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Conflict and cooperation between ants and ant-associated arthropods

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biology

Laboratory of Socioecology
and Social Evolution

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SUMMARY

An intimate association between two species is known as a symbiosis. A symbiotic relationship where both partners benefit is defined as a mutualism. This is in contrast with parasitism, where one partner benefits at the expense of another partner. Commensalism is a third type of symbiotic association and is characterized by one partner that takes advantage of the association and the other is unaffected. These different types of symbioses are widespread in social insects. The largest diversity of social insect symbionts evolved in the group of myrmecophiles, which are arthropods that live inside or in close vicinity of ant nests. Up till now, studies on myrmecophiles focus typically on the association between a host and a single symbiont. In this thesis, ant nests were considered as microcosms with multiple myrmecophiles living together in the same ant nest. We were interested in the biotic and abiotic interactions that affect the symbiotic community as a whole, rather than focusing on single species interactions. In addition, our current knowledge on the symbiosis of arthropods with ants is typically based on very specialized species. Here, we focus on relatively unspecialized myrmecophiles, which probably outnumber the group of specialized species. Our main model system was the myrmecophile microcosm in the nests of red wood ants (*Formica rufa* group). Our second model system was an association of co-inhabiting (= parabiosis) African ant species that also houses a community of myrmecophile species.

In **chapter 1,** we reviewed the diverse group of myrmecophiles that can be found in association with red wood ants (RWAs). In total, 125 species were listed and the biology of the different arthropod groups were discussed. This chapter serves as an introduction to the later chapters.

The main part of this thesis deals with biotic interactions in ant nests, but we examined first in **chapter 2** the effect of abiotic interactions on myrmecophile communities. In accordance with metapopulation theory, myrmecophile species richness per unit volume was negatively correlated with increasing nest mound isolation. We did not find support that the abiotic variables mound moisture, pH, mound size and site size affect the myrmecophile community or its species richness.

To understand the dynamics of a community in nature, it is essential to have an idea of its trophic interactions. In **chapter 4**, a surprising complex food web was found In the RWA microcosm, with most myrmecophiles parasitizing on ant brood and ant prey. In addition, multiple trophic predator-prey links among the myrmecophiles were encountered. The results of the stable isotope analyses complemented the findings of

direct feeding tests and indicated the existence of multiple trophic levels. The presence of large numbers of parasitic myrmecophiles can impose large costs on RWAs. However, RWAs could indirectly benefit from the intraguild predation of brood parasitic myrmecophiles. In **chapter 3,** another mechanism that might counter the high parasitic pressure was shown. In general, large foragers in insect colonies specialize in nest defending against large, extranidal threats. However, we demonstrated that small nurses in RWA colonies are the most aggressive and most efficient defenders of brood against small, intranidal, parasitic myrmecophiles. This group of workers is best suited to defend the brood against these parasites due to their better matching size, high encounter rate in the nest and the high task switching costs that would occur if foragers had to carry out this task.

The RWA myrmecophiles showed a different level of integration, with some species preferring the dense brood chambers, whereas others avoided the brood chambers and lived at the edge of the nest (**chapter 5**). This level of integration was not correlated with the aggression they provoked in their RWA host or their brood predation tendency. Up till now, it is believed that social insect symbionts mainly employ chemical deception by either mimicking the host's cuticular chemical profile (mimicry or camouflage) or being chemical insignificant to sneak into social insect nests. However, in the community of RWA myrmecophiles tested in **chapter 6,** most species had distinct chemical profiles. Some of them carried low concentrations of compounds, but a significant part showed no disguise at all. These results shed new light on the evolution of integration mechanisms in social insect symbionts, and stresses that unspecialized species can integrate in social insect nests by using simple strategies such as hiding, swift movements or emitting defensive chemicals rather than using chemical deception.

Many RWA myrmecophiles are persecuted, but in **chapter 7** we reported that survival of three obligate myrmecophilous beetles over a period of 20 days was not different from a control set-up without ants. However, reduced survival was detected for a facultative myrmecophile in presence of RWAs. Survival analyses in the presence of 9 different ant species showed that the three beetles survived better in presence of larger-bodied ant species, and was highest in presence of its preferred host *F. rufa*, which also has relatively large workers. These results suggests, that in spite of their unspecialized nature, these beetles are optimally adapted to their preferred host and the importance of size asymmetries in host-symbiont interactions.

Finally, the interactions in the parabiotic nests of the large *Platythyrea conradti* and tiny *Strumigenys maynei* ants were described in **chapter 8**. The chemical profile of the

parabiotic partners was very different. The parabiotic association tended to be mutualistic as *P. conradti* benefits from the supreme defence capabilities of *S. maynei* against alien ants and intranidal myrmecophiles. The latter, in turn, benefits from the prey that thrive in the organic material carried to the nest by *P. conradti*.

SAMENVATTING

Een hechte associatie tussen twee soorten is gekend als een symbiose. Een symbiose waar beide partners voordeel uit halen is een mutualisme. Dit is in tegenstelling met parasitisme, waar één partner profiteert van een andere partner. Het derde type van symbiose is commensalisme, waarbij één partner voordeel haalt uit de associatie, maar geen effecten heeft op de andere. Deze drie types zijn wijdverspreid in symbionten van sociale insecten. De grootste diversiteit van deze symbionten vindt men in de groep van de myrmecofielen. Dit zijn symbiotische arthropoden (geleedpotigen) die in of in de nabijheid van mierennesten leven. Myrmecofielen werden tot nu toe vooral apart onderzocht. In deze thesis echter, wordt een mierennest beschouwd als een microkosmos waar gastheer en verschillende myrmecofielen interageren. We zijn geïnteresseerd in de biotische en abiotische interacties die de myrmecofiele gemeenschap in zijn geheel beïnvloeden. Onze huidige kennis is daarnaast voornamelijk gebaseerd op studies met erg gespecialiseerde myrmecofielen. Hier echter, richten we ons op relatief ongespecialiseerde soorten, die waarschijnlijk veel abundanter zijn dan de gespecialiseerde myrmecofielen. Ons belangrijkste modelsysteem is de myrmecofiele gemeenschap die leeft in rode bosmier (*Formica rufa* groep) nesten. Het tweede modelsysteem is de parabiotische (= vredevol samenlevend) associatie van twee Afrikaanse mieren waarmee ook een myrmecofiele gemeenschap was geassocieerd.

We beginnen deze thesis met het samenvatten van de diverse groep van myrmecofielen geassocieerd met rode bosmieren (**hoofdstuk 1)**. We lijsten in totaal 125 geassocieerde soorten op, en bespreken de levenswijze van de verschillende groepen arthropoden. Dit hoofdstuk dient als inleiding op het experimentele deel van de thesis.

Deze thesis handelt grotendeels over biotische interacties in mierennesten, maar eerst onderzoeken we in **hoofdstuk 2** het effect van abiotische op myrmecofiele gemeenschappen. We vinden in overeenstemming met de metapopulatie theorie, dat de soortenrijkdom van myrmecofielen afnam met toenemende isolatie van de bosmierkoepels. We vinden geen effecten van abiotische variabelen op de myrmecofiele gemeenschap soortenrijkdom.

Om de dynamieken van een gemeenschap te begrijpen, is het essentieel om een idee te hebben van de voedselwebrelaties tussen de soorten van die gemeenschap. In **hoofdstuk 4** tonen we aan dat er een complex voedselweb aanwezig is in rode bosmiernesten. De meeste myrmecofielen parasiteren op het broed van de mieren en

aten mee van prooien die de mieren verzamelen. Daarnaast zijn er verschillende trofische interacties tussen de myrmecofielen onderling. Een analyse van de rode bosmiergemeenschap met stabiele isotopen bevestigt het bestaan van meerdere trofische niveaus. Het grote aantal parasieten in bosmiernesten kan een serieuze kost zijn voor de mieren. Anderzijds kunnen deze indirect voordeel halen van het feit dat de parasieten ook andere parasieten opeten. We bespreken in **hoofdstuk 3** een ander mechanisme dat de hoge druk van de parasieten voor de mieren kan verlichten. Bij sociale insecten zijn typisch de grote werksters die buiten foerageren, gespecialiseerd in de verdediging van het nest tegen grote bedreigingen. Hier tonen we echter aan dat kleine werksters die broed verzorgen agressiever waren naar kleine myrmecofielen toe en efficiënter zijn in broedbescherming. Deze groep is beter geschikt om het broed in het nest te beschermen tegen parasieten door hun overeenkomende grootte, hogere kans om de parasieten te ontmoeten en de hoge kosten die gepaard gaan met het wisselen van taak die zouden plaatsvinden als foeragerende werksters deze taak zouden moeten uitvoeren.

Myrmecofielen vertonen een verschillende graad van integratie in bosmiernesten. Deze integratiegraad is niet gelinkt met de agressie die ze opwekten of met hun neiging om broed te eten (**hoofdstuk 5**).

Er wordt tot nu toe algemeen aangenomen dat symbionten van sociale insecten hun gastheer chemisch moeten misleiden door de gastheergeur te imiteren of door lage geurconcentraties te dragen, om te kunnen overleven in hun nesten. De meeste soorten van de myrmecofiele gemeenschap bootsen echter de geur van hun gastheer niet na (**hoofdstuk 6**). Sommigen hebben wel lage concentraties van geurmoleculen, maar het afwijkend chemisch profiel van een significant deel wordt duidelijk herkend door de gastheer. Deze resultaten tonen aan dat ongespecialiseerde symbionten ook kunnen overleven in nesten van hun gastheer door middel van simpele strategieën zoals vluchten en het afweren van de gastheer met chemische stoffen.

In **hoofdstuk 7** vinden we dat de overleving van drie obligate, myrmecofiele kevers niet wordt beïnvloed werd door de aanwezigheid van hun agressieve gastheer. De overleving van een facultatieve gast is wel lager in aanwezigheid van bosmieren. Overleving van de drie kevers bij 9 mierensoorten stijgt met toenemende grootte van de mierensoorten en is het grootste bij de relatief grote bosmieren.

In **hoofdstuk 8** onderzoeken we de interacties in het parabiotische nest van de grote mierensoort *Platythyrea conradti* en de kleine mierensoort *Strumigenys maynei*. De geur van de parabiotische partners is erg verschillend. De parabiotische associatie heeft mutualistische kenmerken, want nestverdediging tegen myrmecofielen en andere mieren wordt vooral uitgevoerd door de agressieve *S. maynei*. Deze kan dan weer profiteren van de prooien die leven in het nestmateriaal aangebracht door *P. conradti*.

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INTRODUCTION

SYMBIOSIS

Symbiosis is the permanent or long-term association between two different species. These associations are very common in nature and can be found in and across all major groups of life (Lewis 1985, Paracer and Ahmadjian 2000). In general, three types of symbiosis can be distinguished depending on the costs and benefits involved for the different partners. In mutualisms, both partners benefit from the association. Parasitism is defined as a symbiosis where one partner benefits at the expense of the other partner. Commensalism is a symbiosis where one partner takes advantage of the association, without harming or benefiting the other partner (Lewis 1985, Paracer and Ahmadjian 2000). Symbiosis plays a tremendous role in the origin of novelties and speciation (Lewis 1985, Smith and Szathmáry 1995). Numerous organisms acquired new capabilities and could exploit novel niches through mutualistic partners (Paracer and Ahmadjian 2000). For example, complete communities of invertebrates can establish around deep sea vents with the help of chemosynthetic bacteria that fix CO_2 in the absence of sun light (Luther et al. 2001). Symbiosis might also be a driver of co-evolution when the symbiotic partners affect reciprocally each other's evolution (Paracer and Ahmadjian 2000). A well-known example of mutualistic co-evolution are the reciprocal adaptations in plants and their pollinators. The co-evolution between host and parasite can lead to an evolutionary arms race when host and parasite are constantly co-evolving in an aggressive way (Dawkins and Krebs 1979). Selection will favour parasites that are highly virulent but at the same time it will favour hosts that are more efficient in excluding the parasites (Paracer and Ahmadjian 2000). The boundaries between the three types of symbioses are not clear-cut. Therefore the three types should be conceptualized as a continuum with mutualism and parasitism as extremes rather than as distinct categories (Johnson et al. 1997). The exact position that symbionts take along this continuum is often hard to determine. Moreover the nature of symbioses is heavily affected by the ecological and environmental context (Daskin and Alford 2012). This is clear in the amphibian disease chytridiomycosis, which is caused by a parasitic fungus. The susceptibility of amphibians to this disease is strongly associated with environmental temperature. The highest amphibian losses occur at high elevations and in cooler seasons, because the parasite has a relatively cool optimal temperature window. Moreover the interaction between amphibian and pathogen is also affected by the prevalence of mutualistic bacteria on the skin of the amphibians that secrete metabolites active against the parasite (Daskin and Alford 2012). Symbioses also differ in the specificity of the association. Symbionts might be specific to one organism, but others can associate with many, often related, organisms.

In general, symbionts that are highly specialized show higher specificity to their host (Paracer and Ahmadjian 2000).

A special case of symbiosis can be found in some small, natural and delineated micro-ecosystems or microcosms. Here, we can find complete ecosystems inhabited by multiple symbionts that are structured and supported by one host. This host provides shelter, food and habitat to multiple symbionts, which are known as inquilines (= lodger, tenant) (Srivastava et al. 2004). Typical examples of these inquiline ecosystems are aquatic communities in phytotelmata or small water habitats formed naturally by a plant (e.g. pitcher plants, tree holes and bromeliads), micro-arthropods in moss patches and fauna associated with insect-induced galls (Sanver and Hawkins 2000, Kitching 2001, Srivastava et al. 2004). These symbiont communities can be conceptualized as metacommunities (sensu Hanski and Gilpin (1991) as symbionts live in spatially distinct and stable patches, susceptible to colonization surrounded by a landscape matrix unsuitable for colonization. The systems have the advantage of small size, small number of symbionts, contained structure and hierarchical and spatial arrangement (Srivastava et al. 2004). Moreover there is a strong interaction between symbionts and host and among the symbionts themselves. Consequently, these microcosm systems have been elaborately used as model systems for testing fundamental questions in ecology studying for example spatial ecology dynamics and food web interactions. In this thesis, we use as a model system the symbiont communities associated with social insects. Social insect nests often support small delineated communities of symbionts and can be regarded as inquiline microcosms as well.

SOCIAL INSECT SYMBIONTS

Organisms ranging from microorganisms such as bacteria, fungi, protozoa and nematodes to macro-organisms such as plants, arthropods and birds, established symbiotic relationships with social insects (Kistner 1982, Hölldobler and Wilson 1990, Schmid-Hempel 2011). The three types of symbiosis are widespread in social insects and especially in ants extremely diverse (Fig. I.1). Again, the nature of the symbiont lies along a continuum between these types of symbiosis and it can move towards another type of symbiosis depending on the abiotic and biotic context (Fig. I.1, Nash and Boomsma 2008, Kronauer and Pierce 2011). From all the social insect symbiont groups, the largest variety of strategies can be found in the group of arthropods. These symbiotic arthropods benefit from the stable conditions, the different food sources and protection against predators (Hölldobler and Wilson 1990, Kronauer and Pierce 2011). In the course of evolution, two different groups of arthropods succeeded to integrate into social insect nests: other social insects and non-social arthropods. The terminology used for the different host-symbiont associations is often ambiguous and several overlapping categories have been proposed (Hölldobler and Wilson 1990, Buschinger 2009). Moreover the nature of the interaction is for most symbionts poorly known, which further complicates the attribution of a symbiont to a single category.

Symbioses among different social insect species

Some social insects are completely dependent on other social insects. Wasmann distinguished mixed nests from compound nests depending on the relationship between symbiont and host (Wasmann 1891). In mixed nests, the brood of both social insect species is nursed in the same chamber and there is intense interaction between the host and symbiont species. In compound nests, however, brood of host and symbiont is kept separated in different chambers and the symbiont raises its own brood (Buschinger 2009).

Compound nests

These relationships are uniquely found among ant species and encompass commensalistic, parasitic and mutualistic interactions. Symbionts are not related with their host and might clearly differ in morphology. The most rudimentary association is known as plesiobiosis and refers to species that simply nest close to each other, but do not interact. When disturbing the nest, brood theft and fighting may occur between the two ant species (Hölldobler and Wilson 1990). Some small ant species build nest chambers adjacent to the nest of larger ant species. They feed on refuse or rob workers that carry food (cleptobiosis) or prey on brood (lestobiosis) of the larger species (Buschinger 2009). Xenobiosis denotes a more advanced form of parasitic association where the symbiont freely moves among its host in the nest without being attacked and regularly obtains food usually by trophallaxis (Hölldobler and Wilson 1990). The best known example is *Formicoxenus nitidulus* which is a tiny ant that lives associated with red wood ants (RWAs) (Donisthorpe 1927). They construct inside RWA mounds little nests separated from the brood chambers of the host and care for their own brood. They often beg for food from their much larger host or steal a food droplet that two host workers share during trophallaxis. Finally, some tropical ant species co-inhabit the same nest and do not exploit the other partner. It is even reported that both ant partners might benefit from the presence of the other species by interspecific trail following, food sharing and nest defence of the other partner (Vantaux et al. 2007, Menzel and

Blüthgen 2010). This apparent mutualistic association is known as parabiosis. It is mainly found in ants that live together in so-called ant gardens (Orivel and Leroy 2010) which are microhabitats formed by arboreal ants that collect seeds of epiphytic plants and let them germinate in their carton nests.

Mixed nests

The symbionts in these nests are obligate parasites and are often referred to as social parasites (Buschinger 2009). Temporary social parasites only exploit the host colony during the founding phase of the parasitic colony. The queen of these parasites invades a host colony where she replaces the host queen. Her eggs and larvae are nursed by workers of the host. A parasitic workforce develops and gradually replaces the host workforce which is no longer replenished. Temporary social parasitism occurs in several groups of ants and is well known in RWAs. Independent colony founding occurs in this group by queens that take over colonies of the *Formica* subgenus *Serviformica* (Seifert 2007). Permanent social parasites fulfil their entire life cycle inside the colony of a closely related species (Emery’s rule). The parasite shares many morphological features with its host, but lost features related to nesting and foraging. This permanent parasitism (also confusingly referred to as inquilinism) is usually characterized by the loss of the worker caste. After usurping the host colony, host workers raise directly sexuals of the parasite. These advanced parasites can be found in bumblebees (*Bombus* subgenus *Psithyrus*), ants (e.g. *Anergates*, *Teleutomyrmex*), wasps (e.g. *Vespula austriaca*) and possibly in termites (*Termes insitivus*) (Schmid-Hempel 1998).

Non-social arthropods

A remarkable legion of arthropods switched in a large number of groups from a free-living state to a strict association with social insects (Kistner 1982, Hölldobler and Wilson 1990, Rettenmeyer et al. 2010). Thousands of species, representing at least 17 orders, 120 families and hundreds of genera formed a strict relationship with social insects (Wilson 1971). There are rough estimates that more than 10000 arthropods are living with ants, which approximates the total number of ant species described (Hölldobler and Wilson 1990). Depending on the targeted host, these arthropods are called myrmecophiles (host: ants), termitophiles (host: termites), melittophiles (host: social bees) or sphecophiles (host: social wasps). Particularly mites (Acari) and rove beetles (Staphylinidae) are preadapted to a life in association with social insects and are the dominant groups among social insect symbionts (Wilson 1971). Most of the symbionts live permanently within the nest of their host and are hence true (non-social) inquilines. However, some arthropods that live outside the nest, are also strictly associated with a social insect host. For example, the ladybird *Coccinella magnifica* is only found on plants near RWAs, as it preys on aphid colonies tended by this ant (Sloggett et al. 1998). In analogy to island biogeography, the largest diversity of arthropod symbionts can be found in species with large colonies, at least in ants, whereas small colonies often have no associates. These colonies provide a larger variety of niches and are often stable, long-living entities (Kronauer and Pierce 2011).

The study of non-social arthropod symbionts was initiated by Erich Wasmann who categorized species in five groups depending on the interaction with their host (Wasmann 1894):

1. <u>Synechtrans - Persecuted guests</u>: These species provoke host aggression and mainly live as scavengers or predators. They can survive in the nest by rapid and swift movements, hiding, retracting beneath a hard exoskeleton and by secreting chemicals that repel their host.
2. <u>Synoeketes - Indifferently tolerated guests</u>: These arthropods are mostly ignored by their host, because they are either very small, too swift, or are apparently neutral in odour. They also live as scavengers or predators inside the nest.
3. <u>Symphiles- True guests</u>: This group comprises the most advanced guests. They are treated by their host as real members of the colony. They species are groomed, carried around and fed by their host.
4. <u>Ectoparasites and endoparasites</u>: These are typical parasites that penetrate or live on the body of their host. It comprises flies, wasps and nematodes whose behaviour is not different from similar parasites targeting non-social hosts.
5. <u>Trophobionts</u>: This group encompasses homopterans and some caterpillars that provide honeydew to their host and in return get protection.

This classification is sometimes ambiguous as some species fall in two or more categories. For example RWA workers treat the rove beetle *Dinarda maerkelii* as a symphile by giving it food during trophallaxis, but these beetles also scavenge and elicit aggression so that they can also be classified as synoeketes. A simpler classification (Kistner 1979) was proposed by David Kistner, a world-leading expert in termitophiles. He distinguished integrated species from non-integrated species. Integrated species are incorporated in the host's social life. This category largely overlaps with the symphiles of Wasmann. Non-integrated species are not incorporated in the social life of the colony, but are adapted to the nest as an ecological niche. This category approximately combines the synechtrans and synoeketes of Wasmann. It is clear that these symbionts greatly differ in strategies and mode of specialization. In contrast with social parasites that are highly specific to one host, non-social arthropods vary in their host specificity. Presumably, the higher the specificity, the more specialized the symbiont is. The myrmecophilous springtail *Cyphoderus albinus* can only be found in ant nests, but shows no preference for a particular ant species (Donisthorpe 1927). In contrast, some specialized symbionts such as the caterpillar of *Maculinae rebeli* are strictly associated with a single host (Akino and Knapp 1999).

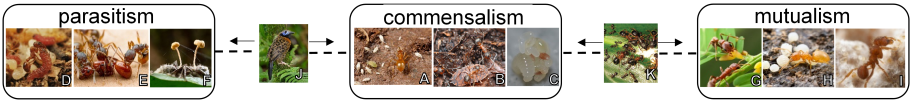

Figure I.1. **Types of symbiotic relationships that ant-associated organisms establish with their ant host. Note that these types can also be found in other social insects.**

Commensalism: A. *Cyphoderus albinus* is an obligate, myrmecophilous springtail that feeds on detritus. B. The common isopod *Porcellio scaber* can be abundant in RWA nests where it feeds on organic nest material. C. The mite *Hypoaspis oophila* lives on egg piles of *Formica* ants. It does not pierce the eggs, but licks the secretions of the egg scales.

