca, Calyx; pd, peduncles; lo, lobula; sog, subesophageal ganglion.

environmental stimulus (mealworm or alien intruder). The pharmacological treatment, however, did not affect the response of the minors to the alien intruders. These results may indicate a limit, perhaps established in larval or early adult development, in the minors' ability to perform defense behavior, which could be tied to the differences in FOR spatial patterning in major and minor brains. The morphological specialization found in these physical castes could, for example, involve differential spatial patterning of major and minor brains.

The possible mechanistic similarity between developed predispositions (caste differences in behavior) and adult flexibility (within-caste behavioral shifts) is implicit in our findings. The same mechanisms that cause majors to defend and minors to forage could also underlie behavioral flexibility within a fully developed, morphologically differentiated caste. Certainly many genes and pathways are involved in the development and performance of foraging and defense behaviors in ants; however, we have shown here that it is sufficient to alter PKG activity to affect behavioral outcomes.

The behavioral effects of *for*-PKG were originally described in *D. melanogaster*, where allelic variation in *for* affects larval and adult foraging behaviors (13, 14). Individuals with a "rover" allele express greater foraging locomotion and eat less than those with only "sitter" alleles (7). In this system, the allelic variation in *for* is important for rover or sitter behavior; however, *for* also affects plasticity; it modulates these behaviors in response to environmental input (7, 15, 16).

A link between *for* and long-term behavioral maturation has been described in social insects where *for* up-regulation in individual workers leads to maturation from performing nursing activities within the colony to foraging outside the honey bee colony (11, 17). Nurses work inside the nest and become foragers who work outside the nest as they mature. Up-regulation of PKG in nurse bees appears to increase the readiness of honey bees to forage. In these honey bees, both age and place of work differ between nurses and forager bees. In *P. pallidula*, major and minor ants reach the same stage of maturity, and both work outside of the nest.

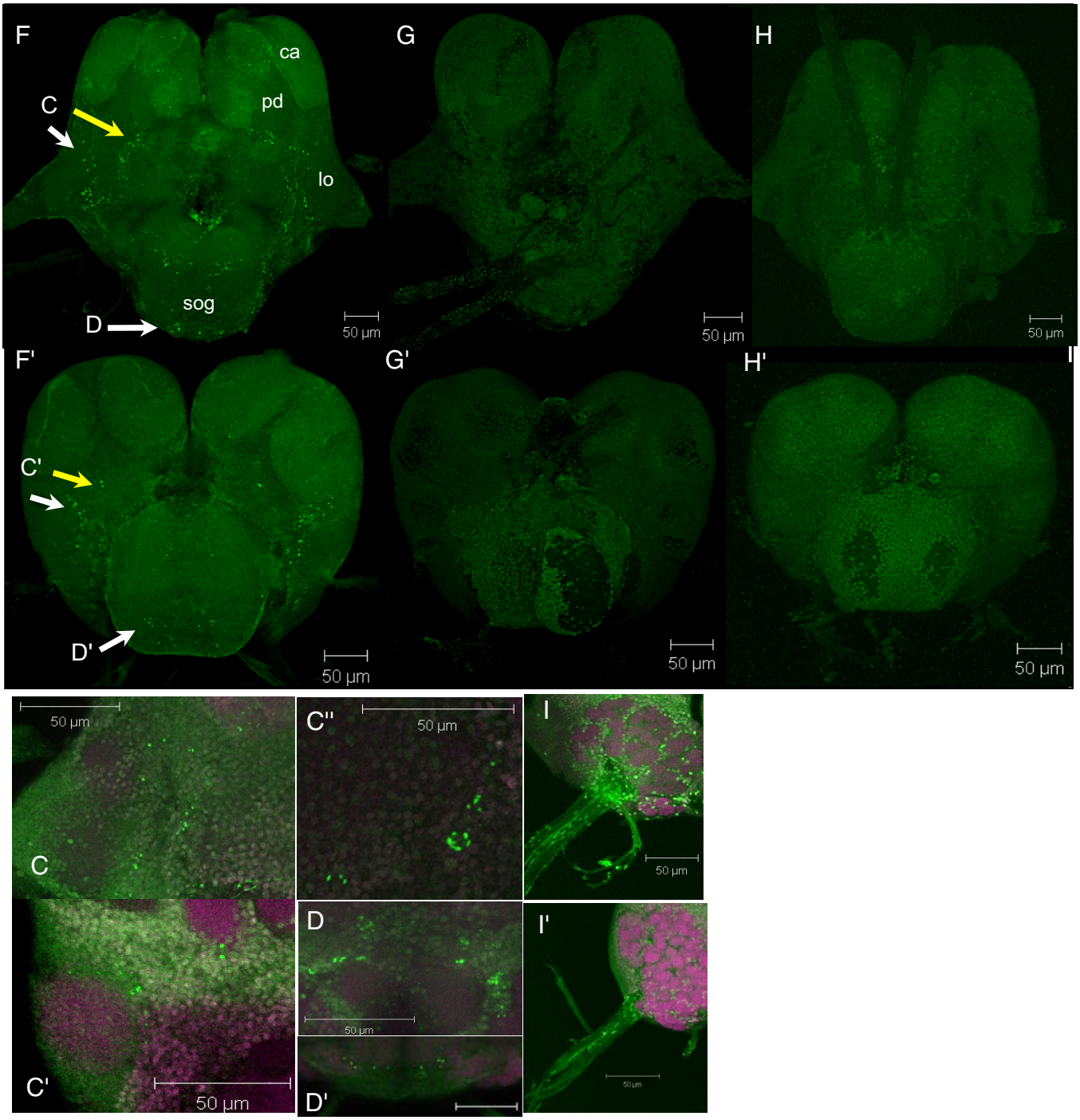


**Fig. 3.** Negative controls (posterior view) and details of PPFOR antibody clusters. Colors are the same as those in Fig. 2. All brain images are 3D reconstructions of 1-$\mu$m optical sections shown in a posterior view with dorsal part at the top. (*F*–*H*″) Anti-PKG staining of major (*F*) and minor (*F*′) whole brains; negative controls using a preabsorption step abolish PKG immunoreactivity in majors (*G*) and minors (*G*′); controls using only the secondary antibody in major (*H*) and minor whole brains (*H*′); 2 clusters, 1 dorsal and on the internal face of the lobula (lo) (white arrows) and 1 posterior and ventral to the peduncle (yellow arrows) of majors (*C*, 20 sections) and minors (*C*′, 20 sections); cluster on the anterior face of the subesophageal ganglion of majors (*D*, 20 sections) and minors (*D*′, 20 sections); (*C*″, 10 sections) single sections taken at higher magnification in the lobula region suggest that PKG is localized to the plasma membrane, cytosol, or both; PKG is also expressed in fiber-like projections in antennal nerves in majors (*I*, 25 sections) and minors (*I*′, 25 sections). ca, Calyx; pd, peduncles; lo, lobula; sog, subesophageal ganglion.

A correlative study on the *for* gene in the ant *Pogonomyrmex barbatus* compares *for* mRNA levels of nurse and forager ants who are different ages and work inside and outside the nest, respectively (18). As is the case in honey bees, foraging gene expression in *P. barbatus* ants is linked to the temporal polyethism (11). The present study provides a role for PKG in defense and caste polyethism.

There are many interesting contrasts and parallels between the behavioral roles of *for*-PKG in *D. melanogaster* and *P. pallidula*. For example, in *D. melanogaster*, genetic polymorphism contributes to the predisposition to behave as a rover or sitter (13). By contrast, in *P. pallidula*, differential feeding of the larvae and social cues influence the development of majors or minors physical caste and behavioral predispositions (6, 19). However,

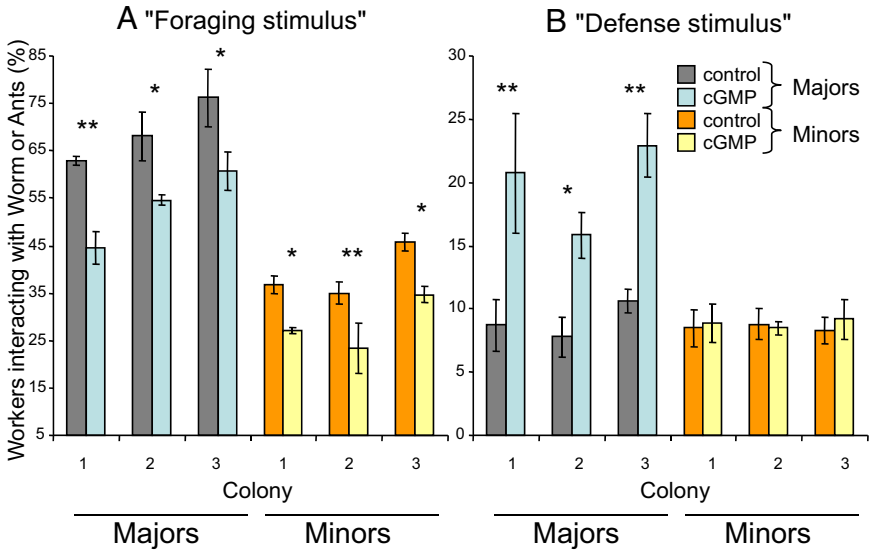


**Fig. 4.** Effect of a PKG activator (8Br-cGMP) on foraging and defense behaviors. (*A*) 8Br-cGMP treatment resulted in a lower percentage of majors ($F_{(3,12)} = 8.05$, $P = 0.003$) and minors ($F_{(3,12)} = 8.06$, $P = 0.003$) interacting with the mealworm compared with the control. Majors respond more to the presence of the worm than minors ($F_{(3,27)} = 69.22$, $P < 0.001$). (*B*) 8Br-cGMP treatment resulted in a higher percentage of majors responding to the presence of aliens ($F_{(3,12)} = 9.15$, $P = 0.002$) but had no significant effect on the percentage of minors interacting with the alien intruders ($F_{(3,12)} = 0.11$, $P = 0.95$). All data are mean percentages ± SE.

even with such predispositions, individuals of either species can exhibit flexible behavioral responses to environmental stimuli. Specifically, rover flies express more sitter-like foraging when they undergo long-term nutrient deprivation (7), and major ants can rapidly exhibit minor-like foraging behaviors in the presence of a prey item and according to the needs of the colony.

The behavioral influence of *for* is conserved across phyla. However, the directional affects of PKG on foraging-related behaviors differs between species: The rover "forager" type arises from higher *for* gene activity in the fly (13) and honey bee (11) and lower activity in *Caenorhabditis elegans* (20) and ants (18). These data suggest that natural selection has harnessed the same gene in different species in somewhat different ways for related behavioral phenotypes despite large differences in their life histories. That spatial differences in expression of PPFOR were found in the *P. pallidula* brain and not in the rover and sitter fly (8) or nurse and forager honey bee brains (11) supports this hypothesis.

## Materials and Methods

**Cloning.** The *P. pallidula for* ortholog *ppfor* was cloned using degenerate primers designed to conserved sequence (sense primer, GTGAACTKAT-CAARGCWGCCATWTTGG; antisense primer, CGATGGCCCAYAGSTKG-CAMTCGG) after alignment of several other species. mRNA was extracted using 50 heads with an Amersham Biosciences kit. Specific cDNA was amplified using a RACE kit from Clontech and cloned with a TOPO TA Cloning kit from Invitrogen. We identified 2 transcripts, 3,924 and 1,836 bp in length (GenBank accession nos. EF999975 and EF999976). The longer *P. pallidula* (T1) transcript has 70% identity with the longest *D. melanogaster for* transcript and 81% identity with the honey bee *Amfor* transcript. The longer *ppfor* transcript resembles a typical PKG (21), sharing the same protein domains found in all of the foraging gene sequences, including: a kinase domain (with a catalytic site), 2 cGMP-binding domains, and a regulatory domain. The short *ppfor* (T2) transcript differs from T1; it only has half of the second cGMP-binding domain and lacks the catalytic site of the kinase domain. Northern blot analyses confirmed the number and size of the *ppfor* transcripts in ant heads.

**PKG Enzyme Assays.** PKG enzyme assay was modified from Kaun et al. (7). Ants were collected, flash frozen in liquid nitrogen, and kept at −80 °C until dissection. Five brains per sample were dissected and homogenized in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM 2-mercaptoethanol, 0.05% Triton X-100, and protease inhibitor mixture (Roche). After 5 min of centrifugation, supernatants were quantified for total amount of protein and analyzed for PKG activity. The final reaction mixture contained 40 mM Tris·HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM [$\gamma$-$^{32}$P]ATP (250–500 cpm/pmol) (Amersham Biosciences), 1 mM EDTA, 2 mM EGTA, 143 $\mu$g/mL of a heptapeptide substrate highly specific to the PKG (RKRSRAE) (Promega), 3 $\mu$M cGMP, and 92 nM highly specific cAMP-dependent protein kinase inhibitor ($K_i$ 50% = 2.3 nM) (5–24, Calbiochem). The reaction mixture was incubated for 10 min at 30 °C and terminated by spotting 70 $\mu$L onto Whatman P-81 filters. The specific PKG activity was expressed as pmol of $^{32}$P-labeled PKG substrate/(min/mg of protein). PKG assays were performed on 5 groups of 5 dissected brains per subcaste per colony with 3 colonies.

**Immunohistochemistry.** Immunohistochemistry was as in Belay et al. (8). Briefly, brains of majors and minors were dissected in PBS (0.1 M, pH 7.4), fixed in 4% paraformaldehyde, and blocked in 4% normal goat serum (Jackson ImmunoResearch) in 0.5% Triton X-100/PBS. Individuals were collected from different colonies, and no differences in expression were found between colonies. The specific guinea pig antibody called anti-FOR, described in Belay et al. (8), was used at 1:100; the neuropile marker mouse mAb nc82 was used at 1:20 (22, 23). Incubation was for 24–48 h at 4 °C. After incubation, brains were washed several times in 0.5% Triton X-100/PBS before adding a goat Cy2-conjugated anti-mouse and a Cy5-conjugated anti-guinea pig Ig (1:100, Jackson ImmunoResearch) for 24 h at 4 °C. For negative controls, brains were incubated in only secondary antibody, in the absence of primary antibody or in preadsorbed anti-FOR serum (Fig. 3). Brains were washed several times, mounted, and examined with a Zeiss LSM 510 confocal laser scanning microscope. The specificity of the primary antibody, anti-FOR antibody generated in guinea pig (8), was measured by using Western blot immunodetection (24) (Fig. S2).

**Ant Collection and Rearing.** *P. pallidula* were collected from the field in southern France in the summer of 2003. This species is monoandrous (only 1 male inseminates a female) and polygynous (2 or more queens per nest) (25). In our experiments, only 1 queen was kept per nest, and experiments were conducted after the ants had been reared in the laboratory for 12 months. All nestmates within a colony shared the same mother and father, which maintained relatedness of the workers around 3/4. Ants were reared and tested under standard laboratory conditions (45% relative humidity, 27–28 °C, under a 12:12 light cycle with lights on at 0900). All colonies were fed the same food on the same schedule (ants were fed water ad libitum, honey, and ≈18 mealworms twice weekly).

**Statistical Analyses.** All data were analyzed byusing nested analyses of variance to take into account the effect of colony of origin (26). For the behavioral analyses, no effect of the repeated testing of each colony was found using a likelihood ratio test. This factor was then removed from analyses. All means are given ± SE. In all figures, *P* levels of $P < 0.001$, $P < 0.01$, and $P < 0.05$ are represented as ***, **, and *, respectively. The number of dead ants was not included as part of the totals.

**Foraging Assays.** The PKG activities of majors and minors given a live mealworm (*Tenebrio* spp., Ward's Natural Science) or a mealworm-shaped plastic model (control) were compared for ants from 3 colonies. Three-hundred minors and 50 majors (the approximate ratios of minors and majors found in our colonies) were collected 4 days before the experiment and placed in a test colony, which consisted of a new plastic Petri dish (140 mm in diameter by 20 mm in height, coated with Teflon on the inner dish wall) containing a watering tube (75 mm, containing 3 mL of distilled deionized $H_2O$, cotton sealed), a nest tube (100 mm, containing 5 mL of distilled deionized $H_2O$, cotton sealed and covered in dark plastic wrap), and a test arena. The test arena, composed of a small (35 mm in diameter by 10 mm in height) Petri dish, was placed in the center of the foraging area of the test colony. For the foraging assays, this arena was constructed so that ants could move freely in and out of the arena with a live mealworm. With a heated needle, 5 equally spaced slits were made around the wall of the Petri dish arena, 1 mm from the bottom of the dish. Each slit was 20 mm long, 1.5 mm wide, and 2 mm spaced apart. The entire outside surface of the dish was painted with white acrylic paint for optimal video recording contrast and counting of individuals within the arena. The colony lid was removed for filming, which began after a 5 min acclimatization period. The mealworm or a control was placed into the center of the arena for 35 min. At the end of this time period, ants that were inside the arena were collected, flash-frozen in liquid nitrogen, and kept at −80 °C until dissection. All colonies were fed 3 mealworms and 6 drops of concentrated honey daily for the 4 days before the experiment.

**Defense Assays.** The brain PKG activity of majors and minors exposed to either alien or nestmate intruder ants (control) were compared for ants from 3 colonies. All alien intruder ants came from the same colony. Test colonies were established as for the foraging assays with 300 minors and 50 majors. The defense arena was constructed so that intruder ants were contained within an area while ants from the colony could encounter the intruders through small slits in the arena. With a heated blade on a dissecting scalpel, 4 rows of 12 equally spaced slits were made around the wall of the Petri dish arena, 1 mm from the bottom of the dish and 1 mm from each successive row. Each slit was 8 mm long, 0.5 mm wide, and 1 mm spaced apart. The slits were made in a staggered brick pattern. The bottom of the arena dish was painted with white acrylic paint for optimal recording contrast. A primary cover was placed over the arena, and the outer wall side surface of the cover was coated with Teflon to prevent outside individuals from climbing over the arena and cover. A 20-mm circular aperture was burned through the top surface of the dish cover for easy insertion of the intruder alien or nestmate ants. A secondary dish cover was made to cover the surface aperture of the primary dish cover constructed from the bottom portion of a Petri dish. Two-hundred minors and 25 majors were collected from an alien source (individuals from another colony) or nestmate source (individuals from the home colony) and introduced into the defense arena. The colony lid was removed for filming, which began after a 5 min acclimatization period. The alien or nestmate intruder ants were placed into the center of the arena for 35 min. At the end of this time period, ants that were touching the side of the arena were collected, flash-frozen in liquid nitrogen, and kept at −80 °C until dissection. All colonies were fed 3 mealworms and 6 drops of concentrated honey daily for the 4 days before the experiment.

**Pharmacological Treatment.** A solution of 2 mM 8Br-cGMP was added daily to the mealworms and honey for the 4 days before the experiment. Treated water with activator was available ad libitum. The control received water, mealworms, and honey without the PKG activator. Feedings were performed in semidark testing room conditions, and test colonies were maintained under a cardboard cover to prevent photodegradation of the PKG activator. No differences were found in the number of dead between the different treatments for the foraging assay ($F_{(3,24)} = 1.23$, $P = 0.32$) and for the defense assay ($F_{(3,24)} = 0.12$, $P = 0.95$).

**Behavioral Effect of Pharmacological Treatment on Foraging and Defense.** The effect of the activator on foraging and defense was measured by using the assays described above. Behavior was recorded with a camera (DM-GL2; Canon). Two spotlights were used for illumination of the recording area (75-W BR30 Plant Light). The colony lid was removed for filming, then a 5-min acclimatization period was given. The stimulus, mealworm or alien ant, was placed in the test colony for 35 min. Data were quantified from the movies by counting the number of test minors and majors interacting with the stimulus every minute for 35 min for each of the 3 colonies. The mean number of ants responding to the stimulus for the last 10 min was used for each experiment. Each colony was tested 3 times over a 4-week period. Ants were scored as responding to the stimulus when they were touching the side of the arena for defense experiments or when they had entered the arena for foraging experiments. At the end of the 35 min, individuals responding to the stimulus were collected and counted under $CO_2$, flash-frozen in liquid nitrogen, and stored in a −80 °C freezer. The numbers of dead individuals were counted daily and at the end of the experiment; no differences in the number of dead ants were found between treatments.

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# Supporting Information

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### SI Text

**SI Results: Biometry.** Majors and minors differ in brain size and morphology; overall, the optic lobes, mushroom body peduncles and calyxes, and remaining neuropile are larger in majors compared with minors. According to the evolution of castes, individual polymorphism within a caste can be determined simply as a consequence of differential growth rates of multiple body dimensions (1, 2). This allometric growth, defined as the precise relation between the dimensions of 2 body parts, should exist within the *P. pallidula* worker subcaste. Wilson (3) suggests that once complete dimorphism is established, the 2 resultant subcastes are capable of diverging further in some parts of the body, with no evident intergradient allometry. We assessed whether the brain size of majors and minors showed an allometric relationship with body size or weight. We made 15 different measures of brain size, body size, and body weight of majors and minors. Body length was significantly positively correlated to the body weight (majors $r = 0.762$, $P < 0.001$; minors $r = 0.662$, $P < 0.001$) and the head length to the head weight (majors $r = 0.852$, $P < 0.001$; minors $r = 0.565$, $P < 0.001$). The brain length (but not brain width) of majors was significantly longer than that of minors [length, majors (473.50 ± 4.80 $\mu$m), minors (355.90 ± 2.30 $\mu$m), $F_{(3,102)} = 214.65$, $P < 0.001$; width, majors (441.87 ± 3.91 $\mu$m), minors (439.68 ± 3.02 $\mu$m), $F_{(3,102)} = 2.16$, $P = 0.096$]. Further analysis of structures within the ant brain suggested differences in morphology between majors and minors. The brain dorsal structures (containing mainly the mushroom bodies) (4) of majors were longer than those of minors (respectively, 190.29 ± 1.92 and 169.34 ± 2.19 $\mu$m, $F_{(3,102)} = 19.40$, $P < 0.001$) and the subesophageal ganglion (SOG) length and width differed significantly between the 2 subcastes [length, majors (219.85 ± 2.36 $\mu$m), minors (185.36 ± 2.71 $\mu$m), $F_{(3,102)} = 35.24$, $P < 0.001$; width, majors (225.75 ± 2.53 $\mu$m), minors (206.99 ± 2.93 $\mu$m), $F_{(3,102)} = 9.39$, $P < 0.001$].

**SI Methods: Biometry.** Majors and minors were collected, weighed, measured and dissected to measure their brain size. Twenty individuals of each caste from 3 different colonies were analyzed. Three measures of the weight were taken (full body, body without head, and head only) using an ultrasensitive balance to ± 1 $\mu$g (MT5; Mettler Toledo). Pictures of each individual in different views were taken using a Leica MZ16FA stereomicroscope connected to a camera and a computer running LAS software. Images were analyzed using ImageJ software with 12 different measures. Brains were measured in a posterior view with dorsal part at the top. The brain length was the maximum distance measured from the top of the dorsal part to the bottom of the ventral part. The brain width was measured as the maximum distance between the 2 sides of the brain at the dorsal face of the lobula. The SOG length was measured as the maximum distance from the dorsal part to the ventral part of the SOG. The SOG width was measured as the maximum distance from the 2 sides of the SOG. The dorsal structures of the brain contain mainly the peduncles and the calyxes of the mushroom bodies (4). Its length was measured as the maximum distance from the top of the dorsal part of the brain to a line marked by the dorsal face of the lobula. The body length was measured from the right side of each individual by a succession of straight lines started at the end of the abdomen, through the postpetiole, the petiole, and the thorax. To complete the body length we added the head length from the right side view, which was measured as the maximum distance from the dorsal face of the head to the ventral face without the mandibles and under the eyes. The head height was measured on the right side view from the top of the posterior part to the bottom of the anterior part at the dorsal front of the eye perpendicular to the head axis. The head was also measured from an anterior and a posterior view. For those measures the lengths represent the maximum distance at the symmetrical middle without the mandibles and the width was measured as the minimum distance across eyes. Individuals with a missing data point for any of the measures were removed from correlation analyses (Statistica software, Statsoft).


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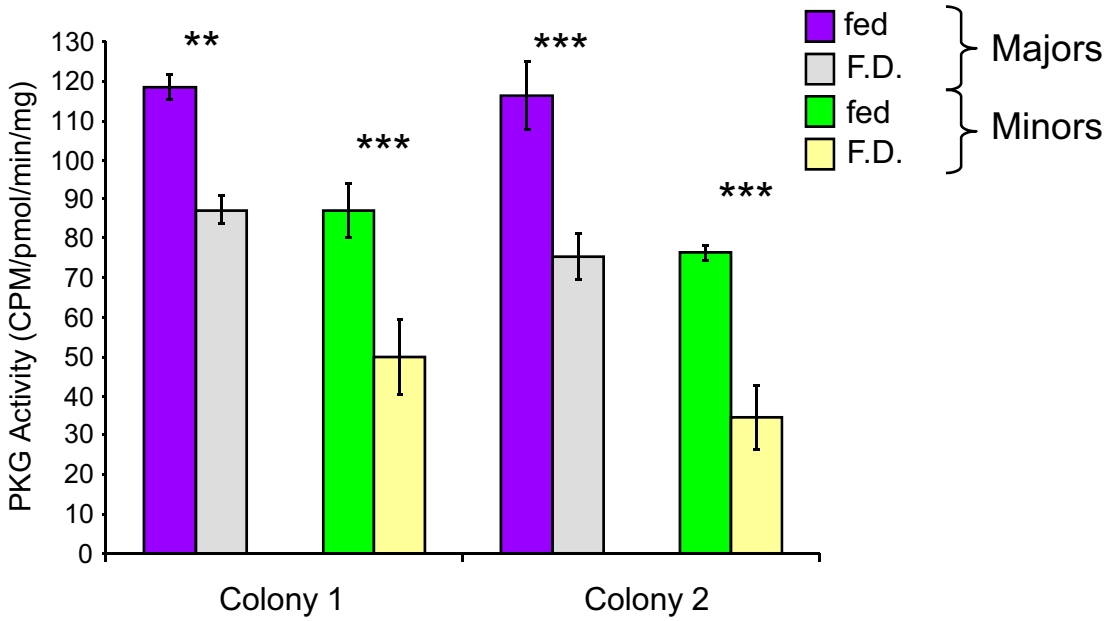


**Fig. S1.** PKG activity of majors and minors in fed or food-deprived (FD) conditions (mean ± SE of 5 groups of 5 dissected brains per subcaste per colony). Testing conditions were as in the foraging assays but majors and minors were food-deprived (fed one-half mealworm and 1 drop of concentrated honey once a week) or fed (fed 4 mealworms and 8 drops of concentrated honey 3 times per week) 21 days before the foraging test. Food deprivation decreased PKG activity in both majors and minors ($F_{(2,29)} = 33.9$, $P < 0.001$); majors maintained higher PKG activity than minors ($F_{(2,29)} = 33.0$, $P < 0.001$). All PKG activity measures are within the normal range (Fig. 1). **,$P < 0.01$; ***, $P < 0.001$.