Parasitism: D. The caterpillar of the myrmecophile *Maculinea alcon* begs for food. This is a highly specialized myrmecophile (= symphile) that is nursed by the *Myrmica* host as its own brood. E. The queen of the social parasitic ant *Aphaenogaster tennesseensis* has usurped a nest of her ant host species *Aphaenogaster rudis*, whose workers unknowingly tend her and begin raising her eggs as their own. As the host queen was killed, this colony will gradually turn into a full nest of *A. tennesseensis*. F. Some fungi are specialized ant parasites, such as *Ophiocordyceps* that has infested and killed a *Camponotus* worker in this figure.

Mutualism: G. Many plant species known as myrmecophytes form mutualistic relationships with ants, in which they provide food bodies, nectar and/or a nesting space in special structures and get in return protection against herbivores. Here, *Pseudomyrmex* workers feed on special food bodies provided by *Acacia*. H. A well-known mutualism is the association between honeydew providing insects (aphids, some caterpillars, scale bugs) and ants. The most intimate association has developed between some root aphids and *Lasius* species. The aphids are treated as cattle and transported to the best feeding locations. I. Leaf-cutter ants are dependent on a fungus for food. The fungus is cultivated on freshly cut leaves brought into the nest by the ants.

Gradations: J. Ant-birds follow raids of army ants and feed on the fleeing insects. However, when the density of the birds is high, the foraging success of the ants will be significantly reduced. The commensalistic by-product relation then shifts towards cleptoparasitism (Wrege et al. 2005). K. Species of the genus *Camponotus* and *Crematogaster* can form parabiotic associations. It was reported that both ant partners benefit from this associations (Menzel and Blüthgen 2010). However, in another study region it was revealed that only one partner takes advantage (Menzel et al. 2014). The other partner gained no benefits and was even exploited to some extent (Menzel et al. 2014).

Photo Courtesy: A: A. Murray, B-C: T. Parmentier, D: Darlyne Murawski, E-F-G-H-I: Alex Wild, J: Bob Gress, K: Florian Menzel

INTEGRATION STRATEGIES OF SOCIAL INSECT SYMBIONTS

In the course of adapting to a life with social insects, symbionts have undergone evolution in different traits. This is most apparent in specialized symbionts who are one of the most spectacular examples of co-evolution to their host (Nash and Boomsma 2008). Surprisingly, different arthropod groups often evolved independently the same traits to promote colony integration. Here we list some of these convergent general traits that can be found in large number of social insect symbionts:

1. Chemical cuticular deception

Social insects possess a complex system of chemical communication which allows the discrimination of nestmates from non-nestmates. In particular, each colony carries a unique blend of chemical cues on their cuticle that forms the “colony odour”. Workers constantly antennate workers that they encounter and compare the perceived odour with the template of the colony. When the odours do not match, the worker will reject or aggress the alien worker rather than behaving altruistically (Lenoir et al. 2001a). In ants, wasps and termites, linear hydrocarbons, i.e. components consisting entirely of C and H atoms, mainly serve as cuticular recognitions cues, whereas in bees fatty acids and wax esters are also important for nestmate recognition (van Zweden and d’Ettorre 2010). The majority of studies on ant nestmate recognition cues report hydrocarbons with chain-lengths between C_{19} and C_{33}. However, heavier hydrocarbons are probably much more frequent, but are underestimated because of the limitations of most GC columns (Martin and Drijfhout 2009). Both social parasites and non-social arthropods are known to crack the host recognition system by mimicking the colony odour. They can actively (chemical mimicry) biosynthesize the compounds to obtain odour congruency or passively acquire (chemical camouflage) the compounds from their host by direct contact with their host (e.g. via allogrooming, trophallaxis, rubbing). In addition, symbionts can be chemically insignificant or “odourless”, by bearing low concentrations of nest recognition cues (Nash and Boomsma 2008, van Zweden and d’Ettorre 2010). Symbionts often combine these deception strategies: caterpillar larvae of *Maculinea rebeli* biosynthesize some recognition cues prior to nest penetration and later acquire passively some additional hydrocarbons from their host, the cleptoparasite *Mutilla europaea* carries lower concentrations of recognition cues prior to nest invasion of the targeted *Polistes* wasp, but matches its hydrocarbon profile after sneaking into the colony (Uboni et al. 2012). Chemical mimicry and camouflage are reported in most non-social arthropod symbionts of which the profile was identified yet (Appendix 6-2: Table A-6.3). A few symbionts can associate with their host with a different chemical profile without provoking aggression. This strategy can be found in some parabiotic symbionts and social parasites (Liu et al. 2000, 2003, Menzel et al. 2008a). It is hypothesized that the host habituates to the alien profile through a learning process (Menzel et al. 2008a).

2. Glands

Many of the specialized symbionts (symphiles) release some attracting or appeasement compounds from special epidermal glands. The symbionts are regularly licked by the host workers. Often they also have yellow brush-like structures, called trichomes, which help in the dissemination of the appeasement substances (Fig. I.2.A and 1.2.C). Non-integrated species release rather repellent substances to deter their host. These glands are already present in free-living relatives (Steidle and Dettner 1993).

3. Morphology

Morphological regression or the reduction or loss of body parts is a typical phenomenon that can be observed. Many species have degenerated mouth parts, shortening or loss of wings and reduction or loss of eyes. Symbionts often have a limuloid (tear-drop) body form with expanded pronota and elytra covering appendages (Wilson 1971). Other typical body forms are flat disks (e.g. *Platyarthrus hoffmannseggii*) and armoured tanks (Histeridae). It is believed that these body forms protects the symbionts from host attacks (Fig. I.2).

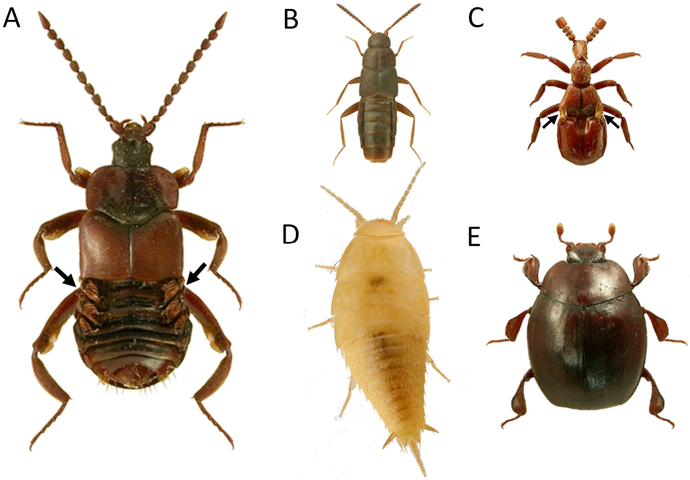

Figure I.2. Morphological variation of some Belgian myrmecophiles. A. *Lomechusoides strumosus* (Staphylinidae): a specialized parasitic myrmecophile that deceives its host *Formica sanguinea* with glandular secretions. Yellow tufts that promote the spread of secretions are indicated by the arrows. B. *Lyprocorrhe anceps* (Staphylinidae): many myrmecophiles have a relative unspecialized morphology that is very similar to free-living relatives. C. *Claviger testaceus* (Pselaphinae): a specialized parasitic myrmecophile with yellow gland tufts and peculiar antennae. D. *Atelura formicaria* (Thysanura): we discovered this species recently in Belgium for the first time (Parmentier et al. 2013). It is a very agile insect with a typical teardrop shape. This shape can also be found in myrmecophilous rove beetles. Another typical myrmecophilous trait is the absence of eyes. E. *Dendrophilus pygmaeus* (Histeridae): This family of beetles is pre-adapted to a live with social insects by its hard and protective exoskeleton. They can retract their appendages in special grooves when aggressed. Photo courtesy: L.Borowiec, D: T. Parmentier.

4. Behaviour

Well-integrated species are treated as true colony members, are intensively nursed and groomed and can be transported by host workers (phoresy). This is often mediated by special appendages or modified antennae of the symbiont that the host workers can grab. Non-integrated species avoid their host by rapid and swift movements, feign death and hide in small crevices inside the nest (Hölldobler and Wilson 1990). A large body of evidence showed that symbionts can follow pheromone trails outside the nest (Akre and Rettenmeyer 1968, Akino 2002). This helps the symbiont to follow colonies that are moving or to locate new colonies. A diverse group of symbionts sollicits for regurgitation of liquid food droplets or directly steals food from two workers in trophallaxis (Hölldobler and Wilson 1990) (Fig. I.3).

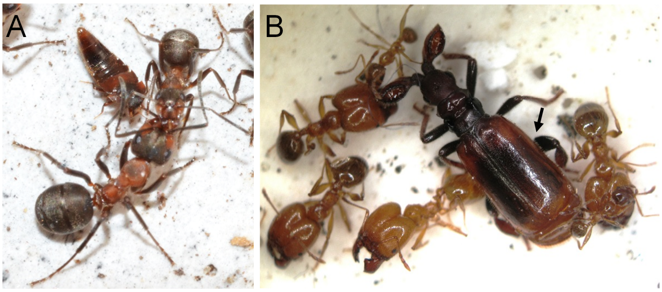

Figure I.3. A. The rove beetle *Dinarda maerkelii* is about to steal a food droplet shared by two unsuspecting *Formica rufa* workers in trophallaxis. B. *Pausus favieri* is one of the most specialized myrmecophiles in Europe and is associated with the smaller ant *Pheidole pallidula*. It mimics the stridulations of the host queen. The arrow indicates the stridulatory organ. They have very peculiar antennae that secrete appeasing compounds. Photo: A-B: T. Parmentier.

5. Sound

In general, chemical cues are considered as the main communication cues in social insects (Hölldobler and Wilson 1990). However some ants communicate by stridulating or drumming the substrate. Recently it was demonstrated that the parasitic beetle *Paussus* mimicks the stridulations of the host queen (Fig. I.3.B) (Di Giulio et al. 2015) and *Maculinea* caterpillars the sound of the host queen larvae (Barbero et al. 2009a), resulting in a royal treatment by the workers.

MODEL SYSTEMS

Red wood ants

Red wood ants (RWAs), often designated as the *Formica rufa* group, are a group of six (*F. rufa*, *F. polyctena*, *F. pratensis*, *F. lugubris*, *F. paralugubris* and *F. aquilonia*) related ant species belonging to the subgenus *Formica* s. str (Goropashnaya et al.

2004). They are distributed across forests, woodlands and heathland across Eurasia (Seifert 2007). The above ground part of the nest are conspicuous mounds made from organic material, twigs and needles (Fig. cover page chapter 1). In spring, the inner part of these mounds starts to heat up to temperatures about 10-20 °C higher than ambient conditions by a combination of efficient solar collection, endogenous metabolic heat production of the ants and microbial decomposition (Rosengren et al. 1987). These high temperatures, which are maintained until the end of autumn, promote rapid brood development throughout a large part of the year in cold and temperate climates and are likely key in the ecological success of RWAs (Rosengren et al. 1987, Gösswald 1989a). RWAs are relatively large ants and the workers differ in size, ranging from 4.5 mm to 9 mm. In spite of their morphological and genetic similarities, RWA species tend to differ in ecological organization (Goropashnaya et al. 2004, Seifert 2007). For example, colonies of *F. polyctena* typically contain multiple queens (polygynous) and their nests consist of a large network of interconnected mounds (polydomous). Colonies of *Formica rufa* and *F. pratensis* are rather headed by a single queen (monogynous) and their nest is limited to a single independent mound (monodomous) (Seifert 2007). In polygynous species, colonies multiply by a group of workers and queens of the mother colony that found a new colony in the neighbourhood (cf. swarming in honey bees). In monogynous species, queens disperse by flight and establish new colonies by temporally parasitizing ants of the *Formica* subgenus *Serviformica*. There are also differences in habitat preference among RWAs (Seifert 2007). *Formica polyctena* prefers to nest in inner forests, whereas *F. rufa* is mainly found along forest edges and *F. pratensis* is distributed in more xerothermic habitats such as heathland (Seifert 2007). In Belgium and Northern France, three species of RWAs can be found: *F. rufa*, *F. polyctena* and *F. pratensis*. We sampled ten sites in Flanders and one in Northern France. Surprisingly, the differences in ecological organization and habitat preference between the RWA species pinpointed above, are less clear-cut in our study sites. Most nests are found along forest edges and all species construct highly polygynous networks of nest mounds, which can be explained by a lack of independent colonization opportunities due to severe habitat fragmentation and a shortage or absence of *Serviformica*'s (Loones et al. 2008). RWA numbers are declining because of habitat destruction and fragmentation, agriculture, commercial collection of pupae for bird food and lack of appropriate habitat management (Dekoninck et al. 2010). All six species of the *F. rufa* group are listed on the IUCN red list and are protected in many European countries. The three RWA species (*F. rufa*, *F. polyctena* and *F. pratensis*) that occur in Flanders are protected under an act of 2009: “het Soortenbesluit”. They gain a lot of attention because of their multi-facetted key-stone role in forest ecosystems (Gösswald 1989a). They are dominant ants affecting the distribution of other ant species and arthropods. They are predators of most arthropods, affect the dynamics of food webs and can control pest insects (Skinner 1980, Gösswald 1989a, Hawes et al. 2002). They can have a positive effect on tree and shrub growth by preying on herbivores. However, they can have a negative effect by tending aphids whose honeydew is the main food source of RWAs (Gösswald 1989b). By collecting huge amounts of organic material, prey and honeydew, they strongly affect chemical, physical and biological properties of the soil and create patchy hotspots for mineralization (Lenoir et al. 2001b, Frouz and Jilková 2008). Wood ants also play an important role as seed dispersers (Gorb and Gorb 1999) and are a predictable food source for a diverse group of species such as woodpeckers and bears in winter (Gösswald 1989b). Finally RWAs support a large group of of strictly and facultatively associated myrmecophiles around or inside their nests (Donisthorpe 1927, Robinson and Robinson 2013), which capitalize on the ideal, thermoregulated nest conditions and the constant supply of food and organic material (Kronauer and Pierce 2011). Diverse aspects of RWA ecology such as their interaction with prey and aphids, social organization, kin recognition, task distribution and response on habitat deterioration have been intensively studied. Surprisingly few studies examined the associated myrmecophiles. At the start of this thesis, there were a few faunistic studies that merely listed RWA myrmecophiles found in particular regions (e.g. Lapeva-Gjonova and Lieff 2012, Robinson and Robinson 2013), but studies exploring the interaction with their host were completely lacking.

Parabiotic microcosm

The ants *Platythyrea conradti* and *Strumigenys maynei* are distributed throughout tropical Africa (Bolton 2000). They normally live not in association with each other, but in the nature reserve of Lamto, Ivory Coast, both species were mosty found together in hollow tree nests (Yéo et al. 2006). Possibly, both species are also associated in other localities, but this requires a careful inspection of the nests. The brood of these species is kept separated in the compound nests. The ant partners behave friendly and have apparently no negative impact on each other (Yéo et al. 2006). Hence this association can be classified as parabiosis. The parabiotic relationship between these ants is remarkable, because of the extreme size differences between the ant partners. *Strumigenys maynei* is a tiny ant (2.5 mm) from the Myrmicinae subfamily, whereas *P. conradti* is a large Ponerine ant (15 mm). Interestingly, these compound nests also house a diverse community of myrmecophiles, including Collembola, Staphylinidae, Pselaphinae, Scydmaeninae, Pseudoscorpiones, Acari, Araneae and Thysanura. Their abundance and diversity might be explained by the organic material that is carried to the nest by *P. conradti* and is found throughout the nest and seals the large nest opening. In analogy to the organic material in RWA mounds, this material might provide shelter, food and help to maintain homeostatic conditions in the nest. These parabiotic nests hence support similar microcosms as RWA nests. However, because of two host ants, we are able to test whether the hosts interact differently with the myrmecophile community.

AIMS AND OUTLINE OF THIS THESIS

During the last 30-40 years, considerable progress has been made in the study of social insect symbionts and the interaction with their host. However, studies typically explored social insect-symbiont interactions by focusing on the interaction between a host and a single symbiont. In this thesis, we tried to investigate ant-symbiont relationships from a community perspective, i.e. exploring the interactions between a host and multiple symbionts, but also among the symbionts themselves. By scaling-up to a community approach, different strategies of symbionts associated with the same host can be compared and linked with other parameters. Moreover, studies were hitherto severely biased towards integrated symbionts, although non-integrated species probably outnumber this very specialized group. Symbionts of RWAs are not very specialized and not well-integrated in the colony. Therefore the RWA model system gave us a unique opportunity to test whether the mechanisms postulated in integrated species also apply for less specialized species. The parabiotic microcosm model system also sheds new light on our understanding of social insect symbionts as two levels of symbiotic interactions, i.e. myrmecophile-host interactions and host-host interactions, are jointly examined for the first time.

This thesis integrates spatial ecology, ethology, trophic ecology and chemical ecology in an evolutionary framework. In particular, the interactions between myrmecophiles and (i) the environment, (ii) the host and non-hosts and (iii) other myrmecophiles were examined. These interactions are schematically summarized in Fig. I.4 and Fig. I.5. We start with **Chapter 1**, where all known arthropods associated with RWAs are reviewed. **Chapter 2** is a classic ecological and hence a rather stand-alone chapter in which we explore the underlying abiotic processes that drive RWA distribution and diversity. In **Chapter 3,** we describe that particular RWA worker groups are specialized in deterring parasitic symbionts. **Chapters 4, 5 and 6** study the interactions between RWAs and the symbionts from a community perspective. They focus respectively on trophic interactions in RWA mounds, the association between level of nest integration, brood predation tendency and aggression elicited and chemical integration mechanisms. In **Chapter 7**, survival of obligate and facultative symbionts was tested in a test set-up with their aggressive RWA host and non-preferred ant hosts. **Chapter 8** deals with the parabiotic model system. We report a putative mutualistic relationship between the two ant species and test chemical integration on two levels: (1) *Platythyrea* vs. *Strumigenys*, (2) myrmecophiles vs. these 2 co-inhabiting ant species. The thesis ends with a discussion, which summarizes the novel findings of this thesis and discuss these in the framework of host-symbiont interactions. I also present some interesting research avenues, which can further stimulate the study of social insect-symbiont interactions.

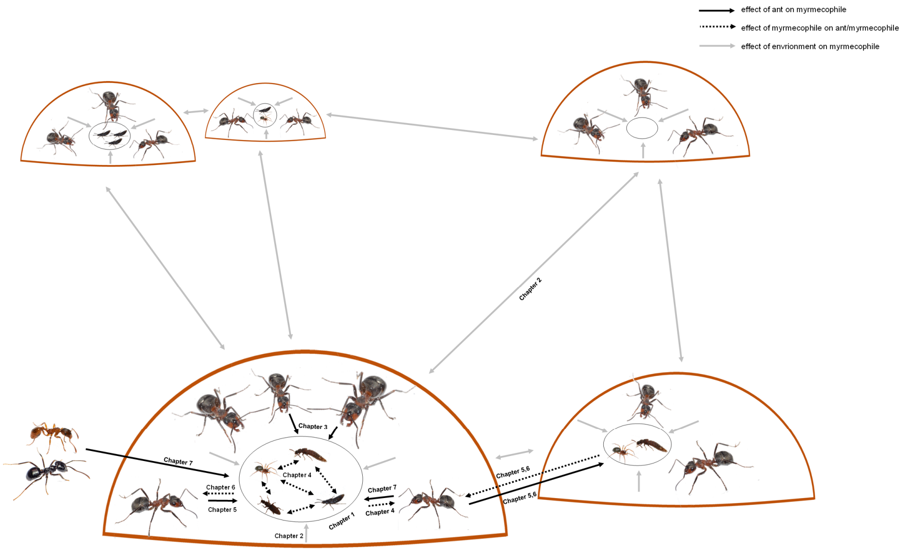

Figure I.4. Overview of the tested interactions in model system 1: Red wood ants. Myrmecophiles are depicted in the black ovals, non-host ant species are represented by figures of *Lasius fuliginosus* (black ant) and *Myrmica ruginodis* (orange-red ant). Photo: T. Parmentier.

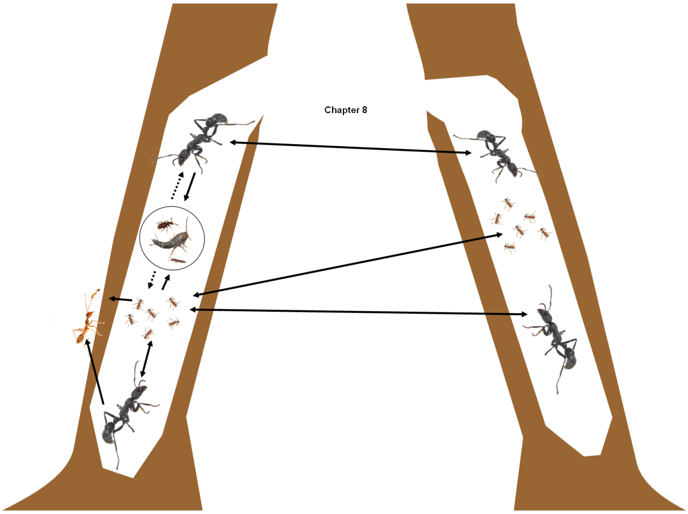

Figure I.5. Overview of interactions tested in model system 2: Parabiotic microcosm. Two different parabiotic nests in hollow trees are depicted. The large black ant is *P. conradti*, the small orange-brown ants *S. maynei*. Myrmecophiles are depicted in the black circle. Alien ant species are represented by two ant species: *Oecophylla longinoda* and *Monomorium pharaonis*. Photo courtesy: T. Parmentier, except for picture of *O. longinoda* and *M. pharaonis* that were adapted from pictures of A. Wild.

CHAPTER 1

A highly diverse microcosm in a hostile world: a review on the associates of red wood ants (*Formica rufa* group)

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ABSTRACT

The importance of Eurasian red wood ants (RWAs) (*Formica rufa* group) in forest and heath ecosystems has long been recognized. One key function of RWAs is the role of their nests in supporting an intriguing ecosystem of a highly diverse group of obligate myrmecophiles and facultative guests. In this review we list 125 obligate arthropod myrmecophiles that occur in RWA mounds or in the near vicinity of the mounds. About half of them are Coleoptera, but also Hemiptera, Diptera, Hymenoptera, Acari and Aranea are well represented. RWAs are estimated to be the primary host for 49 species. 24 species were hitherto only recorded to be associated with RWAs, 12 with both RWAs and other mound building *Formica* species and 9 were found to be associated with both mound building and non-mound building *Formica* species. The remaining associates are less specific and can be found with other ant genera or ant subfamilies. Other mound-building *Formica* ants (*Coptoformica, F. uralensis* and *F*. *truncorum*) support fewer species, most of which are known to also occur with RWAs. We discuss the biology of the different obligate myrmecophilous groups and give general notes on the facultative guests found in RWA mounds. We stress the importance of the conservation of RWAs as hosts of one of the richest and diverse associations known to date in insects.

INTRODUCTION

Eurasian red wood ants (RWAs) (*Formica rufa* group belonging to the subgenus *Formica* s.str.) are represented by six narrowly related and morphologically similar species: *F. rufa*, *F. polyctena*, *F. pratensis*, *F. aquilonia*, *F. lugubris* and *F. paralugubris* (Goropashnaya et al. 2004, Seifert 2007). The mounds of these well-studied species are impressive markers in temperate and boreal forests and heath land across Eurasia. Their key roles have been appreciated since long: they are top predators that have a potential to control outbreaks of pest insects, they create nutrient heterogeneity in forests by concentrating food and organic material in their mounds and structure biotic and abiotic components of forests outside their nests (Gosswald 1989, Frouz 2000, Frouz et al. 2005, Domisch et al. 2008, Wardle et al. 2011). In addition, the presence of RWAs is vital for a large number of associated species living in the mounds or in their vicinity. The unique aspect of these species is that they evolved mechanisms to overcome the aggression of their hosts and benefit from the resources and ideal nest conditions provided by their ant hosts. Since the 19th century naturalists began to focus on the diversity and biology of RWA myrmecophiles. In the last decades, more and more elements of their hidden lifestyle have been revealed and the list of associated species has been growing longer and longer.

The striking diversity of RWA myrmecophiles can mainly be explained by the nest structure of RWAs. Their huge mounds provide stable and long-lasting habitats with controlled temperature and moisture (Rosengren et al. 1987, Frouz and Finer 2007). The mounds are also heterogeneous in temperature, moisture and material (organic thatch material, inorganic soil, central stem) which create a large variety of microhabitats (Coenen-Stass et al. 1980, Rosengren et al. 1987). Furthermore, there is a constant supply of food and organic material which can sustain different trophic groups such as parasites, predators, scavengers, detritivores and mycophages (Skinner 1980). Additionally, some species are attracted by the ant-tended aphid colonies that are typically present in the vicinity of the mounds.

Here we did an exhaustive literature survey on RWA arthropod myrmecophiles. Literature search started from general reviews or studies on myrmecophiles. Then we scanned all groups for more specific published studies on RWA myrmecophiles. We aim to highlight the diversity of arthropods associated with Eurasian RWAs and discuss their biology, distribution and host ant preference. We also give some notes on facultative associates which depend on RWAs and stress the need for RWA conservation and its associated myrmecophile community.

RWA MYRMECOPHILES: OVERVIEW

In our survey, we found reports of 125 arthropod myrmecophiles that have been found in association with RWAs (Table A-1.1 in Appendix 1-1 chapter 1). Most of these live in the nests and are called inquilines. Additionally, some species live in the neighbourhood of the nest or are parasites. Most species occur in nests of several ant hosts, whether or not belonging to different genera. Taxonomic information of the listed host ant species can be found in Table A-1.2 in Appendix 1-1 chapter 1. Most studies focused on myrmecophilous beetles. This sampling bias could contribute to their proportional high diversity. Other groups such as mites, flies and wasps are expected to have much more representatives, but studies on their diversity are hampered by less search effort, taxonomic problems (e.g. cryptic species) and poorly known distribution. The latter makes it hard to judge whether a species is strictly associated with ants or also occurs outside ant nests.

For many myrmecophiles, little is known about the biology or the actual type of interaction with the host ant, i.e. whether it is parasitic, commensalistic or mutualistic. What is known about their biology, however, is reviewed below according to the taxonomic group to which they belong.

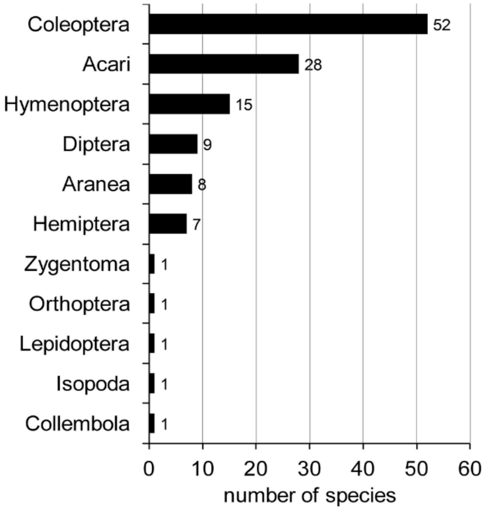

Figure 1.1. Taxonomic distribution of myrmecophiles associated with RWAs.

Coleoptera

More than 40 % RWA myrmecophiles belong to this highly diverse group (Fig. 1.1). Rove beetles and particularly the subfamily Aleocharinae, dominate the list of beetles. Traditionally, taxonomy, distribution and behaviour of the Coleoptera were best studied (Donisthorpe 1927, Janet 1897, Wassmann 1894, Hölldobler and Wilson 1990). Myrmecophilous beetles range from highly integrated guests that are treated as nestmates (licking, feeding), to poorly integrated species that are heavily aggressed by the ants. *Lomechusa* (former *Atemeles*) and *Lomechusoides* (former *Lomechusa*) are text-book examples of highly integrated species. *Lomechusa pubicollis* adults emerge in a RWA nest in autumn and overwinter in a *Myrmica* nest. After hibernation they seek again adoption in a RWA nest to breed. *Lomechusoides* adults, in contrast, integrate in a nest of the same host ant species (Hölldobler and Wilson 1990). Larvae and adults of *Lomechusa* and *Lomechusoides* have special glands that produce highly attractive substances. They live among the brood and are fed, licked and carried by the workers. They also feed on the brood of their host (Hölldobler 1967, Hölldobler 1970). *Hetaerius ferrugineus* is a histerid beetle which is also highly integrated. It is a scavenger, but also solicits for liquid food and occasionally consumes ant brood. It is suggested that adoption is promoted by special trichome glands opening at the margin of the thorax. In case of an attack, it feigns death by oppressing its legs against its body (Hölldobler and Wilson 1990). The rove beetle *Dinarda* is less integrated, but also steals regurgitated food from their ant host (Fig. 1.2.A and Fig. I.3.A in Introduction). When discovered, the beetle raises its abdomen and offers appeasement substances. If ant hostility continues, they still can rely on repellent secretions from the tergal gland (Hölldobler and Wilson 1990). “Poorly” integrated rove beetles avert aggression by swift movements and/or by emitting repellent substances from their tergal gland (Hölldobler and Wilson 1990). Some of them, such as *Quedius brevis* and *Zyras humeralis*, are mostly found in the winter when ant aggression is lowest (unpub. data) (Fig. 1.2.A). Many beetles are hardly noticed by the ants due to their small size and slow movement (e.g. *Monotoma*, Ptiliidae, Scydmaenidae, Pselaphinae) (Fig. 1.2.A). Scydmaenidae, like Staphylinidae and Ptiliidae, are predisposed to a life in ant nests composed of decaying material (RWA, *Lasius brunneus, Lasius fuliginous*) by their preference for moist forest soils and rotten logs (Freude et al. 1974). O’Keefe (2000) mentions no less than 31 Scydmaenidae species associated with RWAs. Most of them, however, can regularly be found in absence of ants in leaf litter, rotten logs and are rather facultative associates than obligate myrmecophiles (Freude et al. 1974, Tykarski 2013). Here we limit Table A-1.1 to Scydmaenidae that are classified as myrmecophiles according to Freude et al. (1974) and Tykarski (2013). Some associated beetles live (partly) outside the mound. Adults and larvae of the ladybird *Coccinella magnifica* are typically found on the vegetation and on the trails near RWA mounds (Fig. 1.2.C). Both feed on the aphid colonies tended by the ants. It was suggested that the adults can follow the trails of RWAs (Godeau 2008). The behaviour of the ladybird is only slightly modified compared with its non-myrmecophilous congener *C. septempunctata*. They overcome ant aggression by running away and ducking down and possibly employ chemical adaptation (Sloggett et al. 1998). *Clytra* are remarkable leaf beetles with red elytra and black patches. Adults live on the trees and herbs near the nest and feed on plant leaves (Fig. 1.2.D). The female drops the eggs near the nest and covers them with her excreta. The covered egg is very similar to plant material and is as a result sometimes carried by the ants and incorporated in the nest (Donisthorpe 1927). The emerged larva permanently lives in the nest and builds a protective case of its own excreta and earth. It mainly feeds on organic nest material and detritus (Fig. 1.2.A). Full grown larvae attach to the central stem or debris and pupate in their larval case (pers. obs. TP). *Protaetia metallica* (sometimes considered as a subspecies of *Protaetia cuprea*: *Protaetia cuprea metallica,* but see Renneson et al. 2012) has a similar alternating life cycle, with free-living adults and larvae confined to the mounds (Donisthorpe 1927, Renneson et al. 2012) (Fig. 1.2.B). The larvae, however, are not protected by a case, but resist attack by their though skin and by boring deeper into the nest (Donisthorpe 1927).

The highly integrated beetle species have special glandular adaptations to a myrmecophilous lifestyle. Adaptations of other beetles are less pronounced. They are morphologically very similar to nonmyrmecophilous relatives. The slender and small size of most beetles protects them of attacks. Still, the antennae of some rove beetles associates (*Thiasophila*, *Notothecta, Dinarda*) are relatively compact to better withstand ant attacks (Freude et al. 1974) (Fig. 1.2.A). Many myrmecophiles are known to mimic the cuticular chemical profile (chemical mimicry) of their ant host or have adaptations to remain undetected (chemical camouflage) (Akino 2008). These strategies have hitherto not been demonstrated in RWA myrmecophilous beetles or in other RWA myrmecophilous groups (but see chapter 6).

Diptera

Syrphid flies of the genus *Microdon* are the best studied myrmecophilous Diptera. Three species with a broad host range are known to be associated with RWAs. Adult flies look like typical flies, whereas the larvae have a unique slug like appearance and locomotion. Young larvae are typically found deep in the nest and feed on detritus and ant brood. Larvae of *M. mutabilis* are ignored or if attacked by an ant worker, they seem unattractive. Nearctic *Microdon* larvae seem to be more integrated as they engage in chemical cuticular mimicry and are transported and licked by their hosts (Howard et al. 1990a, b). Older *Microdon* larvae migrate to the periphery of the nest where they pupate. The adults only live a few days and hover and mate in the near proximity of the nest where they emerged. *Microdon* populations are typically localized while potential hosts are widespread. Elmes et al. (1999) demonstrated that the survival of the eggs of *M. mutabilis* in *Formica lemani* declined dramatically when introduced in conspecific colonies situated only a few hundred meters away. The flies display extreme local adaptation not to one species, but to a local population of ants. Infiltration of *M. mutabilis* in the host ant nest doesn't involve chemical mimicry as demonstrated in Nearctic *Microdon* species (Hovestadt et al. 2012). It is probably mediated by a mimetic chemical coating on the egg scale (Elmes et al. 1999).

Information on the other Diptera is scarce. The adults of *Phyllomyza formicae* and *Forcipomyia myrmecophila* hover over the nests of mound-building *Formica'*s and the larvae live in the nests, probably as scavengers. *Holoplagia transversalis* can be seen running on the trails and nest of its primary host *Lasius fuliginosus*, but it can also be collected with RWAs (Donisthorpe 1927).

Hemiptera

In this order, we find inquilines that live in the RWA nests and species outside the nest and on trees in company with foraging ants. Species living outside the nest are mainly ant mimics gaining protection against their enemies by their resemblance to ants. They typically prey upon aphids or other insects, but also consume plant saps and honeydew (Wachmann et al. 2007). *Pilophorus cinnamopterus* and *Pilophorus perplexus* are rapid ant-like bugs with transverse silvery bands on the wings formed by pale hairs (Donisthorpe 1927, Wachmann et al. 2007). *Myrmecoris gracilis* is a better mimic, with nymphs resembling dark *Lasius* workers and adults *Formica* workers (Wachmann et al. 2007). In constrast to *Pilophorus*, they have a petiolar constriction. The behaviour and appearance of the early stages of *Alydus calcaratus* are also very ant-like. It occurs in heath land often in association with *F. rufa*, but also with other *Lasius* and *Formica* species (Fig. 1.2.E). *Xylocoris formicetorum* and *Notochilus limbatus* are two bugs occurring in the nests of mound-building *Formica'*s. Both species are not ant-like and little is known about their life-history (Donisthorpe 1927, Wachmann et al. 2007). They seem weakly integrated in the nests and probably hunt for mites and other mound associates (Wachmann et al. 2007). *Eremocoris abietis*, which is also not ant-like, can be found outside the nest and in the mounds where it most likely lives as a scavenger (Wachmann et al. 2007). Wasmann (1894) and Donisthorpe (1927) also report an association of *Himacerus mirmicoides, Megacoelum beckeri, Philomyrmex insignis* and *Myrmedobia exilis* with RWAs, but it is unlikely that this represents an obligate association (Wachmann et al. 2007).

Hymenoptera

The best known representative of this order is the inquiline ant *Formicoxenus nitidulus* which lives in the nests of mound-building *Formica*'s. Interestingly, the males are wingless and mating occurs on the mound surface. *Formicoxenus* is a small genus of social parasites with a xenobiotic lifestyle, i.e. they nest in the mound of RWAs, move freely among the hosts and obtain food from them, but their brood is kept separated (Hölldobler and Wilson 1990). *F. nitidulus* is less associated with their hosts than the highly specialized congeneric species *F. quebecensis* and *F. provancheri* who are associated with a single *Myrmica* host and constantly lick their host to acquire cuticular hydrocarbons. In contrast, *F. nitidulus* have 11 hosts (Martin et al. 2007) and do not interact with their host. They are largely ignored and when seized, dropped immediately because of an unattractive cuticular odour. They use a generalist chemical deterrent strategy which can applied to several hosts, as opposed to chemical mimicry directed to one host species (Martin et al. 2007). *Solenopsis fugax* is another ant which can be found in RWA mounds, but also in nests of many other species. This tiny ant gets access to food and brood of their host by small galleries which are too narrow for their host (Janet 1897, Donisthorpe 1927).Several wasps belonging to different families have been found hovering above RWA mounds. *Trichopria fuliginosa* and *Conostigmus formiceti* even live in ant mounds seemingly unharassed. For most species, little is known about their biology. They probably parasitize on the ant workers in or outside the nest, ant brood or other arthropods found in the nests. The oviposition behaviour of some ant parasitoids was recently recorded in detail (Gómez Durán and van Achterberg 2011). *Elasmosoma*, *Kollasmosoma* and *Neoneurus* hover patiently over ant nests, then swiftly strike at an ant worker and finally oviposit with a hook-shaped ovipositor in the ant's gaster. They parasitize mostly *Formica* and it has been hypothesized that formic acid secreted by those ants could be a powerful attractant (Gómez Durán and van Achterberg 2011). *Hybrizon buccatus* was observed while hovering over a *Lasius grandis* trail. Here, no oviposition was found on adult ants. Surprisingly, the wasp grasped a larva being transported on the trail and inserted an egg. The grasping of the ant or larva by the legs and the insertion of the ovipositor are species-specific for the ant parasitoids.

Lepidoptera

From this order, only the moth *Myrmecozela ochraceella* lives in strict association with RWAs (Wasmann 1894, Donisthorpe 1927). The larvae crawl through the nest and feed on the nest material. Similar to the case-building *Clytra* larvae, they spin tubes of silk and nest material in which they live and pupate. The yellowish adults reside in the vicinity of the mounds, but can also be found on and in the nest mounds (Donisthorpe 1927). The well-studied *Maculinea* butterflies are confined to *Myrmica* nests and do not associate with RWAs.

Acari

Mites are the most diverse group found in RWA mounds, both in terms of abundance and number of species (Kielczewski and Wisniewski 1962). The presented list of mites associated with RWAs probably reveals only the tip of the iceberg. *Hypoaspis oophila* is the most conspicuous mite as it exclusively lives in large number on ant eggs. It appears that this mite does not puncture the eggs, but rather feeds on secretions coating the eggs (Donisthorpe 1927). Most species are likely scavengers and some are known to be phoretic (Donisthorpe 1927).

Araneae

The associated spiders can be divided into three groups: species that permanently live inside the mounds (= inquilines), myrmecomorphic species and myrmecophages. *Thyreosthenius biovatus* is a representative of the first group and only occurs in RWA mounds, but is probably abundant and widespread in RWA populations (Fig. 1.2.A and 1.2.F). This spider was found in 80 % of RWA mounds in northern Flanders, Belgium (unpub. data TP). The spider hardly elicits aggression and can walk freely among their ant host. Nymphs and females can be found throughout the year. Males are less abundant than females and probably occur only in spring and summer (pers. obs. TP). The heads of the males are raised in a conspicuous large lobe. *Mastigusa arietina* has a larger host range but is regularly associated with RWAs (Parmentier et al. 2015a). The white egg packets attached to wood pieces in the nest reveal their presence. Those spiders are mostly killed when placed together with RWAs in a cup, suggesting that this species is less integrated than *T. biovatus* (pers. obs. TP). The male palps are remarkably long and whip-like. Sometimes another morphologically similar species, *Mastigusa macrophthalma* is distinguished, but this is likely a subspecies (Heimer and

Nentwig 1991).The primary host of the inquiline spider *Acartauchenius scurrilis* is the small ant *Tetramorium caespitum*, but association with RWAs is also recorded (Donisthorpe 1927). The second group comprises spiders that imitate their ant host morphologically and behaviourally, so-called myrmecomorphic spiders. Three ant-mimicking spiders have been found in the vicinity of RWAs: *Myrmarachne formicaria*, *Phrurolithus festivus* and *Micaria fulgens* (Donisthorpe 1927). *Myrmarachne formicaria* waves its forelegs to imitate antennation and walks very ant-like (Shamble et al. 2013). The chelicerae of the male of this spider are very pronounced. There is little information on the biology of those species, but most myrmecomorphic spiders are considered Batesian mimics (Cushing 1997). Many animals do not prey on ants due to their toxity, distastefulness and aggressive nature. By mimicking ants, myrmecomorphic spiders deceive potential predators and are avoided (Cushing 1997). Recently Davidson reported on the myrmecophagous behaviour of *Dipoena torva*. This spider feeds exclusively on RWAs (*F. aquilonia*) in Scotland. It lives high on the tree stems and spins silk threads between the bark. RWA foragers get tangled with their antennae in the threads and are pierced by the spider in the soft membrane at the base of the antennae. The spider then manipulates the subdued ant away from the bark surface. Finally, the ant corpse hangs freely and is only attached to the stem with a small silk thread. This allows the spider to devour the ant without being attacked by other foragers (Fig. 1.2.G). Simon (1997) reported the occurrence of this spider with RWAs (*F. polyctena*) in Germany, but its dietary preferences and the behaviour of this spider remain unknown. *Dipoena tristis* has a similar hunting strategy and has been found mostly on grass halms near the nest of *Formica* species (Wasmann 1899).

Pan myrmecophilous species

Some obligate myrmecophiles do not show host preference and are associated with almost all ants in their habitat. *Cyphoderus albinus*, *Atelura formicaria*, *Platyarthrus hoffmannseggii* and *Myrmecophilus acervorum* are four typical panmyrmecophilous species that also co-occur with RWAs. They are the only representatives of the orders Collembola, Zygentoma, Isopoda and Orthoptera. They are all well-adapted to a life underground: they lack or have greatly reduced eyes, *C. albinus* and *P. hoffmannseggii* are white in color and *M. acervorum* has lost its wings (Donisthorpe 1927, Junker 1997). *C. albinus* is very characteristic by its erratic movements, *P. hoffmannseggii* by its thick, vibrating antennae (Fig. 1.2.A). A major part of *M. acervorum*'s diet consists of fluids regurgitated (trophallaxis) by the ant host (Junker 1997). Both *C. albinus* and *P. hoffmannseggii* can reach high population densities in RWA mounds (unpub. data).

Figure 1.2. Overview of RWA myrmecophiles. A. A myrmecophile bestiary found in a *Formica rufa* nest in northern Belgium: (1) *Dinarda maerkelii*, (2) *Amidobia talpa*, (3) *Thyreosthenius biovatus*, (4) *Clytra quadripunctata*, (5) *Leptacinus formicetorum*, (6) *Platyarthrus hoffmannseggii*, (7) *Thiasophila angulata*, (8) *Stenus aterrimus*, (9) *Monotoma*, (10) *Quedius brevis*, (11) *Notothecta flavipes*, (12) Lyprorcorrhe anceps, (13) *Myrmetes paykulli*. B. The imago of *Protaetia metallica* (photo courtesy of J.-L. Renneson). C. A *F. pratensis worker* inspects a *Coccinella magnifica* searching for aphids above a nest mound. D. *Clytra quadripunctata* imago above an *F. rufa* nest. E. Nymphs of *Alydus calcaratus* are morphological mimics of *Lasius* and *Formica* ants (photo courtesy of Andreas Haselböck). F. *Thyreosthenius biovatus* with an *F. polyctena* worker. G. The myrmecophagous spider *Dipoena torva* feeds on an *F. lugubris* worker (photo courtesy of Gus Jones BSCG). Photo A, C, D, F by Thomas Parmentier.

FACULTATIVE GUESTS

A vast array of species that are well-known from outside ant nests were recorded in RWA mounds (Kielczewski and Wisniewski 1962, Hlaváč and Lackner 1998, Laakso and Setälä 1998, Stoev and Lapeva-Gjonova 2005, Lapeva-Gjonova and Lieff 2012, Boer 2013, Robinson and Robinson 2013, Härkönen and Sorvari 2014). Those species complete normally their life cycle without ants, but can facultatively be associated with RWAs. Some of the recorded species simply landed coincidentally in the mounds. However, many species across diverse taxa thrive in large numbers in the nests. Those species are attracted by the enrichment of organic material, ideal climatic conditions and constant supply of nutrients in the mounds. A study in Finland showed that the biomass of earth worms was about seven times higher in RWA mounds than in the surrounding soil. Their biomass exceeded the biomass of all other associates (Laakso and Setälä 1997). Earthworms are much rarer in RWA mounds in Northern Belgium. Instead they are dominated by the common woodlouse *Porcellio scaber* (pers. obs. TP, WD). Some species, such as *Xantholinuslinearis* and *Drusilla caniculata*, were designated as myrmecophiles because they often co-occur with ants. They can, however, also live away from ants and are therefore no myrmecophiles in the strict sense. The facultative myrmecophile fauna of RWAs consist mainly of animals associated with decaying vegetable matter and bark. This includes Collembola, Acari, Pseudoscorpionida, Chilopoda, Diplopoda, Isopoda, Nematoda, Ptiliidae, Scydmaenidae, Staphylinidae and Psocoptera (Boer 2013, Robinson and Robinson 2013, pers. obs. TP, WD). Those species are mostly ignored by their size (Collembola, Acari, Psocoptera) or they can avert ant aggression by swift movements (Staphylinidae, Chilopoda). Other ants, such as *Leptothorax acervorum,* have also been recorded in wood ant mounds (Donisthorpe 1927). Isopods and diplopods have a strong exoskeleton, but they are rarely attacked. The concentration of cuticular hydrocarbons, which ant use as kin recognition keys, are probably low in those species as suggested in Kärcher and Ratnieks (2010). When there are few records of a species, it can be troublesome to determine its status as an obligate or facultative associate. For example, Henderickx (2011) described a new myrmecophilous pseudoscorpion species *Allochernes struyvei* based on individuals found in one *F. paralugubris* mound*.* More records, however, are needed to confirm its status of true myrmecophile.