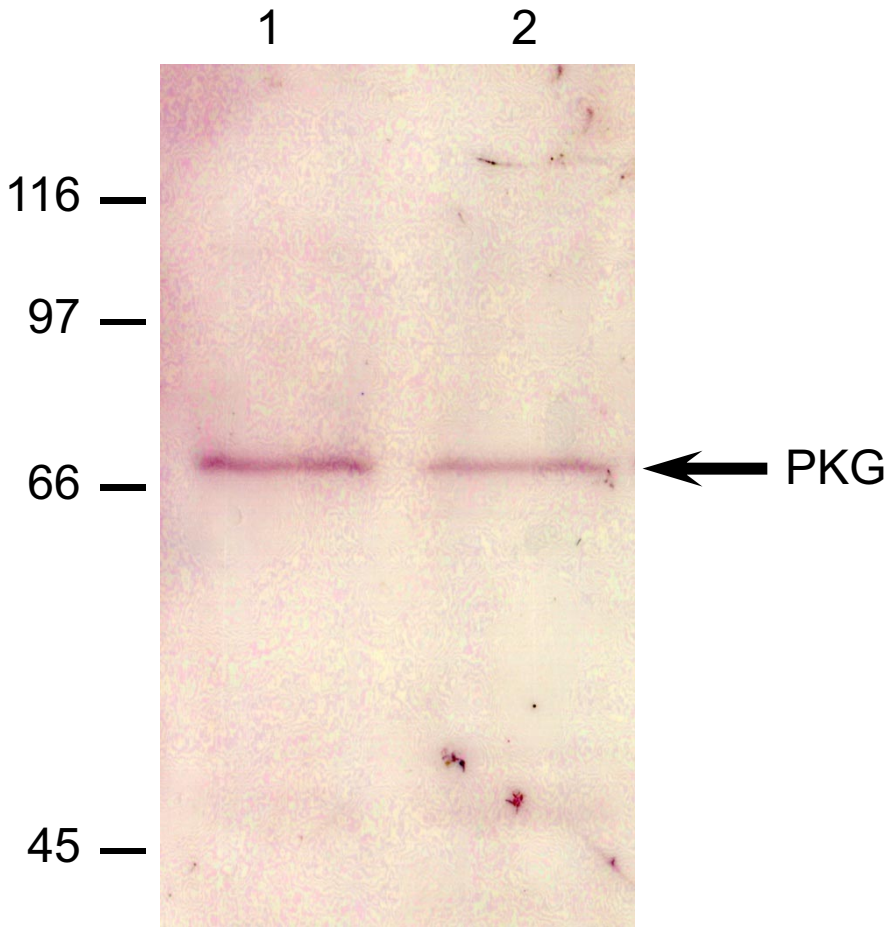


**Fig. S2.** Western blot containing whole body protein extract and using anti-FOR. The bars show the molecular mass in kilodaltons. PKG has an approximate mass of 74 kDa as expected based on the T1 transcript sequence. Protein extractions were performed on 10 and 20 minors (lanes 1 and 2, respectively) and represent a total amount of protein of 20 $\mu$g per lane. Immunodetection was as in Harlow and Lane [Harlow E, Lane D (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY)] using anti-FOR generated in guinea pig [Belay AT, et al. (2007) The foraging gene of *Drosophila melanogaster*: Spatial-expression analysis and sucrose responsiveness. *J Comp Neurol* 504:570–582].

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# Molecular and social regulation of worker division of labour in fire ants

FABIO MANFREDINI,*,† CHRISTOPHE LUCAS,‡,§ MICHAEL NICOLAS,§[1] LAURENT KELLER,§ DEWAYNE SHOEMAKER¶ and CHRISTINA M. GROZINGER*

*Department of Entomology, Center for Pollinator Research, The Pennsylvania State University, University Park, PA 16802, USA, †School of Biological Sciences, Royal Holloway University of London, Egham, TW 20 0EX, UK, ‡Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS, University of Tours, Parc de Grandmont, 37200 Tours, France, §Department of Ecology & Evolution, University of Lausanne, Biophore, Unil-Sorge, Lausanne, Switzerland, ¶USDA-ARS, Gainesville, FL 32608, USA

## Abstract

**Reproductive and worker division of labour (DOL) is a hallmark of social insect societies. Despite a long-standing interest in worker DOL, the molecular mechanisms regulating this process have only been investigated in detail in honey bees, and little is known about the regulatory mechanisms operating in other social insects. In the fire ant *Solenopsis invicta*, one of the most studied ant species, workers are permanently sterile and the tasks performed are modulated by the worker's internal state (age and size) and the outside environment (social environment), which potentially includes the effect of the queen presence through chemical communication via pheromones. However, the molecular mechanisms underpinning these processes are unknown. Using a whole-genome microarray platform, we characterized the molecular basis for worker DOL and we explored how a drastic change in the social environment (i.e. the sudden loss of the queen) affects global gene expression patterns of worker ants. We identified numerous genes differentially expressed between foraging and nonforaging workers in queenright colonies. With a few exceptions, these genes appear to be distinct from those involved in DOL in bees and wasps. Interestingly, after the queen was removed, foraging workers were no longer distinct from nonforaging workers at the transcriptomic level. Furthermore, few expression differences were detected between queenright and queenless workers when we did not consider the task performed. Thus, the social condition of the colony (queenless vs. queenright) appears to impact the molecular pathways underlying worker task performance, providing strong evidence for social regulation of DOL in *S. invicta*.**

*Keywords*: division of labour, fire ants, foraging workers, microarrays, queen pheromone, sociogenomics

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## Introduction

Social groups often exhibit a division of labour (DOL), characterized by a differentiation in the tasks performed by the group members, a process that is believed to enhance the overall efficiency of the group (Duarte *et al.* 2011). While DOL is found in social groups of many species, including birds (Arnold *et al.* 2005), meerkats (Manser 1999), dolphins (Gazda *et al.* 2005) and humans (Durkheim & Coser 1997), the most sophisticated and best studied cases of DOL are found among highly eusocial insects, namely bees, ants, wasps and termites (reviewed in Smith *et al.* 2008). These insect societies exhibit a reproductive DOL, where a few individuals

Correspondence: Fabio Manfredini, Fax: +44 1784 414224; E-mail: fmanfredini79@gmail.com

[1]Present address: Department of Plants Molecular Biology, Centro Nacional de Biotecnologia, C/Darwin 3, Campus de Cantoblanco (UAM), 28049 Madrid, Spain

develop into the reproductive caste, while most of the colony members become nonreproductive workers and perform all tasks related to colony maintenance and growth (Robinson 1992). In addition, social insects display worker DOL, where different individuals specialize on particular colony tasks such as brood care, nest building, defence and foraging (Wilson 1971). Recent studies suggest that similar molecular pathways involved in core biological processes (i.e. reproduction, nutrition and metabolism) are responsible for DOL in different insect lineages with independent origins of eusociality, such as honey bees (Amdam *et al.* 2004), ants (Corona *et al.* 2013) and wasps (Toth *et al.* 2007). This concept, known as the 'groundplan' hypothesis (reviewed in Johnson & Linksvayer 2010), postulates that these molecular pathways derive from physiological traits present in the solitary ancestors that were co-opted and selected to evolve into the queen and worker castes of eusocial insects. However, more studies are needed to test this hypothesis in other systems and to confirm its validity at a broader scale.

Many factors can influence DOL in insect societies, including morphology, genetic variation, developmental and nutritional factors and experience (reviewed in Duarte *et al.* 2011). Further, in many social insect species, pheromones play an important role in regulating both reproductive and worker DOL (Wyatt 2003). Pheromones may have a primer or releaser effect: primer pheromones affect long-term physiological or endocrine processes in the recipient followed by delayed changes in behavioural responses, whereas releaser pheromones elicit immediate behavioural responses (Vander Meer *et al.* 1998; Blomquist & Bagnères 2010). The social insect in which pheromones have been characterized most extensively is the honey bee *Apis mellifera* (reviewed in Grozinger *accepted*; Slessor *et al.* 2005). The pheromones released by the honey bee queen and the developing larvae (brood) can act as primer pheromones, inhibiting worker ovary activation and worker behavioural maturation from nursing/brood care to foraging (Pankiw *et al.* 1998; Hoover *et al.* 2003; Le Conte *et al.* 2006). Both pheromones also may act as releaser pheromones, stimulating attraction in the case of queen pheromone and brood feeding or pollen foraging in the case of brood pheromone (Dreller *et al.* 1999; Keeling *et al.* 2003). Recent microarray studies have revealed that exposure to either queen pheromone or brood pheromone can alter the expression of thousands of genes in the brains of worker honey bees, and these genes are associated with behavioural maturation/worker DOL (Grozinger *et al.* 2003; Alaux *et al.* 2009). Thus, these studies suggest that genomic approaches can be used to both identify primer pheromones and characterize their impacts on behaviour and physiology.

Little is known about the molecular and social mechanisms that regulate worker DOL in ant colonies (reviewed in Libbrecht *et al.* 2013; Lucas *et al.* 2010; Lucas & Sokolowski 2009). One of the best studied ant species is the fire ant *Solenopsis invicta*. The broad knowledge about the basic biology of this insect (reviewed in Tschinkel 2006), together with the understanding of the genetic regulation of social organization (Wang *et al.* 2013) and the recent sequencing of the genome (Wurm *et al.* 2011), makes *S. invicta* an emerging model in the field of sociogenomics (reviewed in Robinson *et al.* 2005). Fire ants live in large colonies with either a single queen (monogyne form) or multiple queens (polygyne form), and tens of thousands of workers organized in several behavioural phenotypes that are associated with size and age (Mirenda & Vinson 1981). Queens produce a pheromone that seems to regulate many of the same behavioural and physiological processes as queen pheromone in honey bees, eliciting both primer and releaser responses. As a primer pheromone, it prevents virgin queens from shedding wings and activating ovaries (Fletcher & Blum 1981; Vargo 1998), it affects caste determination of female larvae (Vargo & Fletcher 1986a,b, 1987), and it may alter aggression levels of workers towards nestmates and acceptance of newly mated queens (Klobuchar & Deslippe 2002; Vander Meer & Alonso 2002). As a releaser, it elicits worker attraction to the queen and induces workers to groom and feed the queen (Vander Meer *et al.* 1980). Finally, in polygyne colonies, the individual egg-laying rate decreases as the number of queens increases owing to a primer pheromone produced by the various queens (Vargo 1992). The active chemical components of queen pheromone have not been characterized, but queens in different reproductive states differ in their venom alkaloid and hydrocarbon profiles (Eliyahu *et al.* 2011). However, the impacts of fire ant queen pheromone on worker DOL and the molecular mechanisms by which queen pheromone alters worker behaviour and physiology are unknown. In particular, given that fire ant workers are permanently sterile, the mode of action of the queen primer pheromone might be significantly different from the honey bee system where workers can activate their ovaries in the absence of a functional queen (Hoover *et al.* 2003).

In this study, we used whole-genome fire ant microarrays (Manfredini *et al.* 2013) to examine (i) the molecular basis for DOL in fire ant workers by comparing foraging and nonforaging workers and (ii) the impact of queen presence on worker gene expression patterns in these two behavioural groups. We hypothesized that similar genes/gene pathways that have been described in other genomic studies to be major regulators of worker DOL, specifically in-nest behaviours vs. foraging, in

social insects such as honey bees and wasps (Alaux *et al.* 2009; Toth *et al.* 2010; Ament *et al.* 2011) would be involved in fire ant worker DOL. We also hypothesized that primer effects of queen pheromone would be evident at the gene expression level and that these effects would differ between the two behavioural groups.

## Materials and methods

### *Insect collection, rearing and sampling*

Monogyne colonies of *Solenopsis invicta* were collected near Athens (GA, USA) in April 2008 for Experiment 1 and in the surroundings of Gainesville (FL, USA) in April–May 2010 for Experiment 2. Fire ant colonies were kept under standard laboratory conditions (Jouvenaz *et al.* 1977) for 3 months before sampling. For Experiment 1, we sampled two groups of ants, that is, foraging (out) and nonforaging (in) workers, while for Experiment 2 the treatment groups were 4: queenright nonforaging (QRin), queenless nonforaging (QLin), queenright foraging (QRout) and queenless foraging (QLout). See Fig. S1 and Appendix S1 (Supporting Information) for a detailed description of rearing condition, group assignment and sampling methodology.

### *Sample preparation for molecular analyses*

Total RNA was extracted from pools of 10 worker ants (whole bodies) using the PicoPure RNA Isolation kit (Applied Biosystems – Life Technologies, Grand Island, NY, USA) combined with an RNase-Free DNase step (Qiagen, Valencia, CA, USA) to remove any possible contamination by genomic DNA. Subsequent steps in the microarray analysis were performed at the Penn State Genomic Core Facility as in Manfredini *et al.* (2013) and see Appendix S1 (Supporting Information).

### *Microarray analysis*

Our microarray, recently developed and validated (Manfredini *et al.* 2013), includes 51 531 probes that match unique transcripts obtained from the sequencing of the *S. invicta* genome (Wurm *et al.* 2011) plus additional sequences from transcriptome studies. Probes were printed in pairs on two 12-plex microarrays (each array had a 135 000 probe capacity, Roche NimbleGen, Inc., Madison WI, USA). We used a loop design with dye swaps incorporated. For Experiment 1, we used 6 arrays of a 12-plex microarray slide which allowed us to hybridize 12 RNA samples: these corresponded to 6 pools of foraging and 6 pools of nonforaging workers from six different colonies (Fig. S2, Supporting Information). For Experiment 2, we used all 12 arrays of a 12-plex microarray slide to hybridize 24 RNA samples: again, we used six different colonies, and for each colony, we processed 1 pool of QRin, 1 of QLin, 1 of QRout and 1 of QLout (Fig. S3, Supporting Information). Array data were analysed using two statistical software packages, namely R 2.11.1 (Team 2009) and SAS (Cary, NC, USA), for both experiments (Appendix S1, Supporting Information).

### *Gene ontology and comparative analyses*

Hierarchical clustering using the Ward method and principal component analysis (PCA) for global patterns of gene expression were performed in JMP 9.0.2 (SAS, Cary, NC, USA), while K-means clustering was performed in GENESIS (Sturn *et al.* 2002). Gene ontology (GO) analyses were performed using functional annotation clustering in DAVID version 6 (Huang *et al.* 2009a,b) with medium stringency and a cut-off of $P < 0.05$. Fire ant genes were matched to their putative *Drosophila* orthologs in FlyBase (Marygold *et al.* 2013). To identify the most overrepresented biological functions (enrichment analysis), we compared the annotation composition in our list of differentially expressed genes to that of a population background composed by all the fire ant transcripts with *Drosophila* orthologs that were included in the statistical analysis. For comparative studies, we used Venny (Oliveros 2007) to overlap lists of differentially expressed genes (only those provided with FlyBase numbers) and we used a hypergeometric test (http://nemates.org/MA/progs/overlap_stats.html) to assess whether genes overlapping between studies occurred significantly more often than expected by chance. Lists of significantly enriched GO terms obtained with functional annotation chart in DAVID (medium stringency and $P < 0.05$) from different experiments were also overlapped in Venny.

### *Validation of differential expression of candidate genes using quantitative real-time PCR*

We examined gene expression levels of seven candidate genes that were differentially expressed in one or both of the two microarray experiments and are known for regulating interesting biological functions in model organisms: *foraging* (*for,* food-related behaviour and polyethism), *hymenoptaecin* (*Hym,* antibacterial response), *myofilin* (*mf,* muscle development), *myosin heavy chain* (*mhc,* locomotion), *ornithine aminotransferase precursor* (*oat,* neurogenesis), *serine protease immune response integrator* (*spirit,* innate immune response) and *synaptotagmin 1* (*syt1,* neurotransmitter secretion). Expression levels were assayed in QRin, QRout and QLin groups by means of

quantitative real-time PCR as in Manfredini *et al.* (2013), see Appendix S1 (Supporting Information).

## Results

### *Experiment 1: gene expression patterns associated with task performed*

The number of differentially expressed transcripts in Experiment 1 between foraging and nonforaging workers ('out' and 'in', respectively) was 1387 at False Discovery Rate (FDR) < 0.05 (Table S1, Supporting Information), representing only 2.7% of the 51 531 transcripts included in the analysis. The global analysis of patterns of expression revealed that the major driver was the task performed by workers. A hierarchical clustering analysis of the 12 individual pools of workers grouped them into two distinct macroclusters, the first encompassing 6 pools of foraging ants and the second 6 pools of nonforaging ants (Fig. 1A). A principal component analysis on the same data set confirmed this result, with the first component (which was associated with the task performed by the workers) accounting for 57.2% of the difference in gene expression. Overall, foraging and nonforaging ants clustered separately into two different portions of the space (Fig. 1B).

We performed gene ontology analysis on the differentially expressed transcripts that have *Drosophila* orthologs with FlyBase annotations (735 of 1387) using DAVID (Huang *et al.* 2009a,b). Six GO terms and 3 KEGG pathways were significantly enriched at $P < 0.05$ (functional annotation clustering, Table S2, Supporting Information): generation of precursor metabolites and energy, myofibril assembly, muscle cell differentiation, multiorganism process, response to oxidative stress, monocarboxylic acid metabolic process, oxidative phosphorylation, pentose and glucuronate interconversions and lysine degradation.

### *Experiment 2: effect of the presence of the queen on gene expression patterns*

We compared gene expression levels between four groups of ants for this experiment: foraging and nonforaging workers from queenright (QRout and QRin, respectively) and queenless colony fragments (QLout and QLin, respectively). Four-hundred transcripts were significantly differentially expressed across the four groups (FDR < 0.05). Because the analysis with SAS in this case detected very few transcripts differentially expressed across treatments (see Appendix S1, Supporting Information), we performed a global analysis of patterns of expression on the whole set of transcripts that were initially included in the analysis. A hierarchical clustering analysis revealed that, similarly to Experiment 1, the task performed was the major driver of gene expression: nonforaging ants (QRin and QLin) formed a separate cluster from foraging workers (QRout and QLout), independent of the presence or absence of the queen (Fig. 2A). This result was confirmed by a principal component analysis, in which the first component corresponded to the task performed (foraging vs. nonforaging) and explained 54% of the differences in gene expression, whereas social environment (presence or absence of the queen, second component) accounted for 25% of the differences. A third component, explaining 21% of the variation, revealed an interaction between task and social environment (Fig. 2B).

Pairwise comparisons of the four groups identified 395 transcripts that were differentially expressed between QRin and QRout at FDR < 0.05 (Table S3, Supporting Information), while there were very few transcripts differentially expressed in the other contrasts at this FDR (Table 1). We performed gene ontology analysis on the set of 395 transcripts significantly differentially expressed between QRin and QRout workers: of these, 248 have *Drosophila* orthologs with FlyBase annotations and were included in the functional annotation clustering in DAVID. We found that 7 GO terms and 2 KEGG pathways were significantly enriched (Table S4, Supporting Information). Among significant GO terms, ageing and cellular respiration survived the Benjamini correction (Benjamini and Hochberg 1995) at $P < 0.05$; the others were skeletal myofibril assembly, aerobic respiration, organic acid metabolic process, signal transduction and larval central nervous system remodelling. Significant KEGG pathways were citrate cycle (TCA cycle), which survived the Benjamini correction at $P < 0.05$, and starch and sucrose metabolism.

Because our analyses did not reveal a significant difference between queenright and queenless workers, we performed a second analysis where we pooled the transcripts that were differentially expressed either in the QRin/QLin or in the QRout/QLout comparisons and used a less stringent FDR (<0.1). This analysis revealed 27 transcripts, of which 22 had *Drosophila* orthologs with FlyBase annotations (Table S5, Supporting Information). Among these genes were *oat*, *hym*, *syt1*, *aspartyl beta-hydroxylase (Asph)*, *cuticular protein 49Aa*, *Niemann-Pick type C-2 (Npc2a)* and *twin of eyeless (toy)*.

### *Comparisons of significantly regulated transcripts in Experiments 1 and 2*

Interestingly, the only contrast that produced differentially expressed transcripts at FDR <0.05 in Experiment 2 was the comparison between foraging and nonforaging workers in queenright colonies, which is analogous to

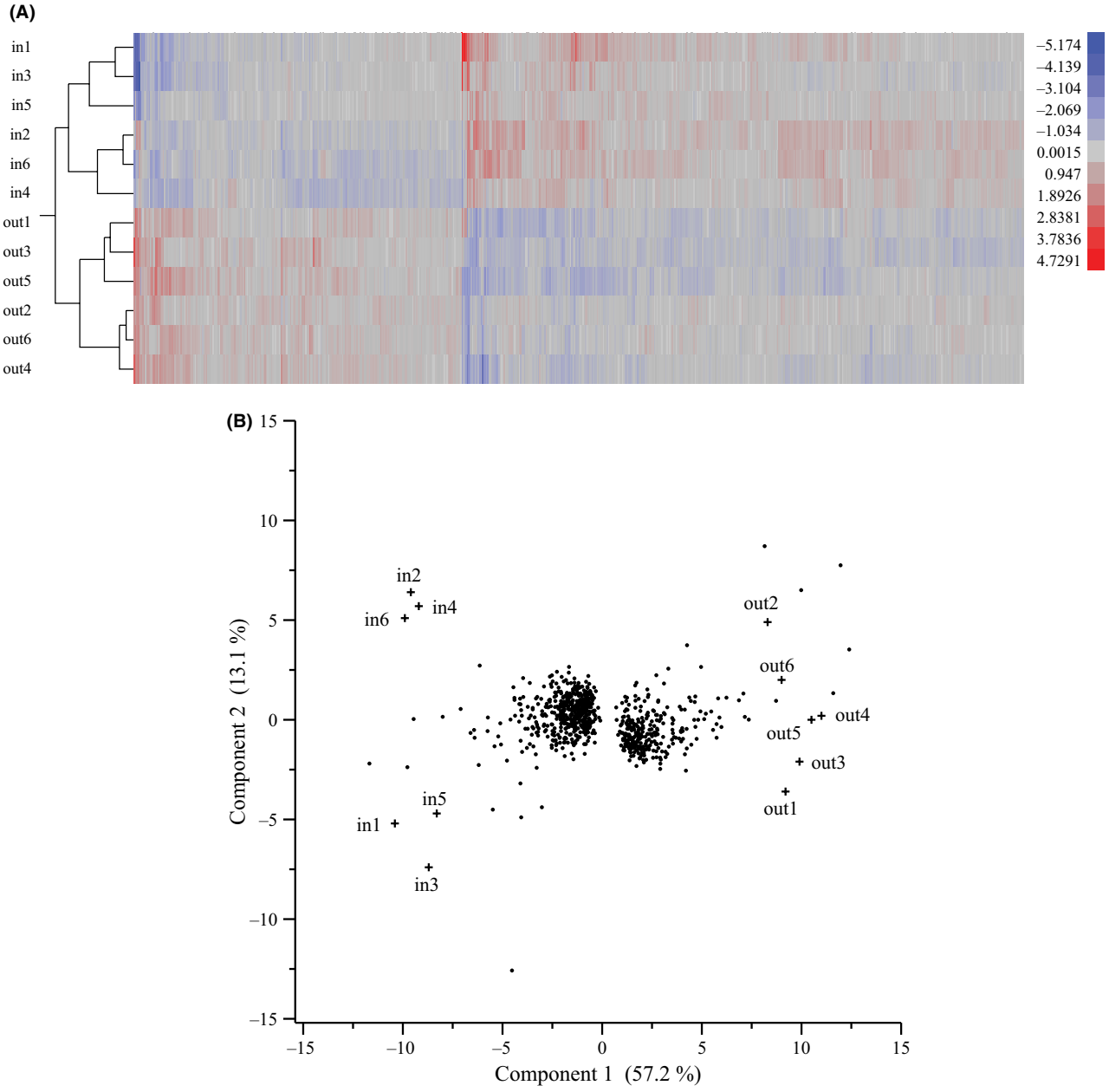


**Fig. 1** Molecular regulation of worker division of labour. Global analyses of significantly differentially expressed transcripts in pools of workers from individual colonies (Experiment 1) show that patterns of gene expression were shared among nonforaging (in) and foraging (out) workers. (A) Heatmap of $\log_2$-transformed and normalized expression values and hierarchical clustering of worker pools according to expression patterns; (B) principal component analysis.

what we tested in Experiment 1. This allowed us to perform an overlap of transcripts that were differentially expressed in Experiments 1 and 2 (Fig. S5, Supporting Information). The overlap analysis identified 89 transcripts that were shared between the two experiments (Table S6, Supporting Information), a number significantly higher than expected by chance (hypergeometric test: representation factor: 8.4, $P < 7.16e^{-55}$). This group includes several genes that are well characterized in *Drosophila*, such as *mf*, *mhc*, *oat*, *spirit*, *syt1*, *adipokinetic hormone receptor (Akhr)*, *I'm not dead yet (Indy)*, *cuticular protein 47Ef*, *cytochrome P450 (Cyp4 g1* and *Cyp4c3)*, *centrosomin (cnn)*, *supercoiling factor (scf)* and *TBP-associated factor 8 (Taf8)*. A gene ontology analysis on the 63 transcripts with *Drosophila* orthologs and FlyBase annotations revealed that none of the 11 clusters produced by

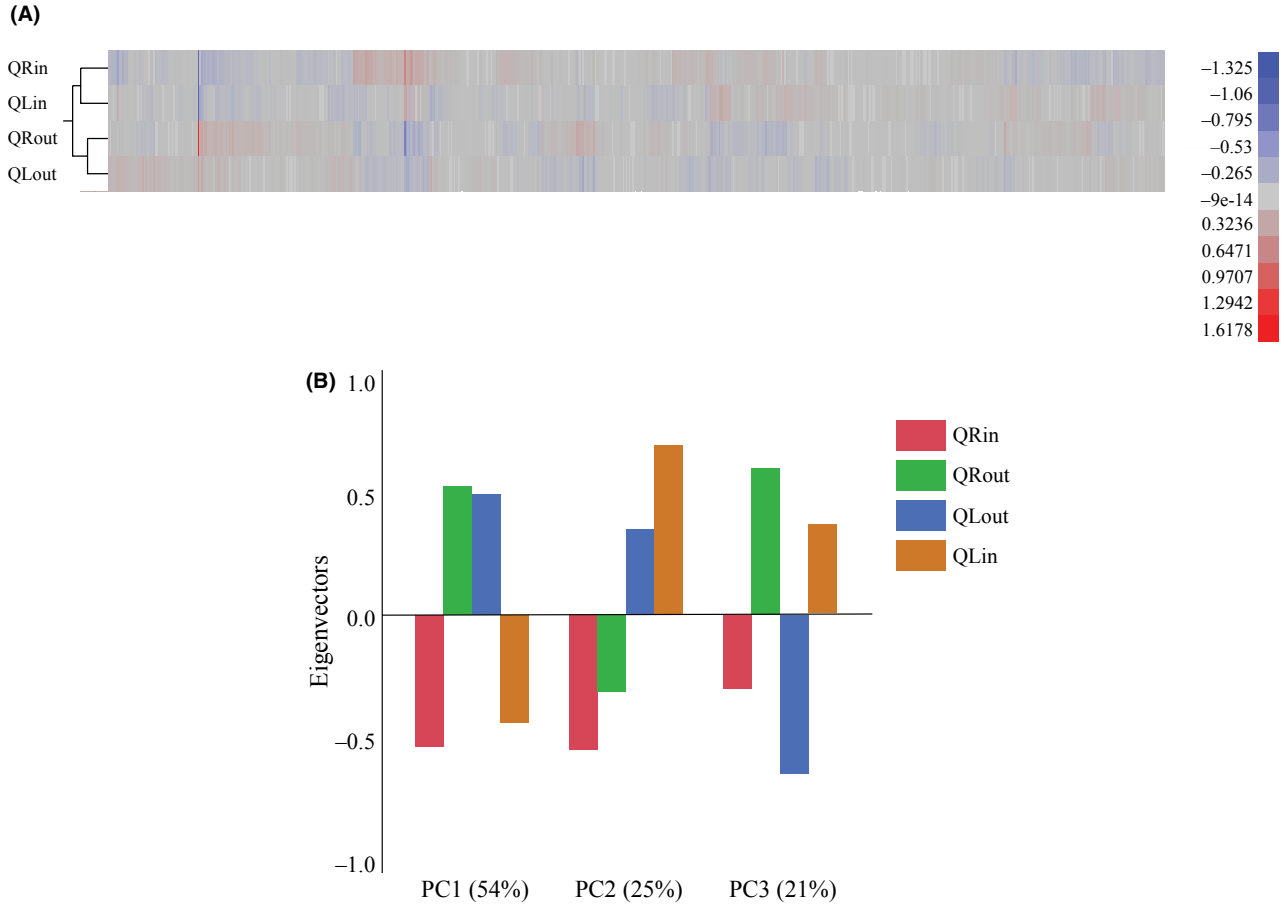


**Fig. 2** Effect of social environment on the regulation of worker division of labour. Global analyses of the complete set of transcripts from Experiment 2 (foraging and nonforaging workers from queenright or queenless colonies). Hierarchical clustering (A) and principal component (B) analyses reveal that patterns of gene expression were more similar in nonforaging workers ('in' groups) and foraging workers ('out' groups) independently on the presence/absence of the queen. QRin, queenright nonforaging; QRout, queenright foraging; QLin, queenless nonforaging; QLout, queenless foraging.

the functional annotation clustering in DAVID was significantly enriched. We performed another overlap analysis on this same data set, this time using significantly enriched GO terms from each experiment instead of individual genes. Twelve GO terms were shared between the two experiments (Table S7, Supporting Information).