DISTRIBUTION

Eurasian RWAs have a very broad distribution covering boreal and temperate Europe and large parts of Russia (Goropashnaya et al. 2004). The distribution of many associated RWA myrmecophiles is concordant with their host ant species. For example the beetles *Thiasophila angulata*, *Amidobia talpa, Monotoma conicollis* are recorded with RWAs in Great Britain, Scandinavia, continental Europe and Siberia. In contrast, some of the listed species have a narrower distribution. *Clytra laeviscula* for example is restricted in Europe to the southern and central part, while the related *Clytra quadripunctata* can found throughout Europe with RWAs. *Atelura formicaria* and *Myrmecophilus acervorum* are also thermophilous species that do not live in northern Europe. The hidden life of the associates makes it hard to estimate their abundance. Some species are fairly common in RWA populations and can attain high local densities (Päivinen et al. 2004, unpub. data), but wasps, flies and true bugs are much rarer and some of them are hardly recorded. This difference however can be partly attributed to a focus on the study of myrmecophilous beetles while other groups are often neglected.

HOST PREFERENCE

RWAs are believed to be the primary host of about 40 % of the species in our survey (Table A-1.1: indicated with *). Moreover, 24 species have hitherto only been recorded with RWAs (Fig. 1.3) (Note that some poorly known species, especially mites, could have a broader host distribution than hitherto recorded). Additionally, there are indications that some RWA myrmecophiles prefer a particular RWA species, e.g. *Oxypoda pratensicola* and *Thiasophila lohsei* typically live in association with *F. pratensis*. Some species are restricted to mound building *Formica*’s, which includes RWAs, *F. truncorum* (*Formica* s. str.), *Coptoformica* and *F. uralensis* (Table A-1.3 in Appendix 1-1 chapter 1). Mound building *Formica* species that do not belong to the *F. rufa* group have a less diverse myrmecophile association: 46 associates (species listed in Table A-1.1 + two panmyrmecophilous species + species Table A-1.3) have been found with *Coptoformica*, *F. truncorum* and *F.uralensis* so far, from which only five species have not recorded with RWAs (Table A-1.3). Conversely, there are 84 RWA myrmecophiles not found with other mound building *Formica*’s. Some species such as *Dinarda hagensii* and *Thiasophila canaliculata* have *F. exsecta* (*Coptoformica*) as primary host. The lower diversity of myrmecophiles associated with non-RWA mound building *Formica*’s can be explained by a smaller geographic range, smaller nests and possibly also by a sampling bias. A few species can be found with several species of the genus *Formica*, including both mound-building *Formica* ants as well as *Raptiformica* (*F. sanguinea* sometimes builds small mounds, but is here not considered as mound building because it nests can regulary be found under stones, in the ground or in tree trumps) and *Serviformica* ants. RWA queens found new colonies by parasitizing *Serviformica* colonies (Hölldobler and Wilson 1990). This take-over behaviour could promote the association of myrmecophiles both with *Serviformica* and mound building *Formica*'s. A large part of the species has also been found with *Camponotus* and/or *Lasius*, two other genera of the subfamily Formicinae. RWAs share many myrmecophiles especially with *L. fuliginosus* and *L. brunneus*, probably because they all construct nests with decaying organic material. About a quarter of the myrmecophiles has also been found with other subfamilies of the Formicidae. These include the panmyrmecophilous species, but also other species with more restricted host range across non-related taxa (e.g. *Lomechusa* species that switch host in winter). Many myrmecophiles succeeded to integrate in the wood ant mounds, but few are host specific (24 species exclusively found in RWAs). It can be expected that most species associated with RWAs rather apply general strategies, such as swift movements, defence chemicals (Staphylinidae: tergal gland), small compact size, hard exoskeleton, chemical insignificance, death feigning, ducking and avoidance. These general strategies facilitate easy host switching.

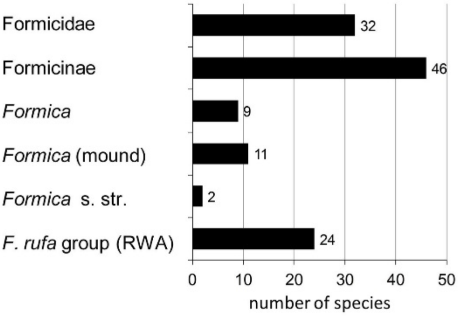

Figure 1.3. Taxonomic distribution of all recorded hosts of myrmecophiles associated with RWAs (based on column 4 in Table A-1.1).

CONSERVATION

RWAs populations are under increasing pressure by intensive agriculture, habitat fragmentation, deforestation, urbanization, habitat deterioration (e.g. overgrowing shrubs) and recreation (Gyllenstrand and Seppä 2003, Sorvari and Hakkarainen 2005, 2007, Mäki-Petäys et al. 2005, Dekoninck et al. 2010). The six species of the *F. rufa* group are listed on the IUCN Red List of Threatened species (IUCN 2013) and are protected in most European countries. Initially, the awareness of the role of RWAs in controlling pest insects stimulated conservationists (Gösswald 1989b). Later their importance for nutrient soil cycles and their complex social organization has encouraged conservation measures. However, the importance of their nests for myrmecophiles and other associated species has often been overlooked. Population sizes and prevalences of myrmecophiles decrease when RWA mounds become more isolated (Päivinen et al. 2004, Härkönen and Sorvari 2014, chapter 2). It can be expected that myrmecophiles strictly bound to RWAs are affected the most by the deterioration of population densities of their host. However, myrmecophiles that also occur with other ant hosts likely suffer from a decline in wood ant nests as well. For those species, the rich organic, thermoregulated and stable mounds are likely source habitats in which they can attain high population densities (unpub. data). Dispersal from those patches to surrounding nests of other ant hosts, which are often of lower quality and short-lived, can be vital in the population dynamics of those species. RWAs can thus be considered as typical flagship species and their protection should be primordial to conserve a highly diverse group of associated species.

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APPENDIX CHAPTER 1

Appendix 1-1. Red wood ant associates

Table A-1.1. Myrmecophiles associated with RWAs (*F. rufa* group). Host ant: * indicates primary host of myrmecophile are RWAs (as mentioned in at least one reference in right column or when the myrmecophile have hitherto only been found with RWAs). RWA species are underlined, primary host in bold when known. Abbreviations and taxonomy of host ant see table 3. Host ant: all known host ants reported in references on the right. Taxonomic relation: based on list of host ants. Biology: I = inside nest, E = outside nest, A = alternating life cycle with larval stage inside mound, and adult stage extranidal, D = different summer and winter host, P = larva parasitic inside ant worker or ant larva. Names of species of the *F. rufa* group correspond to the original species name given in the reference.

		Host ant	Taxonomic relation host ants	Biology		References
ACARI						
→MESOSTIGMATA						
Androlaelaps myrmecophila (Evans and Till, 1966)	*	*Fprat Frufa Frufa*	*F. rufa* group	I		10 , 31, 40
Hypoaspis cuneifer Michael 1891		*Caeth Cherc Clign Fcuni Fexse Ffusc Frufa Frufib Fsang Lalie Lbrun Lflav Lfuli Lmixt Lumbr Mscab Ppall Sfuga Terra*	Formicidae	I		1, 2, 4, 5, 6, 10 , 18, 32
Hypoaspis montana Berlese, 1904		*Ffusc Frufa Lnige Myrmica*	Formicidae	I		5, 10 , 40
Hypoaspis myrmecophila (Berlese, 1892)		*Aphaenogaster Clign Fcuni Frufa Frufib Fsang Lflav Lnige MSbarb Mlaev Mrubr Mrugi Mscab Messor*	Formicidae	I		1, 2, 4, 5, 6, 10, 18
Hypoaspis neocuneifer (Evans and Till, 1966)	*	*Frufa*	*F. rufa* group	I		10, 40
Hypoaspis oophila (Wasmann 1897)		*Fcuni Ffusc Fpoly Fprat Frufa Frufib Fsang*	*Formica*	I	lives on the eggs	1, 2, 4, 5, 10 , 18, 40, 45
Laelaps laevis Michael, 1891		*Fprat Frufib Fsang Mbarb Tcaes*	Formicidae	I		4, 5, 32
Myrmozercon acuminatus Berlese, 1903		*Frufa MScapi*	Formicidae	I		5, 32
RHODACARIDAE						
Punctodendrolaelaps formicarius Hugta and Karg, 2010	*	*Frufa* s.l.	*F. rufa* group	I		31
TRACHYTIDAE						
Uroseius myrmecophilus Wisniewski 1979	*	*Fpoly*	*F. rufa* group	I		32
TRACHYUROPODIDAE						
Urojanetia coccinea (Michael, 1891)		*Fexse Ffusc Fpoly Frufa Frufib Fsang Lnige Lflav Mrubr Mscab*	Formicidae	I		4, 5, 17, 32
Urojanetia coccinea var. sinuate		*Atest Caeth Ffusc Frufa Frufib Fsang Lflav Lnige Mscab*	Formicidae	I		5 , 32
URODINYCHIDAE						
Urodinychus janeti Berlese, 1904	?	*Ffusc Frufa*	*Formica*	I		5
UROPODIDAE						
Oodinychus ovalis (C.L. Koch, 1839)		*Camponotus Formica Lasius*	Formicinae	I		17
Oplitis pandata (Michael, 1894) ssp. n. “A”	?	*Fexse Frufa*	Formicamound	I		17
Trematurella elegans (Berlese, 1916)	?	*Fpoly Lnige*	Formicinae	I		17
Urodiscella ricasoliana (Berlese, 1889)		*Clign Frufa Lfuli Lumbr*	Formicinae	I		5, 6
Uroseius koehleri Wisniewski, 1979	*	*Frufa*	*F. rufa* group	I		17
→ SARCOPTIFORMES						
ACARIDAE						
Forcellinia wasmanni (Monniez, 1892)		*Ffusc Frufa Fsang Lalie Lfuli Lnige Mrugi Tcaes*	Formicidae	I		6
Tyrophagus formicetorum Volgin, 1948	*	*Frufa*	*F. rufa* group	I		16
→ TROMBIDIFORMES						

NANORCHESTIDAE						
Speleorchestes formicorum Trägårdh, 1909	*	*Frufa*	*F. rufa* group	I		5
PYGMEPHORIDAE						
Petalomium sawtschuki (Sevastianov, 1967)	*	*Frufa*	*F. rufa* group	I		13
Pygmephorus samsinaki Mahunka, 1967	*	*Frufa*	*F. rufa* group	I		12
Siteroptes bohemicus Mahunka, 1967	*	*Frufa*	*F. rufa* group	I		12
SCUTACARIDAE						
Disparipes nudus Berlese, 1886	?	*Lfuli Frufa*	Formicinae	I		1
Imparipes atypicus Karafiat, 1959	?	*Frufa Mrugi*	Formicidae	I	phoretic	9, 15
Imparipes pennatus Karafiat, 1959	?	*Frufa Terra*	Formicidae	I	phoretic	9
Scutacarus rotundatus (Berlese, 1903)	?	*Frufa Lnige*	Formicinae	I		6
ARANEA						
AGELENIDAE						
Mastigusa arietina (Thorell, 1871)		*Ffusc Fpoly Frufa Lfuli Lbrun Lumbr*	Formicinae	I		1, 5 , 44, 45
CORINNIDAE						
Phrurolithus festivus (C.L.Koch, 1835)		*Ffusc Frufa Fsang Lbrun Lflav Lfuli Lnige*	Formicinae		mimicry	1, 5
GNAPHOSIDAE						
Micaria fulgens (Walckenaer, 1802)	?	*Frufa*	*F. rufa* group		mimicry	2
LINYPHIIDAE						
Acartauchenius scurrilis (O.P.-Cambridge, 1872)		***Tcaes** Frufa Lflav*	Formicidae	I		1, 2, 5, 45
Thyreosthenius biovatus (O. P.-Cambridge, 1875)	*	*Ffusc Flugu Fpoly Fprat Frufa*	*Formica*	I		1, 2, 5 , 19, 40, 41, 44, 45
SALTICIDAE						
Myrmarachne formicaria (de Geer 1778)		*Mrubr Mscab Fcuni Frufa Frufib*	Formicidae	E	mimicry	5
THERIDIIDAE						
Dipoena torva (Thorell, 1875)	*	*Faquil Fpoly*	*F. rufa* group	E	myrmecophagous	20, 33
Dipoena tristis (Hahn, 1833)		*Ffusc Frufa Fsang Frufib*	*Formica*	E	myrmecophagous	3
COLLEMBOLA						
Cyphoderus albinus Nicolet, 1842		Panmyrmecophilous	Formicidae	I	loss of pigment, blind	1, 5 ,26, 40, 41, 45
ISOPODA						
Platyarthrus hoffmannseggii Brandt, 1833		panmyrmecophilous	Formicidae	I	loss of pigment, blind	1, 5, 7, 26, 40, 41, 45
ZYGENTOMA						
Atelura formicaria Heyden, 1855		Panmyrmecophilous	Formicidae	I	loss of pigment, blind trophallaxis	1, 2, 18, 45
COLEOPTERA						
CETONIIDAE						
Protaetia (Potosia) metallica (Herbst, 1782)	*	*Fpoly Fprat Frufa Fural Fsang Lfuli*	Formicinae	A	larva though skin	1,5,24, 38, 41, 45
COCCINELLIDAE						
Coccinella magnifica Redtenbacher, 1843	*	*Flugu Fpoly Fprat Frufa*	*F. rufa* group	E	feeds on aphids colonies tended by rwas / trail following / ducking down	5, 19, 41, 45
CHRYSOMELIDAE						
Clytra laeviuscula Ratzeburg, 1837		*Asubt Frufa Fsang Lalie Lnige*	Formicidae	A	larva in case	1, 5, 39
Clytra quadripunctata (Linnaeus, 1758)	*	*Clign Faqui Fexse Ffusc Flugu Frufa F.prat Fsang Fgaga*	Formicidae	A	larva in case	1, 5, 19, 24, 39, 41, 45
CRYPTOPHAGIDAE						
Emphylus glaber (Gyllenhal, 1808)	*	*Faqui Fpoly Frufa Fural*	Formicamound	I		1, 3, 24, 44

Hypocoprus latridioides Motschulsky, 1839		***Fexse*** *Flugu Fobsc Frufa*	Formicamound	I		1, 39
HISTERIDAE						
Abraeus perpusillus (Marsham, 1802)		*Lfuli Lbrun Frufa*	Formicinae	I		24
Dendrophilus pygmaeus (Linnaeus, 1758)	*	*Faqui Fexse Flugu Fpoly Fprat FrufaLfuli*	Formicinae	I	death feigning	1, 5, 14, 19, 21, 24,39, 41, 44, 45
Hetaerius ferrugineus (Olivier, 1789)		*Formica Fcine Fexse Ffusc Fprat Frufa Frufib Fsang Lasius Lflav Lfuli Lnige Leptothorax Lacer Myrmica Mscab Prufe Terra*	Formicidae	I	glandular adaptations trophallaxis	1, 2, 3, 14, 21, 24, 39, 41
Myrmetes paykulli Kanaar, 1979	*	*Faqui Flugu Fpoly Fprat Frufa Lasius sp.*	Formicinae	I	death feigning	1, 2, 5, 14, 19, 21, 24, 41, 45
LATRIDIIDAE						
Corticaria longicollis (Zetterstedt, 1838)	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Lnige Tcaes*	Formicidae	I		24, 34, 39, 44
Corticaria inconspicua Wollaston, 1860	*	*Fprat Frufa*	*F. rufa* group	I		24
MONOTOMIDAE						
Monotoma angusticollis (Gyllenhal, 1827)	*	*Faqui Fexse Flugu Fprat Fpoly Frufa*	Formicamound	I	death feigning	1, 5, 19, 24, 40, 41,44, 45
Monotoma conicicollis Aubé, 1837	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Fural*	Formicamound	I	death feigning	1, 5, 19, 21, 24, 39, 40, 41, 44, 45
PTILIIDAE						
Ptilium myrmecophilum (Allibert, 1844)	*	*Faqui Flugu Frufa Fprat Fsang Ftrun Lasius*	Formicinae	I		1, 5 , 24, 39, 40, 41
Ptenidium formicetorum Kraatz, 1851	*	*Faqui Fexse Fpoly Frufa Fprat Lbrun Lfuli*	Formicinae	I		1, 5, 24, 41, 44
STAPHYLINIDAE						
ALEOCHARINAE						
Amidobia talpa (Heer, 1841)	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Ftrun Lfuli*	Formicinae	I		5, 19, 24, 29, 40, 44, 45
Atheta confusa (Märkel, 1844)		*Frufa* ***Lfuli***	Formicinae	I		24
Dinarda dentata (Gravenhorst, 1806)		*Faqui Fcine Fexse Ffusc Fpoly Frufib* ***Fsang***	*Formica*	I	trophallaxis brood parasite	24, 29
Dinarda hagensii Wasmann, 1889		***Fexse*** *Fprat*	Formicamound	I	trophallaxis brood parasite	24, 37, 39
Dinarda maerkelii Kiesenwetter, 1843	*	*Flugu Fpoly Fprat Frufa Fsang Ftrun*	*Formica*	I	trophallaxis brood parasite	1, 2, 5, 19, 24, 37, 39, 45
Euryusa optabilis Heer, 1839		*Frufa Lbrun Lnige Lfuli*	Formicinae	I		24
Lomechusa emarginata (Paykull, 1789)		*Fcine* ***Ffusc*** *Frufa Fsang Lasius Mrubr Mrugi Mrugu Mscab Msabu Msulc*	Formicidae	D	glandular adaptations trophallaxis brood parasite	5, 24, 37
Lomechusa pubicollis Brisout de Barneville, 1860	*	*Ffusc Fpoly Fprat Frufa Frufib Ftrun Lfuli Lalie Lflav Lnige Lumbr Mrubr Mrugi Msulc Tcaes*	Formicidae	D	glandular adaptations trophallaxis brood parasite	1, 4, 5, 11, 24, 41
Lomechusoides inflatus (Zetterstedt, 1828)	?	*Fexse Fgaga Fprat Frufa Fural*	*Formica*	I	glandular adaptations trophallaxis brood parasite	24, 25
Lomechusoides sibiricus Motschulsky, 1844		*Frufa Fsang*	*Formica*	I	glandular adaptations trophallaxis brood parasite	25
Lomechusoides strumosus (Fabricius, 1792)		*Fprat Frufa* ***Fsang***	*Formica*	I	glandular adaptations trophallaxis brood parasite	1,24
Lomechusoides welleni (Palm, 1949)	?	*Flugu Frufa Fural*	Formicamound	I	glandular adaptations trophallaxis brood parasite	24
Lyprocorrhe anceps (Erichson, 1837)	*	*Faqui Fexse Flugu Fnigr Fpoly Fprat Frufa Ftrun Lfuli*	Formicinae	I		1, 5, 19, 24, 37, 39, 40, 41, 44, 45

Notothecta confusa (Märkel, 1844)		*Frufa* ***Lfuli***	Formicinae	I		24
Notothecta flavipes (Gravenhorst, 1806)	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Fsang Ftrun Lflav*	Formicinae	I		1, 5, 19, 24, 29, 37, 39, 40, 41, 44, 45
Oxypoda formiceticola Märkel, 1841	*	*Faqui Fexse Ffusc Flugu Fpoly Frufa Lasius*	Formicinae	I		24, 37, 39, 40, 41, 44
Oxypoda pratensicola Lohse, 1970	*	*Fexse* ***Fprat***	Formicamound	I		24, 37 , 39
Oxypoda recondita Kraatz, 1856		*Frufa Fsang Lbrun*	Formicinae	I		5
Oxypoda rugicollis Kraatz, 1856		*Fexse Fprat Fpres Frufa Lasius*	Formicinae	I		24, 37, 39
Oxypoda vittata Märkel, 1842		*Frufa Lbrun* ***Lfuli***	Formicinae	I		24, 29, 45
Thiasophila angulata (Erichson, 1837)	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Fsang Ftrun Fural Lbrun Lfuli*	Formicinae	I		1, 5, 24, 29, 37, 40, 41, 44, 45
Thiasophila canaliculata Mulsant and Rey, 1875		***Fexse*** *Frufa*	Formicamound	I		1, 24, 37, 39
Thiasophila inquilina Märkel, 1844		***Lfuli*** *Frufa Fprat*	Formicinae	I		5, 24
Thiasophila lohsei Zerche, 1987	*	*Fprat*	*F. rufa* group	I		14, 24, 37 , 39
Zyras (Zyras) haworthi (Stephens, 1832)		*Asubt Clign Frufa Lfuli*	Formicidae	I		5
Zyras (Pella) humeralis (Gravenhorst, 1802)		*Faqui Fpoly Fprat Frufa Lbrun* ***Lfuli*** *Lumbr*	Formicinae	I	winter association	1, 5, 24, 29, 39, 45
PSELAPHINAE						
Batrisodes venustus (Reichenbach, 1816)		*Clign Ffusc Fpoly Frufa Ftrun Mscab Lbico Lbrun Lfuli Lnige*	Formicidae	I		1, 5, 24, 29
SCYDMAENINAE						
Euconnus claviger (P.W.J.Müller and Kunze, 1822)		*Clign Ffusc Frufa Fsang Lbrun Lfuli Lnige "rufagroup"*	Formicinae	I		14, 21, 22, 42
Euconnus maeklinii (Mannerheim, 1844b)		*Fpoly Frufa Lbrun Lfuli Lnige "rufagroup" Lasius*	Formicinae	I		1, 14, 22, 24, 42, 44
Euconnus pragensis Machulka, 1923		*Clign Fcine Frufa Lbrun Lnige Lfuli Lasius*	Formicinae	I		14, 21, 22, 24, 42
STAPHYLININAE						
Quedius brevis Erichson, 1840		*Faqui Fexse Flugu Fpoly Frufa Fsang Lbrun Lfuli*	Formicinae	I	death feigning	1 , 5, 19, 24, 37, 40 , 39, 41, 45
STENINAE						
Stenus aterrimus Erichson, 1839	*	*Fpoly Fprat Frufa Ftrun*	*Formica* s. str.	I		1, 2, 24, 29, 37, 39, 41, 45
TACHYPORINAE						
Lamprinodes saginatus (Gravenhorst, 1806)		*Acanthomyops Fexse Ffusc Fpoly Frufa Fsang Lflav Lfuli Msabu Mrubr Mrugi Mscab Ponera*	Formicidae	I		1, 5, 24, 41, 45
XANTHOLININAE						
Gyrohypnus atratus (Heer, 1839)	*	*Faqui Flugu Fpoly Fprat Frufa Lfuli Mrubr*	Formicidae	I		1, 5, 19, 24, 29, 40, 41, 44
Leptacinus formicetorum Märkel, 1841	*	*Faqui Fexse Flugu Fpoly Fprat Frufa Frufib Fural Lbrun*	Formicinae	I		1, 5, 19, 24, 37, 39, 40, 41, 44, 45
TENEBRIONIDAE						
Myrmechixenus subterraneus Chevrolat, 1835	*	*Faqui Fcine Fexse Ffusca Flugu Fprat Fpres Fpoly Frufa Ftrun Lnige Lfuli*	Formicinae	I		1, 14, 19, 24, 39, 41, 44
DIPTERA						
CHIRONOMIDAE						
Forcipomyia myrmecophila (Egger, 1863)	?	*Fexse Frufa*	Formicamound	A		5, 19, 41
MILICHIIDAE						
Phyllomyza formicae Schmitz, 1923	*	*Flugu Frufa Fprat*	*F. rufa* group	A		5, 19
MYTHICOMYIIDAE						
Glabellula arctica (Zetterstedt, 1838)	?	*Faqui Fexse Fpoly Fprat*	Formicamound	A		14b, 44
SCATOPSIDAE						
Colobostema infumatum (Haliday, 1833)		*Lfuli Frufa*	Formicinae			5
Colobostema nigripenne (Meigen, 1830)		*Myrmica* RWA	Formicidae	?		19, 43
Holoplagia transversalis (Loew, 1846)		***Lfuli*** *Frufa*	Formicinae	A		5
Scatopse leucopeza Meigen, 1818		*Frufa Lbrun Lfuli*	Formicinae	?		1
SYRPHIDAE						
Microdon mutabilis (Linnaeus, 1758)		*Ffusc Flema Frufa Frufib Lnige Lbrun Lflav*	Formicinae	A	brood parasite	1, 2, 5, 35

Microdon devius (Linnaeus, 1761)		*Ffusc Frufa Fsang Lflav Lfuli*	Formicinae	A	brood parasite	1, 5
Microdon analis (Macquart, 1842)		*Fexse Ffusc Flema Frufa Fsang Lfuli*	Formicinae	A	brood parasite	30
HEMIPTERA						
ALYDIDAE						
Alydus calcaratus (Linnaeus, 1758)		*Ffusc Fprat Frufa Frufib Fsang Lflav Lnige Myrmica Mrubr*	Formicidae	E	mimicry	1, 2, 5, 28
ANTHOCORIDAE						
Xylocoris formicetorum (Boheman, 1844)	*	*Fexse Ffusc Flugu Fpoly Fprat Frufa Fsang Ftrun Lflav*	Formicidae	I		2, 19, 23b, 28, 41, 44
LYGAEIDAE						
Notochilus limbatus Fieber, 1870		*Frufa Myrmica*	Formicidae	I		1, 28
Eremocoris abietis (Linnaeus, 1758)	*	*Frufa Camponotus*	Formicinae	E		1, 28, 41
MIRIDAE						
Myrmecoris gracilis (R.F. Sahlberg, 1848)		*Ffusc Frufa Lnige*	Formicinae	E	mimicry	1, 5, 28
Pilophorus cinnamopterus (Kirschbaum, 1856)	*	*Fprat Frufa*	*F. rufa* group	E	mimicry	1, 5, 28
Pilophorus perplexus Douglas and Scott, 1875		*Ffusc Frufa Fsang Lbrun Lemar Lfuli Lnige*	Formicinae	E	mimicry	5, 28, 43
HYMENOPTERA						
BRACONIDAE						
Elasmosoma berolinense Ruthe, 1858		*Camponotus Cvagus Ffusc Fjapo Fprat Frufa Fsang Formica Lnige Polyergus*	Formicinae	P	ant parasite	1, 3, 5, 36
Fachylomma [Eurijpterna) creinierl		*Lfuli Frufa*	Formicinae	?		3
Neoneurus auctus (Thomson, 1895)	*	*Frufa Fprat*	*F. rufa* group	P	ant parasite	36
Neoneurus clypeatus (Förster, 1862)	*	*Frufa*	*F. rufa* group	P	ant parasite	36
Kollasmosoma marikovskii (Tobias, 1986)	*	*Fprat*	*F. rufa* group	P	ant parasite	36
DIAPRIIDAE						
Trichopria fuliginosa (Wasmann, 1899)		*Frufa Lfuli*	Formicinae	I		5
EUCHARITIDAE						
Chalcura sp.	*	*Frufa*	*F. rufa* group	P	ant parasite	1, 5, 36
Eucharis bedeli (Cameron, 1891)		*Cataglyphis Fjapo Frufa*	Formicinae	P	ant parasite	36
Eucharis adscendens (Fabricius, 1787)		*F rufa Fcuni Messor*	Formicidae	P	ant parasite	36
FORMICIDAE						
Formicoxenus nitidulus (Nylander, 1846)	*	*Faqui Fexse Flugu Fpoly Fprat Fpres Frufa Fsang Ftrun Fural*	Formicamound	I	xenobiosis with chemical deterrent strategy	1, 2, 5, 27, 41, 44, 45
Solenopsis fugax (Latreille, 1798)		*Fcine Fcuni Ffusc Fprat Frufa Frufib Fsang Lflav Lmixt Lnige Mscab Prufe Tcaes*	Formicidae	I	lestobiosis	2, 5
ICHNEUMONIDAE						
Hybrizon buccatus (de Brébisson, 1825)		*Formica Fprat Frufa Lasius Myrmica Tapinoma*	Formicidae	P	ant parasite	5, 36
Eurypterna cremieri (Romand,1838)		*Frufa Lasius*	Formicinae	P	ant parasite	36
MEGASPILIDAE						
Conostigmus inquilinus (Erichson, 1844)	*	*Frufa*	*F. rufa* group	?		1
Conostigmus formiceti (Erichson, 1844)	*	*Frufa*	*F. rufa* group	I		1, 5
LEPIDOPTERA						
TINEIDAE						
Myrmecozela ochraceella (Tengström, 1848)	*	*Flugu Fprat Frufa*	*F. rufa* group	A	larva in case	1, 5
ORTHOPTERA						
MYRMECOPHILIDAE						
Myrmecophilus acervorum (Panzer, 1799)		Panmyrmecophilous	Formicidae	I	trophallaxis	1, 2, 8, 23

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45 = pers.l obs. TP and/or unpub. data.

Table A-1.2. Taxonomic classification of RWA and ants listed in Table A-1.1 and Table A-1.3.

Dolichoderinae				
Tapinoma Förster, 1850				
Formicinae				
Camponotus Mayr, 1861	*C. aethiops* (Latreille, 1798)	Caeth		
	C. herculeanus Linnaeus, 1758	Cherc		
	C. ligniperda Latreille, 1802	Clign		
Formica Linnaeus, 1758				
	F. uralensis Ruzsky, 1895	Fural		mound building
Formica s.str.	*F. aquilonia* Yarrow, 1955	Faqui	*F.rufa* group	mound building
	F. lugubris Zetterstedt, 1839	Flugu	*F.rufa* group	mound building
	F. polyctena Förster, 1850	Fpoly	*F.rufa* group	mound building
	F. pratensis Retzius, 1783	Fprat	*F.rufa* group	mound building
	F. paralugubris Seifert, 1996	Fpara	*F.rufa* group	mound building
	F. rufa Linnaeus, 1761	Frufa	*F.rufa* group	mound building
	F. dusmeti Emery, 1909			mound building
	F. frontalis Santschi, 1919			mound building
	F. obscuripes Forel, 1886	Fobsc	*F. rufa* group (North-America)	mound building
	F. truncorum Fabricius, 1804	Ftrun		mound building
Coptoformica	*F.exsecta* Nylander, 1846	Fexse		mound building
	F. pressilabris Nylander, 1846	Fpres		mound building
	F. suecica Adlerz, 1902	Fsuec		mound building
Raptiformica	*F. sanguinea* Latreille, 1798	Fsang		
Serviformica	*F. cinerea* Mayr, 1853	Fcine		
	F. cunicularia Latreille, 1798	Fcuni		
	F. fusca Linnaeus, 1758	Ffusc		
	F. gagates Latreille, 1798	Fgaga		
	F. lemani Bondroit, 1917	Flema		
	F. japonica Motschoulsky, 1866	Fjapo		
	F. rufibarbis Fabricius, 1793	Frufib		
Lasius Fabricius, 1804	*L. alienus* (Förster, 1850)	Lalie		
	L.brunneus (Latreille, 1798)	Lbrun		
	L. emarginatus (Olivier, 1792)	Lemar		
	L. flavus (Fabricius, 1782)	Lflav		
	L. fuliginosus (Latreille, 1798)	Lfuli		
	L. mixtus (Nylander, 1846)	Lmixt		
	L. niger (Linnaeus, 1758)	Lnige		
	*L. umbratus (*Nylander, 1846)	Lumbr		
Polyergus Latreille, 1804	*P. rufescens* (Latreille, 1798)	Prufe		
Myrmicinae				
Aphaenogaster Mayr, 1853	*A. subterranea* (Latreille, 1798)	Asubt		
	A. testaceopilosa (Lucas, 1849)	Atest		
Leptothorax Mayr, 1855	*L. acervorum* (Fabricius, 1793)	Lacer		
Messor Forel, 1890	*M. barbarus* (Linnaeus, 1767)	MSbarb		
	M. capitatus (Latreille, 1798)	MScapi		
Myrmica Latreille, 1804	*M. rubra* (Linnaeus, 1758)	Mrubr		
	M. ruginodis Nylander, 1846	Mrugi		
	M. rugulosa Nylander, 1842	Mrugu		
	M. sabuleti Meinert, 1861	Msabu		
	M. *scabrinodis* Nylander, 1846	Mscab		
	M. sulcinodis Nylander, 1846	Msulc		
Pheidole	*P. pallidula* Westwood, 1840,	Ppall		
Solenopsis	*S. fugax* Latreille, 1798	Sfuga		
Tetramorium Mayr, 1855	*T. caespitum* (Linnaeus, 1758)	Tcaes		

Table A-1.3. Myrmecophiles associated with other mound building Eurasian wood ants and not recorded with RWAs.

Myrmecophile	Host ant	Taxonomic relation host ants	References
Thiasophila bercionis Bernhauer, 1926	Fexse Fural	Formicamound	(Päivinen et al. 2002)
Zyras cognatus (Märkel, 1842)	Ffusc Fexse Lbrun Lnige Lfuli	Formicinae	(Päivinen et al. 2002)
Zyras limbatus (Paykull, 1789)	Ffusc Fsang Fexse Lfuli Lflav Lbrun Lnige Mrubr Mscab	Formicidae	(Päivinen et al. 2002)
Rhyncholophus phalangloides Moniez 1894	Fexse	*Coptoformica*	(Uppstrom 2010)
Micaria pulicaria (Sundevall, 1831)	Fcuni Fexse Ffusc Fsang Lfuli Lnige Lumbr Tcaes	Formicidae	(Donisthorpe 1927)

CHAPTER 2

Metapopulation processes affecting diversity and distribution of myrmecophiles associated with red wood ants

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ABSTRACT

Red wood ants (RWAs) support a diverse community of myrmecophiles in their nest mounds. Given that nest mounds provide fairly constant and distinct habitat patches for myrmecophiles, metapopulation and metacommunity dynamics can be expected to play an important role in structuring myrmecophile communities. Here, we investigate how site, site size (i.e. number of mounds per site), mound isolation, mound size, moisture, pH and RWA host (*Formica rufa* and *Formica polyctena*) affect the (meta)community composition and species richness. We demonstrate that community composition is structured by site and within-site isolation. In addition, species richness per unit volume is negatively correlated with increasing nest mound isolation. Mound size and site size at a higher spatial scale had no effect on community composition or diversity. The latter suggests that few mounds are required to support the minimum viable metapopulation size. We did not find support that the environmental variables mound moisture and pH affect the myrmecophile community or its species richness. Finally, the communities of the two closely related wood ant species *F. rufa* and *F. polyctena* were very similar. Overall, our results demonstrate, in accordance with metapopulation theory, that isolated mounds support fewer myrmecophile species. Diverse myrmecophile metacommunities also occur in small RWA sites, with well connected nest mounds. We discuss the powerful potential of ant nests, and particularly RWA mounds, for metapopulation and metacommunity research.

INTRODUCTION

A diverse group of arthropods is strictly associated with ants (Hölldobler and Wilson 1990). They benefit from the resources provided by their host and the homeostatic nest conditions. Myrmecophiles are confined to ant nests, but differ in degree of host specificity. While some species are restricted to one ant species or narrowly related species, others occur with different ant taxa and few even show no preference at all (Hölldobler and Wilson 1990). Myrmecophiles live thus in small, spatially distinct and stable patches (= ant nests of associated host ant taxa) susceptible to colonization surrounded by a large landscape matrix unsuitable for colonization. Hence, the populations of myrmecophiles can be expected to be organized as metapopulations (sensu Hanski and Gilpin 1991) wherein local dynamics in the ant nest interact with dispersal among the ant nest patches. When multiple myrmecophile species live in the same set of distinct ant nests, their community can be described as a community of metapopulations or a metacommunity (Hanski and Gilpin 1991). Metapopulation theory has proven to be a successful concept to study fragmented populations connected through dispersal. A key prediction of metapopulation theory is that populations in small and isolated patches are more likely to get extinct because of smaller carrying capacities and smaller odds to get rescued by new colonisations. Consequently, those patches support fewer species at the metacommunity level. Local environmental characteristics of the patch have been demonstrated as a third factor to affect patch occupation probability in metapopulations (Ranius 2000, Thomas et al. 2001, Jeffries 2005, Chisholm et al. 2011).

Because of their hidden life style, the distribution and abundance of myrmecophiles are unclear and likely underestimated. In this study, we investigate which (metapopulation) processes structure myrmecophiles associated with European red wood ants (RWAs) (*Formica rufa* group). RWAs are dominant and aggressive arthropod predators in European woodlands (Skinner 1980, Laakso and Setälä 2000, Hawes et al. 2002). Still, many arthropods managed to evade ant aggression and live successfully in or around their nest mounds in one of the largest associations of arthropods including Coleoptera, Hymenoptera, Diptera, Lepidoptera, Heteroptera, Isopoda, Collembola, Acari and Araneae (chapter 1: Parmentier et al. 2014).

Two RWA species, *Formica rufa* and *Formica polyctena*, co-occur in western Flanders, Belgium (Dekoninck et al. 2010). Their populations are isolated units due to forest fragmentation. They vary considerably in size, but the majority of the populations is relatively small. Nest mounds differ in local ecological characteristics, size and relative position to other mounds of the site. Nests persist likely by budding or by accepting related new queens (pers. observations TP). Those distinct and small RWA sites are thus ideal subjects to test factors classically structuring the dynamics and affecting the composition and richness of metapopulations and metacommunities.

Studies in large forest complexes in Finland by Päivinen et al. (2004) and Härkönen and Sorvari (2014) demonstrated that isolation of mounds of the RWAs *F. aquilonia* and *F. polyctena* negatively affected the diversity of myrmecophiles. Lower beetle diversity was also demonstrated in smaller mounds (Päivinen et al. 2004). These findings agree thus with metapopulation theory. Yet, it is not understood whether the same processes structure myrmecophile metacommunities in highly fragmented and impoverished, small, RWA sites. Moreover, it remains unknown whether local patch (i.e. mound) characteristics and factors at a larger spatial scale affect myrmecophile metacommunities. Therefore, we want to test in-depth potential factors structuring the myrmecophile metacommunity in fragmented RWA sites. More specifically, we assess the effect of site, isolation and multiple mound characteristics (size, pH, moisture, host ant) on: a) myrmecophile metacommunity composition and b) myrmecophile species richness.

MATERIAL AND METHODS

Study area

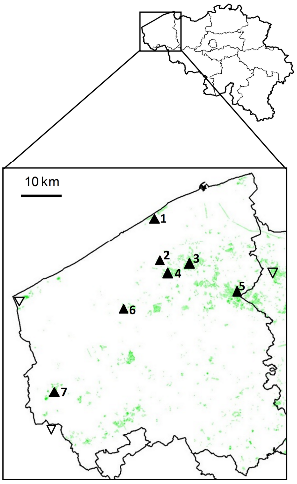

Figure 2.1. Overview of red wood ant sites (1 = De Haan, 2 = Roksem,3 = Beisbroek, 4 = Aartrijke, 5 = Beernem, 6 = Vladslo, 7 = West-Vleteren) in West Flanders, the westernmost province of Belgium.Unsampled red wood ant sites in West Flanders and nearby regionsare indicated by inverted open triangles. The mapped sites compriseall red wood ant mounds in this area. Forest fragments indicated in green. Detailed maps per site see Appendix 2-1.

The study area is situated in northwest Belgium (province: Western Flanders) (Fig. 2.1). This is a highly urbanized region with only few fragmented woodland patches remaining. Two RWA species, *Formica rufa* Linnaeus, 1761 and *Formica polyctena* Förster, 1850 persist in those isolated and small forests (Dekoninck et al. 2003). The two ant species are closely related and are even known to hybridize (Seifert et al. 2010). They can be distinguished based on degree of pilosity. Moreover they tend to have different ecological preferences (Seifert 2007). *Formica rufa* usually forms monodomous (one mound per colony) and monogynous (one queen) colonies along forest edges, while most *F. polyctena* colonies are polydomous (multiple mounds in a colony) and polygynous (multiple queens in a mound) typically established in inner forests (Seifert 2007). However, in the study area these differences are less clear-cut with *F. rufa* often forming strong polygynous and polydomous colonies and with *F. polyctena* mounds regularly lining forest edges (pers. observations TP, Dekoninck et al. 2010). Nevertheless, the two species can unambiguously be separated based on their pilosity and no hybrids occur in the study area.

We surveyed 83 mounds (29 *F. rufa*, 54 *F. polyctena*) in seven RWA sites and recorded the presence of myrmecophiles (Fig. 2.1) (detailed maps per site in Appendix 2-1). Two sites (West-Vleteren, Vladslo) support *Formica rufa*, three *Formica polyctena* (Beernem, Roksem, Aartrijke) and in the two remaining sites (De Haan and Beisbroek) both species occur sympatrically. We use the word “site” to describe a population of *F. rufa* and/or *F. polyctena* in a particular forest complex.

The distribution of the RWA mounds in the study area was already well recorded during previous studies (Loones et al. 2008, Dekoninck et al. 2010, Parmentier 2010) Additionally, the woodlands were intensively scanned prior to sampling to record new, moved or disappeared nests. Therefore we were able to map all nest mounds of the seven sites.

Inventory of myrmecophiles and nest mound variables

During the summer of 2012 and 2013, we collected all myrmecophiles in a 2-L nest sample by successively inspecting small portions of that sample spread out in a large white tray. Afterwards, nest material, ants and their brood were gently put back into the nest to minimize disturbance. The 2-L samples were taken from the central core of the 83 mounds. Beetles were identified following Freude et al. (1964, 1974), spiders following Roberts (2001). We identified 13 beetle species (including 8 rove beetles), two spiders, one springtail and one isopod.

For each mound we recorded the following variables: host species, site size, mound size, isolation, moisture and pH. The host ant species was either *F. rufa* or *F. polyctena*. The seven RWA sites were assigned to two size classes based on total number of RWA mounds (small: < 15 mounds, large: 25-48 mounds). There was a large variation in mound height (and mound depth) corresponding rather with sun exposure than with colony size. Therefore we used nest surface (ellipse: $\pi ab/4$ with a, b the largest and smallest diameter of the mound) following Liautard et al. (2003) who demonstrated that this is a good measure for mound size and productivity in mound building ants. Dispersal distance and frequency is species specific and isolation for several species in one study system is consequently difficult to quantify with one parameter (Kindlmann and Burel 2008). Therefore mound isolation was estimated as the sum of the surface areas of other nest mounds within a 100-m radius (S_{100}) of the focal nest mound or by the nearest neighbor distance (d_{min}). An additional nest sampling of the central core was done to measure environmental variables. These samples (ca. 10 g) were collected after three consecutive dry days during the summer and brought to the lab. PH was recorded (Lutron sensor PH-223) on 1:5 soil suspensions. Additionally soil samples were dried overnight at 60 °C in an oven (Memmert) to quantify moisture content gravimetrically.

The possible host ant species of the observed myrmecophiles are listed in Table 2.1. The main secondary hosts are *Lasius fuliginosus*, *Formica sanguinea*, *Lasius brunneus* and *Formica fusca*. *F. fusca* was observed interspersed among the RWA sites of De Haan, Beisbroek and Roksem but in very low densities. *F. sanguinea* was only recorded at the edge of the site in Beisbroek and *L. fuliginosus* at the edge of Beisbroek and De Haan. Those nests were located farther than 100 m from the nearest RWA mound. *L. brunneus* was not observed near the RWA sites. Therefore we assume that the used isolation proxies calculated from only RWA mounds are accurate estimations for most myrmecophiles. Exceptions are the "pan"myrmecophilous species *Platyarthrus hoffmannseggii* and *Cyphoderus albinus* which also thrive in ant nests of common species (belonging to the genera *Lasius*, *Myrmica*, *Leptothorax* and *Tetramorium*) found in all RWA sites.

Data analyses

Multivariate analysis

In this analysis we were interested which factors structured the myrmecophile community as a whole and assessed their relative importance. Hence, we examined which variables could affect the species composition in a myrmecophile community.

We grouped the independent variables in three subsets: a) nest mound variables (= moisture, pH, nest size, ant species) b) within-site isolation variables (= S_{100} and d_{min}), and c) site variables (site size and site identity). For every category, we ran an RDA (Redundancy Analysis) with the presence-absence data of myrmecophile species as dependent community matrix. Then we selected a minimal number of significant variables by applying the *foward.sel* function in R package packfor for the three subsets of variables. Thereafter we applied variation partitioning following the methods of Peres-Neto et al. (2006) with the *varpart* function in the R package vegan. In this approach, the total variation (expressed in R^2) explained by the model is partitioned into unique and shared fractions of the subsets of predictors. Adjusted R^2 values were calculated for each fraction and provide unbiased estimates of the variation explained by those fractions (Peres-Neto et al. 2006). The significances of the fractions were tested by a permutation test (n=1000) using the function *anova*. Significant variables were plotted on an unconstrained Principal Component Analysis (PCA) with the presence-absence data as community matrix. D_{min} and mound size were ln transformed and S_{100} was square rooted. Continuous variables were centered and divided by their standard deviations.