The lack of any significant difference in gene expression between foraging and nonforaging workers in queenless colonies from Experiment 2 suggests that in the absence of the queen, there is a breakdown in the performance of defined tasks (foraging vs. nonforaging) in workers. In honey bees, loss of the queen results in accelerated behavioural maturation, and workers enter the foraging state faster (Pankiw & Page 2001). We investigated whether the gene expression patterns in queenless workers from Experiment 2 were more similar to foraging or nonforaging ants from Experiment 1 by using a directional overlap analysis of lists of transcripts obtained with the SAS protocol. We overlapped the complete set of 17 960 transcripts that were upregulated (but not necessarily statistically significant) in queenless workers in Experiment 2 with significantly upregulated transcripts in nonforaging and foraging workers from Experiment 1 (Table S8, Supporting Information). For the queenless/foraging workers comparison, 292 transcripts were upregulated in both groups, while 351 were not (total of 643 transcripts); for the queenless/nonforaging workers comparison, 243 transcripts were upregulated in both groups, while 470 were not (total of 713 transcripts). This difference was highly significant (chi-square, $P < 0.0001$), indicating that queenless workers were more 'forager-like' in terms of gene expression.

### *Comparative studies across species*

To investigate whether mechanisms of division of labour are conserved across social insects, we performed

**Table 1** Pairwise comparisons of transcripts that were differentially expressed in the four groups of workers at two different False Discovery Rate (FDR) after analysis with R

| | FDR < 0.05 | | FDR < 0.1 | |
|---|---|---|---|---|
| | Up | Down | Up | Down |
| Nonforaging | | | | |
| QL vs. QR | 0 | 0 | 14* | 1* |
| Foraging | | | | |
| QL vs. QR | 0 | 4 | 7* | 5* |
| Queenright | | | | |
| In vs. out | 89 | 306 | 256 | 681 |
| Queenless | | | | |
| In vs. out | 1 | 0 | 26* | 2* |

Up = transcripts upregulated in the first term of the comparison; Down = transcripts downregulated in the first term of the comparison; *for these comparisons, the LIMMA method 'nested' was used instead of 'separate' to control for multiple testing across comparison because it was more powerful in detecting significantly regulated transcripts.

overlap analyses between all significantly differentially expressed genes from Experiment 1 (foraging vs. nonforaging ants in queenright colonies, 735 transcripts with FlyBase numbers) and the differentially expressed genes from previous studies on honey bees (Alaux *et al.* 2009; Ament *et al.* 2011) and paper wasps (Toth *et al.* 2010) that also investigated transcriptomic differences between foraging vs. nonforaging workers. Statistical analysis revealed that in both cases there was less overlap than expected by chance (see Appendix S1 and Table S9, Supporting Information). Only 16 genes were shared between *S. invicta*, *A. mellifera* and *Polistes metricus*, including *mf, thiolester-containing protein II (Tep2)*, *Rab-protein 7 (Rab7)*, *probable cytochrome P450 6 g2*, *larval serum protein 2 (Lsp2)*, *histone (His3.3A* and *His3.3B)* and *epidermal stripes and patches (Esp)* (Fig. 3). We can add to this list *for* and *syt1*, both of which were differentially expressed in our study and in wasp (Toth *et al.* 2010) and in other honey bee studies (Ben-Shahar *et al.* 2002; Whitfield *et al.* 2003). The patterns of expression of these 16 genes in foraging and nonforaging workers were not consistent across the three social insects (data not shown).

## Discussion

We investigated the molecular and social mechanisms underpinning worker division of labour (DOL) in the fire ant *Solenopsis invicta.* Our first major finding confirms that worker DOL in fire ants is associated with important changes at the transcriptomic level: 1387 transcripts in Experiment 1 and 395 transcripts in Experiment 2 were differentially expressed between foraging and nonforaging workers, and there was a significant overlap in the suites of differentially expressed transcripts between the two experiments. Furthermore, pools of ants from individual colonies clearly clustered into one of two groups based on expression profiles in Experiment 1. It is worth noting that despite the significant overlap, there were clear differences in the suites of genes differentially regulated between foraging and nonforaging workers in the two experiments. These differences are likely due to extrinsic factors related to the experimental conditions (e.g. year and site of collection in the field, colony genetic backgrounds, nest design, time of sampling) which were similar but not identical in the two experiments (see 'Insect collection, rearing and sampling' in the Appendix S1, Supporting Information).

Interestingly, the transcriptional differences between foraging and nonforaging workers completely disappeared in queenless conditions (Experiment 2), where only one gene (*hypothetical protein SINV_01841*) was differentially expressed between foraging and nonforaging workers. Despite the fact that social environment (presence/absence of the queen) contributed to 25% of the variation in gene regulation at a genomic scale, there were only a handful of genes significantly differentially expressed between queenless and queenright workers. Overall, these results suggest that DOL is transcriptionally regulated in fire ants and is associated with easily distinguishable behavioural (foraging/nonforaging) and spatial (inside/outside the nest) phenotypes. Furthermore, the queen does have primer effects on workers and appears to impact DOL, because because a queen's results in a more 'forager-like' gene expression patterns in workers.

Finally, comparative studies across species revealed a large variability in the proportion of transcripts differentially expressed according to foraging/nonforaging behaviour: these were 2.7% in fire ant whole bodies (Experiment 1), 4% in paper wasp brains, 13% in honey bee brains and 20% in honey bee fat bodies. Comparative studies also suggest that there is no substantial conserved suite of 'DOL genes' across these three species: overlap analyses were not supported by statistical significance and the directionality of the expression patterns of the 16 common genes varied from species to species. However, our results show some conservation of gene ontology categories when comparing fire ants with honey bees, while no GO terms were shared with paper wasps, possibly because for paper wasps only brain tissue was used. An alternative explanation for the negative results of our comparative analyses across social insects could be associated with the methodology of analysis itself. In fact, in order to directly compare gene regulation in different species of insects, we rely

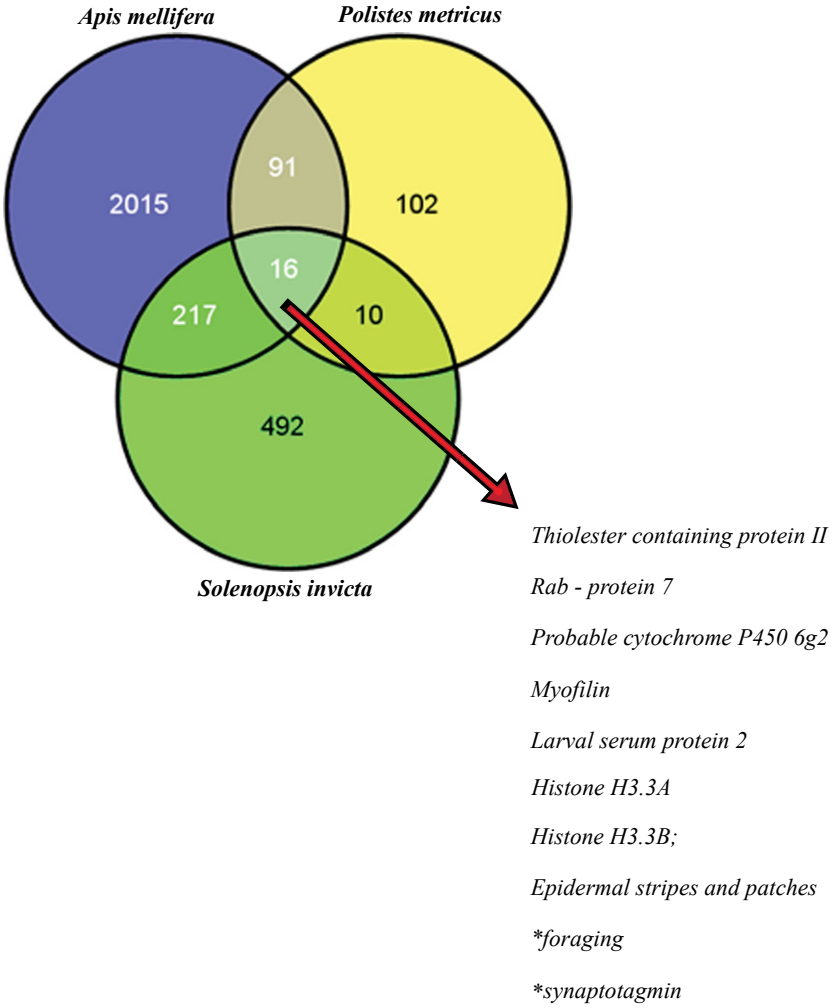


**Fig. 3** Comparative analysis of transcripts associated with worker division of labour across social insect species. Overlap analysis of FlyBase numbers corresponding to transcripts that were differentially expressed between foraging and nonforaging workers in *Apis mellifera* (brain tissue, Alaux *et al.* 2009 and fat bodies Ament *et al.* 2011), *Polistes metricus* (brain tissue, Toth *et al.* 2010) and *S. invicta* whole-body samples from Experiment 1. *These genes were not listed in Alaux *et al.* 2009 and Ament *et al.* 2011, but were differentially expressed in *A. mellifera* based on different studies (Ben-Shahar *et al.* 2002; Whitfield *et al.* 2003).

on annotations from *Drosophila melanogaster*, and while doing this, we might miss groups of genes that have specific functions in social insects or gene families that are expanded in this group but have not been characterized in *D. melanogaster*. Finally, comparisons between more closely related species (other ants) would likely reveal greater conservation of DOL genes.

### *Molecular regulation of worker division of labour*

Gene ontology analyses in foraging and nonforaging workers revealed that muscle structure development and metabolic process recurred as differentially regulated across both experiments. Muscle activity is likely associated with differential motility and locomotory behaviour in the two groups of ants, where foraging workers actively search for food sources in the foraging area, while nonforaging workers perform nursing tasks within the nest. Muscle development also depends on the size and the age of an ant and these two features are good predictors for foraging and nonforaging behaviours in fire ant workers (Mirenda & Vinson 1981). However, the correlation of size and age with foraging behaviour is not straightforward, because age of foraging largely depends on size, whereby minor workers are recruited as foragers much earlier than majors, but they are short-lived so that the total amount of time spent foraging by the two types of workers is similar (Tschinkel 2006). Interestingly, in a recent study on the ant *Camponotus fellah* where individual workers were monitored for 41 days with a tracking system, it has been observed that there is great overlap among groups of workers, with some nurses being older and some foragers being younger than the average workers' age (Mersch *et al.* 2013). As for metabolism, differential regulation of metabolic genes in workers with different tasks has been found in previous studies on other social insects such as honey bees (Whitfield *et al.* 2003; Ament *et al.* 2008; Alaux *et al.* 2009) and paper wasps (Sumner *et al.* 2006). In particular, Ament *et al.* (2011) observed

that honey bee nurses maintain high levels of lipid and protein metabolism which presumably relate to brood food production, whereas carbohydrate and energy metabolism are held consistently high in foragers to support energy expenses due to flight behaviour. In support of these observations, we found that several GO terms and KEGG pathways related to carbohydrate and energy metabolism varied with foraging/nonforaging behaviours across our two experiments (Table S2 and S4, Supporting Information). For a discussion of genes of interest associated with the metabolism of lipids and carbohydrates, see Appendix S1 (Supporting Information).

Whitfield *et al.* (2003) reported that honey bee nurses had higher expression of genes associated with neurogenesis, probably due to their role in changing brain structure before the shift to foraging activity, and that nurses and foragers differed for the regulation of genes involved in intracellular signalling in the brain. Consistent with this hypothesis, we found that *S. invicta* workers with foraging/nonforaging tasks significantly differed in expression of genes associated with larval central nervous system remodelling and signal transduction [see Appendix S1 (Supporting Information) for a discussion of some of these genes].

### *Effect of social environment on regulation of worker division of labour*

Interestingly, the distinction between the two worker phenotypes was no longer visible at the transcriptional level in queenless colonies, despite the fact that behaviourally and spatially the two phenotypes were still recognizable (see Fig. S1B, Supporting Information). One possible explanation for this apparent contradiction is that queenless workers were not performing the two behaviours reliably, as a result of a loss of specialization and/or spatial organization. Under this scenario, some foraging workers may have spent time inside the nest (therefore, they were assigned to the nonforaging group) and, vice versa, some nonforaging workers may have occupied the outside portion of the nest; hence, they were assigned to the foraging group. Alternatively, after removing the queen (and potentially interrupting the direct effect of primer pheromone on gene regulation in workers), the behavioural and spatial differences between foraging and nonforaging workers may no longer be regulated at the level of gene expression, but rather rely on other factors such as neuropeptides or metabolites that were previously secreted in the hemolymph. An additional interpretation is that the two phenomena are uncoupled, that is, the differences in gene expression associated with queen removal are not directly involved in the regulation of task behaviours: however, our analysis of gene expression levels in queenright vs. queenless workers independently of the task performed does not support this hypothesis because no significant differences between the two macrogroups were detected. This analysis instead suggests that there might be increased foraging behaviour in queenless workers because their patterns of gene expression were most similar to those found in queenright foragers (see Table S10, Supporting Information). Similar effects are observed in honey bees, where workers exhibit accelerated behavioural maturation (i.e. increase in the number of workers performing foraging) in the absence of the queen or brood (Page *et al.* 2012). Finally, it is possible that the observed changes in gene expression are not a direct result of the loss of the queen on workers, but rather on the brood, because the brood in queenless colonies is likely reduced and skewed towards an older age distribution. Regardless of the exact mechanism, the results suggest that the sudden loss of the queen can impact the social and spatial organization of a fire ant colony. Additional studies will be necessary to confirm that loss of the queen does indeed result in behavioural changes in workers in fire ant colonies.

Surprisingly, despite this effect on DOL, the presence of the queen had little effect on gene expression patterns in general. Only a handful of genes were differentially expressed between queenright and queenless workers at FDR < 0.1 [see Appendix S1 (Supporting Information) for a discussion of these genes]. Our results suggest that while queen presence may impact fire ant worker DOL, the effects at the transcriptional level are still quite limited in comparison with the effects of queens on honey bee workers. In honey bees, exposure to queen pheromone triggers changes in expression of thousands of genes in the worker brain (Grozinger *et al.* 2003). However, in honey bees, there are several profound primer effects of queen pheromone on workers, including inhibition of ovary activation (Hoover *et al.* 2003) and reduction in juvenile hormone titres (Pankiw *et al.* 1998), in addition to reduced behavioural maturation. In fire ants, the queen pheromone has significant effects on gynes within a 24-h window, resulting in large-scale changes in gene expression (Wurm *et al.* 2010) and physiology (wing shedding and ovary activation, Fletcher & Blum 1981), but no primer effects have been reported in workers. Because caste differences are highly canalized in fire ants, the primer effects of queen pheromone in this system may have been reduced. The presence of a worker caste that is irreversibly sterile prevents the occurrence of mechanisms of queen-worker conflict for the production of males, as observed in other social insects (Bulmer 1981). This fundamental life history trait

might have selected for a reduction in the direct control of the queen over the workers' physiology through pheromones. Alternatively, it is possible that other social cues, such as brood presence, compensate for the loss of the queen. Brood (in particular fourth-instar larvae) are known to play an important role in regulating the pace of colony activity in fire ants (including queen's egg-laying rate, Tschinkel 1988) and the presence of equal amounts of brood in both colony fragments in Experiment 2 may have masked or buffered the impact of queen loss. Indeed, in honey bees, brood pheromone has similar primer effects as queen pheromone (reviewed in Grozinger *accepted*). Finally, it is possible that the treatment time (5 days) was not long enough to see differences in workers at the transcriptional level, although changes in gynes are observed within 24 h (Wurm *et al.* 2010) and behavioural changes in workers are observed after 48 h (Klobuchar & Deslippe 2002) and within 5 days (Vander Meer & Alonso 2002) after queen removal.

## Conclusions

Our results indicate that there are indeed consistent transcriptional differences associated with DOL in fire ant workers. Interestingly, there was no significant overlap in the sets of genes associated with DOL in fire ant, honey bee and paper wasp workers, although there is some indication that core physiological processes, such as carbohydrate, protein and lipid metabolism, are similarly regulated across these species. We also provide evidence for the first time that social context, namely presence or absence of the queen, can impact worker DOL in fire ants. While the effect of queen presence on gene expression was limited, it nonetheless suggests that the queen may be producing a primer pheromone that impacts worker behaviour and physiology. These findings further confirm the power of a genomic approach for identifying the subtle effects of primer pheromones. In future, it would be of great interest to test whether the addition of a queen extract (which presumably would contain the queen pheromone) is able to 'rescue' expression profiles in queenless workers and make them more similar to queenright workers. Furthermore, it will be necessary to extend this type of approach to encompass the complex social structure of fire ant colonies, where both monogyny and polygyny occur and are determined by two alternative variants of the same genomic element (Wang *et al.* 2013). In particular, it will be noteworthy to examine how the interaction between social structure and genetic background impacts worker DOL and gene expression in both monogyne and polygyne colonies and in workers of different size and age.

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F.M., C.L., L.K., D.S. and C.M.G. designed research. F.M., C.L., M.N. and D.S. performed research. F.M. and C.M.G. analysed the data. F.M. and C.M.G. wrote the manuscript. C.L., L.K. and D.S. provided comments on the manuscript.

## Data accessibility

The array data were deposited on the ArrayExpress database (www.ebi.ac.uk/arrayexpress) according to MIAME standards (accession numbers: E-MTAB-2094 for Experiment 1 and E-MTAB-2108 for Experiment 2). Gene expression data are provided as Appendix S1 (Supporting Information) with this article.

## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Experimental set-up for Experiment 2. A) Four mother colonies were split into 2 colony fragments each. All colony fragments were provided with a nesting chamber, a foraging area with a cricket, water and sugar water, and equal amounts of workers and brood. B) Queenless colony fragment. The queen is no longer inside the nesting chamber, but some workers continue to perform in-nest tasks. QR = queenright; QL = queenless; in = nonforaging workers; out = foraging workers.

**Fig. S2** Microarray hybridization scheme for Experiment 1. For each group of workers, 6 pools were hybridized in a loop design: 3 pools were labelled with the Cy3 dye and other 3 with the Cy5 dye. We used 6 arrays of a 12-plex array slide with 135 000 probe capacity designed by Roche NimbleGen, Inc. (Madison WI).

**Fig. S3** Microarray hybridization scheme for Experiment 2. For each group of workers, 6 pools were hybridized in a loop design: 3 pools were labelled with the Cy3 dye and other 3 with the Cy5 dye. We used a whole 12-plex array slide with 135 000 probe capacity designed by Roche NimbleGen, Inc. (Madison WI).

**Fig. S4** Validation of analysis of gene expression with R in samples from Experiment 1. Analysis with R produced almost twice the number of transcripts differentially expressed between foraging and nonforaging workers at FDR<0.05 than analysis with SAS (1387 vs. 771, respectively). However, a comparative analysis between the two set of transcripts revealed large overlap confirming that the choice of either analysis would not affect the biological significance of the results.

**Fig. S5** Comparative analysis of sets of transcripts differentially expressed at FDR <0.05 in both Experiments 1 and 2. A pool of 89 transcripts were shared (more than expected by chance, hypergeometric test: representation factor: 8.4, $P < 7.16e\text{-}55$), but these did not produce any significantly enriched GO terms. GO terms indicated in the figure refer to the two sets of transcripts analysed separately.

**Fig. S6** Quantitative real-time PCR validation of expression levels of genes of interest. A) Expression levels of the following genes associated with GO terms of interest were analysed using quantitative real-time PCR (see Table S10 for detailed information about these genes and the primers we used): *For* (food-related behaviour and polyethism), *Hym* (antibacterial response), *mf* (muscle development), *mhc* (locomotion), *oat* (neurogenesis), *spirit* (innate immune response) and *syt1* (neurotransmitter secretion). Mean expression levels in QLin and QRout were normalized to levels of expression in QRin workers. We used 9 pools for QLin, 8 for QRin and 5 for QRout, each pool being composed by 10 workers from the same colonies used for Experiment 2 (but different individuals). Statistical analysis was performed with nonparametric Wilcoxon comparisons for each pair of treatments: * = $P < 0.05$; ** = $P < 0.01$. #For a better visualization of the results, the bar associated with the gene *Hym* is not represented in full length in

QRout: average relative expression for this gene was 3.95 with S.E. ±0.94; QRin = queenright nonforaging workers; QLin = queenless nonforaging workers; QRout = queenright foraging workers. B) $Log_2$-transformed and normalized expression values for the same genes as above after microarray analysis.

**Table S1** Experiment 1: differentially expressed transcripts between foraging vs. nonforaging workers at FDR <0.05.

**Table S2** Experiment 1: significantly enriched GO terms and KEGG pathways (functional annotation chart, $P < 0.05$).

**Table S3** Experiment 2: differentially expressed transcripts between queenright nonforaging (QRin) and foraging (QRout) workers at FDR <0.05.

**Table S4** Experiment 2: significantly enriched GO terms and KEGG pathways (functional annotation chart, $P < 0.05$).

**Table S5** Experiment 2: differentially expressed transcripts between queenright and queenless workers at FDR <0.1. The list includes transcripts that were differentially expressed either in the QRin-QLin or in the QRout-QLout comparisons.

**Table S6** Differentially expressed transcripts that were shared at FDR <0.05 between Experiment 1 and Experiment 2.

**Table S7** Comparisons of significantly enriched GO terms from Experiment 1 and Experiment 2 ($P < 0.05$).

**Table S8** Overlap analysis between 17 960 transcripts upregulated in queenless workers and transcripts that were upregulated in foraging and nonforaging workers.

**Table S9** Comparative analysis of the 735 transcripts provided with FlyBase annotations that were differentially expressed in foraging vs. nonforaging workers in Experiment 1 and in other previous transcriptome studies.

**Table S10** Quantitative real-time PCR validation of expression levels of genes of interest in workers from Experiment 2: gene lists and primers' sequences.

**Appendix S1** Supporting information for online publication including further details (with relevant references) on the following aspects: insect collection, rearing and sampling; sample preparation for molecular analyses; protocols for microarray analysis; validation of differential expression of candidate genes using quantitative real-time PCR; comparative studies across species; discussion of genes of interest.

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# Recognition in Ants: Social Origin Matters

**Joël Meunier[1,2]*, Olivier Delémont[3], Christophe Lucas[1,4]**

**1** Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland, **2** Zoological Institute, Evolutionary Biology, University of Basel, Basel, Switzerland, **3** Institut de Police Scientifique, Ecole des Sciences Criminelles, University of Lausanne, Lausanne, Switzerland, **4** Institut de Recherche sur la Biologie de l'Insecte (UMR 6035), CNRS, University of Tours, Tours, France

## Abstract

The ability of group members to discriminate against foreigners is a keystone in the evolution of sociality. In social insects, colony social structure (number of queens) is generally thought to influence abilities of resident workers to discriminate between nestmates and non-nestmates. However, whether social origin of introduced individuals has an effect on their acceptance in conspecific colonies remains poorly explored. Using egg-acceptance bioassays, we tested the influence of social origin of queen-laid eggs on their acceptance by foreign workers in the ant *Formica selysi*. We showed that workers from both single- and multiple-queen colonies discriminated against foreign eggs from single-queen colonies, whereas they surprisingly accepted foreign eggs from multiple-queen colonies. Chemical analyses then demonstrated that social origins of eggs and workers could be discriminated on the basis of their chemical profiles, a signal generally involved in nestmate discrimination. These findings provide the first evidence in social insects that social origins of eggs interfere with nestmate discrimination and are encoded by chemical signatures.

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* E-mail: joel.meunier@unibas.ch

## Introduction

The evolution of sociality requires recognition mechanisms allowing group members to direct cooperative or aggressive behaviours towards the correct individuals. In particular, the ability to discriminate against non-group members is important as it may help to prevent colony invasion, to avoid exploitation of group resources by outsiders and to limit erosion of relatedness between group members [1], [2]. Identifying which factors influence nestmate discrimination is therefore fundamental to gain a better understanding of social evolution.

In social insects, nestmate recognition is generally mediated by the blend of chemical compounds (CC) present on the cuticle of individuals [3]. These CC are mainly hydrocarbons, which can have genetic bases and also be acquired from the environment [4]. Repeated grooming, trophallaxis and body contacts between individuals regularly homogenize chemical profiles among colony members. This blend of chemicals produce a colony-profile, which compared to intruders' odours, allows resident workers to assess colony membership and engage correct behavioural responses [3], [5–7]. Colony social structure is generally thought to influence abilities of resident workers to discriminate between nestmates and non-nestmates. In particular, the presence of multiple resident queens is predicted to broaden the mix of genetically determined chemical cues composing the colony-profile, which in turn is expected to increase discrimination errors between nestmate and non-nestmates individuals, and therefore to inhibit aggression against conspecific foreigners [8], but see [9].