Univariate analysis

In this analysis, we analysed the effect of multiple variables on the number of myrmecophile species found. Predictor variables (moisture, pH, mound size, ant species, isolation, site size and site) were regressed with (a) total species richness, (b) Staphylinidae species richness and (c) restricted myrmecophyle species richness (total species richness minus the panmyrmecophilous species *C. albinus* and *P. hoffmannseggii*), per 2 L volume fitting poisson generalized models with log link function. Goodness-of-fit tests based on likelihood ratio confirmed that models were Poisson error distributed.

We used the *dredge* function (package MuMIn) to rank models based on AICc (corrected Akaike's Information Criteria). The model with the lowest AICc was considered the model with the best support ('best model'). Other models for which the AICc difference (Δ AICc) with the best model were ≤ 2, are argued to have substantial support as well and were selected with the best model (Burnham and Anderson 2002). We calculated Akaike weights (wi) for those models, which represent the relative probability (ranging from 0 to 1) that a model is the best among the subset of candidate models. We used a model-averaging approach to estimate averages, standard errors and confidence intervals of parameters for the selected set of models. Estimates were weighted by the model's Akaike Weight (Burnham and Anderson 2002). Confidence intervals of those model-averaged estimates excluding 0 are significant at the $\alpha = 0.05$ level (Nakagawa and Cuthill 2007). In addition, we tested significance of factors of the 'best' models (lowest AICc) with Type II likelihood ratio tests using function *Anova* in R package car.

Mound size was ln transformed and S_{100} was square rooted. Continuous variables were centered and divided by their standard deviations. Sites were nested within site size classes in our models. We used S_{100} (total nest surface of other mounds within a radius of 100 m) as proxy for isolation in these univariate analyses. The effect of isolation on species richness was similar when employing d_{min} (nearest distance to other mound) as isolation measure, but models had higher AICc-values.

RESULTS

Distribution

Table 2.1 shows the mean abundances and proportions of nests occupied per species and indicates whether the myrmecophile was found with *F. rufa*, *F. polyctena* or both. Almost all myrmecophiles were observed with both host ant species. Exceptions were the spider *Mastigusa arietina* and the histerid beetle *Dendrophilus pygmaeus*, which both only occurred with *F. polyctena*. This is likely caused by the small number of individuals recorded (26 and 2, respectively). The most abundant species is the ant springtail *Cyphoderus albinus*, which occurred in more than 90% of the ant nests. This species can reach enormous abundances up to 1362 individuals per 2-L sample. The spider *Thyreosthenius biovatus* and the rove beetle *Thiasophila angulata* were also recorded in more than 50% of the sampled nests. The spider *Thyreosthenius biovatus* was only recorded three times in Belgium (pers. communication Dr. L. Baert). Yet, we found this spider in 80% of the mounds and in all sampled RWA sites. Table 2.2 gives an overview of the seven RWA sites: number of mounds, average species richness and number of RWA specific myrmecophiles (*Stenus aterrimus*, *Dinarda maerkelii*, *Clytra quadripunctata*, *Monotoma angusticollis*, *Monotoma conicicollis*, *Thyreosthenius biovatus*) in the study region. In the larger sites, more myrmecophile species were detected. This is a sampling effect (cf. number of sampled mounds), because average species richness per mound in small sites equals that of large sites. RWA specific myrmecophiles also occur in the smallest and highly isolated RWA sites (Fig. 2.1). On average, there was a comparable diversity of those RWA specifics in large and small sites (Table 2.2).

Table 2.1. List of observed species, total recorded individuals (N), mean number of individuals (Mean), proportion of occupied nests (%), maximum number of individuals found in a 2-L sample (Max). Host: myrmecophile associated with host *Formica rufa* (R) and/or *Formica polyctena* (P) in this study, Literature hosts: other host ant species occurring in the study area based on chapter 1: Parmentier et al. 2014, RWA = RWA species, Ffusc = *Formica fusca,* Fsang = *Formica sanguinea,* Lfuli = *Lasius fuliginosus*, Lbrun = *Lasius brunneus,* Lflav = *Lasius flavus,* L sp. = *Lasius* species).

Species	N	Mean	%	Max	Host	Literature hosts
COLEOPTERA						
Staphylinidae						
Stenus aterrimus Erichson, 1839	17	0.20	16.5	2	R+P	RWA
Thiasophila angulata (Erichson, 1837)	131	1.56	54.1	22	R+P	RWA/Fsang/Lfuli/Lbrun
Nothotecta flavipes (Gravenhorst, 1806)	12	0.14	11.8	2	R+P	RWA/Fsang/Lflav
Lyprocorrhe anceps (Erichson, 1837)	46	0.55	18.8	16	R+P	RWA/Lfuli
Amidobia talpa (Heer, 1841)	106	1.26	32.9	36	R+P	RWA/Lfuli
Dinarda maerkelii Kiesenwetter, 1843	10	0.12	10.6	2	R+P	RWA/Fsang
Quedius brevis Erichson, 1840	7	0.08	7.1	2	R+P	RWA/LFuli/Lbrun/Fsang
Leptacinus formicetorum Märkel, 1841	119	1.42	35.3	16	R+P	RWA/Lbrun
Chrysomelidae						
Clytra quadripunctata (Linnaeus, 1758)	159	1.89	45.9	23	R+P	RWA/Ffusc/Fsang
Monotomidae						
Monotoma angusticollis (Gyllenhal, 1827)	114	1.37	47.1	16	R+P	RWA
Monotoma conicicollis (Gyllenhal, 1827)	39	0.47	21.2	5	R+P	RWA
Histeridae						
Myrmetes paykulli Kanaar, 1979	14	0.17	15.3	2	R+P	RWA/(L sp.)
Dendrophilus pygmaeus (Linnaeus, 1758)	2	0.02	3.5	1	P	RWA/Lfuli
ARANEAE						
Thyreosthenius biovatus (O. P.-Cambridge, 1875)	450	5.36	80.0	24	R+P	RWA/(Ffusc)
Mastigusa arietina (Thorell, 1871)	26	0.31	10.6	7	P	RWA/Lfuli/Lbrun/Ffusc
ISOPODA						
Platyarthrus hoffmannseggii Brandt, 1833	259	3.1	16.5	109	R+P	all ants
COLLEMBOLA						
Cyphoderus albinus Nicolet, 1842	4500	54.2	91.8	1362	R+P	all ants

Table 2.2. RWA (RWA) site size (indicated by number of mounds), number of sampled mounds and total species richness and average species richness per mound of myrmecophiles and myrmecophiles specific to RWAs (*Thyreosthenius biovatus*, *Monotoma angusticollis*, *Monotoma conicicollis*, *Clytra quadripunctata*, *Stenus aterrimus* and *Dinarda maerkelii*).

Site	Total mounds	Sampled mounds	Total species	Total species specific to RWAs	Average species ± SE	Average species specific to RWAs ± SE
Beernem	49	20	16	5	5.30 ± 0.58	1.70 ± 0.30
West-Vleteren	37	20	15	6	5.60 ± 0.49	2.65 ± 0.25
Beisbroek	27	19	15	6	4.21 ± 0.38	2.05 ± 0.27
De Haan	14	11	13	6	5.27 ± 0.39	2.36 ± 0.32
Roksem	10	8	11	5	4.75 ± 0.61	2.25 ± 0.29
Aartrijke	3	3	8	2	5.67 ± 0.27	2.00 ± 0.00
Vladslo	2	2	10	5	6.50 ± 0.19	3.00 ± 0.71

Multivariate analysis

Forward selection of the mound characteristics subset retained the variables moisture, pH and host species. S_{100} and d_{min} of the within-site isolation subset were both selected. Site identity was selected, but site size was eliminated from the site subset. Fig. 2.2 illustrates the explained variation (based on adjusted R^2 values) of the myrmecophile community by the different subsets. Explained variation (12.9%) was

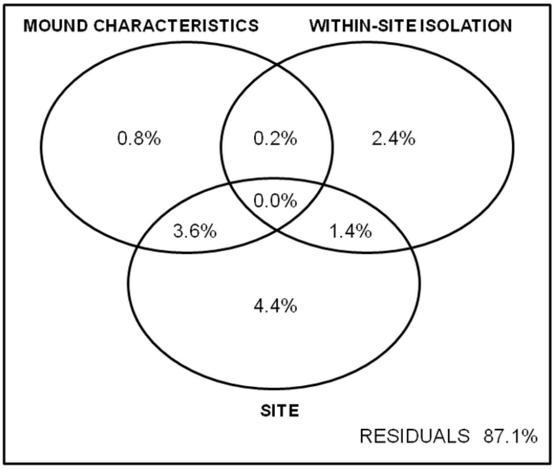

Figure 2.2. Variation partitioning based on adjusted R^2. Total variation is 100% and numbers represent proportions of explained variationby each fraction. Mound characteristics subset = pH, moisture, host, within-site isolation subset = d_{min} and S_{100}, site subset = site identity.

relatively low, indicating that random processes and possibly unrecorded variables have a large effect on species composition. Pure within-site isolation (isolation conditioned for site and mound characteristics) (explained variation = 2.4%, $P = 0.002$) and pure site identity (conditioned for isolation and mound characteristics) (explained variation = 4.4%, $P = 0.004$) structured significantly the myrmecophile community. Pure mound characteristics (conditioned for isolation and site identity) ($P = 0.151$) had no effect on the composition of the myrmecophile community. Mound characteristics in particular sites also explained a large fraction (3.6%). The PCA plot depicts that most myrmecophiles are correlated with increasing S_{100} and/or decreasing d_{min}, so their occurrence increases with decreasing isolation (Fig. 2.3). Mounds of different sites are not separated in distinct clusters, but show some structuring corresponding with the results of variation partitioning. Mounds of large RWA sites are similar in species composition to those of small RWA sites (95% confidence ellipses overlapping). The species composition of the community associated with *F. rufa* is only slightly different from (95% confidence ellipses slightly distinct) the community associated with *F. polyctena*. These differences could result from the correlation between site and host ant species, i.e. some sites supported one RWA or had a majority of one species. Therefore site differences in myrmecophile prevalence could cause differences in host species preference. In the variation partitioning analysis, this variation could be captured by the fraction shared by site and mound characteristics.

Univariate analysis

Table 2.3. Overview of model selection for models explaining (a) total myrmecophile species richness, (b) Staphylinidae species richness and (c) restricted myrmecophile species richness (total species richness minus the panmyrmecophilous species *C. albinus* and *P. hoffmannseggii*). Models are ranked from the lowest AICc value (= 'best' model) to higher AICc values (decreasing likelihood). Only models with Δ AICc ≤ 2 are selected. Akaike's weight (wi) indicate the likelihood of a model, given the set of models being considered (Burnham and Anderson 2002).

Species richness		Model	df	AICc	Δ AICc	wi
(a)	Total	(intercept)+S_{100}	2	349.78	0.00	0.35
		(intercept)+S_{100}+pH	3	350.50	0.71	0.25
		(intercept)+S_{100}+moisture	3	350.51	0.73	0.25
		(intercept)+S_{100}+site size	3	351.47	1.69	0.15
(b)	Staphylinidae	(intercept)+S_{100}	2	270.3	0.00	0.54
		(intercept)+S_{100}+moisture	3	271.8	1.52	0.25
		(intercept)+S_{100}+mound size	3	272.2	1.91	0.21
(c)	Restricted myrmecophiles	(intercept)+S_{100}	2	343.12	0.00	0.38
		(intercept)+S_{100}+moisture	3	343.66	0.54	0.29
		(intercept)+S_{100}+pH	3	344.52	1.40	0.19
		(intercept)+S_{100}+site size	3	345.04	1.91	0.15

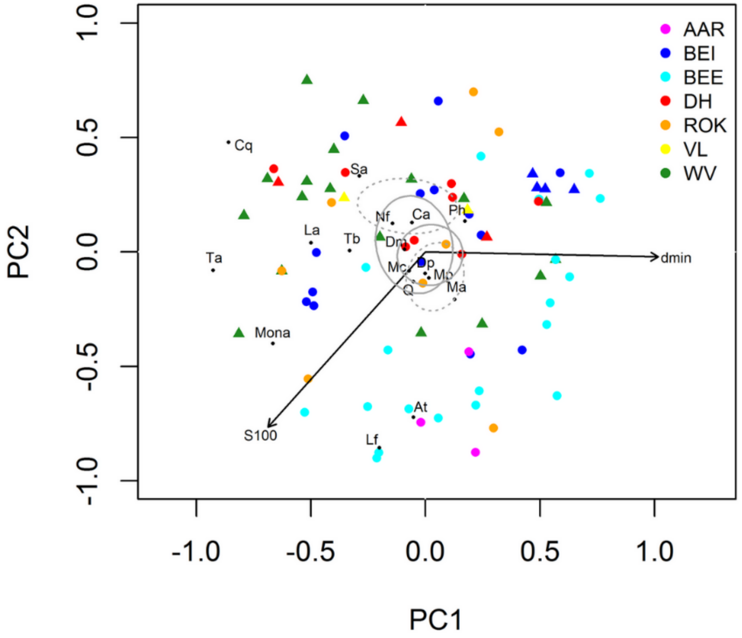

Figure 2.3. PCA diagram with species, mounds and significantvariables of RDA analyses plotted. Mounds are sorted according to host ant species (*F. rufa* = triangle, *F. polyctena* = circle) and site (different colors). Isolation decreases with higher S_{100} but increases with higher d_{min}. Most species are positively correlated with S_{100} and/or negatively with d_{min}. At = *Amidobia talpa*, Cq = *Clytra quadripunctata*, Ca = *Cyphoderus albinus*, Dend = *Dendrophilus pygmaeus*, Dm = *Dinarda maerkelii*, Lf = *Leptacinus formicetorum*, La = *Lyprocorrhe anceps*, Ma = *Mastigusa arietina*, Mona = *Monotoma angusticollis*, Mc = *Monotoma conicicollis*, Mp = *Myrmetes paykulli*, Nf = *Notothecta flavipes*, Ph = *Platyarthrus hoffmannseggii*, Q = *Quedius brevis*, Sa = *Stenus aterrimus*, Ta = *Thiasophila angulata*, Tb = *Thyreosthenius biovatus*. Host ant species 95% confidence ellipses indicated with gray dotted ellipses: upper ellipse *F. rufa*, lower ellipse *F. polyctena*.Site size 95% confidence ellipses indicated with gray full ellipses: left ellipse small site, right ellipse large site.

Table 2.4. Overview of model-averaged estimates for the log linear Poisson models explaining the log of the response variables: (a) total myrmecophile species richness, (b) Staphylinidae species richness and (c) restricted myrmecophile species richness. Estimates are averaged for all models with ΔAICc ≤ 2 and weighted by each model's Akaike weigth (wi). Relative variable importance (w_{ip}) of a particular variable is the sum of all wi's of models incorporating that variable. Isolation decreases with higher S_{100}. Thus positive S_{100} slopes correspond with higher diversity in less isolated mounds. 95% CI of predictors not encompassing 0 are given in bold.

Species richness		predictor variable	estimate	95% CI	w_{ip}
(a)	total	(intercept)	1.62	1.52 to 1.72	
		S_{100}	0.14	0.05 to 0.24	1.00
		moisture	-0.02	-0.16 to 0.04	0.25
		pH	0.02	-0.04 to 0.17	0.25
		host ant			
		mound size			
		site size	0.01	-0.14 to 0.29	0.15
		site			
(b)	Staphylinidae	(intercept)	0.55	0.38 to 0.72	
		S_{100}	0.33	0.16 to 0.49	1.00
		moisture	-0.02	-0.24 to 0.10	0.25
		pH			
		host ant			
		mound size	-0.01	-0.22 to 0.14	0.21
		site size			
		site			
(c)	Restricted myrmecophiles	(intercept)	1.37	1.25 to 1.48	
		S_{100}	0.20	0.09 to 0.31	1.00
		moisture	-0.02	-0.18 to 0.04	0.29
		pH	0.01	-0.07 to 0.17	0.19
		host ant			
		mound size			
		site size	0.01	-0.18 to 0.30	0.15
		site			

Table 2.3 reports the selected set of models for which Δ AICc ≤ 2. The best model explaining either (a) total species richness, (b) Staphylinidae richness or (c) restricted myrmecophile species richness (total species richness minus the panmyrmecophilous species *C. albinus* and *P. hoffmannseggii*), was a model with an intercept and only the predictor variable S_{100} incorporated. Other well supported models (Δ AICc ≤ 2) always incorporated S_{100} and one other predictor variable. Parameter averaging across those models for which Δ AICc ≤ 2 and respectively explaining (a) total species richness, (b) Staphylinidae richness or (c) restricted myrmecophile species richness is given in Table 2.4. For the three species richness measures, only the 95% CIs of S_{100} do not encompass 0. This indicates that this factor is significant in the averaged model. The relationship between increasing S_{100} and species richness of total myrmecophiles, Staphylinidae and restricted myrmecophiles is given in Fig. 2.4. The S_{100} effect size is higher for restricted myrmecophile richness compared with total species richness (Table 2.4). The effect of isolation is hence lower on species richness when also considering the panmyrmecophilous species. This is logical because *C. albinus* and *P. hoffmannseggii* also occupy nests of other ant species among the nest mounds in the

study sites. Staphylinidae species richness is more severely affected by isolation compared with the average effect of isolation on restricted myrmecophiles. The factor S_{100} was also highly significant in the 'best' models (lowest AICc) with only S_{100} as predictor variable ((a) total species richness: $P = 0.005$, (b) Staphylinidae species richness: $P < 0.001$ (c) restricted myrmecophile species richness: $P < 0.001$).

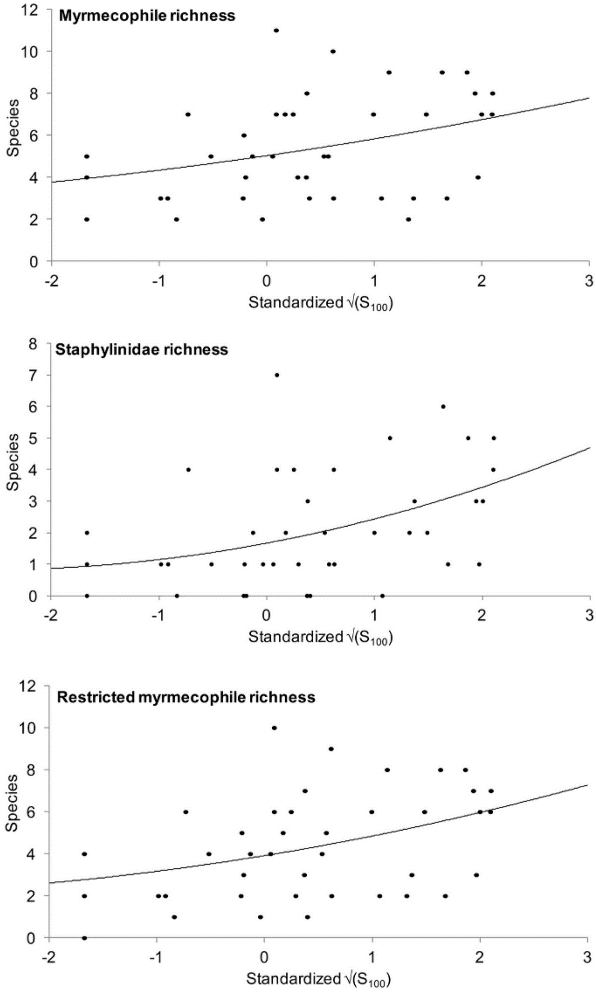

Fig. 2.4. Relationship between increasing S_{100} (increasing amount of nest surface of other mounds within 100 metres = decreasing isolation) and species richness of total myrmecophiles, Staphylinidae and restricted myrmecophiles (= total myrmecophile species richness minus panmyrmecophilous *Cyphoderus albinus* and *Platyarthrus hoffmannseggii*). Fitted models are based on model-averaged coefficients.

DISCUSSION

Our study demonstrates that severely fragmented wood ant populations still support a relatively diverse group of myrmecophiles. We found in this study 17 obligate myrmecophile species including six specialist RWA associates. Studies in large forest complexes in Finland recorded a similar diversity: Päivinen et al. (2004) found 16 beetle species in 49 mounds of *F. aquilonia*, Härkönen and Sorvari (2014) reported 22 myrmecophiles in 12 mounds with *F. polyctena.* In this study, *F. rufa* and *F. polyctena* did not differ in total myrmecophile species richness and staphylinid species richness and their community composition was similar. The myrmecophiles in this study are also associated with other RWA species (*F. lugubris*, *F. aquilonia, F. pratensis*) suggesting that the myrmecophile community is probably similar for all six European RWAs (Päivinen et al. 2004, Lapeva-Gjonova and Lieff 2012, Robinson and Robinson 2013, chapter 1: Parmentier et al. 2014).

RWA mounds as patches in a myrmecophile metapopulation / metacommunity

Myrmecophiles perceive ant nests as small suitable patches distributed in a hostile landscape matrix. In the studied sites, RWA mound distribution is highly heterogeneous ranging from highly isolated to well connected and ultimately to polydomous aggregations. In accordance with the predictions of metapopulation theory, we found very strong evidence that myrmecophile and rove beetle diversity was positively correlated with mound connectivity. These results are akin to Päivinen et al. (2004) and Härkönen and Sorvari (2014) who reported a negative correlation between myrmecophile diversity and nest isolation in *F. aquilonia* and *F. polyctena*. Variation partitioning demonstrated that among site differences explained more variation in the myrmecophile community than within-site isolation. This suggests that processes at a larger spatial scale than the myrmecophile metacommunity in a particular RWA site are important as well. These processes could include the spatial distribution and isolation of the sites (i.e. myrmecophiles are organized in a metacommunity of metacommunities). Site isolation, however, is hard to estimate as most species occur with more general ant species as well.

Larger patches can support larger populations and are more likely to be colonized in metapopulation models. Therefore large patches have a higher occupation probability in classical metapopulation models (Hanski 1994). In contrast with Päivinen et al. (2004) we did not find a relationship between mound size and diversity or prevalence. However, we sampled a fixed amount of nest material from all nests, rather than the whole mound. So we measured density rather than population sizes. Therefore total species richness and population sizes are probably higher in large nests.