Odours of intruders may also influence nestmate discrimination through distinct pathways. One way is to present a relatively small global-amount of CC, so that resident workers are not able to compare intruders' odours to colony-profile [10]. Recent studies in wasps and ants support this hypothesis, showing that lure presenting relatively small global amount of cuticular hydrocarbons elicit fewer aggressions from foreign workers [11–13]. The other possibility is to present a specific blend of CC that interferes with nestmate discrimination. For instance in the ant *Camponotus floridanus*, queens and queen-laid eggs presenting CC associated with high fertility rates elicit fewer aggression from foreign conspecific workers [14], [15].

Signals associated with the social origin of intruders are also known to influence discrimination against foreign queens and workers. In the fire ant *Solenopsis invicta*, workers from multiple-queen (= polygyne) colonies tolerated foreign conspecific queens only if these queens came from polygyne colonies [16]. In the ant *Messor barbatus*, workers from single-queen (= monogyne) colonies accepted more foreign conspecific workers from polygyne than monogyne colonies [17]. Finally in the ant *Formica selysi*, resident workers were less aggressive towards foreign workers originating from colonies with similar than alternative social forms [18]. Chemical analyses suggested that discrimination between intruders from alternative social origins could be based on chemical profiles, as *S. invicta* queens and *M. barbatus* workers present subtle differences of chemical profiles when originating from monogyne and polygyne colonies [19], [20]. Whether chemical profiles of *F. selysi* workers reflect their social origin remained however untested so far.

Contrary to adult intruders, the influence of social origin of foreign eggs (we always refer to queen-laid eggs in this manuscript) on their elimination by resident workers has never been explored in social insects. The elimination of foreign eggs can be important to maintain colony integrity, as subsequent brood from infiltrated

queens may dilute relatedness among nestmates, monopolise colony resources, parasitize host colonies and hence can decrease inclusive fitness of resident workers and induce colony collapse [2], [21]. In *F. selysi*, recent works suggest that social origin of foreign eggs could interfere with their elimination rate. In particular, monogyne workers rejected eggs from foreign monogyne colonies, while polygyne workers accepted eggs from foreign polygyne colonies [22]. These results called for further studies disentangling whether social origin of introduced eggs or recipient workers influenced the acceptance of foreign eggs, and testing potential association between chemical profiles and social origin of eggs.

Our present study was conducted in the ant *F. selysi* (i) to test the influence of social origin of eggs on their acceptance by foreign workers, and (ii) to investigate the association between chemical profiles and social origins of workers and eggs. Using egg-acceptance bioassays, we first investigated whether monogyne and polygyne workers discriminated between eggs from their own colony, foreign monogyne colony and foreign polygyne colony. Then, we determined whether social origin of eggs and workers could be discriminated on the basis of their chemical profiles by testing qualitative and quantitative differences between chemical profiles of individuals (i.e. eggs and workers) sampled in 23 monogyne and 23 polygyne field colonies.

## Materials and Methods

### Model species

The study population of *F. selysi* is located between Sierre and Susten along the river Rhône in central Valais, Switzerland (7°36′30″E, 46°18′30″N, altitude 565 m). In this population, monogyne and polygyne colonies live in close proximity with no sign of genetic differentiation or mating incompatibilities between social forms [23], [24]. The social structure (monogyne or polygyne) of each colony involved in this study had been previously determined by genotyping eight to 100 workers per colony at nine microsatellite markers (method described in [23]). Repeated sampling and genotyping of individuals in the same colonies over several years confirmed that the colonies have stable social structures with very low rates of queen turnover [23], [25], [26].

### Egg acceptance bioassays

We estimated the survival rate of eggs introduced into 93 recipient groups of workers using the set up described in Meunier *et al.* [22]. Workers and eggs were sampled from 25 monogyne and 18 polygyne field colonies during the first week of May 2008. Each recipient group was composed of 100 nestmate workers placed in a fluon-lined plastic box (15×15×15 cm) with access to standard ant food *ad libitum* (food composition in [27]). The day of field sampling, each recipient group of workers was set up and received a set of 30 eggs (8 out of the 93 tested groups of workers received (mean ± SD) 17.5±4.2 eggs due to the small quantity of eggs found) from either (i) nestmate colonies, (ii) foreign monogyne colonies or (iii) foreign polygyne colonies. Prior to introduction, eggs were placed in small plastic trays (3×3 cm) and observed under a stereomicroscope to check that they were not damaged. Twenty-four hours after introduction, the number of undamaged eggs in each recipient group of workers was counted. Egg survival rate was the ratio between the number of undamaged eggs counted after 24 hours and the total number of introduced eggs.

### Chemical analyses

Extractions of the CC were made on groups of 10 workers and 30 eggs. Workers were randomly collected under large flat stones covering the 23 monogyne and 23 polygyne colonies used for chemical analyses [28], and immediately frozen on dry ice. Eggs were collected at the same place than workers and individually observed to exclude damaged ones from extractions. On the field, groups of frozen workers and sampled eggs were placed into glass vials (2 ml, Sigma-Aldrich, Buchs, Switzerland) filled with 500 µl of hexane (Sigma 52765, Buchs, Germany). Five minutes later, workers and eggs were removed from hexane, and their respective vials were sealed and stored at −20°C. Both eggs and workers were collected and chemical profiles extracted on the 7$^{th}$ and 15$^{th}$ of April 2009.

Chemical analyses were made on the hexane extracts described above, which were previously evaporated and reconstituted in 100 µl of hexane with 10 ng/µl of eicosane (*n*C20; not present in *F. selysi*) as an injection internal standard. A 2 µl sample of the extract was injected on a Agilent 7890 gas chromatograph fitted with a HP-5MS fused silica capillary column (0.25 mm×30 m, 0.25 µm film thickness; Agilent, Morges, Suisse) linked to a mass analyzer (Agilent 5975 mass spectrometer). The injector was used in splitless mode with a splitless time of 2 min. Injector temperature was held constant at 250°C. An oven program that began at 70°C (1 min) and was ramped at 20°C/min to 140°C, then 3°C/min to 230°C, 2°C/min to 260°C and 3°C/min to 300°C (10 min). Carrier gas was Helium at a flow rate of 1 ml/min. Electron impact positive ions at 70 eV were recorded in the scanning mode (mass range scanned 40–550 amu). The mass spectra were interpreted by fragmentation analysis and comparison to previous publications [29–32]. Retention indices based on a series of *n*-alkane standards (C24–C30, Grace GR-628008) were compared to published data. MSD Chemstation Agilent Technologies software was used to calculate the retention time and total area of each peak for subsequent analysis.

### Statistical analyses

The survival of eggs was tested using mixed-effect models (GLMMs) within groups of monogyne and polygyne workers. In these analyses, the origin of eggs (nestmate, foreign from monogyne colonies or foreign from polygyne colonies) was used as a fixed factor and the arcsine-transformed proportion of eggs still alive after 24 hours (egg survival rate) entered as response variable. The normality of residuals were tested using Shapiro Wilcoxon tests (all $p>0.05$), and pairwise comparisons between egg origins tested using post-hoc Tukey HSD tests. Because eggs or workers originating from the same field colony were sometimes used twice in the analyses (albeit once per egg origin), we included the colony of origin of eggs and workers as random factors in all the analyses.

Whether social origin of eggs and workers could be discriminated on the basis of their chemical profiles was tested using two linear discriminant analyses (DA). The significance of each DA was evaluated (i) by testing difference between groups using Wilks' Lamba tests and (ii) through the percentage of correct assignment of eggs or workers to their social origin, which was given by statistical models and cross-validations (Leave-one-out method). The structure coefficients (i.e. correlations between discriminating variables and discriminant functions) were used to assess the importance of each peak in discriminating social origin of eggs and workers. According to Mardia's criterion [33], coefficient of correlations above 0.7 times the largest coefficient in a discriminant function were considered to have contributed significantly. Coefficients of correlations were obtained from Spearman rank correlation tests.

Because a sample size of at least three times the number of variable is recommended for multivariate analyses [34], DAs were done on 15 out of all the extracted peaks (table 1). We selected the 15 peaks with the highest variations between social forms rather than the ones with the highest relative amount, because no clear evidence exists about positive associations between relative amount of CC and importance of information [35]. Variation between social forms was estimated using Mann-Whitney *U*-tests. To avoid limitations inherent to analyses of compositional data, the area of each peak was transformed according to Aitchison formula [36] prior to DAs. In this formula, $Z_{i,j} = \ln\left(\frac{Y_{i,j}}{g(Y_j)}\right)$, $Z_{i,j}$ is the transformed area of peak *i* for colony *j*, $Y$ is the area of the peak *i* for colony *j*; and $g(Y_j)$ is the geometric mean of the areas of all peaks of the colony *j*. To apply this formula in profiles containing undetected components, the constant 1750 (= one-tenth of the smallest area measured) was added to all peak areas [35]. DA on the absolute quantity of each peak provided comparable results.

Differences in the total amount of CC extracted from groups of monogyne and polygyne eggs or workers were investigated using *t*-tests. For each sampled colony, the total absolute quantity of extracted CC was calculated using the formula $Tot_i = \sum \frac{Abs_{ij} \times ISq}{ISa_i}$, where $Tot_i$ is the total absolute quantity of CC extracted from colony *i*, $Abs_{ij}$ is the GC-MS area of the peak *j* for colony *i*, $ISq$ is the quantity of internal standard introduced in the sample (here 20 ng) and $ISa_i$ is the GC-MS area of the internal standard in the colony *i*.

## Results

### Egg discrimination

The social origin of foreign eggs significantly influenced their survival rate in both groups of monogyne and polygyne workers (figure 1, GLMMs, monogyne workers: $F_{2,30.96} = 5.19$, $p = 0.011$; polygyne workers: $F_{2,17.94} = 13.75$, $p < 0.001$). Overall, foreign eggs originating from monogyne colonies survived significantly less

**Table 1.** Mean relative amount (%) of the chemical compounds used in discriminant function analyses.

| | | | | Workers | | | | | Eggs | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Peaks** | **RT** | **Data set** | | **Mo** | **Po** | ***P*-value** | $r_s$ | | **Mo** | **Po** | ***P*-value** | $r_s$ | |
| 1 | 4.56 | W | | **0.261** | **0.056** | **0.0004** | *−0.52* | | 0.076 | 0.053 | 0.0106 | | |
| 2 | 6.42 | W | | 0.068 | 0.043 | 0.0255 | *−0.33* | | – | – | – | | |
| 4 | 8.23 | W | | 0.119 | 0.078 | 0.0381 | *−0.32* | | – | – | – | | |
| 10 | 15.17 | | E | 0.035 | 0.045 | 0.0448 | | | 0.107 | 0.088 | 0.0009 | *−0.54* | * |
| 15 | 19.48 | | E | 0.104 | 0.084 | 0.3712 | | | 0.086 | 0.073 | 0.0009 | *−0.55* | * |
| 22 | 25.87 | | E | 0.010 | 0.008 | 0.3596 | | | 0.038 | 0.027 | 0.0016 | *−0.52* | * |
| 27 | 28.03 | | E | 0.050 | 0.055 | 0.5276 | | | **0.318** | **0.486** | **0.0003** | *0.58* | * |
| 29 | 30.05 | W | E | 1.049 | 1.572 | 0.0146 | *0.40* | | **1.473** | **2.180** | **0.0003** | *0.58* | * |
| 30 | 30.22 | | E | 0.051 | 0.061 | 0.6013 | | | **0.084** | **0.141** | **<0.0001** | *0.70* | * |
| 31 | 30.82 | | E | 1.808 | 1.708 | 0.4199 | | | 8.884 | 12.572 | 0.0015 | *0.49* | * |
| 34 | 30.77 | | E | 0.012 | 0.009 | 0.0826 | | | 0.036 | 0.023 | 0.0009 | *−0.56* | * |
| 35 | 31.74 | W | | **0.105** | **0.506** | **<0.0001** | *0.76* | * | 0.858 | 1.035 | 0.0505 | | |
| 36 | 31.92 | W | | 0.059 | 0.095 | 0.0015 | *0.46* | | – | – | – | | |
| 37 | 32.11 | W | | 0.062 | 0.093 | 0.0039 | *0.39* | | 0.168 | 0.130 | 0.0546 | | |
| 39 | 32.70 | W | E | 1.024 | 1.452 | 0.0121 | *0.43* | | **0.211** | **0.297** | **0.0002** | *0.57* | * |
| 52 | 36.23 | W | | **9.286** | **5.241** | **<0.0001** | *−0.61* | * | 29.213 | 24.053 | 0.0130 | | |
| 56 | 36.99 | W | | 0.298 | 0.539 | 0.0092 | *0.40* | | 0.959 | 0.873 | 0.1064 | | |
| 61 | 38.06 | | E | 2.090 | 2.139 | 0.9307 | | | **0.738** | **0.930** | **0.0006** | *0.55* | * |
| 65 | 39.01 | W | E | **0.776** | **1.276** | **<0.0001** | *0.78* | * | **0.285** | **0.451** | **<0.0001** | *0.69* | * |
| 77 | 43.67 | W | E | 1.301 | 2.031 | 0.0080 | *0.45* | | 0.838 | 1.028 | 0.0009 | *0.49* | |
| 79 | 44.68 | W | E | **0.904** | **2.308** | **<0.0001** | *0.81* | * | 0.755 | 0.992 | 0.0007 | *0.53* | * |
| 85 | 46.79 | | E | 4.422 | 4.440 | 0.6013 | | | 4.320 | 5.161 | 0.0009 | *0.53* | * |
| 89 | 48.30 | W | | 0.269 | 0.207 | 0.0240 | *−0.29* | | 0.355 | 0.333 | 0.4485 | | |
| 95 | 50.41 | W | | **0.305** | **0.779** | **<0.0001** | *0.72* | * | 0.380 | 0.335 | 0.0018 | | |
| 98 | 52.25 | | E | 0.504 | 0.509 | 0.5713 | | | **1.040** | **1.480** | **<0.0001** | *0.64* | * |

Mean retention times (RT) are given in minutes. Discriminant analyses were computed on data set including groups of (*W*) workers or (*E*) eggs originating from (*Mo*) monogyne and (*Po*) polygyne colonies. Values in **bold** remained significant after Bonferroni correction (reported *P*-values are uncorrected). Correlations between the relative amount of each peak and the respective discriminant function are provided ($r_s$). Asterisks (*) indicate chemical compounds that significantly contribute in discriminating social origins of workers or eggs [33]. (Peak 1) Nonanal; (Peak 2) *n*C13; (Peak 4) Tridecanol;(Peak 10) Butyl dodecanoate; (Peak 15) *n*C19; (Peak 22) Nonadecanal; (Peak 27) *n*C22; (Peak 29) 9-C23:1; (Peak 30) 7-C23:1; (Peak 31) *n*C23; (Peak 34) Heneicosanal; (Peak 35) 11-,9-MeC23; (Peak 36) 7-MeC23; (Peak 37) 5-MeC23; (Peak 39) 3-MeC23 + Decyl dodecanoate; (Peak 52) *n*C25; (Peak 56) 13-,11-,9-MeC25; (Peak 61) 3-MeC25 + Decyl tetradecanoate + Dodecyl dodecanoate; (Peak 65) 3,9-+ 3,7-di-MeC25 + Decyl pentadecanoate + Undecyl tetradecanoate + Dodecyl tridecanoate; (Peak 77) 3-MeC27 + Dodecyl tetradecanoate + Decyl hextadecanoate; (Peak 79) x,y-diMeC28 (mainly) + *n*C28; (Peak 85) 9-C29:1 ; (Peak 89) 11-,9-MeC29; (Peak 95) 12-,10-,8-MeC30; (Peak 98) 9-C31:1 (mainly) + 9,23-C31:2.

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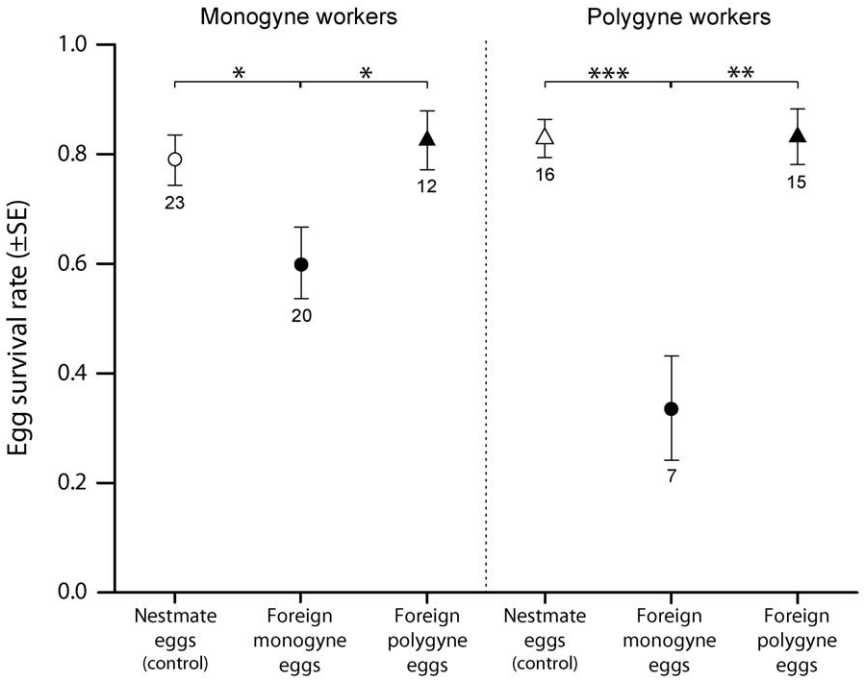


**Figure 1. Survival rate of eggs introduced in groups of monogyne and polygyne workers.** In both groups of workers, foreign monogyne eggs (*black circle*) had a significantly lower survival rate than both foreign polygyne eggs (*black triangle*) and nestmate eggs (*white circle* and *white triangle*), whereas there was no significant difference between the survival rates of foreign polygyne eggs and nestmate ones. The number of recipient group is indicated below the SE bars. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
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than nestmate eggs (Tukey HSD tests, monogyne workers: $p=0.026$; polygyne workers: $p<0.001$) or foreign eggs from polygyne colonies (monogyne workers: $p=0.029$; polygyne workers: $p<0.001$). By contrast, there was no significant difference between the survival rates of nestmate eggs and foreign polygyne eggs (monogyne workers: $p=0.868$; polygyne workers: $p=0.892$). A lower intrinsic viability of monogyne than polygyne eggs is unlikely to explain these results, as there was no significant difference between the survival rates of both types of eggs introduced with nestmate workers (figure 1, *white circle* and *white triangle*, *t*-test, $t=0.62$, d.f. = 37, $p=0.54$).

## Chemical profiles of eggs and workers

Chemical profiles of eggs and workers contained qualitative differences. Thirty-two peaks are exclusively present in workers and one is present in eggs only (table S1). Chemical profiles generally contained a majority of hydrocarbons like alkanes, methylalkanes, odd di-methylalkanes and linear unsaturated hydrocarbons (mono- and di-enes). Chain lenghts are even- or odd-numbered and range from 9 to 33 carbons atoms. Major peaks (more than 5% of total area) are composed of linear alkanes such as tricosane (*n*C23), pentacosane (*n*C25) and heptacosane (*n*C27) or 9-monoenes (9-C25:1, 9-C27:1, 9-C29:1) (respectively peaks 31, 52, 72, 48, 70 and 85). Peaks 1 to 9 are very volatile compounds and are usually associated with the Dufour gland [29], [37]. They are present only in small quantities in workers and eggs from both social forms. A few other compounds rarely described in *F. selysi* were found in large quantities like aldehydes, alcohols and acid esters [29], [37]. Because of the presence of acids, peaks were generally more complex in workers than eggs, whatever the social form. Indeed, acids are present only in workers not in eggs (except in peak 10, 24 and 53).

## Social origins and chemical signatures

After Bonferonni correction, the relative amount of 7 out of 67 peaks extracted from eggs and 6 out of 98 peaks extracted from workers were significantly different between monogyne and polygyne colonies (tables 1 and S1). Those peaks were mainly composed of linear alkanes (peaks 15, 27, 31, 52), branched alkanes (peaks 35, 39, 61, 77, 95), mono alkenes (peaks 29, 30, 85, 98), aldehydes (peaks 1, 22 and 34) and dimethyl alkanes (peak 65, 79).

Discriminant analyses clearly separated workers and eggs according to their social origins. Discriminant scores from the two DAs were significantly larger in polygyne than monogyne groups of workers and eggs (workers: *Wilks' lambda* = 0.117, $F_{1,30}=15.05$, $p<0.0001$; eggs: *Wilks' lambda* = 0.225, $F_{1,30}=6.90$, $p<0.0001$). Moreover, 97.8% of workers and eggs were correctly assigned to their social origins by statistical models, and respectively 91.3% and 84.8% by cross-validation method. According to Mardia's criterion, five and 14 out of the 15 selected peaks significantly contributed in discriminating social origin of workers and eggs, respectively (table 1).

The total amount of extracted CC was significantly larger in polygyne than monogyne eggs (polygyne: 388.2±20.2 ng (mean ± SE); monogyne: 335.3±14.7 ng; *t*-test, $t=2.11$, d.f. = 44, $p=0.040$), and significantly smaller in polygyne than monogyne workers (polygyne: 1836.7±94.2 ng; monogyne: 2092.2±79.0 ng; *t*-test, $t=2.08$, d.f. = 44, $p=0.044$). Despite these differences, the social origins of both eggs and workers were not associated with the presence/absence of specific CC (table S1). Also, the total number of peaks extracted by group was not significantly associated with social origins (*t*-tests; eggs: $t=1.24$, d.f. = 44, $p=0.22$; workers: $t=0.61$, d.f. = 44, $p=0.54$).

## Discussion

Understanding which proximate factors influence the ability of group members to discriminate against foreigners is fundamental to gain a better understanding of the evolution and maintenance of complex social systems. Our egg-acceptance bioassays provide the first evidence in social insects that social origin of queen-laid eggs is associated with cues that interfere with nestmate discrimination. In particular, all *F. selysi* workers discriminated against foreign monogyne eggs, whereas they did not discriminate between nestmate and foreign polygyne ones. Chemical analyses confirmed that discrimination between eggs from alternative social origins could be based on quantitative differences in their chemical profiles. Finally, we showed that chemical profiles of *F. selysi* workers reflected their social origin, a result in accordance with studies in *M. barbatus* and *S. invicta* [19], [20]. Hence, this study supports the view that chemical profiles specific to each social form exist in both eggs and workers, and possibly interfere with nestmate discrimination by conspecific workers.

A somewhat surprising result of our bioassays was that both monogyne and polygyne workers rejected foreign monogyne eggs, whereas they accepted foreign polygyne ones. First, the similar behavioral response of monogyne and polygyne workers towards foreign eggs stands in contrast with the distinct behavioral response of both types of *F. selysi* workers towards foreign conspecific workers, where the level of aggression decreases with matches between social origins [38]. Second, the discrimination against foreign monogyne eggs contrasts with results in most of the Hymenopteran species studied so far, where workers tolerate eggs originating from foreign colonies, and this independently from their social origins [39-42], but see [43]. Altogether, these results indicate that information sources used to discriminate nestmates from non-nestmates differ when experimental intruders are eggs and workers, and reveal that eggs present (i) colony-specific cues that are used by workers to assess colony membership [22] and (ii) socially-related cues that may condition their acceptance by foreign workers.

Ultimately, the risk of egg elimination by foreign workers is unlikely to have specifically selected for the socially-related cues interfering with nestmate discrimination of eggs, as in this species, mated queens introduced in foreign colonies are generally killed before any egg production [44]. However, our artificial introduction of foreign eggs revealed that such cues exist and could be by-products of traits that are under alternative selection pressures between monogyne and polygyne colonies [28]. For instance, an egg-signal that would prevent workers from recognising maternal origin of resident eggs could have been selected in polygyne colonies to limit the costs of nepotistic behaviours [45], and by doing so, it could favor the general acceptance of polygyne eggs by foreign conspecific workers.

Proximately, the influence of social origin of eggs on their tolerance by foreign workers indicates either that (i) eggs lack reliable colony-recognition cues when they are produced in polygyne colonies, or that (ii) signals associated with polygyne origins prevent workers to perceive cues of colony-membership. The first hypothesis seems less likely, since the global-amount of extracted CC was larger in polygyne than monogyne eggs and the chemical profiles of eggs presented some differences across polygyne colonies (Mean variance in the relative amount of each peak across polygyne colonies ($\pm$ SE) = $0.439 \pm 0.049$). By contrast, a signal interfering with nestmate discrimination against queens has already been described in the monogyne ant *C. floridanus*, wherein workers accepted foreign conspecific queens only if they presented signals associated with high fertility [14]. Although this finding is in a different context than egg recognition, it is consistent with our second hypothesis and call for furthers studies investigating the nature of the signals (e.g. fertility signals) specific to monogyne and polygyne eggs in *F. selysi*.

Results from behavioural tests of nestmate discrimination towards eggs and workers imply that resident workers detect signals associated with the social origin of conspecific intruders in *F. selysi* (this study), [18]. Our chemical analyses revealed that monogyne and polygyne origins of eggs and workers could be discriminated on the basis of their chemical profile, a keystone in insects' communication system [3]. In social insects, divergences in chemical profiles generally result from specific genetic background or life-history traits between colonies [19], [46], [47]. In *F. selysi*, the genetic background of monogyne and polygyne colonies is unlikely to produce specific chemical signatures, as no sign of genetic differentiation or mating incompatibilities have been found between social forms in the studied populations [23], [24]. However, the number of queen per colony is associated with several life-history traits that could explain the observed differences in chemical profiles [28]. For instance, the larger volume of polygyne eggs and larger body size of monogyne workers could explain the larger global-amount of CC extracted in these two groups [27], [26]. Similarly, monogyne and polygyne colonies may suffer from different levels of queen-queen and queen-workers competitions [2], which could have selected for distinct chemical signals in their colony members. Finally, divergences between chemical profiles could be by-products of microenvironments specific to monogyne and polygyne colonies [48], [49], albeit the two types of colonies are geographically mixed in the studied population [23]. Setting up laboratory colonies wherein queen number could be manipulated might help to further disentangle whether the relative amount of CC required to influence nestmate discrimination results from the number of queen in the colony (i.e. social environment) or from the social origin of resident queens (i.e. genetic background).

Studies assessing or manipulating the availability of informations are a necessary instrument in unravelling the diversity of outcomes of social evolution and adaptive behaviour in general [50], [51]. By focusing on the social origin of intruders rather than recipient individuals, our findings shed light on the influence of social origin of eggs on their acceptance by foreign workers, and the presence of chemical compounds signalling social origins of both eggs and workers. This reveals that association between colony social structure and nestmate recognition do not necessarily reflect alternative ways for recipient workers to process colony-specific cues, but can rather result from alternative socially-influenced signals presented by conspecific intruders. The proximate and ultimate reasons for the presence of specific chemical profiles in each social form of *F. selysi* remain, however, open for further investigations.

## Supporting Information

**Table S1 Chemical compounds extracted from eggs and workers in *F. selysi*.** Relative amount (%) of all the chemical compounds extracted from groups of workers or eggs originating from monogyne (*Mo*) and polygyne (*Po*) colonies. Retention time (*RT*) is the mean retention time of CC across all extractions. The 15 peaks with the largest variation of relative amount between Mo and Po colonies were included in the discriminant function analyses for workers (*W*) and eggs (*E*). *P*-values remaining significant after Bonferonni corrections are indicated in **bold** (only uncorrected values are shown). Unknown compounds with identical numbers correspond to the same family of compounds.

Main compounds contained in complex peaks are represented in **bold**.
(DOC)

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## Author Contributions

Conceived and designed the experiments: JM CL. Performed the experiments: JM CL. Analyzed the data: JM CL. Contributed reagents/materials/analysis tools: OD. Wrote the paper: JM CL.

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Table S1 - **Chemical compounds extracted from eggs and workers in *F. selysi***

| | | | | Workers | | | Eggs | | |
|---|---|---|---|---|---|---|---|---|---|
| Peaks | Name | RT | Data set | Mo | Po | *P*-value | Mo | Po | *P*-value |
| 1 | Nonanal | 4.56 | W | **0.261** | **0.056** | **0.0004** | 0.076 | 0.053 | 0.0106 |
| 2 | *n*C13 | 6.42 | W | 0.068 | 0.043 | 0.0255 | - | - | - |
| 3 | *n*C14 | 7.74 | | - | - | - | 0.066 | 0.064 | 0.5862 |
| 4 | Tridecanol | 8.23 | W | 0.119 | 0.078 | 0.0381 | - | - | - |
| 5 | *n*C15 + Unknown | 9.42 | | 0.185 | 0.139 | 0.1679 | 0.188 | 0.170 | 0.9300 |
| 6 | *n*C16 + Unknown | 11.55 | | 0.126 | 0.115 | 0.2560 | 0.105 | 0.102 | 0.8618 |
| 7 | Tetradecanal | 11.86 | | 0.045 | 0.047 | 0.2127 | - | - | - |
| 8 | *n*C17 | 13.97 | | 0.199 | 0.203 | 0.7940 | 0.108 | 0.101 | 0.4199 |
| 9 | Xi-MeC17 | 14.26 | | 0.023 | 0.027 | 0.8962 | 0.079 | 0.061 | 0.1893 |
| 10 | Butyl dodecanoate | 15.17 | E | 0.035 | 0.045 | 0.0448 | 0.107 | 0.088 | 0.0009 |
| 11 | Xi-MeC17 | 15.39 | | 0.032 | 0.033 | 0.5420 | 0.108 | 0.075 | 0.0519 |
| 12 | Dodecyl butyrate | 16.31 | | 0.019 | 0.037 | 0.0448 | - | - | - |
| 13 | *n*C18 | 16.65 | | 0.045 | 0.053 | 0.5566 | 0.210 | 0.196 | 0.1195 |
| 14 | Hexadecanal | 17.11 | | 0.032 | 0.031 | 0.4721 | - | - | - |
| 15 | *n*C19 | 19.48 | E | 0.104 | 0.084 | 0.3712 | 0.086 | 0.073 | 0.0009 |
| 16 | Heptadecanal | 20.00 | | 0.015 | 0.014 | 0.3154 | 0.053 | 0.044 | 0.5457 |
| 17 | Unknown 1 | 20.16 | | 0.034 | 0.032 | 0.9826 | 0.129 | 0.084 | 0.4588 |
| 18 | Octadecanol | 20.49 | | 0.123 | 0.104 | 0.0867 | - | - | - |
| 19 | *n*C20 (St) | 22.38 | | 0.979 | 1.144 | 0.3154 | 6.072 | 5.335 | 0.0340 |
| 20 | Octadecanal | 22.94 | | 0.072 | 0.065 | 0.8109 | - | - | - |
| 21 | *n*C21 | 25.21 | | 0.051 | 0.049 | 0.7607 | 0.402 | 0.626 | 0.0223 |
| 22 | Nonadecanal | 25.87 | E | 0.010 | 0.008 | 0.3596 | 0.038 | 0.027 | 0.0016 |
| 23 | Unknown 1 | 26.28 | | 0.055 | 0.080 | 0.9826 | 0.184 | 0.156 | 0.9387 |
| 24 | 2-Me Propyl hexadecanoate | 26.48 | | 0.093 | 0.132 | 0.9307 | 0.144 | 0.105 | 0.0036 |
| 25 | 3-MeC21 + Nonyl undecanoate + Decyl decanoate | 27.26 | | 0.038 | 0.041 | 0.0714 | 0.065 | 0.076 | 0.0026 |
| 26 | Unknown 1 | 27.43 | | 0.041 | 0.038 | 0.7940 | 0.191 | 0.105 | 0.0493 |
| 27 | *n*C22 | 28.03 | E | 0.050 | 0.055 | 0.5276 | **0.318** | **0.486** | **0.0003** |
| 28 | Eicosanal | 28.63 | | 0.049 | 0.040 | 0.0583 | - | - | - |
| 29 | 9-C23:1 | 30.05 | W E | 1.049 | 1.572 | 0.0146 | **1.473** | **2.180** | **0.0003** |
| 30 | 7-C23:1 | 30.22 | E | 0.051 | 0.061 | 0.6013 | **0.084** | **0.141** | **<0.0001** |
| 31 | *n*C23 | 30.82 | E | 1.808 | 1.708 | 0.4199 | 8.884 | 12.572 | 0.0015 |
| 32 | Nonyl tridecanoate + Unknown2 | 31.12 | | 0.086 | 0.072 | 0.0997 | 0.087 | 0.066 | 0.0042 |
| 33 | Unknown 2 | 31.27 | | 0.109 | 0.124 | 0.0750 | 0.623 | 0.453 | 0.0018 |
| 34 | Heneicosanal | 30.77 | E | 0.012 | 0.009 | 0.0826 | 0.036 | 0.023 | 0.0009 |
| 35 | 11-,9-MeC23 | 31.74 | W | **0.105** | **0.506** | **<0.0001** | 0.858 | 1.035 | 0.0505 |
| 36 | 7-MeC23 | 31.92 | W | 0.059 | 0.095 | 0.0015 | - | - | - |
| 37 | 5-MeC23 | 32.11 | W | 0.062 | 0.093 | 0.0039 | 0.168 | 0.130 | 0.0546 |
| 38 | 2-MeC23 | 32.45 | | 0.040 | 0.042 | 1.0000 | - | - | - |
| 39 | 3-MeC23 + Decyl dodecanoate | 32.70 | W E | 1.024 | 1.452 | 0.0121 | **0.211** | **0.297** | **0.0002** |
| 40 | 5,13-diMeC23 | 32.96 | | 0.136 | 0.150 | 0.4456 | 0.074 | 0.066 | 0.4684 |
| 41 | Unknown 1 | 33.18 | | 0.029 | 0.031 | 0.9653 | - | - | - |
| 42 | *n*C24 | 33.44 | | 0.315 | 0.303 | 0.5276 | 1.724 | 1.674 | 0.8605 |
| 43 | Xi-MeC24 + Decyl tridecanoate + Undecyl dodecanoate | 33.73 | | 0.802 | 0.887 | 0.6476 | 0.091 | 0.107 | 0.1663 |
| 44 | X-Me Decyl dodecanoate | 33.99 | | 0.802 | 0.871 | 0.7114 | - | - | - |
| 45 | X-Me Nonyl tridecanoate + X-Me Decyl dodecanoate | 34.56 | | 0.449 | 0.487 | 0.9826 | - | - | - |
| 46 | Undecyl tridecanoate + Decyl tetradecanoate | 34.79 | | 0.146 | 0.115 | 0.2293 | - | - | - |
| 47 | X-C25:2 + X-Me Decyl tridecanoate | 35.02 | | 1.494 | 1.401 | 0.5276 | 0.080 | 0.049 | 0.4857 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 48 | 9-C25:1 + X-Me Undecyl dodecanoate | 35.45 | | | 4.980 | 6.167 | 0.2127 | 4.576 | 5.433 | 0.0134 |
| 49 | 7-C25:1 + X-Me Nonyl tetradecanoate + Xi-Me Undecyl dodecanoate | 35.58 | | | 0.736 | 0.807 | 0.3049 | 0.395 | 0.414 | 0.7170 |
| 50 | 5-C25:1 + X,X'-diMe Nonyl tridecanoate | 35.68 | | | 0.152 | 0.120 | 0.4857 | 0.036 | 0.042 | 0.4857 |
| 51 | X,X'-diMe Nonyl tridecanoate | 35.87 | | | 0.253 | 0.251 | 0.9134 | - | - | - |
| 52 | *n*C25 | 36.23 | W | | **9.286** | **5.241** | **<0.0001** | 29.213 | 24.053 | 0.0130 |
| 53 | X,X'-diMe Nonyl tridecanoate | 36.38 | | | 0.751 | 0.687 | 0.2947 | 0.059 | 0.031 | 0.0381 |
| 54 | X-Me Undecyl tridecanoate | 36.59 | | | 0.405 | 0.384 | 0.9653 | - | - | - |
| 55 | X-Me Decyl tetradecanoate | 36.86 | | | 0.694 | 0.606 | 0.6476 | - | - | - |
| 56 | 13-,11-,9-MeC25 | 36.99 | W | | 0.298 | 0.539 | 0.0092 | 0.959 | 0.873 | 0.1064 |
| 57 | 7-MeC25 | 37.21 | | | 0.275 | 0.315 | 0.6633 | 0.128 | 0.110 | 0.0751 |
| 58 | 5-MeC25 + X-Me Decyl tetradecanoate + X-Me Undecyl tridecanoate | 37.46 | | | 0.404 | 0.377 | 0.6476 | - | - | - |
| 59 | X-Me Decyl tetradecanoate | 37.62 | | | 0.139 | 0.199 | 1.0000 | - | - | - |
| 60 | X-Me Undecyl tetradecanoate | 37.75 | | | 0.048 | 0.052 | 0.8448 | - | - | - |
| 61 | 3-MeC25 + Decyl tetradecanoate + Dodecyl dodecanoate | 38.06 | | E | 2.090 | 2.139 | 0.9307 | **0.738** | **0.930** | **0.0006** |
| 62 | 5,9-diMeC25 + X,X'-diMe Nonyl tetradecanoate | 38.23 | | | 0.526 | 0.625 | 0.8278 | 0.419 | 0.162 | 0.5566 |
| 63 | X,X'-diMe Decyl tetradecanoate | 38.44 | | | 0.273 | 0.255 | 0.9480 | - | - | - |
| 64 | *n*C26 | 38.79 | | | 0.285 | 0.235 | 0.2380 | 1.376 | 1.091 | 0.0069 |
| 65 | 3,9-+ 3,7-di-MeC25 + Decyl pentadecanoate + Undecyl tetradecanoate + Dodecyl tridecanoate | 39.01 | W | E | **0.776** | **1.276** | **<0.0001** | **0.285** | **0.451** | **<0.0001** |
| 66 | X-Me Decyl tetradecanoate | 39.35 | | | 1.283 | 1.222 | 0.5566 | - | - | - |
| 67 | X-Me Nonyl pentadecanoate | 40.01 | | | 1.809 | 1.820 | 0.7441 | - | - | - |
| 68 | Xi-C27:2 + X-Me Decyl pentadecanoate | 40.26 | | | 0.827 | 1.008 | 0.2469 | 0.083 | 0.108 | 0.1241 |
| 69 | Xi'-C27:2 | 40.52 | | | 0.272 | 0.372 | 0.1249 | - | - | - |
| 70 | **9-C27:1** + 7-C27:1 + X-Me Decyl tetradecanoate | 41.24 | | | 28.318 | 28.033 | 0.5276 | 13.900 | 14.655 | 0.1565 |
| 71 | 5-C27:1 + X-Me Decyl pentadecanoate | 41.51 | | | 2.134 | 2.049 | 0.5862 | 0.551 | 0.527 | 0.6367 |
| 72 | ***n*C27** + X,X'-diMe Decyl pentadecanoate | 41.80 | | | 3.327 | 2.515 | 0.1820 | 8.816 | 7.258 | 0.0305 |
| 73 | ***n*C27:2** + X,X'-diMe Decyl pentadecanoate | 42.09 | | | 0.914 | 0.802 | 0.5420 | - | - | - |
| 74 | 13-,11-,9-MeC27 | 42.59 | | | 0.671 | 0.645 | 0.8448 | 1.034 | 0.894 | 0.0211 |
| 75 | 7-MeC27 + X,X'-diMe Decyl pentadecanoate | 42.85 | | | 1.980 | 1.818 | 0.5134 | 0.258 | 0.241 | 0.5384 |
| 76 | 5-MeC27 + X-Me Undecyl pentadecanoate | 43.10 | | | 0.863 | 0.837 | 0.8448 | 0.173 | 0.188 | 0.2186 |
| 77 | 3-MeC27 + Dodecyl tetradecanoate + Decyl hextadecanoate | 43.67 | W | E | 1.301 | 2.031 | 0.0080 | 0.838 | 1.028 | 0.0009 |
| 78 | Unknown 1 | 43.95 | | | 0.553 | 0.434 | 0.2653 | - | - | - |
| 79 | *n*C28 + **x,y-diMeC28** | 44.68 | W | E | **0.904** | **2.308** | **<0.0001** | 0.755 | 0.992 | 0.0007 |
| 80 | Undecyl hextadecanoate + Dodecyl pentadecanoate + Tridecyl tetradecanoate | 44.05 | | | 0.307 | 0.274 | 0.9480 | - | - | - |
| 81 | X-Me Undecyl pentadecanoate | 44.67 | | | 0.313 | 0.365 | 0.7441 | - | - | - |
| 82 | 9,19-C29:2 | 45.90 | | | 2.270 | 1.984 | 0.2209 | 0.231 | 0.225 | 0.7170 |
| 83 | 9,21-C29:2 | 46.16 | | | 3.622 | 3.853 | 0.8448 | 0.480 | 0.549 | 0.1838 |
| 84 | 9,23-C29:2 | 46.43 | | | 3.097 | 3.169 | 0.7441 | 0.670 | 0.488 | 0.4684 |
| 85 | 9-C29:1 | 46.79 | | E | 4.422 | 4.440 | 0.6013 | 4.320 | 5.161 | 0.0009 |
| 86 | 7-C29:1 | 46.89 | | | 2.011 | 1.907 | 0.4721 | 2.095 | 2.391 | 0.1305 |
| 87 | 5-C29:1 | 47.11 | | | 0.810 | 0.862 | 0.9653 | 0.438 | 0.468 | 0.2186 |
| 88 | *n*C29 + Unknown | 47.38 | | | 0.374 | 0.291 | 0.2469 | 1.830 | 1.671 | 0.2966 |
| 89 | 11-,9-MeC29 | 48.30 | W | | 0.269 | 0.207 | 0.0240 | 0.355 | 0.333 | 0.4485 |
| 90 | 7-MeC29 | 48.55 | | | 0.090 | 0.097 | 0.6792 | 0.137 | 0.168 | 0.1093 |
| 91 | 9,19-C30:2 | 48.84 | | | 0.038 | 0.018 | 0.3712 | - | - | - |
| 92 | 9,21-C30:2 | 49.07 | | | 0.129 | 0.123 | 0.9134 | - | - | - |
| 93 | 3-MeC29 + Dodecyl hextadecanoate + Decyl octodecanoate | 49.33 | | | 0.122 | 0.145 | 0.2560 | 0.102 | 0.154 | 0.0040 |
| 94 | *n*C30 + Unknown 1 | 49.67 | | | 0.104 | 0.133 | 0.1546 | - | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 95 | 12-,10-,8-MeC30 | 50.41 | W | **0.305** | **0.779** | **<0.0001** | 0.380 | 0.335 | 0.0018 |
| 96 | 9,19-C31:2 | 51.64 | | 0.409 | 0.363 | 0.5134 | 0.036 | 0.037 | 0.3561 |
| 97 | 9,21-C31:2 | 51.89 | | 2.775 | 2.687 | 0.4995 | 0.300 | 0.335 | 0.0634 |
| 98 | **9-C31:1** + 9,23-C31:2 | 52.25 | E | 0.504 | 0.509 | 0.5713 | **1.040** | **1.480** | **<0.0001** |
| 99 | 9,21-C33:2 | 56.65 | | 0.125 | 0.126 | 0.7607 | - | - | - |

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Research article

# Hydrocarbon distribution and colony odour homogenisation in *Pachycondyla apicalis*

**V. Soroker [1], C. Lucas [2], T. Simon [3], D. Fresneau [4], J. L. Durand [4] and A. Hefetz [3]**

[1] *Department of Entomology, Institute for Plant Protection, Volcani Center, Beit Dagan 50250, Israel, e-mail: sorokerv@agri.gov.il*
[2] *Méchanismes de Communication, Université Paris Sud, CNRS-UMR 8620, 91405 Orsay Cedex, France*
[3] *Department of Zoology, George S. Wise Faculty of Life Science, Tel Aviv University, Ramat Aviv 69978, Israel*
[4] *Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris Nord, CNRS-FRE 2413, 93430 Villetaneuse, France*

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**Summary.** Within and between individuals hydrocarbon (HC)-circulation was studied in *Pachycondyla apicalis* workers, using radioactive labeling. Newly synthesized HCs occurred both in the PPG and on the epicuticle in appreciable amounts, lesser quantities were found in the crop. The front basitarsal brush contained a greater amount of radiolabeled HCs than could be predicted from its surface area, suggesting preferential secretion to these organs. We propose that the newly synthesized HCs are secreted primarily to the front basitarsal brushes and are thereafter either distributed throughout the body surface, or cleared via the PPG and the alimentary canal.

Using labeled HCs as a model, we tracked the time-dependent dispersion of cuticular lipids among 11 workers, one of which was prelabeled for 24 hours. Distribution among the recipients became progressively uniform, reaching near homogenization between 5–10 days. The mean HCs transfer of *P. apicalis* to the PPG was substantially lower compared to that of *Camponotus fellah* or *Aphaenogaster senilis*. In contrast, transfer to the cuticle in this species was superior. We attribute the low transfer to the PPG to the inefficacy of passive body contact characteristic of *P. apicalis*, as opposed to trophallaxis and/or allogrooming that typify the other two species. The higher occurrence of radiolabeled HCs in *P. apicalis* cuticle can be attributed to their accumulation in the basitarsal brushes. The impact of cuticular lipid transfer and formation of uniform colony odour, as opposed to the maintenance of an idiosyncratic caste-specific composition, are discussed.

*Key words:* Colony odour, hydrocarbons, *Pachycondyla apicalis,* nestmate recognition.

## Introduction

It has long been postulated that nestmate recognition in populous societies relies on a uniform colony odour (Gestalt), rather than individual recognition through sharing recognition cues among nest members (Crozier and Dix, 1979). Colonial specificity of cuticular hydrocarbons (HCs) indicated that they may constitute the recognition labels in social insects (Vander Meer and Morel, 1998), which was corroborated by direct experiments in several ant species (Lahav et al., 1999; Thomas et al., 1999; Wagner et al., 2000). Moreover, splitting colonies results in divergence of HC profiles of the two groups, probably because the flow of cues between the groups is interrupted (Boulay et al., 2000; Lahav et al., 2001; Lenoir et al., 2001). These changes in profile are often, but not always, accompanied by a disruption in the recognition of original nestmates, thus, providing an experimental evidence for the Gestalt Model (Boulay et al., 2000; Lahav et al., 2001) and the significance of cue exchange.

Studies using *Cataglyphis niger* have demonstrated that HCs are biosynthesized in the fat body and secreted to the haemolymph where they are transported both to the postpharyngeal gland (PPG) and the epicuticle (Soroker and Hefetz, 2000). It was further shown in a number of ant species that the newly synthesized HCs circulate between the cuticle and the PPG, both within and between individuals (see Lenoir et al., 1999 for review). Recent findings in *C. niger* showed that the crop contains appreciable amounts of HCs that are congruent with that of the PPG (Soroker et al., 1998). These HCs originate from the PPG rather than being sequestered from the haemolymph by the crop. It is hypothesized that the HC-flow from the PPG to the gut provides a mechanism for clearing away surplus HCs (Soroker and Hefetz, 2000). Hydrocarbon circulation seems to be slightly different in the ponerine *Pachycondyla apicalis.* Workers of *P. apicalis* possess a well-developed front basitarsal cuticular brush that they frequently

pass through the mouth while grooming (Hefetz et al., 2001). Prevention of contact between the mouth and the brushes by mouth blocking or leg restrain resulted in an increase in brush hydrocarbon amount with a concomitant decline in PPG HCs, suggesting that in this species PPG-HCs originate mostly by sequestration from the basitarsal brushes.

Inter-individual hydrocarbon exchange between nestmates provides a major mechanism by which uniform colony odour can be obtained. The magnitude of these exchanges seems to be correlated with the mode of cue exchange. In species exercising trophallaxis transfer is intensive and rapid. Species that do not perform trophallaxis but exhibit intensive allogrooming i.e. *Aphaenogaster senilis*, have also moderate to high levels of transfer, whereas species that exhibit no trophallaxis and almost no allogrooming have rather low levels of inter-individual HC transfer (Soroker et al., 1995). Thus, in *Camponotus fellah* which exhibits high rates of HC transfer, newly synthesized HCs were uniformly distributed among nestmates attaining normality after 24 hours while in *A. senilis*, in which HCs transfer is at moderate rates, the process was much slower (Lenoir et al., 1999).

Similar to many other ponerine ants, *P. apicalis* workers do not perform trophallaxis with nestmates, and allogrooming occurs at a relatively low frequency of 0.5% (Soroker et al., 1995). Indeed, HC transfer in dyadic encounters is low and can be explained solely by physical contact followed by selfgrooming. Colonies of *P. apicalis* are typically composed of less than 100 individuals. Theoretically, even this low amount of cue exchange should still be sufficient to update the gestalt colony odour in these relatively small size colonies, but it is also possible that odour homogeneity is never attained and that recognition in this species is based on individual odours (Crozier, 1987). The objective of this study was to test whether this low inter-individual HC transfer results in odour homogenization in groups of *P. apicalis* ants, as well as to investigate the respective roles of the PPG and basitarsal brushes in this process.

## Materials and methods

### *Collection and maintenance of the ant colonies*

Colonies of *P. apicalis* were collected from Petit Saut French Guyana (1 colony), from Bahia, Brazil (2 colonies), and from Los Tuxtlas, Mexico (1 colony). In the laboratory, the ants were kept under standard conditions: 24–28 °C, 50% humidity and a photoperiod of 14:10 L/D, and fed three times a week with an identical diet of sugar water and a mixture of minced insects.

### *Radiotracer experiments*

The partition of newly synthesized HCs in the various body parts of workers was assessed following injections of 1 µCi [1–$^{14}$C] sodium acetate into the abdomens of 10 workers as described earlier (Soroker et al., 1995). Twenty-four hours post-injection (25 ± 3 °C), the PPG and crop of each ant was dissected and immersed individually in 100 µl of pentane. For assessing the amount of newly synthesized cuticular HCs, the front legs of each of the ants were removed and individually extracted for 5 minutes in 100 µl pentane, while thoraces, including the median and rear legs of the individual ants were extracted by immersing them in 400 µl pentane for 5 minutes.

To assess HC transfer between workers the 4 colonies were divided into groups of 11 ants, a prelabeled donor and its 10 nestmates (group encounters; total number of groups = 21). Donor ants were injected with 1 µCi [1-$^{14}$C] sodium acetate as described above and kept in isolation for 24 hours. Thereafter each donor was placed with its 10 nestmates in a plastic box lined with plaster. The ants were supplied with food and water (Lenoir et al., 2001). Five to six groups were established for each time period. Groups were constructed to obtain representatives of the different colonies for each time period assayed. The groups were maintained for 1 (n = 5), 3 (n = 5), 5 (n = 6), and 10 (n = 5) days. All the dead ants were immediately removed and were not taken into account for transfer calculations. When the donor died the whole group was discarded. At the end of each time period, the entire group was sacrificed for quantification of radioactive hydrocarbons.

The occurrence of labeled HCs in the PPG and the thoracic and legs epicuticle was monitored for all group members, donors and recipients, following extraction as described above. The samples were stored at –20 °C until analysis. HCs were separated by TLC and their radioactivity monitored by IP Autoradiography as described earlier (Soroker et al., 1995). Transfer of labeled HCs was calculated as the percentage labeled HCs found in each recipient out of total labeled HCs found in the donor(s) and recipient(s) combined.

### *Morphometric measurements*

The surface area of the front legs including the basitarsal brushes was estimated from scanning electron micrographs of 5 legs from 3 individuals, prepared as described in (Hefetz et al., 2001). First, we counted the number of hairs per µm$^2$ using a sample of 5 squares (ca 9000 µm$^2$ each) randomly selected throughout the brush. This was multiplied by the total surface of the tarsus divided by 2 (since the brush occurs only dorsally) assuming a cylinder, and by the average area of a single hair (n = 10). To the area of the brush we added the area of the other leg parts assuming a hairless cylinder, to obtain the total surface area of the front legs.

The areas of the middle and hind legs were calculated as hairless cylinders, while the thorax area was calculated considering it as a symmetric ellipsoid that revolves around its large axis (Lahav et al., 1998).

### *Inter-individual interactions*

To assess the total and inter-individual distribution of contacts in a group of untreated, 11 individually marked workers (each ant received a letters code, Table 2) were videotaped for consecutive 24 hrs. Contact durations of each ant with its 10 nestmates were measured for 5 minutes of every hour, for a total of 120 minutes over the 24 hours. Contact was defined when any body parts of two ants were touching for at least 2 seconds. The index of interaction-dispersion among the interacting individuals was calculated using the sum and standard deviations of contact duration of each ant with its 10 nestmates, as described in statistical analyses.

### *Statistical analyses*

To assess when the distribution of newly synthesized HCs among the recipient group-mates reaches homogeneity an index of inequality in radioactive HCs repartition among the recipients was constructed. This index constitutes values between 0–1 and is calculated using the quadratic concentration rate (*qcr*) (Durand, 1998):

$$qcr = \frac{s}{T}\sqrt{N}$$

where $N$ is the number of recipient ants in the group; $T$ is the total of radioactive HCs found on all recipients of a particular group (for the

PPG and cuticle respectively), and $s$ is the standard deviation:

$$s = \frac{\sqrt{\bullet\ (x - \text{Mean})^2}}{N - 1}$$

$x$ represents the amount of radioactive HCs (for the PPG and cuticle respectively) in individual ants.

The index is 0 when there is a uniform distribution among nestmates, and 1 when there is complete inequality in distribution i. e., transfer was to only one recipient.

Assessment of the distribution of inter-individual contacts was made using the above index, but in this case $x$ represents the mean contact duration of each ant with its 10 nestmates.