Local patch characteristics have been demonstrated as a third factor affecting metapopulation and metacommunity dynamics (Ranius 2000, Thomas et al. 2001, Jeffries 2005). Moisture and pH are two key abiotic variables that structure the soil arthropod community and might thus determine the quality of wood ant mounds for myrmecophiles as well (Giller 1996). Generally, dry and/or acid soils tend to have lower diversity and abundances of soil fauna (Giller 1996, Tsiafouli et al. 2005). Additionally, there are indications of differential niche preference amongst soil organisms, whereby related species favor different conditions along the soil pH and soil moisture gradients (Giller 1996). The sampled RWA mounds varied considerably in acidity (pH: 3.10 - 6.33) and moisture content (5% - 67%), but this had no effect on total diversity or community composition.

Metapopulations need a minimum number of patches for long-term persistence, commonly referred to as the minimum viable metapopulation size (MVM) (Hanski et al. 1996). A key result of this study is that (very) small and isolated RWA populations can have a very diverse myrmecophile community, suggesting that MVM is low for RWA myrmecophiles. This can be partly explained by other ant host nests which can serve as stepping stones. However, alternative host ant nests were rare for most myrmecophiles. Moreover RWA specific myrmecophiles such as *T. biovatus* and *M. angusticollis* were also found in the small RWA sites, indicating that those myrmecophiles could persist in very small metapopulations. A RWA mounds provide a warm and moist environment with plenty of food resources (Skinner 1980, Rosengren et al. 1987, Frouz et al. 2005). Moreover, large numbers of queens occur in single mounds and regularly new mounds bud from the nest (pers. observations TP). Therefore the extinction risk of the mound, colony and population at a larger scale is relative low without major disturbances. Hence, a small number of highly connected mounds might support small, stable myrmecophile metapopulations for a long period.

Ant nests and their associates as terrestrial model systems in metapopulation and metacommunity research

Ant nests provide suitable systems to test metapopulation and metacommunity theory. Nests of ants are clearly delineated islands in a matrix of unsuitable habitat for obligate myrmecophiles. Therefore “patch” dimensions, “patch” isolation and connectivity measures are easy to quantify. Because of their small size, homogenous and representative samples can be easily collected and rapidly extended to a large number of patches. Ant nests are abundant and many myrmecophiles or myrmecophile communities are widespread. This allows hypothesis testing on both a broad spatial scale (e.g. regional effects on metacommunity dynamics) and on a local scale (e.g. the effect of altitudinal and environmental variables on the metacommunity dynamics of panmyrmecophilous species when multiple hosts are available in a site). Ant nests vary in longevity and regularly new nests are founded independently or budded from other nests (e.g. RWA nest life span ranges from less than 1 year to more than 70 years (Klimetzek 1981, Gösswald 1989). This nest dynamism facilitates the tracking of colonization, succession and competition (cf. competition-colonization trade-off). Promising ant hosts to test metacommunity hypotheses are especially RWAs (*F. rufa* group), *Formica sanguinea* and the shiny black wood ant *Lasius fuliginosus*. Their nests are easy to track, they have a wide distribution and they support a large diversity of species in one nest (Päivinen et al. 2003, chapter 1: Parmentier et al. 2014). Overall, the properties of ant nests correspond thus with classic theoretical metapopulations and metacommunities. They are valuable tools to broaden our knowledge on general questions in evolution and community functioning.

ACKNOWLEDGEMENTS

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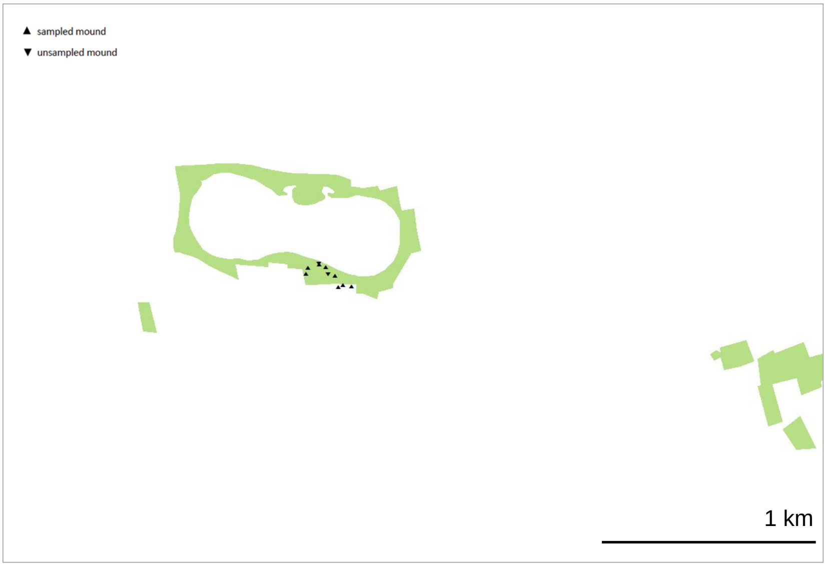
APPENDIX CHAPTER 2

Appendix 2-1: Detailed maps of different RWA sites.

site numbers correspond to overview map of Fig. 2.1

1. DE HAAN

2. ROKSEM



3. BEISBROEK

4. AARTRIJKE

5. BEERNEM

6. VLADSLO

7. WEST-VLETEREN

CHAPTER 3

Context-dependent specialization in colony defence in the red wood ant *Formica rufa*

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ABSTRACT

In many species, specialized defence traits and strategies are crucial for surviving enemy attacks or securing resources. In numerous social insect lineages, a morphologically and behaviourally distinct soldier caste specializes in colony defence, with larger foragers typically engaging most in the aggressive defence of the colony against external threats. We hypothesized, however, that specialization in aggression could show vastly different patterns in the context of the defence against small intranidal parasites that prey on brood. This is because we expected that small, intranidal nurse workers could be better suited to defend against these parasitic myrmecophiles (= ant associates) due to their better matching size, high encounter rate and the high task switching costs that would occur if foragers had to carry out this task. Here, we present data that support this hypothesis from a study on specialization in defence against two parasites in the red wood ant *Formica rufa*. In particular, we show that small workers displayed the strongest aggressive behaviour towards the parasitic rove beetle *Thiasophila angulata* and the spider *Thyreosthenius biovatus*, and present evidence that small workers were better at preventing brood predation than larger workers. In addition, there was worker task specialization in defensive behaviour, with nurses and workers at nest entrances being more aggressive towards *T. angulata* than extranidal foragers. We argue that this context-dependent specialization in aggression and nest defence was likely to be important in favouring the pronounced worker polymorphism observed in both this and other ant species and discuss our findings in relation to models for the evolution of division of labour and caste polymorphism in insect societies.

INTRODUCTION

Across the animal kingdom, aggression is the predominant form of behaviour to acquire or defend vital resources (Krebs and Davies 2012, Grether et al. 2013) and both intraspecific and interspecific animal contests are frequently settled on the basis of size asymmetries (Reichert 1998). In group-living organisms defence can be shared by group members or in some cases allows for task specialization. Specific members will then act as specialized defenders as demonstrated in, for example, cichlid fishes, spiders and naked mole-rats (Lacey and Sherman 1991, Bruintjes and Taborsky 2011, Pruitt and Riechert 2011). Social insects are especially good models for exploring defence specialization as they have such distinct morphological and behavioural specializations. In numerous social insect lineages, the size advantage of large individuals has led to the evolution of a morphologically distinct caste of larger and more aggressive soldiers, which specialize in defending the colony (Hölldolber and Wilson 1990, Nowbahari et al. 1999). Indeed, an evolved soldier caste occurs not only in some ants, bees and termites but also in eusocial aphids, gall-dwelling thrips and snapping shrimps (Hölldobler and Wilson 1990, Tian and Zhou 2014). When physical castes occur, their presence generally benefits the productivity or survival of the colony (Hölldobler and Wilson 1990, Billick and Carter 2007, Modlmeier and Foitzik 2011). Nevertheless, a morphological caste system may also have costs, as it may prevent a colony from rapidly adjusting caste ratios, increase the energetic rearing cost or limit the task repertoire (Oster and Wilson 1978, Hölldobler and Wilson 1990). These costs may explain why, in the majority of social insect species, workers only specialize behaviourally in different tasks, usually in relation to their age (‘age polyethism’, Oster and Wilson 1978, Hölldolber and Wilson 1990). In this case, young workers typically perform safe tasks inside the nest first, such as nursing the brood, and only later in life move on to more risky tasks outside the nest, such as foraging or territorial defence (Hölldobler and Wilson 1990, Sturgis and Gordon 2013), a configuration that has been shown to optimize colony efficiency (Tofilski 2002).

Although the studies cited above demonstrate that in many social insect species, a morphologically and behaviourally distinct soldier caste may specialize in colony defence, and that larger and older foragers typically engage most in the aggressive defence of the colony (Hölldolber and Wilson 1990, Nowbahari et al. 1999, Wilgenburg et al. 2010), this pattern has been demonstrated almost exclusively in relation to the defence against outside threats by large enemies, such as competitor ants or vertebrates. We hypothesized, however, that specialization in aggression could show a directly opposite pattern in the context of defence against small intranidal parasites preying on brood. This is because we expected that small, intranidal nurse workers could be better suited to defend against such enemies than large foragers due to their better matching size, their more frequent interaction with the brood and the parasites and the high costs that would occur if foragers had to regularly switch to carrying out defensive tasks inside the colony (Duarte et al. 2011, Goldsby et al. 2012). The aim of this study was to provide the first test of this adaptive theory on context specificity in task specialization in aggression. To do so, we used the red wood ant (RWA), *Formica rufa*, and two associated brood parasitic myrmecophiles, the rove beetle, *Thiasophila angulata*, and the linyphiid spider *Thyreosthenius biovatus*, as a model. In this size-polymorphic ant, large foragers have been shown to be more involved in hunting and defence against other colonies (Higashi 1974, Herbers 1979, McIver and Loomis 1993, Wright et al. 2000, Tanner 2008, Parmentier 2010, Batchelor and Briffa 2011). In our study, however, we tested whether a different pattern holds in terms of worker size and task group (nurse, forager, mound worker) and specialization in aggression in the context of defence against intranidal, myrmecophile parasites preying on brood. In addition, we assessed for one of the parasites whether small nurses were better brood defenders than large nurses.

MATERIAL AND METHODS

Study species

Red wood ants

Red wood ants (*Formica rufa* group) are moderately polymorphic, displaying a pronounced size variation (4.5-9 mm), but lack discrete subcastes with shape specialization (van Boven 1986). RWA workers have been reported to perform different roles according to both age and size (Higashi 1974, Herbers 1979, McIver and Loomis 1993, Wright et al. 2000, Tanner 2008, Parmentier 2010)(Higashi 1974, Tanner 2008, Parmentier 2010). Young workers nurse the brood (mainly small, young workers) or do not participate in tasks (mainly large, young workers). Workers of intermediate age are engaged in intranidal building (mainly small workers) or repairing the nest surface (mainly large workers). Finally, the oldest workers tend to forage for food. Small, old workers are mainly allocated to aphid tending close to the nest, whereas large, old workers mainly hunt for prey and tend aphids at larger distances. Large RWA workers are more aggressive towards conspecific workers (Batchelor and Briffa 2011). Ant workers can switch task depending on the needs of the colony (Hölldobler and Wilson

1990). However, foragers in RWAs, in particular, are rather consistent in doing their task (Rosengren and Fortelius 1986, Parmentier et al. 2012).

Red wood ant-associated myrmecophiles

Myrmecophiles or ‘ant guests’ live in close association with ants and are able to penetrate into the deepest parts of the nests. Myrmecophiles’ life strategies are very diverse, with some being commensals, some ecto- or endoparasites and others parasites that prey on brood (here also referred to as brood predators), steal food (kleptoparasites) or feed on ants (myrmecophages) (Hölldobler and Wilson 1990). Myrmecophiles can integrate into ant colonies based on the presence of specific chemical (e.g. adoption of the host colony odour, emitting repellent compounds, appeasement glands), behavioural (e.g. swift movements, death feigning) and morphological (e.g. small, slender body, short appendages, myrmecomorphic) adaptations (Hölldobler and Wilson 1990). These adaptations prevent ants deterring or killing these myrmecophiles. Parasites of ants are widespread and could impose high costs, although their long-term impact is poorly understood (Hölldobler and Wilson 1990, von Beeren et al. 2011, Hovestadt et al. 2012). An especially rich community of myrmecophiles, of which some are parasites that prey on brood, can be found living inside European RWA colonies (chapter 1: Parmentier et al. 2014).

As model species we used the parasitic brood predators *T. angulata* and *T. biovatus*. The rove beetle, *T. angulata*, is a typical scavenger that feeds on prey items collected by the ants. It has been observed eating ant eggs and resides in the ants’ brood chambers. We confirmed this in preliminary nest location tests (full tests see chapter 5) with artificial nests of six connected pots (9 cm diameter, 5 cm height) filled with 1 cm plaster and nest material. We transferred 360 workers, 150 pupae and 90 larvae to the nest. After 1 day, ant workers concentrated all brood in one chamber (hereafter called the brood chamber). Worker density was also highest in this chamber. Then *T. angulata* individuals were randomly divided over the six chambers. After 3 days, 22 beetle individuals were found in the brood chamber and 18 in the other five chambers. he small linyphiid spider *T. biovatus* is also strictly associated with RWAs (chapter 1: Parmentier et al. 2014). It also occurs in the brood chambers as demonstrated by similar nest location tests as explained above, in which eight individuals were located in the brood chamber and eight in the other five chambers. The spider was observed eating ant eggs and small larvae. Both species can be common in the RWA nests in our study population. For example, in one nest, we found 24 *T. biovatus* spiders and in another 22 *T. angulata* beetles in a 2-litre sample.

Sample collection

Figure 3.1. Size-polymorphic *F. rufa* red wood ant workers shown alongside the two parasites studied here, the rove beetle *T. angulata* and the spider *T. biovatus*. Photo: T. Parmentier

We collected *F. rufa* workers and associated *T. angulata* rove beetles of five distinct colonies originating from two populations (West-Vleteren: WV1, WV2, WV3, Vladso: VL) in western Flanders, Belgium and one population (Boeschepe: BOE) in northern France during 2012-2014. *Thyreosthenius biovatus* spiders were taken from WV1, WV2 and WV3 in the same period. We collected the myrmecophiles by spreading out nest material on a large tray in the field. Beetles and spiders were kept together with workers and nest material of the colony of origin until the start of the experiments (Fig. 3.1).

Experiments

Experiment 1: specialization in aggression towards parasites

We sampled workers and classified them as performing one of three different tasks. Workers following pheromone trails heading towards trees with aphids (which are milked for food) were classified as foragers (> 5 m from nest). Mound workers were workers that stayed near the nest openings. They differed from returning and outgoing foragers, which walked determinedly in straight lines to or away from the nest openings on the mound. Finally, we took a nest sample from the deep underground part of the nest and spread it out on a tray. These samples consisted of an enormous amount of eggs, larvae and queens, which indicated that we took samples of the deep brood chambers. We classified workers in these deep samples as nurses when they carried eggs or larvae into safety in the tray in the field. For each task, we selected workers over the complete size range of the colony. Mound workers found in this study were expected to have an intermediate age. All three tasks are done by workers over the entire range (see x-axis range in Fig. 3.2 for three tasks). However, the average size of nurses is smaller than the average size of foragers and mound workers (Higashi 1974, Herbers 1979, Parmentier 2010). Workers were placed in a circular, plastic cup (7 cm diameter, 5 cm height) with a bottom layer of plaster of Paris (ca. 1 cm) and with the inner side coated with Fluon. After an hour of acclimatization, a *T. angulata* rove beetle found in the same nest as the focal ant was added. After 30 s, 15 consecutive interactions (in some trials 14) between ant and beetle were recorded. Ant-parasite aggression was quantified as the proportion of interactions that were aggressive (either biting, snapping and opening of mandibles) out of the total interactions. This experiment was done blind with respect to task and trials were tested in three colonies (WV1, WV2, VL). We used 40 beetles in 274 trials in total (total for three colonies: $N_{\text{nurse}} = 106$, $N_{\text{forager}} = 88$, $N_{\text{mound worker}} = 80$), but with at least 1 h between consecutive trials. Head width was used as a proxy for size and was measured after the experiment with a binocular microscope (40X).

Similar aggression tests were conducted between mound workers and *T. biovatus*. Here we only tested the effect of size variation and not the effect of worker task on aggression. Aggression trials were tested in three colonies (WV1, WV2, WV3). Spiders (18 in total) were used in 90 trials in total, but with at least 1 h between consecutive trials. The beetle and spider behaviour did not seem to change after spiders were reused. Individuals were also not wounded during the interactions.

Experiment 2: defence against brood predation

Here we tested whether small nurses were better defenders of the brood. To do so, a set of either five small nurses (head width < 1.4 mm, average mean per set ± SE = 1.09 ± 0.11 mm) or five large nurses (head width > 1.6 mm, average mean per set ± SE = 1.81 ± 0.12 mm) were placed in a small vial (4.5 cm diameter) filled with ca. 1 cm of moistened plaster of Paris. Subsequently, we placed five RWA eggs in the centre and introduced a *T. angulata* rove beetle, after which we counted the eggs eaten after 24 h. Additionally, a control with a beetle and without ants was performed. These three treatments (control - small nurses - large nurses) were repeated in two *F. rufa* colonies (WV1, BOE, total for two colonies: $N_{\text{control}} = 36$, $N_{\text{large}} = 37$, $N_{\text{small}} = 36$). Nurses, brood and *T. angulata* beetle in a trial originated from the same colony. A different beetle was used for every test. Workers readily started to nurse, lick and transport the eggs when introduced in the arena. For the spider *T. biovatus*, we also used a control experiment with five eggs to validate its status as brood predator and compare the potential impact of both myrmecophiles.

Data analyses

Experiment 1: specialization in aggression towards parasites

The probability of aggressive acts occurring towards *T. angulata* was modelled using a GLMM (generalized linear mixed model) with binomial error distribution and logit link function using package lme4 version 1.1-6 in R version 3.0.1 (R Core Team 2014). The full model included head width, task (nurse, forager and mound worker) and the interaction between these two variables as fixed factors, and colony and the individual beetle used as nested random factors (beetle nested in colony). We also included an observation level random factor to account for overdispersion (Browne et al. 2005). Backward model selection was performed with the drop1 function (Wald chi-square test), to remove nonsignificant fixed predictors from the model. Similarly, the proportion of aggressive acts of mound workers towards the spider *T. biovatus* were fitted with a binomial GLMM, but here only size was modelled as a fixed factor. Colony and individual spider were coded as nested random factors (spider nested in colony), and an observation level factor was again incorporated to take into account possible overdispersion. Significance of the fixed factors in both (final) models was tested with likelihood ratio tests (LRT, mixed function, package afex, version 0.9-109) in R. Fisher's LSD tests were used as post hoc tests in the *T. angulata* model to compare pairwise the three tasks (glht function, package multcomp 1.3-3).

Experiment 2: defence against brood predation

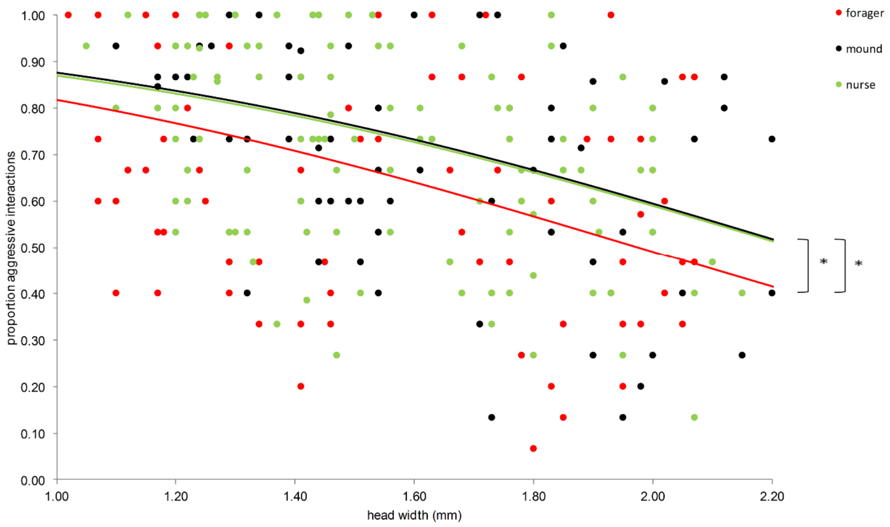
The proportion of the eggs that were eaten by *T. angulata* was compared among treatment conditions (with control, small or large workers) and colonies, as well as their interaction using a fixed-factor GLM (generalized linear model) with binomial error distribution. We accounted for overdispersion by using a model of the quasibinomial family. Backward model selection was performed with the drop1 function (Wald chi-square test) to remove nonsignificant predictors from our model. Significance of the predictor of the final model was assessed using LRTs (Anova function, package car) in R (R Core Team 2014). Fisher's LSD tests were used as post hoc tests (glht function, package multcomp 1.3-3).

RESULTS

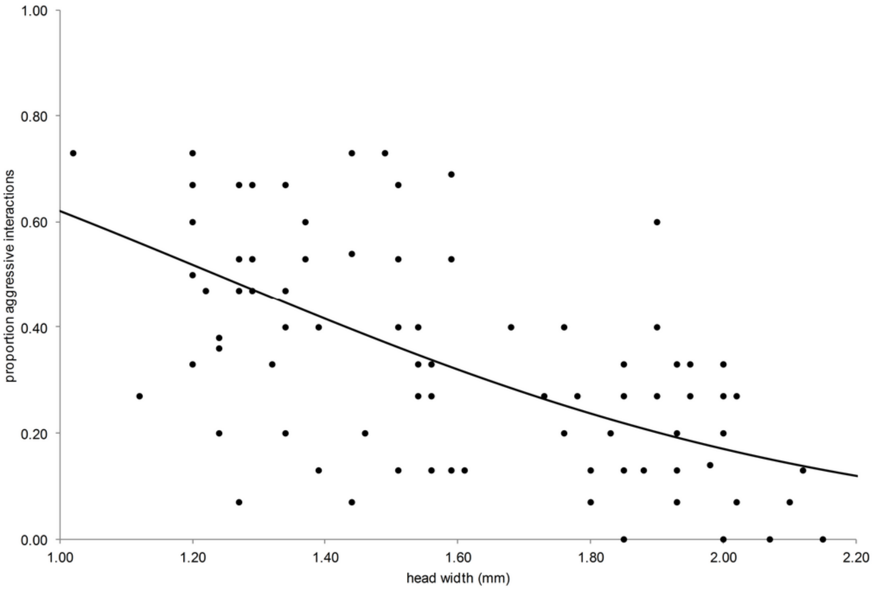
Experiment 1: specialization in aggression towards parasites

The rove beetle *T. angulata* escaped ant aggression by quickly running away. It often bent its abdomen, which is a typical defence strategy of rove beetles by which they emit defensive chemicals. The beetle never attacked ant workers. In terms of size and task group specialization in defence against this beetle, model selection resulted in a model in which worker size and task were included as main effect factors. As expected by our hypothesis, small workers showed significant specialization in defence against this beetle, as the likelihood of aggression of ant workers towards the beetle declined with increasing worker size (binomial GLMM, LRT: $\chi^2 = 40.11$, $P < 0.0001$, Fig. 3.2). In addition, worker aggression was affected by the task group to which the worker belonged (binomial GLMM, LRT: $\chi^2 = 6.85$, $P = 0.033$, Fig. 3.2), with foragers being less aggressive than either nurses ($z = 2.366$, Fisher LSD: $P = 0.018$) or mound workers ($z = 2.212$, Fisher LSD: $P = 0.027$), but with the aggression of mound workers and nurses not being significantly different from one another ($z = -0.0003$, Fisher LSD: $P = 0.999$). The spider *T. biovatus* avoided ant aggression by running away and the spider never attacked ant workers. Workers, however, also showed a clear size specialization in aggression towards this spider, with small workers once again being more aggressive (binomial GLMM, LRT: $\chi^2 = 39.65$, $P < 0.0001$, Fig. 3.3).