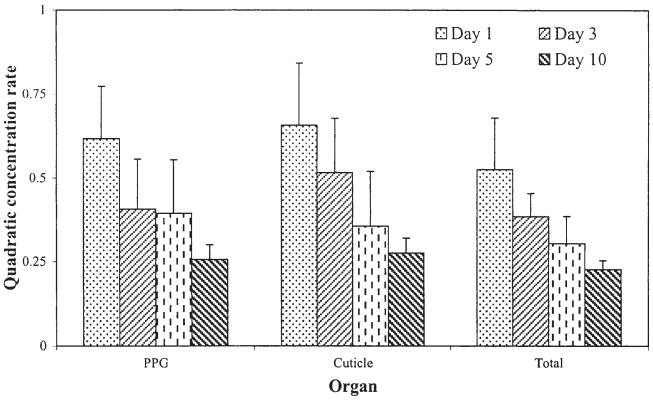


**Figure 1.** Homogeneity of newly synthesized hydrocarbons among group mates *P. apicalis* (expressed as index of inequality repartition, mean ± SD). Four to five groups of workers, constructed from 4 different colonies, were used for each time period. The index ranges from 0–1. An index of 0 represents uniform distribution among nestmates while 1 indicates total inequality in distribution (transfer only to one ant) *P. apicalis* housed together nest mates for various time lengths. Groups consisted of 11 individuals, 1 donor and several recipients (5–10, at the end of experiment).

## Results

### *The distribution of newly synthesized HCs in individual ants*

The distribution of newly synthesized HCs in various body parts of individual workers 24 hours post-injection is shown in Table 1. Appreciable amounts of newly synthesized HCs are found in the PPG and only small amounts in the crop. Most interesting was the distribution of cuticular HCs. The front legs had about 1.5 times more radioactivity per unit surface-area than in the thorax and the 2 rear pair of legs combined.

### *Transfer of newly synthesized HCs from the prelabeled donor to its group-mates*

Figure 1 shows the qcr index of intra-group dispersion of newly synthesized HCs after 1, 3, 5, and 10 days, respectively. Lower index values indicate a more uniform dispersion of the newly synthesized HCs among individuals within a group. As can be seen from the figure there was a gradual decrease in the index as time progressed both for the cuticle and the PPG, indicating that dispersion of HCs within the group of 11 ants becomes progressively more even. After 10 days the index reached 0.25 ± 0.04 and 0.27 ± 0.04 for the PPG and cuticle, respectively.

When the distribution of newly synthesized HCs becomes homogeneous within the group of 11 individuals, it is expected that each individual possess about 9% of total radioactivity. Considering that the PPG contains 35% and the cuticle 56% of total newly synthesized HCs per ant (Table 1), each ant in a group of 11 should theoretically possess about 3% and 6% of labeled HCs in its PPG and cuticle, respectively. This expectation was met for the PPG that reached 3% after 10 days but not for the cuticle that showed only about 2%.

### *Within-group behavioural interactions*

The duration and percentage of time that each ant spent in contact with other ants are shown in Table 2. Except for one extraordinary ant, most ants spent between 9–19% of their time in contact with their nestmates. We used the qcr index of dispersion for assessing how homogeneous was the distribution of contacts among group-mates. As seen in Table 2, this index was rather low (between 0.25–0.42; Table 2) indicating that contacts were more or less evenly distributed among the different ants. The exceptional ant (tjar in Table 2) had recently started to lay eggs and was often groomed (70% of her total contact time) by 7 of her nestmates.

**Table 1.** Distribution of newly synthesized HCs in various body parts of workers *Pachycondyla apicalis*. Radioactive data are mean ± sem of 10 workers. Morphometric data are mean ± sem of 3 workers for front legs measurements (5 legs), and 10 workers for thorax and rear legs measurements.

| Body part | Surface area ($mm^2$) | Radioactivity DPM*/ body part (mean ± sem) | % of total radioactivity | Radioactivity DPM/$mm^2$ |
|---|---|---|---|---|
| PPG | ND | 2219 ± 250.5 | 35 | ND |
| Crop | ND | 658 ± 187.5 | 9 | ND |
| Front legs (pair) | 10.5 ± 0.3 | 991 ± 150.8 | 15 | 94 |
| Thorax + 2 rear leg-pairs | 40.7 ± 2.4 | 2740 ± 291.4 | 41 | 67 |

* DMP-disintegrations per minute.

**Table 2.** Interactions involving contacts (any body parts of two ants touching for at least 2 seconds) and allogrooming among 11 workers *P. apicalis* housed as a group for 24 hours. Data are the sum, mean, and SD of interactions performed by each ant with its 10 nestmates during 24 observations of 5 minutes of each hour (total of 24 observations = 120 min).

| Ant identity | Duration of interactions | | | | Index of dispersion |
|---|---|---|---|---|---|
| | Total (min) | % Time | Mean | SD | |
| nm | 16.1 | 13.4 | 1.46 | 1.59 | 0.31 |
| tj | 16.6 | 13.8 | 1.51 | 1.49 | 0.28 |
| aj | 10.7 | 8.9 | 0.97 | 1.42 | 0.42 |
| tjaj | 14.6 | 12.2 | 1.33 | 1.81 | 0.39 |
| tjar | 48.0 | 40.0 | 4.36 | 3.02 | 0.20 |
| tb | 22.2 | 18.5 | 2.02 | 2.80 | 0.40 |
| ab | 13.0 | 10.9 | 1.18 | 1.42 | 0.35 |
| tbab | 23.1 | 19.2 | 2.10 | 2.75 | 0.38 |
| tr | 12.5 | 10.4 | 1.13 | 0.99 | 0.25 |
| ar | 12.8 | 10.7 | 1.16 | 1.35 | 0.33 |
| trar | 21.5 | 17.9 | 1.95 | 1.73 | 0.26 |

*Comparison of transfer dynamics between species*

To evaluate the impact of transfer mode on transfer dynamics, we compared the mean transfer rates (Fig. 2, bar graphs) and its Coefficient of Variance (Fig. 2, line graphs) in three species with different behaviours. *P. apicalis* has relatively small colonies, does not perform trophallaxis and performs little allogrooming; *A. senilis* comprises populous colonies, does not perform trophallaxis, but performs intensive allogrooming; *C. fellah* comprises populous colonies, and practices both intensive trophallaxis and allogrooming. The data for *P. apicalis* are from this study while those for *A. senilis* and *C. fellah* were obtained from a previous study (Lenoir et al., 2001) that was conducted the same way as the present one.

In general in all species the mean HCs-transfer to the PPG increased with time while the Coefficient of Variance decreased, but at different rates. Workers of *C. fellah* showed the highest transfer rate and the lowest Coefficient of Variance. Conversely, the lowest rates and the highest variance characterized *P. apicalis*. Moreover, *C. fellah* reached almost the maximal value of transfer (6%) already by day 5, whereas in *P. apicalis* transfer did not surpass 3% even after 10 days. Both transfer rate and variance in *A. senilis* showed intermediate values. Transfer to the cuticle had a somewhat more complex pattern (Fig. 2B). In these analyses we measured the transfer to the thorax including the legs, which roughly constitutes about a third of the actual transfer (excluding the head and the gaster). In *C. fellah* the trend was similar to that in the PPG. Variance was rather low and levelled by day 6, reaching about 1.5% (actual transfer of approximately 4.5%). In *A. senilis* and *P. apicalis* the variance was initially high, but declined to the *C. fellah* levels by day 6. It is worthwhile noting that the transfer to the cuticle in both species was higher than that of *C. fellah.* In *A. senilis* we attribute this difference to the highly extensive allogrooming that directs the newly synthesized HCs preferentially to the cuticle. In *P. apicalis* where allogrooming is rare we attribute the higher amount of newly synthesized HCs to their accumulation in the front basitarsal brushes.

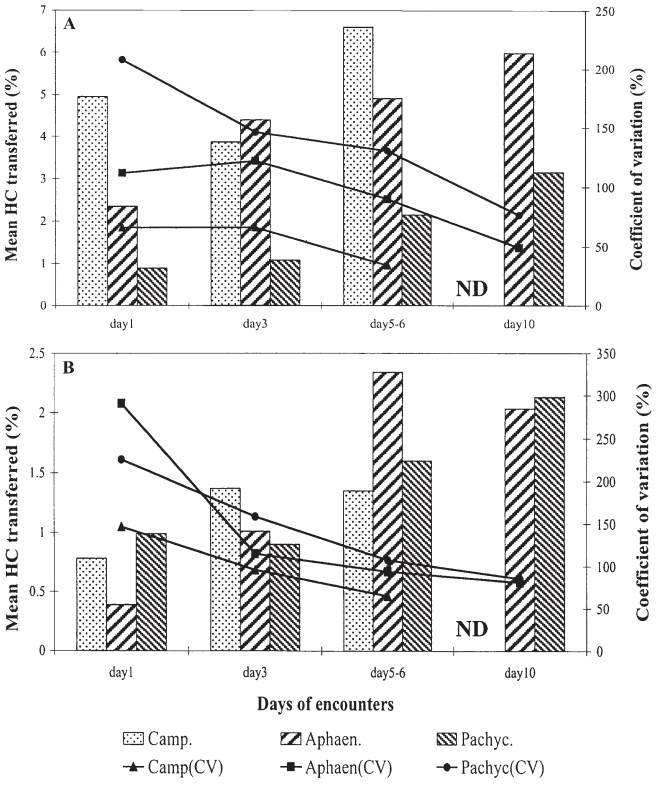


**Figure 2.** Comparison of hydrocarbon transfer between species: A) HC distribution in the PPG; B) HC distribution on the cuticle of the different species. Data for A. *senilis* and *C. fellah* are taken from similar transfer experiments reported by Lenoir et al. (2001). In case of *P. apicalis* number of individuals equalled (day 1 = 48; day 3 = 49; day 5 = 49; day 10 = 35). In case of A. *senilis*, day 1 = 90; day 3 = 69; day 6 = 77; day 10 = 73 and in case in *C. fellah,* day 1 = 68; day 3 = 68; day 6 = 60. ND = not determined. Bars represent mean transfer and the line curve the coefficient of variance.

## Discussion

The chemical basis of nestmate recognition in ponerine is little understood. It is assumed that, as in other species of ants (Lenoir et al., 1999; Vander Meer and Morel, 1998), cuticular HCs are implicated. On the other hand there are numerous reports that indicate that either the complete hydrocarbon profile (Liebig et al., 2000) or some of its constituents (Peeters et al., 1999) act as fecundity signals or are involved in the establishment of dominance hierarchies. Whether disparate HCs are involved in each system or they interact in a complex manner is still unclear. Another open question is whether in small colonies such as in *P. apicalis* nestmate recognition is based on individual recognition or a common colony odour. If the latter prevails it is clear that a mechanism

facilitating the formation of a uniform colony odour should exist. In several ant species such a uniform colony odour is achieved by cue transfer between nestmates (Soroker et al., 1998; Lenoir et al., 2001).

In the present study we examined whether cue transfer is possible by determining the distribution of newly synthesized HCs among groups of 11 workers of *P. apicalis*. The size of the group was selected because it is manageable for radioactive work yet large enough to provide a close simulation of the dynamics of HCs distribution in an intact colony.

First, we investigated the magnitude of newly synthesized HCs 24 hours after the ants were injected with the radioactive precursor. Considerable quantities of newly synthesized hydrocarbons were detected both on the cuticle and in the PPG, with a particular accumulation in the front tarsal brushes, which possessed about 50% more newly synthesized HC per unit area than any other cuticular part measured. This is consistent with the hypothesis that the front brushes play a major role in the distribution of HC to other body parts (Hefetz et al., 2001). We cannot determine at the moment whether the elevated radioactivity is the result of higher rates of HC secretion into the frontal brush, or preferential accumulation during selfgrooming. However, previous studies have shown that when the front brushes are restrained so that they have no contact with other body parts, their HC content increases. This lends credence to the higher secretionary rate hypothesis. Another organ that had considerable amount of radioactive HC was the PPG. Their origin may be from internal transport, but we suggest that the bulk comes from external sources. While grooming, the ants extensively pass their front tarsal brushes through their mouth, possibly discharging HC into the PPG. The small amounts of radioactive HC that were found in the crop further suggest that they are eliminated from the PPG via the alimentary canal, as was also suggested for *C. niger* (Soroker and Hefetz, 2000). Thus, we propose the following HC-circuit for *P. apicalis* workers. They are biosynthesized by the fat body or associated oenocytes and transported mostly to the cuticle, but also possibly to the PPG. The front basitarsal brushes are the site for preferential secretion, from which the HCs are distributed to other body surfaces through the extensive use of these brushed during selfgrooming. HCs turnover is achieved by selectively transporting them into the PPG and thereafter clearing them through the gut.

Whether or not HCs function as nestmate recognition cues, being cuticular components they may serve as a model for the behaviour of recognition cues. Our study indicates that newly synthesized HCs are transferred between individuals, and that after 10 days every member of the group tested has received a share of these HCs. Thus, also in *P. apicalis* achieving a uniform colony odour through mutual cue exchange is possible. It is worthwhile noting that all the mother colonies from which the groups were created had been queenless for several weeks. They have had time to stabilize as a queenless group, and there was no apparent overt conflict regarding dominance hierarchy, all interactions being amicable.

When we compare the dynamics and rate of newly synthesized HCs distribution within the colony of *P. apicalis* to other species, the impact of the mode of transfer becomes apparent (Fig. 2, and Lenoir et al., 2001). In *C. fellah*, due to extensive trophallaxis and allogrooming newly produced HCs are quickly disseminated, for both the PPG and the cuticle, throughout the nest with little variance between the receiving workers. In the two species that do not perform trophallaxis the dynamics of HCs-transfer to the PPG and the cuticle differed. In *A. senilis* transfer rates and variance involving the PPG were intermediate. Behavioural observations in this species have shown very high rates of allogrooming that may compensate, in terms of HCs transfer to PPG, for the lack of trophallaxis. Transfer to the cuticle was initially lower, but by day 5 was appreciably higher than that of *C. fellah*. This emphasizes the importance of allogrooming in HC-distribution in this species. Transfer rates involving the PPG in *P. apicalis* were inferior to both species but, nevertheless, show at the end of 10 days little variance among the recipient workers in the amount of hydrocarbon received. As in *A. senilis*, higher levels of transfer to the cuticle were observed, but since allogrooming is a rare event in this species, it can be attributed to accumulation in the front basitarsal brushes. Moreover, the lack of trophallaxis and the rarity of allogrooming suggest that most of the transfer observed was due to physical contacts and may be considered as passive transfer. Behavioural observations on a group of 11 ants during a 24 hrs period corroborate this idea. Although, at some period, particular ants had more contact with group-mates than did other ants, the overall index of interactions was similarly low for all the ants in the group. This indicates that physical contacts are more or less evenly distributed among nestmates.

The passage of recognition chemicals between individuals to generate a uniform colony odour may create a problem when caste specific signals are also present on the cuticular surface (Sledge et al., 2001; Tenschert et al., 2001). How could idiosyncrasy be maintained if these caste specific compounds are transferred to all colony members? In honeybees for example the caste specific queen mandibular pheromone is spread on its cuticle, but it constitutes a unique set of chemicals. While it is actively spread throughout the colony by the workers, each worker carries the signals only temporarily because it is quickly eliminated either by allogrooming or metabolism (Naumann et al., 1991). This may also be the case in gamergates of the ponerine ant *Dinoponera quadriceps* that possess large quantities of (Z) 9-hentriacontene while workers have only minute amounts of this compound, albeit active transport of these compounds among workers was not reported (Peeters et al., 1999). Cases where caste specificity is not expressed through the production of unique chemicals, but by possessing idiosyncratic profiles of common compounds are harder to reconcile with cuticular chemical-flow among nest members. One way of using cuticular compounds to signal idiosyncrasy is to avoid contact with other nest members. This may be the case in *Harpegnathos saltator* (Liebig et al., 2000) or *Diacamma ceylonense* (Cuvillier-Hot et al., 2001). Avoidance of contact bears also

consequences on nestmate recognition system. If these species use cuticular HCs for nestmate recognition, it implies that either the ants use individual recognition, or the gamergates are excluded from the recognition circle. Alternatively, recognition cues in these ants may be different from cuticular HCs, or that only part of the hydrocarbon profile is used for recognition (Kaib et al., 2000). In the latter case, its composition must be rather constant over time or the ants must have the ability to learn and memorize newly emerging odours.

Among ponerine ants, hydrocarbon transfer between individuals has been investigated thus far only in *P. apicalis*, making any generalization difficult. However, since within the confinement of the nest, ants are always in contact, it is not impossible that phenomenon described here represents a general one. Demands for efficient colony insularity together with the necessity to maintain social structure have undoubtedly selected for the evolution of various mechanisms of cue transfer. Selection would operate both on the behavioural modalities pertaining to cue transfer and the exocrine glands that may serve as gestalt organ to enable the adaptive manipulation of cues transfer-rates and cue-distribution among nestmates. Ponerine ants that show mostly primitive caste differentiation accompanied by developed hierarchical systems provide excellent study cases for testing these hypotheses.

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# Cues of Maternal Condition Influence Offspring Selfishness

**Janine W. Y. Wong[1], Christophe Lucas[2], Mathias Kölliker[1]***

**1** Department of Environmental Sciences, Zoology and Evolution, University of Basel, Basel, Switzerland, **2** Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS, University of Tours, Tours, France

## Abstract

The evolution of parent-offspring communication was mostly studied from the perspective of parents responding to begging signals conveying information about offspring condition. Parents should respond to begging because of the differential fitness returns obtained from their investment in offspring that differ in condition. For analogous reasons, offspring should adjust their behavior to cues/signals of parental condition: parents that differ in condition pay differential costs of care and, hence, should provide different amounts of food. In this study, we experimentally tested in the European earwig (*Forficula auricularia*) if cues of maternal condition affect offspring behavior in terms of sibling cannibalism. We experimentally manipulated female condition by providing them with different amounts of food, kept nymph condition constant, allowed for nymph exposure to chemical maternal cues over extended time, quantified nymph survival (deaths being due to cannibalism) and extracted and analyzed the females' cuticular hydrocarbons (CHC). Nymph survival was significantly affected by chemical cues of maternal condition, and this effect depended on the timing of breeding. Cues of poor maternal condition enhanced nymph survival in early broods, but reduced nymph survival in late broods, and vice versa for cues of good condition. Furthermore, female condition affected the quantitative composition of their CHC profile which in turn predicted nymph survival patterns. Thus, earwig offspring are sensitive to chemical cues of maternal condition and nymphs from early and late broods show opposite reactions to the same chemical cues. Together with former evidence on maternal sensitivities to condition-dependent nymph chemical cues, our study shows context-dependent reciprocal information exchange about condition between earwig mothers and their offspring, potentially mediated by cuticular hydrocarbons.

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* E-mail: mathias.koelliker@unibas.ch

## Introduction

Parental care evolved due to its fitness benefits to offspring, and it often comes at a cost for parents [1,2]. Offspring that vary in condition are expected to differ in the fitness gain per unit of provisioning obtained from their parents [3,4,5,6,7], and parents that differ in condition should experience differential costs of provisioning [8,9]. Because parents and offspring are closely related, there is an evolutionary (kin selected) incentive for parents to adjust their provisioning to offspring condition (i.e., need or quality [5,6,10,11,12]) in order to maximize their returns on investment. However, it should also pay off to offspring to adjust their demand to parental condition to moderate the cost of investment that offspring impose on their parents [8]. As a consequence, it is in the overall interest of both parents and offspring to be sensitive to variation in each other's condition, and selection may favor the exchange of information about condition between parents and offspring through cues or signals (see [13] for definitions of terms). The evolutionary conflict between parents and offspring over parental investment [5,10,12,14] may have a modulating effect in the evolution of the signals, leading to "information warfare" [15] between parents and offspring and the evolution of exaggerated and costly signals.

Previous research has focused on offspring begging signals conveying information about offspring condition as signals of need or quality [3,6,10,16,17]. The reverse expectation that offspring should be sensitive to cues of parental condition [8], or that parents even may have evolved signals to convey honest information about their condition to their offspring, has received less theoretical or empirical scrutiny. We may ultimately often expect a reciprocal form of parent-offspring communication where parents and offspring exchange information about their respective condition (and maybe even beyond, an information exchange among all family members in a communication network; [18]; see also [19,20]). Based on these arguments, one may expect offspring to adjust their demand or selfishness to cues or signals of parental condition. The question how selfish offspring should be, how much resources they should demand from their parents, and how competitive they should be against their siblings is at the heart of parent-offspring conflict theory [5,7,10,12,14]. In its most extreme form, offspring selfishness leads to siblicide, that is, the killing and possible consumption of a sibling offspring [12,21,22,23]. So, if parents provide cues or signals about their condition to their

offspring (either as inadvertently released information or as evolved signal of parental condition), and offspring are sensitive to these cues, how should offspring respond in terms of their selfishness? The prediction partly depends on the consequences of the parent's condition on the amount of obtained care, and on whether sibling interactions are purely competitive or if there is scope for cooperation among siblings (see [24] for review of evidence of sibling cooperation). Under pure competition over limited resources, offspring perceiving that their parents are in poor condition, which therefore will provide low levels of care, should compete more intensely and maybe even attempt to kill their siblings earlier (or, alternatively, disperse). This is because the poor condition of the parents would indicate insufficient resources for all offspring, enhanced sibling competition and threat of mortality. In contrast, when cooperation between offspring can compensate partly for reduced care provided by parents in poor condition, offspring perceiving cues of poor parental condition may reduce their competitive drive due to the advantage of maintaining a larger number of siblings to cooperate with. Sibling cooperation may occur for example if larger groups/broods of young are better in predator defense, have enhanced foraging efficiency or directly cooperate for example by sharing food [25,26,27].

Parental condition is often related to the timing of breeding, for example because individuals in good condition are able to breed early [28]. Furthermore, early breeders may face quite different ecological conditions compared to late breeders in terms of population density, food availability, predation pressure, temperature, etc., which are all factors that may also contribute to variation in their condition, in the benefits/costs of parental care (e.g., [29,30]) and in the pay-off of sibling competition versus sibling cooperation. Correspondingly, parental cues/signals of condition and/or offspring sensitivities to these cues/signals may be expected to vary with the timing of breeding. Few studies investigated such context-dependent parent-offspring communication, but there is some evidence for different responses of parents to variation in offspring signals of quality by early and later breeders [31,32].

The European earwig (*Forficula auricularia*) is an insect species with uniparental maternal care including egg- and offspring attendance and food provisioning [33,34,35,36,37,38]. The offspring (nymphs) signal their condition by solicitation pheromones in the form of cuticular hydrocarbons (CHC) to which the females show two distinct responses: When exposed to CHC extracts from well-fed nymphs (as compared to poorly fed nymphs, or controls) females increase their food provisioning [39] and modify the timing of second clutch production [31]. This latter response depends on the timing of breeding, with early females advancing and late females delaying second clutch production. Furthermore, females in poor condition provide food to fewer nymphs [40] and they negatively affect their nymphs' survival under conditions of limited food availability, probably because of mother-offspring competition over the scarce food [41]. This is in contrast to the beneficial effects of maternal presence under conditions of plentiful food where female food provisioning enhances nymph survival [34]. Finally, siblicide and cannibalism are a primary cause of mortality throughout nymph development [42,43], which makes *F. auricularia* an ideal model system to test the influence of maternal condition cues on offspring selfishness.

## Materials and Methods

The animals used in this experiment originated from a laboratory population held according to our standard laboratory rearing protocol and based on a large founder population [44,45]. In brief, groups of approximately 80 males and 80 females (randomly selected from the breeding stocks) were set up for mating in two plastic containers (37×22×25 cm) lined with Fluon to prevent the insects from escaping, humid sand as a substrate, and egg-cardboard and plastic tubes as shelters. The food consisted of an artificial diet [45] and was changed twice a week. The containers were kept in a climatic chamber at 60% humidity and 14 h/10 h 20°C/20°C light/dark photoperiod cycle ("summer conditions"). Upon observation of the first oviposition on 21 January 2011, all females were set up individually in Petri-dishes (10×2 cm) with humid sand as substrate and plastic shelters as nests and ad libitum food. All females were then transferred to "winter conditions", which consisted of one week at 10°C to trigger egg-production, and 15°C afterwards and 80% humidity (throughout without light). The females were held under these conditions until the eggs hatched (= day 0). Food was changed twice a week from isolation to oviposition. No food was provided from oviposition to hatching [34]. One day after hatching the number of hatched nymphs was counted, and the clutches were standardized to a maximum of 25 nymphs in preparation for the experimental set up (see below). The female and five randomly selected nymphs were weighed to the nearest 0.001 mg using a Mettler-Toledo MT5 Micro-balance (Mettler, Roche, Basel), provided with ad libitum food and transferred to summer conditions (see above).

### Experimental Design

The aim of the experimental design was to allow the earwig mother to release chemical cues in the substrate and to expose the nymphs to these cues over an extended time period, but preventing physical contact between mother and nymphs. We achieved this by keeping mothers and nymphs in separate Petri dishes and swapping them daily between the two Petri dishes. This treatment ensured that nymphs were exposed continuously to any chemical cues females released and left in the substrate, and that the maternal cues were renewed every other day.

The experiment was initiated on day 2 after hatching. The female and 20 nymphs (between 15 and 19 nymphs when brood size was smaller; 7 out of 37 cases) were separated and transferred to a pair of Petri-dishes (10×2 cm) containing humid sand as substrate and plastic shelters, respectively. At this stage, the females were randomly assigned either to the high food (HF) or to the low food (LF) treatment. To obtain females in HF or in LF condition, while keeping nymph condition constant, we manipulated the degree of female food access (pollen pellets [36]) and kept it constant for nymphs. HF females had daily access to large amounts of food (approx. 10 mg) for 3 hours. LF females had access only every second day to a smaller amount of food (<1 mg) for a period of 3 h (see also [40]). The nymphs had daily access to ad libitum food (pollen pellets) during these 3 h of female treatment. In all samples, the remaining food was removed after the 3 h feeding period.

Because HF females had access to larger amounts of food for a longer total amount of time, we expected them to produce more frass, which would have biased nymph food intake through allocoprophagy and, hence, potentially nymph condition. To prevent such an effect, female frass was removed daily before swapping females and nymphs between Petri-dishes. The number of nymphs alive was counted daily. In this species, deaths due to siblicide and cannibalism cannot easily be directly observed because the attacked nymphs are consumed quickly and completely. The number of nymphs alive is therefore mostly a consequence of nymph cannibalism (only 33 dead bodies were observed over the

course of the experiments; out of 721 nymphs set up in total). On day 40 after hatching, we counted the number of surviving nymphs, and we took again the weight of the female and of five randomly chosen nymphs (or fewer, depending on the number of survivors).

The sample size consisted of 37 replicates (Petri dish pairs), 18 females and their broods in the HF treatment and 19 females and their broods in the LF treatment. The experimental treatments were properly randomized as there were no significant differences between treatments in female egg-laying date (means ± s.e.; HF: 17.500±3.607, LF: 19.684±3.511; $t_{35} = 0.434$, $p = 0.667$), clutch size (HF: 67.556±2.501, LF: 63.526±2.434; $t_{35} = -1.155$, $p = 0.256$), hatching success (HF: 0.826±0.042, LF: 0.810±0.041; $t_{35} = -0.279$, $p = 0.782$), female body weight at hatching (HF: 52.178±1.805, LF: 49.826±1.757; $t_{35} = -0.933$, $p = 0.357$), or nymph body weight at hatching (HF: 1.585±0.076, LF: 1.580±0.074; $t_{35} = -0.045$, $p = 0.965$).

## Extraction and Quantification of Cuticular Hydrocarbons (CHC)

After termination of the experiment on day 40, all females were individually frozen at −30°C for later CHC extraction. For extraction, each female was immersed for 10 minutes in 800 µl of the extraction solution which consisted of n-Heptane (Rotisolv 99% pure, Carl Roth AG, Arlesheim, Switzerland) and 2.5 ng/µl n-Octadecane as an internal standard ($C_{18}H_{38}$; Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland). The female was then removed from the vial and the extract stored at −30°C. Chemical analysis was carried out using Gas-Chromatography/Mass-Spectrometry (Agilent GC 7890A/5975C MSD; electron impact: 70 eV). For analysis, 2 µl extract were injected in the GC (containing 2×2.5 ng = 5 ng of the internal standard) in splitless mode (splitless time = 2 min.) and a constant inlet temperature of 250°C. The GC-MS system was equipped with a HP-5MS fused silica capillary column (length: 30 m, inner diameter: 0.250 mm, film thickness: 0.25 µm; Agilent J & W GC columns, Agilent Technologies, USA). The GC temperature program started with a temperature of 70°C (held for 2 min), then increased at 15°C/min to 232°C (held for 11 min), and then at 5°C/min to 300°C (held for 7 min). The column helium flow rate was 1 ml/min, ion detection started after a five minute solvent delay, and the MSD was set to a scan range of 40–550 m/z. For quantification of the CHC profiles, we integrated 31 peaks (of which one was the internal standard octadecane; nC18) from the chemical chromatogram using Chemstation software (Agilent Technologies, Inc.). For quantification, we divided the area of each peak by the area of the internal standard in the same chromatogram and multiplied this ratio by 5 ng to obtain an estimate of the quantity for each peak in ng. We provide peak identifications based on comparison with previous unpublished CHC identification from earwigs (Wong et al. submitted) and using fragmentation analysis [46,47,48] with MassHunter B.06.00 software (Agilent Technologies, Inc.). Kovats retention indices were calculated according to [49] based on a series of n-alkane standards (C8–C40, Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland).

## Statistical Analysis

We analyzed the effect of the female condition treatment on the proportion of nymphs alive using a generalized linear model with a logit link, a binomial error distribution (correcting for over-dispersion), the number of nymphs alive as the dependent variable, the number of nymphs originally present at experimental set up as denominator, and the female condition treatment, hatching date and their interaction as fixed effects.

The measures of peak quantities were transformed using the power transformation $y = x^{0.2}$ which yielded approximately normal distributions. The values $y$ of each peak were then standardized to a mean = 0 and standard deviation = 1 ($z_i = (y_i - \bar{y})/\sigma_y$) [as recommended in 46]. Given the large number of peaks in the CHC data (k = 30 peaks) relative to sample size (n = 37), and in order to take into account tight correlations among individual peak quantities, we used a variable clustering approach as implemented in JMP®Pro 10.0.1 to reduce data dimensionality (for more information about variable clustering, see e.g. [50] or the SAS/STAT User's Guide, SAS Institute Inc., Cary, NC). Variable clustering is analogous to principle component analysis, but joins highly correlated variables (pointing in a similar direction in multivariate space) in clusters [47], facilitating biological interpretation of the experimental results. After forming the clusters, the peak of each cluster that showed the strongest correlation with its own cluster as compared to the next closest cluster was used as the cluster representatives for further analysis [46].

To analyze the effects of the female condition treatment and hatching date on the female's CHC profile we used a MANOVA with the cluster representatives as dependent variables (repeated measurements), and the treatment, hatching date and their interaction as fixed factors. To directly test for a quantitative relationship between the proportion of nymphs alive and maternal CHC we used a step-wise linear regression approach with hatching date dependent survival (see results for details on how this variable was calculated) as dependent variable and the cluster representatives as candidate explanatory variables. The model with the lowest value for the Bayesian Information Criterion (BIC) was chosen as the final model and confirmed using both forward and backward variable selection procedures. All statistical analyses were carried out using JMP®Pro 10.0.1 statistical software (SAS Institute Inc.) and all reported p-values are two-tailed.

# Results

As intended, females from the HF treatment gained significantly more weight over the course of the experiment (mean ± s.e.; 12.879 mg ±1.254) than females from the LF treatment (3.945 mg ±1.225; $t_{35} = -5.088$, $p<0.0001$), but the female food treatment did not affect nymph weight gain (from day 1 to day 40) (HF: mean ± s.e.; 3.993 mg ±0.331; LF: 4.258 mg ±0.322; $t_{35} = 0.575$, $p = 0.569$). Thus, our food manipulation successfully generated variation in female condition while keeping nymph condition unaffected.

The proportion of nymphs alive on day 40 was affected by the female condition treatment through an interaction with hatching date (GLM; LR-$\chi^2_1 = 6.177$, $p = 0.013$; Figure 1), while the main effects of the female condition treatment (LR-$\chi^2_1 = 0.899$, $p = 0.343$) and hatching date (LR-$\chi^2_1 = 0.014$, $p = 0.907$) were not significant. The interaction was due to a significantly higher proportion of nymphs alive in the LF treatment among early hatching broods (contrast; LR-$\chi^2_1 = 7.016$, $p = 0.008$) and the opposite, marginally non-significant, trend among late hatching broods (contrast; LR-$\chi^2_1 = 3.456$, $p = 0.063$) (see Figure 1).

The statistical clustering of the 30 peaks resulted in six clusters of highly correlated peaks (summarized in Table 1) jointly explaining 80.2% of the total variance in compound quantities. Entering the representative chemical compounds for each cluster (see Table 1) as repeated measures in a MANOVA with female condition treatment, hatching date and their interaction as fixed terms revealed a significant effect of female condition (but not hatching date or their interaction) on the relative CHC quantities and, hence, the composition of the CHC profile (Table 2; within-

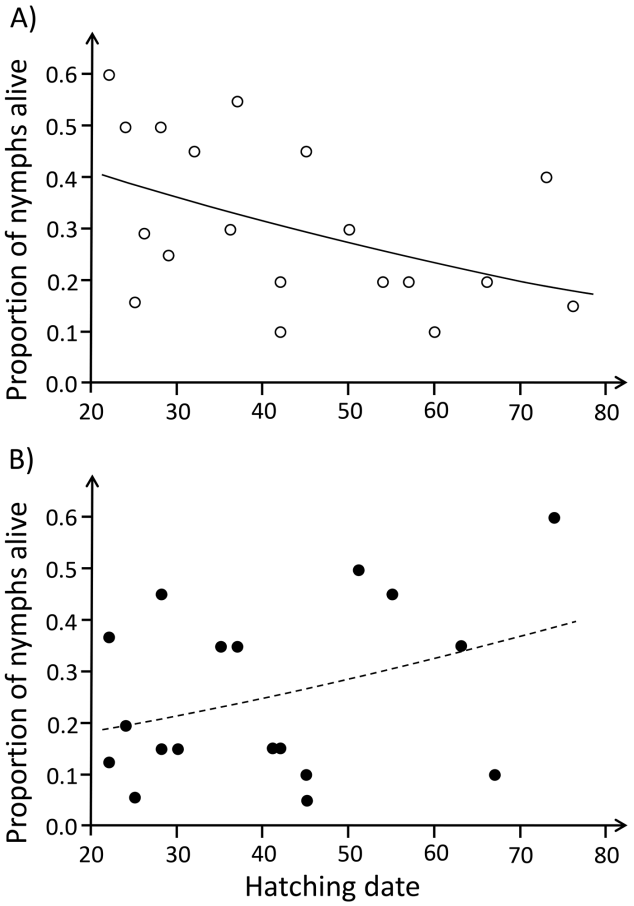


**Figure 1. Relationship between the proportion of nymphs alive and brood hatching date for the two female condition treatments.** A) low-condition treatment, B) high-condition treatment. Female condition was manipulated by varying experimentally the quantity of food to which the females had access (see Materials and Methods). On the x-axis, a julian date is provided with 6.2.2011 corresponding to day 1.
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subjects effects: compound × treatment interaction). Conversely, the total CHC quantity was not significantly affected by female treatment, but dependent on hatching date (Table 2; between-subjects effects: hatching date).

In order to correlate nymph survival patterns (Figure 1) to maternal CHC, a new variable for hatching date dependent survival was computed as the product of the standardized residuals (with respect to treatment means) of the proportion of nymphs alive and of hatching date. Positive values for this variable contribute to a positive covariance, negative values to a negative covariance between survival and hatching date. Hatching date dependent survival was significantly different between the HF and LF treatment ($t_{35} = -2.151$, $p = 0.038$; see Figure 2). To test if maternal CHC predict nymphs survival patterns, we used hatching date dependent survival as the dependent variable in a step-wise linear regression with the female condition treatment and the six compound cluster representatives as predictor variables. The final model included CHC clusters 3 and 6 (Table 1) as the sole significant linear predictor variables (positive and negative respectively; Table 3). The female condition treatment dropped from the model as its formerly significant effect was explained by these two predictors.

The quantity ratio of cluster 3 and cluster 6 CHC was affected by the maternal condition treatment ($F_{1,33} = 11.618$, $p = 0.002$; Fig. 2), but it was not significantly related to hatching date ($F_{1,33} = 0.371$, $p = 0.546$) or to an interaction between hatching date and treatment ($F_{1,33} = 0.026$, $p = 0.874$). Thus, the relative quantity of cluster 3 CHC compared to cluster 6 CHC was a cue for female condition and significantly predicted the hatching date dependent nymph survival pattern, but it was not in itself significantly related to hatching date.

## Discussion

Parents may transmit information about their condition or environmental conditions through pre-birth maternal effects, through their behavioral interactions with offspring, the provisioning of resources [2,51,52], but also through specific signals as part of a reciprocal exchange of information between parents and offspring. For example, treehopper (*Umbilia crassicornis*) nymphs signal predator threat to their tending mother through vibrational signals [53], and the mothers produce vibrational signals to reduce the likelihood of falls alarms among her nymphs [54]. In this study, we provided evidence in the European earwig *Forficula auricularia* that condition-dependent chemical cues/signals from the mother, as encoded in her CHC profile, predict offspring survival, and that the direction of this effect depended on the timing of breeding. The nymphs from both treatments had access to equal amounts of food throughout and did not differ in their weight, and cannibalism occurred in almost all cases of nymph death. Thus, the difference in survival between treatments was most likely due to variation in nymph siblicidal and cannibalistic drive, induced by cues of maternal condition.

The information transfer about female condition was not direct through a behavioral interaction from mothers to their offspring. We experimentally prevented any physical (visual, tactile, or other) contact between mothers and nymphs by keeping the mother and her nymphs in separate Petri dishes (and swapping them daily) to ensure that only chemical information about maternal condition, and not her behavior or the amount of maternal food provisioning, could mediate the observed effects on nymph siblicide and cannibalism. Thus, females must have released chemical cues in the form of non-volatile contact pheromones in the substrate, and the nymphs were exposed to these cues when subsequently placed in the same environment. Under natural conditions this indirect substrate-born signaling would occur in the breeding burrows during the period of maternal care. Female earwigs "mark" their breeding burrow with pheromone secretions (shown for the sand earwig *Labidura riparia*; [55]; pers. obs. for *F. auricularia*), to which the nymphs are then exposed while in the burrow.

The effect of maternal chemical cues of condition on nymph siblicide and cannibalism depended on the timing of breeding. Among early broods, nymphs exposed to maternal cues of poor condition showed a significantly and about two-fold higher survival rate than nymphs exposed to maternal cues of high condition. Interestingly, the effect was in the opposite direction among late broods. This effect could be either due to a quantitative or qualitative difference in the condition-dependent chemical cues among early and late breeding females or a difference in the response to the same condition-dependent cues among nymphs from early and late broods. Although our data does not allow us to fully disentangle the two possibilities, our further analyses indicate that the latter is the more likely explanation. Variation between females in CHC profiles was quantitative in nature. Early and late breeding females, and females in poor and good condition, had qualitatively the same

**Table 1.** Summary of peaks, chemical identity of maternal cuticular hydrocarbons and their statistical clustering.

| Cluster | Ret. Time | Kovats Index[2] | *Peak-ID*: Compounds | $r^2_{own\ cluster}/r^2_{next\ closest\ cluster}$/ 1- $r^2$ ratio | Prop. Var. explained[3] |
|---|---|---|---|---|---|
| 1 | 13.95 | 2098 | *CC1*: nC21 | 0.865/0.434/0.238 | 0.805 |
| | 14.40 | 2146 | *CC2*:5-MeC21 | 0.824/0.757/0.724 | |
| | 14.61 | 2168 | ***CC3*:3-MeC21** | 0.914/0.606/0.219 | |
| | 14.88 | 2198 | *CC4*: nC22 | 0.903/0.663/0.289 | |
| | 15.79 | 2275 | CC5: X,X′-nC23:2+ X′′-nC23:1 | 0.502/0.195/0.619 | |
| | 16.05 | 2298 | CC6: nC23 | 0.824/0.594/0.433 | |
| 2 | 16.59 | 2333 | *CC7*:11-, 9-, 7-MeC23 | 0.939/0.698/0.201 | 0.884 |
| | 16.77 | 2345 | *CC8*:5-MeC23 | 0.734/0.515/0.549 | |
| | 17.11 | 2368 | *CC9*:3-MeC23 | 0.941/0.757/0.241 | |
| | 17.29 | 2380 | *CC10*: X-nC24:1 | 0.865/0.451/0.246 | |
| | 17.73 | 2407 | ***CC12*: unknown HC** | 0.939/0.698/0.201 | |
| **3** | 17.56 | 2397 | *CC11*: nC24 | 0.833/0.557/0.377 | 0.845 |
| | 19.52 | 2498 | *CC14*: nC25 | 0.704/0.338/0.447 | |
| | 20.34 | 2529 | **CC15:13-, 11-, 9-MeC25** | 0.932/0.574/0.160 | |
| | 20.56 | 2537 | *CC16*:7-MeC25 | 0.860/0.573/0.329 | |
| | 21.34 | 2567 | *CC17*:3-MeC25 | 0.914/0.672/0.261 | |
| | 22.26 | 2602 | *CC18*: unknown HC | 0.779/0.667/0.663 | |
| | 23.33 | 2635 | *CC19*:13-, 11-, 9-MeC26 | 0.892/0.625/0.289 | |
| 4 | 26.30 | 2734 | *CC22*:13-, 11-, 9-MeC27 | 0.815/0.382/0.299 | 0.837 |
| | 26.51 | 2742 | *CC23*:7-MeC27 | 0.789/0.569/0.491 | |
| | 27.10 | 2764 | *CC24*:7,15-; 7,19-; 11,15-; 11,17-; 11,19-diMeC27 | 0.874/0.308/0.183 | |
| | 27.29 | 2772 | ***CC25*:2,17-; 2,19-; 2,21-; 2,23-diMeC27** | 0.871/0.269/0.176 | |
| 5 | 25.35 | 2698 | *CC21*: nC27 | 0.199/0.011/0.809 | 0.704 |
| | 28.86 | 2838 | *CC26*:13-, 11-, 9-, 7-MeC28 | 0.791/0.433/0.369 | |
| | 30.94 | 2938 | ***CC28*:11-, 9-, 7-MeC29** | 0.946/0.140/0.062 | |
| | 31.50 | 2964 | *CC29*:7,19-; 9,19-; 11,17-; 11,19-diMeC29 | 0.878/0.144/0.142 | |
| **6** | 19.04 | 2473 | *CC13*: X,X′-nC25:2+ X′′-nC25:1 | 0.572/0.324/0.633 | 0.681 |
| | 24.60 | 2675 | *CC20*: X,X′-nC27:2+ X′′-nC27:1 | 0.775/0.508/0.458 | |
| | 29.67 | 2875 | ***CC27*: X,X′-nC29:2** | 0.785/0.272/0.295 | |
| | 33.38 | 3075 | *CC30*: X-nC31:1 | 0.590/0.077/0.444 | |

The representative peak for each cluster is highlighted in bold[1]. Clusters, peaks within clusters and chemical compounds within clusters are numbered according to the order of their retention times. Clusters 3 and 6 (bold) were condition dependent and significant predictors of nymph survival patterns.

[1]The compound with strongest correlation with its own cluster compared to the next closest cluster (i.e., compounds with lowest 1-$r^2$ ratio) were chosen as cluster representatives.

[2]Index computed according to [49], and using a series of n-alkane standards (C8–C40).

[3]Variance explained by the cluster divided by the total variance among the peaks of this cluster.

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CHC profiles. The composition of the female CHC profile (in particular the quantity ratio between cluster 3 and cluster 6 CHC) varied quantitatively with female condition, but not with timing of breeding, and it explained the treatment effect on nymph survival. We cannot fully rule out that other cues not measured by CHC extraction and GC-MS analysis (e.g., peptides or proteins) may be the causal agents underlying this effect, but any such cue would have had to be correlated with maternal CHC. Thus, our results indicate that nymphs born early and nymphs born late had opposite responses to maternal substrate-born cues of condition, expressing behavioral reaction norms [56] of opposite sign. The ratio of cluster 3 CHC quantities (mostly composed of nC25 alkanes with linear and methylated pentacosane; Table 1) to cluster 6 CHC quantities (composed of a mix of monoenes and dienes of C25, C27, C29 and C31; Table 1) was lower in females of poor condition, and was associated with lower cannibalism rates among early broods *and* higher cannibalism rates among late broods (and vice versa for higher ratios). This is evidence for context-dependence of offspring responses to maternal cues/signals. If variation in hatching date has a genetic component, these results would show genotype × family environment interactions [57] with the maternal chemical cues of condition being a component of the family environment to which the nymphs are sensitive. G × E is an important factor in the maintenance of heritable variation of phenotypic traits [58,59] and in the present case would contribute to maintained variation in cannibalistic tendencies.

We previously showed that the same manipulation of female food access affected the food provisioning rate of earwig mothers, with females in poor condition providing food to fewer nymphs

**Table 2.** Effect of female nutritional condition on cuticular hydrocarbon profiles.

| **Between-subjects effects** | $F_{1,33}$ | p |
|---|---|---|
| Condition treatment | 0.254 | 0.617 |
| Hatching date | 6.568 | 0.015 |
| Condition treatment × hatching date | 0.131 | 0.720 |
| **Within-subjects interactions** | $F_{5,29}$ | p |
| Compound × condition treatment | 5.222 | 0.002 |
| Compound × hatching date | 1.643 | 0.180 |
| Compound × condition treatment × hatching date | 0.411 | 0.837 |

Results from MANOVA with the six compound cluster representatives (see Table 1) as dependent variables (i.e., within-subjects effect) and the female condition treatment and hatching date as between-subjects effects.
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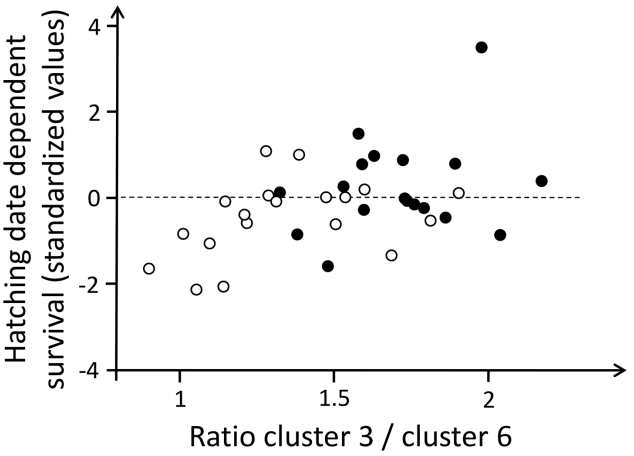


**Figure 2. Hatching date dependent nymph survival in relation to the ratio of cluster 3/cluster 6 CHC.** The hatching date dependent survival rate was computed as the product of the standardized residuals (with respect to treatment means) of the proportion of nymphs alive and of hatching date. Positive values imply lower than average survival in early hatching broods or higher than average survival in late hatching broods. Negative values imply higher than average survival in early hatching broods or lower than average survival in late hatching broods. The CHC clusters 3 and 6 were selected based on variable clustering and a step-wise linear regression (see Tables 1 & 3).
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than females in high condition [40]. Furthermore, the presence of a mother can reduce nymph survival when the mother is in poor condition and food is scarce, because mothers in poor condition compete with offspring for access to the limited available resources [41]. As a consequence, nymphs should associate poor maternal condition with low expected food provisioning by their mother, and more costly interactions with her, and they should respond to the corresponding cues of maternal condition accordingly. Based on the predictions we formulated in the introduction, the higher cannibalism rate among late broods when exposed to chemical cues/signals of poor maternal condition fits a scenario of such enhanced competition when the mother is in poor condition. Conversely, the lower cannibalism rate among early broods when exposed to cues of poor maternal condition would then suggest a differential benefit of living in larger sibships and/or of sibling cooperation when the mother is in poor condition. Recent experiments demonstrated that earwig nymphs not only compete (including siblicide) [42], but that they are also very gregarious over large parts of their juvenile development [40,60], and that they cooperate by sharing food, a behavior particularly pronounced in the absence of physical interactions with their mother [27]. Thus, there is scope for both sibling competition and cooperation in *F. auricularia*. But why should the benefits of cooperative versus competitive strategies vary with the timing of breeding? In earwigs, early broods are the first to emerge from their winter burrows and experience low densities, less cannibalism threat by other earwigs and more time for development before the next winter starts. The low density could imply that the costs of dispersing and self-foraging (to escape from a mother in poor condition with which nymphs would otherwise have to locally compete for food; [41]) may be lower for early brood nymphs. Concurrently, maintaining larger sib groups by keeping the level of siblicide low may be beneficial for self-foraging, for example because larger groups of nymphs are more efficient at foraging or provide a better protection against predators (see [61] for a review). However, further studies are required to test this hypothesis.

Our results showed that the maternal CHC profile contained reliable information about condition and was associated with time-dependent behavioral responses in offspring (i.e., cannibalistic drive) that have immediate fitness consequences in terms of survival. Thus, there is selection on this cue, and it seems likely that variation in maternal CHC profiles may have evolved to some extent due to its signaling function. We do not know if the observed variation in CHC profiles carries strategic costs (i.e., is a signal of condition) or if it rather reflects a constraint of limited food intake (i.e., is an index of condition; [13,62]). Given that CHC derive from the fat-metabolism (which necessarily partly depends on the quality and quantity of ingested food [63]), it is possible that limitation in food intake directly constrains the quantitative production of CHC influencing CHC profiles in turn. However, the female condition treatment did not affect the overall quantity of CHC, only its composition, implying that some CHC

**Table 3.** Relationship between hatching date dependent nymph survival and female CHC.

| **Final model** | **Regression coefficient (± s.e.)** | $F_{1,34}$ | p |
|---|---|---|---|
| CHC cluster 3 | 0.479 (0.165) | 8.362 | 0.007 |
| CHC cluster 6 | −0.355 (0.165) | 4.593 | 0.039 |
| **Rejected terms** | | **F** | |
| Condition treatment | – | 0.332 | 0.568 |
| CHC cluster 1 | – | 0.734 | 0.398 |
| CHC cluster 2 | – | 0.152 | 0.699 |
| CHC cluster 4 | – | 0.060 | 0.808 |
| CHC cluster 5 | – | 0.598 | 0.445 |

Results from step-wise linear regression with hatching date dependent nymph survival as dependent variable (see main text for definition) and the six compound cluster representatives (see Table 1) and the female condition treatment as dependent variables. The final model (confirmed using both forward and backward model simplification) had BIC = 112.05, and $r^2 = 0.229$ (null-model BIC = 114.43).
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decreased (cluster 3 CHC - nC25 alkanes with linear and methylated pentacosane; Table 1) but others increased (cluster 6 CHC - monoenes and dienes of C25, C27, C29 and C31; Table 1) under food restriction.

CHC are well known for their multitudes of functions in insect communication, especially their role as cues in insect (kin) recognition [64,65,66]. A comparably well studied example in the context of parental care are burying beetles (*Nicrophorus vespilloides*), where adult CHC profiles display information about breeding status (breeding versus non-breeding), and to a lesser extent also about their sex and nutritional condition [67]. Male and female parents in this biparental beetle recognize each other based on these CHC [67,68], and CHC of adults in breeding status act as a trigger of begging behavior in the larvae [69]. However, it is not known in burying beetles if larvae modulate their begging in response to condition-dependent variation in parental CHC. CHC have been invoked as signals of quality in other social contexts. For example, in black garden ants (*Lasius niger*) it was shown that ant queen CHC convey information about queen reproductive potential, and inhibits worker ovarian development and aggression [62]. While these studies previously showed that CHC can display information about various aspects of individual condition/quality, our study suggests that CHC act as maternal condition cues mediating offspring siblicide and cannibalism and, hence, their selfishness.

## Conclusions

Taken together, our results on the effect of maternal condition-dependent cues on nymph siblicide and cannibalism reported here, and the former findings in *F. auricularia* showing that earwig nymphs express condition-dependent CHC profiles that affect maternal behavior [39] and reproductive physiology [31], we provided to our knowledge the first evidence for CHC variation to be involved in a reciprocal information exchange about nutritional condition between parents and offspring in insects. The CHC exposure effects on nymph selfishness and maternal reproductive physiology both depend on the timing of breeding. Although the ultimate causes of this variation remain to be illuminated, our findings that behavioral consequences of information exchange depend on the timing of breeding suggest that adaptive responses in communication can be strongly context-dependent and include responses that are in opposite direction.

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## Author Contributions

Conceived and designed the experiments: JWYW MK. Performed the experiments: JWYW. Analyzed the data: MK CL. Wrote the paper: MK JWYW.

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**Author for correspondence:**
Joël Meunier
e-mail: meunier.joel@gmail.com

†These authors contributed equally to this study.

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# Paternal signature in kin recognition cues of a social insect: concealed in juveniles, revealed in adults

Janine W. Y. Wong[1,†], Joël Meunier[2,†], Christophe Lucas[3] and Mathias Kölliker[1]

[1]Department of Environmental Sciences, Zoology and Evolution, University of Basel, Basel, Switzerland
[2]Zoological Institute, Evolutionary Biology, Johannes Gutenberg University Mainz, Mainz, Germany
[3]Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS, University of Tours, Tours, France

Kin recognition is a key mechanism to direct social behaviours towards related individuals or avoid inbreeding depression. In insects, recognition is generally mediated by cuticular hydrocarbon (CHC) compounds, which are partly inherited from parents. However, in social insects, potential nepotistic conflicts between group members from different patrilines are predicted to select against the expression of patriline-specific signatures in CHC profiles. Whereas this key prediction in the evolution of insect signalling received empirical support in eusocial insects, it remains unclear whether it can be generalized beyond eusociality to less-derived forms of social life. Here, we addressed this issue by manipulating the number of fathers siring clutches tended by females of the European earwig, *Forficula auricularia*, analysing the CHC profiles of the resulting juvenile and adult offspring, and using discriminant analysis to estimate the information content of CHC with respect to the maternal and paternal origin of individuals. As predicted, if paternally inherited cues are concealed during family life, increases in mating number had no effect on information content of CHC profiles among earwig juveniles, but significantly decreased the one among adult offspring. We suggest that age-dependent expression of patriline-specific cues evolved to limit the risks of nepotism as family-living juveniles and favour sibling-mating avoidance as group-living adults. These results highlight the role of parental care and social life in the evolution of chemical communication and recognition cues.

## 1. Introduction

The evolution of group living selects for recognition mechanisms ensuring that cooperative and aggressive behaviours are directed towards the appropriate individuals, but also that adult group members avoid the costs of sibling-mating. In insects, information about encountered individuals is typically displayed by the chemical cues present on the waxy layer covering their cuticle: the cuticular hydrocarbons (CHCs) [1–3]. CHC profiles have been shown to reflect information about different aspects of an individual's identity, such as the species [4] or the sex [5]. Inter-individual variation in CHC profiles is common in nature and typically due to various not mutually exclusive sources. For instance, CHC profiles have been shown to change over the course of an individual's life cycle, e.g. owing to aging [6] or to changes in individual tasks within colonies of eusocial insects [7], they can be influenced by the environment, such as the nesting substrate [8,9], nutritional condition [10,11] or social interactions with conspecifics, which mediates the active or passive transfer of chemical compounds between individuals [12–14]. Finally, CHC profiles can also vary owing to genetic differences between individuals (e.g. [15,16]). A heritable component to variation in CHCs is important for long-term similarities of CHC profiles among individuals originating from the same family or colony and thus, for CHCs to represent informative and sufficiently stable cues for individual identity and kin recognition (e.g. [3,17]).

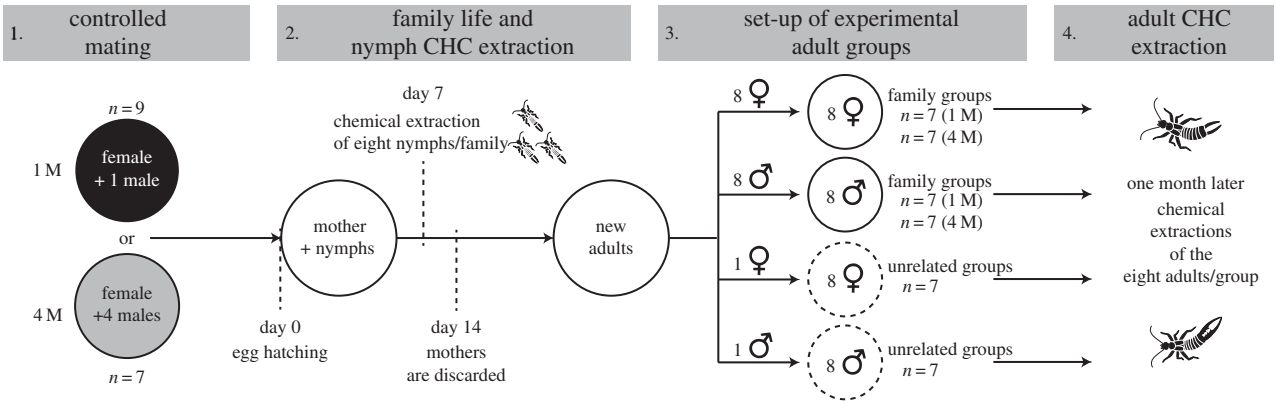


**Figure 1.** Experimental design used to extract the CHC profiles from nymph and adult earwigs.

The importance of social conflicts on the expression of parent-of-origin specific cues in offspring has been a central and often controversial point in the research on the evolution of insect communication and social life (e.g. [18,19]). In particular, it has been suggested that polyandrous colonies of eusocial insects (ants, some bees and wasps) select against the expression of patriline-specific signals in their offspring (e.g. eggs, larvae and workers), because the expression of such signals could enhance the risks of paternally driven nepotistic conflicts between colony members and thus ultimately reduce colony efficiency and the fitness of group members [18,20]. Whereas this prediction received empirical support in several Hymenoptera species (e.g. reviewed in [21]; but see [22–24]), it remains unclear whether this process is specific to the derived eusocial systems or a more widespread phenomenon involved in the early evolution of social life. Disentangling these issues therefore requires investigating the occurrence of such a mechanism in insect species expressing non-derived forms of social life, such as the ones with temporary family life and maternal care. In families with uniparental female care and where multiple paternity occurs within a female's clutch, paternally inherited genes are predicted to select for the expression of patriline-specific signals in their offspring, which would favour cooperation between their own descendants and competition against the paternally unrelated half-siblings. Conversely, sibling competition generally reduces the number and/or quality of a mother's offspring [25], so that tending females (and/or the maternally inherited genes) could benefit from limiting the expression of patriline-specific cues in the offspring. Importantly under this hypothesis, the maternal concealment of information about the paternal origin of offspring should be limited to juveniles. When offspring become reproductive adults, they could otherwise suffer from the concealment of cues that are possibly used to limit the risks of inbreeding depression. Although this age-specific expression of patriline-specific cues in offspring is of key importance to better understand the joint evolution of insect communication and social life, its occurrence remained surprisingly unexplored so far.

In this study, we investigated whether variation in CHC profiles contains information allowing kin recognition in the European earwig *Forficula auricularia* L. (Dermaptera: Forficulidae), and whether the profiles are associated with an age-specific expression of paternally inherited cues in offspring. In this species, clutches are often sired by multiple males [26,27]. The offspring (nymphs) live in family groups for several weeks, during which females provide multiple forms of care, such as egg and offspring attendance and food provisioning [27,28]. Previous work has shown that sibling competition and cannibalism are common in this species [29] and occurs significantly earlier and more often between unrelated nymphs from different clutches [29]. Thus, kin recognition cues seem to be present and used, and cannibalism is a potential form of nepotistic interactions among young nymphs. Once adult, *F. auricularia* individuals live in mixed-sex groups [27]. Inbreeding (sibling-mating) was shown to entail substantial fitness costs in this species [30], which could have thus selected for the expression of maternally and/or paternally inherited recognition cues to allow individuals avoiding mating with close relatives and thus limiting inbreeding depression.

We addressed the four following questions to test the predictions on the information content of CHC profiles in *F. auricularia*. (i) Is variation in CHC profiles smaller among individuals from the same than from different families, as expected if chemical signatures are family specific? (ii) Is within family variation larger in broods sired by multiple males compared with broods sired by a single male, as expected if the cues are heritable and display a signature of paternal origin? (iii) Is the expression of a paternal signature, i.e. higher variation in CHC profiles among offspring in multiply sired clutches, absent in offspring, but present in adults, as predicted under age-dependent concealment of paternally inherited cues? Finally, (iv) does the environment shared by adults also contribute to variation in their chemical profiles and thus possibly hamper family recognition after family disruption?

## 2. Material and methods

### (a) Experimental design

The chemical signatures of 112 nymphs and 329 adults of *F. auricularia* were extracted from 16 experimental clutches (figure 1). These clutches were second clutches of either nine females mated to a single male ('singly mated females' = 1 M-treatment) or seven females mated to four successive unrelated males (each male was used only once across all the mating trials; 'multiply mated females' = 4 M-treatment). The 16 mothers (and their mates) were from a second laboratory-born generation of individuals sampled in May 2009 in Dolcedo (Italy). They were reared under standardized laboratory conditions until each female produced her first clutch (see details in [31]). Sixteen days after their first clutch hatched, all 16 females were isolated individually in a small Petri dish (10 cm diameter) for a second clutch production. The Petri dishes were kept in a

climate chamber at 15°C, 60% humidity and complete darkness until egg laying and hatching.

One day after hatching of their second clutch (day 1), each mother and 37.6 ± 1.28 nymphs (mean ± s.e.) of her second clutch were transferred into new Petri dishes and subsequently reared at 20°C, 60% humidity and 14 L : 10 D cycle. On day seven, eight nymphs per clutch (from seven 1 M and seven 4 M clutches) were randomly sampled, singly isolated in glass-vials (300 µl) and immediately frozen at −20°C for later chemical extractions. The remaining nymphs were kept with their mothers until day 14. Then mothers were removed and all nymphs were transferred to large Petri dishes (14 cm diameter) until their adulthood [31]. Just after moulting into adults, males and females of each family were separated in two new large Petri dishes to prevent sibling-mating. Once all individuals became adults, eight males and eight females were randomly sampled in each family and set up in new large Petri dishes (called family groups) with seven 1 M groups and seven 4 M groups per sex. We used the same seven 4 M families to sample nymphs and adults. But owing to small clutch sizes, we used five 1 M families to sample both nymphs and adults, two 1 M families to sample only nymphs and two different 1 M families to sample only adults.

To test the influence of shared environment (i.e. the Petri dish) on variations in adult chemical profiles, we mixed adults from 12 experimental clutches (both 1 M and 4 M) to form seven groups of eight unrelated females and seven groups of eight unrelated males (called unrelated groups, figure 1). One month later, all adults were frozen during 2 h at −20°C, then individually transferred to a 2 ml glass vial and kept at −20°C until chemical extractions. Except when mentioned, all Petri dishes contained humid sand as a substrate, one plastic tube as shelter and received ad libitum food changed twice a week [31].

## (b) Chemical extraction

CHCs from nymph, female and male were extracted individually for 10 min using 60 µl (nymphs) or 800 µl (adults) of *n*-Heptane (Carl-Roth AG, Arlesheim, Switzerland) as solvent, and *n*-Octadecane as internal standard (concentration of 2.5 ng $\mu l^{-1}$, Fluka Analytical, Sigma-Aldrich, Buchs, Switzerland). The extracts were subsequently analysed by gas chromatography-mass spectrometry. Full description of chemical analyses are provided in the electronic supplementary material.

## (c) Statistical analyses

Chemical extraction resulted in a total of 19 peaks of CHCs in nymphs and 19 in adults. Peaks 18 (nymphs) and 25 (adults) were excluded from the analyses because peak 18 was collinear to peak 19 in nymphs (table 1; Pearson correlation, $t_{110} = 29.55$, $r = 0.94$, $p < 0.0001$) and peak 24 to peak 25 in adults (table 1; Pearson correlation; $t_{327} = 54.53$, $r = 0.95$, $p < 0.0001$), resulting in 18 peaks in adults and nymphs. The results remained unchanged when peaks 19 and 25 were excluded instead. We subsequently conducted a series of linear discriminant analyses (DA) to investigate the degree to which the chemical signature of nymphs and adults reflected their family of origin, and how this information content varied with the number of fathers that sired the clutch (table 1). The significance of each DA was evaluated both using Wilks' λ tests and prediction success (by estimating the percentage of correct assignment of individuals to their family of origin) through cross validation (leave-one-out method). The cross validation allowed us to control for potential overfitting of the data by the statistical models. We used 18 peaks for adults and 18 for nymphs, which corresponded to the recommendation in multivariate statistics like DA, that sample size should be at least three times the number of variables used [11]. To avoid limitations inherent to analyses of compositional data (as is the case for the CHC profiles), the area of each peak was transformed according to Aitchison formula [32] prior to DA (for details, see [13]). Comparable results were found when the DA were done on an estimation of the absolute quantity of each peak using a known internal standard.

We first tested the degree to which the chemical signature of nymphs and adults generally reflected their family of origin using two DA based on the chemical signatures of either all nymphs or all adults reared in family groups. We then analysed whether these DA remained significant when taking into account the mating treatment and the age (and the adult sex) of the tested individuals by conducting a series of six DA based on each combination of nymphs, males and females sampled in 1 M and 4 M groups (table 1). Finally, we tested whether the chemical signature of adults at least partly reflected their shared environment (i.e. the shared Petri dish) using two DA based on the chemical signatures of either males or females from unrelated groups.

The prediction successes obtained from the cross-validation method were compared using general linear models (GLMs) with binomial error distribution. To this end, the prediction success obtained from the cross-validation method on each DA was converted into a binomial vector (1 or 0 values) of a length equal to the number of individuals involved in the DA and wherein the proportion of 1 was equal to the prediction success obtained from the cross-validation method. The prediction successes of nymphs from 1 M and 4 M families was compared using a GLM wherein 1 M/4 M was entered as fixed factor, and the two binomial vectors reflecting the respective prediction success concatenated to form the response variable (we used the same process to generate the response variables in all the following GLMs). The prediction successes of adults from 1 M and 4 M families were then compared using a GLM wherein mating treatment, sex and their interaction were entered as fixed factors. Adult sex was included in the model to control for potential sex-specific CHC profiles in earwig adults. Finally, whether the chemical signature of adults at least partly reflects their shared environment was tested by comparing the prediction successes of adults between 1 M families and unrelated groups and the ones of adults between 4 M families and unrelated groups using two GLMs, in which the type of group (family versus unrelated), the sex of the adults and their interactions were entered as fixed factors. Adults from 1 M and 4 M groups were analysed separately because mating treatment influenced the prediction success of adults (see Results). All statistical analyses were conducted using the software R. 3.0.2 (http://www.r-project.org/).

# 3. Results

The 19 CHCs present on the cuticular extracts of individual earwigs did not only exhibit quantitative differences between life-stages (table 1), but also qualitative ones with eight CHCs specific to nymphs and eight to adults. Overall, the chemical signatures of nymphs and adults significantly predicted their family of origin (nymphs: Wilk's $\lambda < 0.0001$, approx. $f = 11.06$, $p < 0.0001$; adults: Wilk's $\lambda = 0.0001$, approx. $f = 4.70$, $p < 0.0001$). The prediction successes were 92.0% and 64.1% using cross validation for nymphs and adults, respectively. Interestingly, these predictions remained significant when taking into account the sex of the tested adults and/or the mating treatment (table 2 and figure 2, all $p < 0.0001$), with the corresponding successes ranging from 52.7 to 81.8% (figure 2).

As predicted under the age-dependent concealment of paternally inherited cues in offspring, the mating treatments did not affect variation in the nymph chemical profiles, but affected the ones in adult chemical profiles. The prediction success of nymphs was not significantly different between the mating treatments (figure 2; GLM, Likelihood ratio (LR) $\chi^2_1 = 0.44$, $p = 0.506$), but the one of adults was significantly higher in the 1

**Table 1.** Mean relative peak area (%) of the CHCs extracted from 112 nymphs, 163 females and 166 males of the European earwig. (KI, mean Kovats retention index; IS, internal standard.)

| | CHC | KI | males | females | nymphs |
|---|---|---|---|---|---|
| (1) | nC13 | 1300 | — | — | 7.24 |
| (2) | nC15 | 1500 | — | — | 1.18 |
| IS | nC18 | 1801 | — | — | — |
| (3) | nC21 | 2100 | — | — | 4.77 |
| (4) | X,X′-nC23 : 2 + X″-nC23 : 1 | 2275 | — | — | 10.68 |
| (5) | X‴-nC23 : 1 | 2281 | — | — | 2.86 |
| (6) | nC23 | 2300 | 0.14 | 0.15 | 9.33 |
| (7) | 11-, 9-MeC23 | 2334 | — | — | 1.15 |
| (8) | 5-MeC23 | 2348 | — | — | 0.68 |
| (9) | 3-MeC23 + (X-nC24 : 1) | 2370 | — | — | 1.45 |
| (10) | X,X′-nC25 : 2 + X″-nC25 : 1 | 2474 | 0.08 | 0.22 | 38.51 |
| (11) | nC25 | 2501 | 1.37 | 2.20 | 3.84 |
| (12) | 13-, 11-, 9-MeC25 | 2533 | 1.03 | 2.60 | 2.77 |
| (13) | 3-MeC25 + (X-nC26 : 1) | 2570 | 0.20 | 0.88 | 1.19 |
| (14) | 13-, 11-, 9-MeC26 | 2633 | 0.10 | 0.29 | — |
| (15) | X,X′-nC27 : 2 + X″-nC27 : 1 | 2675 | 0.44 | 0.63 | 3.36 |
| (16) | nC27 | 2701 | 1.62 | 1.58 | 0.37 |
| (17) | 13-, 11-, 9-, 7-MeC27 | 2743 | 25.91 | 35.94 | 2.99 |
| (18) | 7,15-; 7,19-; 11,15-; 11,17-; 11,19-diMeC27[a] | 2769 | 6.87 | 14.33 | 0.58 |
| (19) | 2,17-; 2,19-; 2,21-; 2,23-diMeC27 | 2776 | 4.25 | 8.30 | 0.70 |
| (20) | 13-, 11-, 9-, 7-MeC28 | 2836 | 0.94 | 1.15 | — |
| (21) | 9,15-diMeC28 | 2865 | 1.30 | 1.76 | — |
| (22) | 13-, 11-, 9-, 7-MeC29 | 2943 | 24.46 | 11.38 | 0.47 |
| (23) | 7,19-; 9,19-; 11,17-; 11,19-diMeC29 | 2966 | 22.94 | 13.22 | — |
| (24) | 15-, 13-, 11-, 9-MeC30 | 3037 | 3.69 | 2.00 | — |
| (25) | 15-, 13-, 11-, 9-MeC31[b] | 3138 | 1.58 | 0.39 | — |
| (26) | 7,19-; 9,19-; 9,21-diMeC31 | 3167 | 1.49 | 0.36 | — |
| (27) | 13-, 11-MeC33 | 3335 | 0.17 | 0.09 | — |

[a]Excluded from the DA on nymphs owing to collinearity.
[b]Excluded from the DA on adults owing to collinearity.

**Table 2.** Results of discrimination analyses of earwig individuals according to their life stage, their sex (only for adults), the type of rearing group (family or unrelated) and the mating treatment (1 M or 4 M). (The table indicates the number of families used (*N* fam) and the total number of individuals (*N* ind) per type of experimental group.)

| life stage | rearing group | mating treatment | *N* fam | *N* ind | $\lambda$ | approx. *f*-value | *p*-value |
|---|---|---|---|---|---|---|---|
| nymphs | family | 1 M | 7 | 56 | <0.0001 | 11.05 | <0.0001 |
| | family | 4 M | 7 | 56 | <0.0001 | 15.2 | <0.0001 |
| males | family | 1 M | 7 | 55 | 0.0002 | 5.69 | <0.0001 |
| | family | 4 M | 7 | 56 | 0.0003 | 4.87 | <0.0001 |
| | unrelated | — | 7 | 55 | 0.0095 | 1.97 | <0.0001 |
| females | family | 1 M | 7 | 54 | 0.0009 | 3.67 | <0.0001 |
| | family | 4 M | 7 | 55 | 0.0033 | 2.67 | <0.0001 |
| | unrelated | — | 7 | 54 | 0.0083 | 1.98 | <0.0001 |

M compared with the 4 M groups (figure 2; GLM, LR $\chi^2_1 = 5.55$, $p = 0.018$). The prediction success of adults was also significantly higher among males than females (figure 2; GLM, LR $\chi^2_1 = 5.13$, $p = 0.024$), but not significantly influenced by an interaction between the mating treatments and the sexes of the adult individuals (GLM, LR $\chi^2_1 = 0.49$, $p = 0.485$).

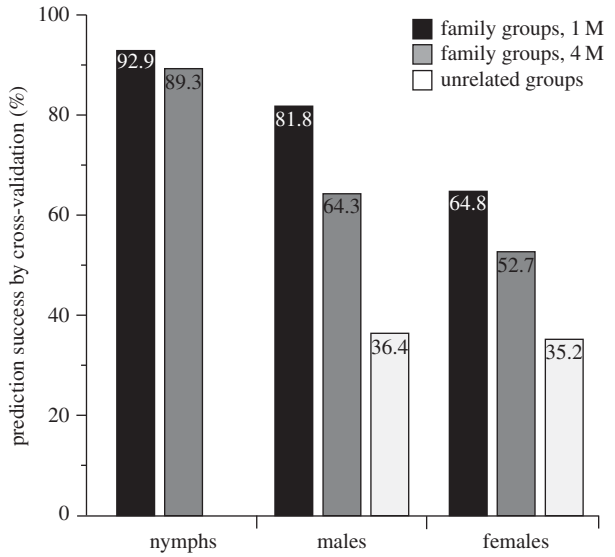


**Figure 2.** Prediction success by jack-knife cross validation of earwig individuals according to their life stage, their sexes (only for adults), the type of rearing groups (family or unrelated) and the mating treatments (1 M or 4 M). The corresponding values are given at the top of each bar.

Independently from the mating treatments, our results also showed that the chemical signature of adults partly reflected the environment in which they had been reared. The DA performed on the groups of unrelated adults significantly separated each sex according to their experimental groups (table 2 and figure 2), with 36.4% of males and 35.2% of females correctly assigned to their experimental groups by cross-validation method. Nevertheless, adult chemical profiles reflected more their clutch of origin than their groups/environments, as the prediction successes were lower among unrelated groups than 1 M family groups (GLM, group type: LR $\chi^2_1 = 18.19$, $p < 0.0001$; sex: LR $\chi^2_1 = 2.16$, $p = 0.142$; interaction: LR $\chi^2_1 = 1.34$, $p = 0.247$) or 4 M family groups (GLM, group type: LR $\chi^2_1 = 18.54$, $p < 0.0001$; sex: LR $\chi^2_1 = 1.77$, $p = 0.183$; interaction: LR $\chi^2_1 = 0.91$, $p = 0.341$).

## 4. Discussion

In social insect systems where progeny are sired by different males, potential conflicts between patrilines emerge and may select against the expression of patriline-specific signatures in the CHC profiles of offspring. These conflicts are thought to constrain information content in the cues displayed by each group member and to limit the scope for nepotism between progeny of the same sire [18,19]. While previous research focused on eusocial systems (e.g. reviewed in [21]; but see [22–24]), we showed here that such constraints can also be found in an insect species with simpler forms of social life (maternal care and family life). In particular, our results demonstrated that mate number did not influence the inter-individual diversity of CHC profiles expressed among young earwig offspring, whereas it increased such diversity in the resulting groups of adult offspring. In other words, there was no significant information content on mate number among young nymphs, but this information was expressed among adult males and females. Our study also demonstrated that even if the CHC profiles of nymphs, adult males and adult females contained a heritable component that could mediate the recognition patterns formerly reported in this species in terms of cannibalism and food sharing [29,33], they also reflect to a lower extent the environment and social group experienced by the individuals.

Our results supported the prediction that family life and multiple mating should favour the concealment of paternally inherited cues only in the young offspring (i.e. during family life). We did not find evidence for a paternal signature in the form of increased variability in the CHC profiles of nymphs from multiply sired clutches but found such an increase among adult offspring. Because earwig males in our experiment never encountered the eggs or the offspring they sired [27], any paternal signature in offspring CHC profiles would reflect paternally inherited variation at least partly, irrespective of the proximate mechanisms underlying the expression of heritable variation in CHC profiles. Proximately, the inherited variation can be expressed due to, for example, genetic variation in the fat metabolism, the preference for certain micro-environments or food intake behaviour, which in turn may affect CHC profiles. Different potential expression pathways may affect the temporal stability of the expressed heritable variation, but it does not change the ultimate effect that heritable information about maternal and/or paternal origin is displayed.

One hypothesis to explain the observed lack of paternally inherited cues in the chemical profiles of the nymphs is that their expression is developmentally constrained at this stage. For instance, nymphs might only be able to express immature profiles, because the paternal-cue-coding part of their genotype can only be fully expressed after a certain maturation time. In line with this hypothesis, it was shown in several dipterous insects (*Cucilidae*, *Muscidae* and *Drosophilidae*) that the CHC profile does not remain constant throughout their life [3]. In the ant *Cataglyphis niger*, the amounts of hydrocarbons in the postpharyngeal gland increased with maturation, especially in the first 7 days after emergence [34]. An alternative hypothesis is that mothers conceal information about their offspring's paternal origin by transferring CHCs to the eggs during oogenesis, as reported in the German cockroach *Blatella germanica* [35], or continuously to the nymphs during the period of maternal care. In the European earwig, the continuous transfer of CHCs to the eggs [36] and the frequent maternal grooming of nymphs [37] could allow females to progressively shape nymph CHCs by applying hydrocarbons. Ultimately, maternal concealment of paternal signatures in the nymph CHC profiles may either reflect a side effect of maternal behaviour (e.g. body contact, grooming and food provisioning), or an evolved maternal strategy to limit nepotistic/antagonistic sibling interactions among the different patrilines inside her brood. Further research on the mechanism and adaptive function of the found patterns is needed. But consistent with the hypothesis that selection on mothers favoured concealment of paternally inherited signatures on her offspring, previous experiments showed discrimination in cannibalism among nymphs from different clutches (i.e. with different mothers) [29], but lack of effect of multiple mating on cannibalism rate within clutches [38].

Our results further demonstrated that the CHC profiles of *F. auricularia* adults not only have a heritable component, but also partly reflected the shared environment and social group. In particular, experimental groups of unrelated adults were successfully assigned to their new group (although at a significantly lower success rate compared with the family

groups). It was shown before that multiple abiotic factors can influence the chemical profiles of individuals, such as temperature [39], nesting substrate [8,40] or diet [10]. As we kept abiotic factors constant between our groups, we consider them an unlikely influence on group-specific profiles. Hence, the most likely explanation for the reported result is that social interactions passively (e.g. body contacts) and/or actively (e.g. allogrooming) mediated the transfer of chemical compounds between adults and thus contributed to the partial homogenization of odours within groups, a common process in colonies of eusocial insects [41,42]. In *F. auricularia*, old nymphs and adults are known to aggregate densely for foraging, resting and mating [43,44], as well as to express allogrooming [45], which both offer scope for social transfer of chemical compounds. Social transfer of recognition cues within groups may be beneficial for instance because it can facilitate the expression of group-directed forms of social behaviour [46].

A somewhat surprising result from our analyses was that the family-specificity of CHC profiles was higher among male than female adult family groups, suggesting a sex-difference in the expressed heritable variation in CHCs or an enhanced CHC exchange between males as compared with females, e.g. owing to higher levels of allogrooming and close physical contacts [12,41,47]. Because we found no difference in the group-specificity of CHC of unrelated males versus unrelated females (figure 2), the sex difference in the expressed CHC variation is more likely owing to intrinsic differences between the sexes, that is, a difference in the expression of heritable variation as was found in *Drosophila simulans* [48].

The discovered patterns of expressed CHC variation in *F. auricularia* are consistent with a scenario where mothers conceal any paternal signature in their offspring's chemical profiles to minimize antagonistic interactions among patrilines inside her brood, and where later in life information about both maternal and paternal origin are expressed potentially to avoid costs of sibling-mating in adults. However, the mechanisms of how paternal signatures in kin recognition cues of juveniles are concealed, and their adaptive function, require further investigation. Overall, our results provide insight into the role of parental care and social life in the evolution of chemical communication and recognition cues.

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Data accessibility. Data are deposited in the Dryad repository (doi:10.5061/dryad.73180).

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Dédié à PP/M/L/B/A/C/F/P/S et aux futurs…l'éternité, c'est long, surtout vers la fin (WA).