Both myrmecophiles occurred in the brood chambers of the ants (see also chapter 5). In none of the cases in which they were attacked were they killed or wounded. The beetle escaped by rapid movements or by emitting chemicals from the abdomen. Its slender body hampered the ants' attempts to grasp the beetle. The spider elicited less aggression and could often walk freely among the ants. It avoided being bitten by quickly running away. These observations suggest that the spider may use chemical mimicry (displaying a chemical profile similar to the ant host) or chemical insignificance (expressing a small amount of recognizable odour cues) to avoid detection (van Zweden and d'Ettorre 2010).



Figuur 3.2. Proportion of aggressive interactions performed by three *F. rufa* task groups (‘nurse’, ‘mound worker’ and ‘forager’) against the rove beetle *T. angulata* in relation to the workers' size. Binomial GLMM: effect of worker size on the probability of aggression: P < 0.001, effect of task group on the probability of aggression: P ¼ 0.033. Fisher's LSD post hoc tests of the three tasks: *P < 0.05. Note that lines with fitted model predictions for nurses and mound workers are overlapping on the figure.



Figuur 3.3. Proportion of aggressive interactions performed by *F. rufa* workers against the parasitic spider *T. biovatus* in relation to the workers' size. Binomial GLMM: effect of worker size on the probability of aggression: P < 0.001.

Experiment 2: defence against brood predation

The rove beetle was found to heavily prey on the ant eggs in the control treatment without workers (proportion of eggs eaten: 0.85, 95% confidence interval, CI: 0.73-0.92). The spider imposed lower costs in terms of brood predation (proportion of eggs eaten: 0.19 ± 0.06 SE), which could explain why the beetle also elicited more aggression than the spider (see results above and Scharf et al. 2011 and von Beeren et al. 2011. In terms of specialization in protection against brood predation by the rove beetle, model selection resulted in a model in which treatment (control, small or large workers) was included as the main effect factor. The proportion of eggs eaten by the rove beetle was significantly reduced in the presence of ants (binomial GLM, LRT: $\chi^2 = 35.636$, $P < 0.0001$, Fig. 3.4). Both small (proportion of eggs eaten: 0.389, 95% CI: 0.27-0.53, $z = -4.605$, Fisher LSD: $P < 0.0001$) and large workers (proportion of eggs eaten: 0.639, 95% CI: 0.50-0.76, $z = -2.422$, Fisher LSD: $P = 0.015$) reduced the proportion of eggs eaten compared with the control treatment without ants. Nevertheless, in support of our a priori hypothesis, small nurses were significantly more efficient than large nurses in protecting the brood against *T. angulata* ($z = -2.592$, Fisher LSD: $P = 0.010$).

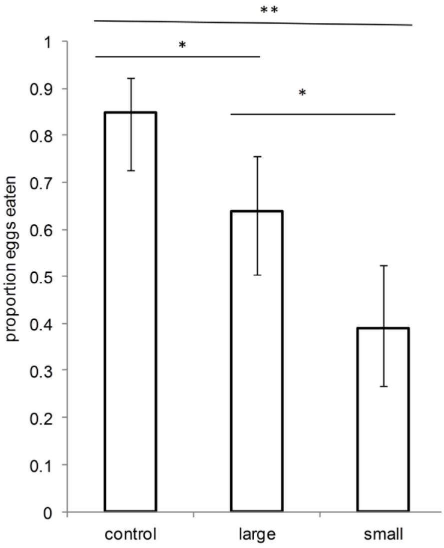


Figure 3.4. Proportion of eggs eaten by *T. angulata* in relation to the size of *F. rufa* workers. Bars show the proportion of ant eggs (± 95% confidence intervals) that were eaten in an arena by a *T. angulata* individual in the presence of five large nurses (large) and five small nurses (small) as well as in the absence of any ants (control). Binomial GLM: *P < 0.05, **P < 0.001.

DISCUSSION

Overall, our results demonstrate that specialization in defence against intranidal parasites preying on brood in the RWA *F. rufa* shows vastly different patterns from those documented previously in the context of defence against large, external enemies, such as vertebrates or other ants (Lamon and Topoff 1981, Moffett 1985, Hölldolber and Wilson 1990, Batchelor et al. 2012), and we discuss these results in the context of the evolution of division of labour and caste polymorphism in insect societies (Oster and Wilson 1978, Hasegawa 1997, Beshers and Fewell 2001).

A first key result was that there was significant size specialization in aggression, but that small workers were more aggressive towards the parasites than large ones, which contrasts with the traditional results of large workers generally being more aggressive and efficient in nest defence in size-polymorphic ant species (Lamon and Topoff 1981, Moffett 1985, Hölldolber and Wilson 1990, Batchelor et al. 2012). Two observations supported this conclusion: worker aggression towards the myrmecophile parasites *T. angulata* and *T. biovatus* were negatively correlated with worker size and small nurses were more efficient at decreasing egg predation by *T*. *angulata* than large nurses. These results diverge from earlier results that showed that large *F. rufa* workers were supreme fighters in clashes with other colonies (Batchelor et al. 2012), being more aggressive, living longer in fights and compensating for the poor fighting capabilities of small workers in fights between rival groups of workers (Batchelor and Briffa 2011, Batchelor et al. 2012). Based on this, we hypothesize that small workers detect small myrmecophiles more efficiently. Indeed, both myrmecophiles studied are fairly small and match the size of the smallest workers (Fig. 3.1). Small workers bear their antennae closer to the soil surface which could promote the detection rate of small animals. In addition, small workers of polymorphic *Camponotus* ants have been shown to bear more sensillae on their antennae and have more antennal glomeruli in their brain to process olfactory stimuli compared with medium and large workers (Mysore et al. 2009, 2010). A similar morphological adaptation could promote the detection of small intruders by small RWA workers. Most parasites (both kleptoparasites and brood predators) associated with RWAs have similar small sizes (chapter 1: Parmentier et al. 2014). Consequently, nest defence against intranidal parasitic myrmecophiles is likely to be allocated to small workers based on increased ergonomic efficiency (Oster and Wilson 1978, Hasegawa 1997).

A second key result was that worker aggression also differed between different task groups and again contrasted with the typical pattern observed in relation to defence against external threats. In particular, we found that workers that performed tasks inside the nest (i.e. that were nursing or present at the nest entrances) were, over their entire size range, more aggressive towards *T. angulata* than workers foraging outside the nest. Again, this pattern is opposite to that documented in other studies on task cohort and age specialization in aggression in the context of defence against extranidal threats (Hölldobler and Wilson 1990). For example, foragers of the leafcutter ant *Acromyrmex echinatior* have been shown to more rapidly display aggressive behaviour than within-nest workers (Norman et al. 2014) and a similar pattern has been observed in the ant *Cataglyphis cursor* (Nowbahari and Lenoir 1989). This pattern has been explained on the basis that division of labour in these ants is partly based on age polyethism, whereby only older workers engage in risky foraging and defence tasks. By performing risky tasks at older age, workers extend their life expectancy and improve colony efficiency (Duarte et al. 2011).

The contrasting pattern of task group specialization in the defence against external enemies versus parasites preying on brood can be interpreted in adaptive terms in the context of models of division of labour (Oster and Wilson 1978, Hasegawa 1997, Beshers and Fewell 2001), and could have several reasons. First, the parasites studied here were found not to attack ant workers, but rather to avoid any interaction. Hence, defence against these parasites may not be very risky compared to defence against other ants or vertebrates, and thereby favour the performance of intranidal defence by young nurses as well (Tofilski 2002). Second, workers inside the nest interact more frequently with the myrmecophile parasites than foragers, and prior encounter and greater experience in attacking these parasites could cause nurses and mound workers to recognize them more rapidly as a threat than foragers and to have a lower threshold to initiate aggression. In fact, it is well known that prior fighting experience may intensify future aggressive encounters, both in animals in general (Hsu et al. 2006) and more specifically in ants (Van Wilgenburg et al. 2010). Finally, a third explanation of the contrasting patterns in aggression that we found is that foragers would incur significant switching costs if they had to regularly switch to carrying out defensive tasks inside the colony, owing to the travel time between different task locations, or energy costs owing to shifts in behavioural state (Duarte et al. 2011, Goldsby et al. 2012). Indeed, previous studies suggested that wood ant foragers do not readily switch to other tasks and specialize purely in foraging over extended periods of time (Rosengren and Fortelius 1986, Parmentier et al. 2012). In combination, it is clear that these three factors make intranidal nurse workers ideally suited to perform non-risky defence strategies against intranidal myrmecophiles. Intranidal workers over the complete worker size range have higher aggression propensities than similar-sized foragers. Wood ant nurses inside the nest are on average smaller than workers foraging, especially at large distances, outside the nest (Higashi 1974, Herbers 1979, Parmentier 2010). As a result, the most optimal size and task cohorts to deter the parasites are represented inside the nest. Large nurses in the colony are outnumbered by small nurses. However, these large nurses are still more aggressive than similarly sized foragers. So apparently this 'task' effect enhances the low defence capabilities of large workers engaged in intranidal defence.

Our results demonstrate that nest defence specialization in wood ants is surprisingly context-dependent. Whereas large foragers are specialized in territorial defence and in defence against other external threats, small nurse workers appear most efficient in chasing away small parasites inside the nest. Earlier, a similar context-dependent specialization in aggression has been demonstrated in leafcutter ants. For example, in the leafcutter ant *Atta laevigata*, large workers attack vertebrate predators but small workers are recruited to defend their territory against rival ant colonies, presumably because of their better ergonomic size match (Whitehouse and Jaffe 1996). Small workers of the leafcutter ant *Atta colombica* hitchhiking on leaves are also specialized in defending, and ergonomically better suited to protect workers carrying leaves against small parasitic flies and reducing bacterial and fungal loads on the leaves (Feener and Moss 1990, Griffiths and Hughes 2010). Finally, small workers of the leafcutter ant *Acromyrmex octospinosus* are specialized in the removal of spores of parasitic *Escovopsis* fungus that colonizes their mutualistic fungus gardens, while large workers rather remove large pieces of *Escovopsis*-infected fungus garden (Abramowski et al. 2011). These findings and our results suggest that small ant workers (especially small nurses) are vital in some aspects of nest defence and are key in the defence against 'small' threats such as small myrmecophiles, bacteria and fungus spores in the colony. We believe that this context-dependent aggression response may be widespread among polymorphic social insects and could be a contributing factor for the evolution and maintenance of adaptive size polymorphism in these insects (Oster and Wilson 1978, Hasegawa 1997, Beshers and Fewell 2001). Furthermore, based on our results, it is likely that even in monomorphic social insect species, young nurse workers would specialize in intranidal defence, and that the presence of parasites would therefore affect the optimal allocation of roles as a function of age (Tofilski 2002).

Aggression and specialization in defence in RWA workers is highly context-dependent. We have shown that small workers inside the nest are best suited to attack intranidal parasites, and previous studies reported that large foragers are better suited to defend the colony against external threats. This context-dependent specialization in aggression can be interpreted in the context of adaptive models of the evolution of task specialization and caste polymorphism, and is argued to potentially be one of the key factors in promoting and maintaining size or caste polymorphism in both these ants and other social insects alike (Oster and Wilson 1978, Hasegawa 1997, Beshers and Fewell 2001).

ACKNOWLEDGEMENTS

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CHAPTER 4

Trophic interactions in an ant nest microcosm: a combined experimental and stable isotope ($\delta^{13}C/\delta^{15}N$) approach.

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ABSTRACT

Living in close association with other organisms has proven to be a widespread and successful strategy in nature. Some communities are completely driven by symbiotic associations and therefore, intimate relationships among the partners can be expected. Here, we analysed in-depth the food web of a particularly rich community of arthropods found in strict association with European red wood ants (*Formica rufa* group). We studied the trophic links between different ant-associated myrmecophiles and food sources associated with the host ant, but also tested predator-prey links among myrmecophiles themselves. Our approach combined direct feeding tests and stable carbon and nitrogen isotope analyses for a large number of myrmecophiles. The results of the direct feeding tests reveal a complex food web. Most myrmecophiles were found to parasitize on ant brood. Moreover, we encountered multiple trophic predator-prey links among the myrmecophiles. The results of the stable isotope analyses complement these findings and indicate the existence of multiple trophic levels and trophic isotopic niche compartmentalization. $\delta^{15}N$ values were strongly correlated with the trophic levels based on the direct tests, reflecting that $\delta^{15}N$ values of myrmecophiles increased with higher trophic levels. This strong correlation underlines the strength of stable isotopes as a powerful tool to assess trophic levels. In addition, the stable isotope data suggest that most species only facultatively prey on ant brood. The presence of numerous trophic interactions among symbionts clearly contrasts with the traditional view of social insects nests as offering an enemy-free space for symbionts. Interestingly, the ant host can indirectly benefit from these interactions because brood predators are also preyed upon by other myrmecophiles. Overall, this study provides unique insights into the complex interactions in a small symbiont microcosm system and suggests that the interactions between host and symbiont might be mediated by other symbionts in the same community.

INTRODUCTION

A highly diverse range of organisms lives in intimate association or symbiosis with other organisms (Paracer and Ahmadjian 2000). This association can take different forms - ranging from commensalism, where one partner benefits without costs for the other, to mutualism where both partners take benefits of the association, and parasitism, where one partner is exploited for the benefit of the other. The establishment of symbioses is thought to have driven the evolution of species, communities and even entire ecosystems (Paracer and Ahmadjian 2000). Some of those symbiont communities are microcosms centered on one keystone species which provides resources, shelter and habitat to associated species. Animals that live in such systems are known as inquilines. Typical examples of such so-called “inquiline” communities are the fauna associated with the water filled leaves of pitcher plants and bromeliads, and fauna associated with insect-induced galls (Sanver and Hawkins 2000, Kitching 2001, Srivastava et al. 2004). Such small, delineated microcosms have been considered as models to study ecological and evolutionary processes (Srivastava et al. 2004). In particular, the unravelling of trophic relationships in these communities has advanced our understanding of local ecosystem dynamics and structuring (Kitching 2001, Kneitel and Miller 2002, Trzcinski et al. 2005).

Nests of social insects can also be inhabited by a diverse community of inquiline guest species (Donisthorpe 1927, Kistner 1979, Hölldobler and Wilson 1990, Kronauer and Pierce 2011). These inquilines have developed mechanisms to circumvent colony aggression and thrive in a unique habitat characterized by ideal homeostatic conditions and a constant supply of food (Hölldobler and Wilson 1990). Moreover, it has been suggested that such nests provide an enemy-free space with low predation-pressure from the perspective of the associate (Kronauer and Pierce 2011). In spite of the taxonomic and life strategy diversity of species strictly associated with social insect nests and their potential use as model systems to study ecosystem and evolutionary processes (chapter 2: Parmentier et al. 2015a), little is known about the local community dynamics and interactions between symbiont-host and among symbionts themselves. Food web studies, in particular, are essential to understand local community functioning and its dynamics. It is well known that many social insect inquilines prey on brood or steal food from their host (Hölldobler and Wilson 1990, von Beeren et al. 2010, Hovestadt et al. 2012). Witte et al. (2008) demonstrated different strategies in the myrmecophile community associated with the army ant *Leptogenys distinguenda* ranging from kleptoparasites that steal food from the ant host to detrivores and brood predators. However, most studies only report the trophic interaction between the host and a single inquiline. In addition, (feeding) interactions among social insect symbionts have been even less studied, or at most been described based on occasional observations of single attacks (Donisthorpe 1927). De Visser et al. (2008) provides a rare case study using natural abundance stable isotope signatures to describe food web interactions among spiders and other invertebrates found in termitaria, but the reported species might not all have been strict associates of termites.

Our knowledge of the trophic interactions in inquiline microcosms associated with social insects is thus very fragmentary. The aim of the present study was to carry out an integrated study of the trophic interactions among red wood ant associates based on carbon (C) and nitrogen (N) stable isotope analyses as well as direct preference tests. This results in the first fine-scale study of the effect of ant associates on host fitness, and their effect on community functioning.

MATERIAL AND METHODS

Red wood ants and the myrmecophile community

Red wood ants (RWAs) are known to support a diverse group of associated arthropods in their nests (chapter 1: Parmentier et al. 2014). Some of these are strictly confined to ant nests and are thus considered obligate myrmecophiles. This group mainly consists of beetles, and especially rove beetles, but spiders, flies, hemipterans, an isopod and a springtail are also often reported. Others only live facultatively in association with RWAs and are typical soil organisms mostly found in the absence of ants (e.g. the common isopod *Porcellio scaber*) (chapter 1: Parmentier et al. 2014). The RWA species *Formica polyctena* and *Formica rufa* have a similar colonial organization in the fragmented woods of Flanders (Belgium) and they are even known to hybridize (Seifert et al. 2010, chapter 2: Parmentier et al. 2015a. The associated myrmecophile community is likely to be identical in both species and is highly similar to other European RWAs (chapter 2: Parmentier et al. 2015a).

Experiments

Our approach combines direct feeding tests with stable isotope analyses, which both can explain different attributes of a food web.

Inference of trophic interactions via direct feeding tests

Here we aimed to test directly trophic links in the RWA myrmecophiles community. This is a rather time-consuming technique, which strength depends on the number of food sources tested. With this technique we can determine potential trophic interactions and estimate the number of trophic levels, but we cannot define the relative importance of the trophic interactions.

Myrmecophiles for this experiment were collected in several nests of five RWA populations (West-Vleteren, De Haan, Roksem, Aartrijke, Beernem, description see chapter 2: Parmentier et al. 2015a) in Western Flanders, Belgium and in Boeschepe, France from December 2012 to April 2015. We took nest material out of different parts of the nest (outer layer mound, central part mound, earth nest under mound). Myrmecophiles were subsequently collected by spreading out this nest material on a large white tray in the field. Ants, their brood and nest material were gently placed back in the nest after collecting myrmecophiles. Tested organisms originate from both *F. polyctena* and *F. rufa* colonies. During tests, host species origin was not accounted for, because all tested myrmecophiles have been found in nests of both ant species. Hence, trophic relations were assumed to be similar in both *F. polyctena* and *F. rufa* mounds.

We offered different food sources to myrmecophiles associated with RWAs, analysing both trophic sources associated with the RWA host (eggs, larvae, pupae, dead ants, trophallaxis, ant prey), and studying the predator-prey relationships among symbionts themselves. We used nine staphylinid beetle species (*Quedius brevis*, *Dinarda maerkelii*, *Pella humeralis*, *Thiasophila angulata*, *Notothecta flavipes*, *Lyprocorrche anceps, Amidobia talpa*, *Leptacinus formicetorum*, *Stenus aterrimus*), two spiders (*Thyreosthenius biovatus*, *Mastigusa arietina*), one isopod (*Platyarthrus hoffmannseggii*), one springtail (*Cyphoderus albinus*), and three non-staphylinid beetle species: *Clytra quadripunctata* (Coleoptera: Chrysomelidae), *Monotoma angusticollis* (Coleoptera: Monotomidae) and *Myrmetes paykulli* (Coleoptera: Histeridae). We used the adult stage for all species, except for *C. quadripuncta* where the late instar larvae were tested, since the adults of the latter leave the nest directly after emergence and live on plants in the vicinity of wood ant nests where they mate and drop their eggs near the host nest. The larvae live permanently in the nest and make a case where they can hide (Donisthorpe 1927). All species used in the direct feeding tests are strictly associated with ants (Donisthorpe 1927, chapter 1: Parmentier et al. 2014).

First, feeding preference was tested directly by offering food sources associated with wood ants: RWA eggs, RWA larvae, RWA pupae, dead RWA workers, trophallaxis and ant prey. Engagement of myrmecophiles in trophallaxis, which is the transfer of regurgitated food among workers in social insects, was tested by offering 15 RWA workers sugar water (30%) stained with blue colorant (E131, i.e. Patent Blue V, Cook and Bake). After 6 h, these workers were placed in a darkened arena with 15 starved workers of the same colony to promote trophallaxis among workers. Myrmecophiles found in the same mound of the workers were then added and their gut was dissected after 48 h. The presence of blue colorant then indicates that the myrmecophile engaged directly in trophallaxis or stole a sugar droplet of two workers in trophallaxis. In some tests, dead workers were found. To rule out the possibility that the myrmecophile obtained the blue colorant by devouring the ant gut directly, we placed dead ant workers with several myrmecophiles in an arena, but none of the guts of the myrmecophiles were found to colour blue. Diptera larvae are an important part of the diet of wood ants (Punttila et al. 2004). Dead larvae of *Phaenicia sericata* were therefore chosen as a proxy for ant prey brought into the ant nests. Secondly, living myrmecophiles co-inhabiting with the focal myrmecophile were offered and acceptance tested: *C. albinus*, young *P. hoffmannseggii* isopods*, M. angusticollis, A. talpa*, *T. biovatus* spiderlings, rove beetle larvae (Aleocharine subfamily), Ptiliidae and mites found in the mounds. We lumped the obligate myrmecophile *Ptenidium formicetorum* and the facultative myrmecophiles of the genus *Acrotrichis* together in Ptiliidae prey*.* The staphylinid *A. talpa* was selected because it is the smallest and slowest staphylinid living in wood ant nests and therefore has the highest potential among staphylinids to be a prey item.

Food items were offered to a myrmecophile in snap lid vials filled with a ca. 1 cm bottom of moist plaster of Paris. The behaviour of myrmecophiles, except for *C. albinus* and *P. hoffmannseggii*, towards dead ant workers and ant prey was recorded in a darkened room with a camera (SONY HDR-XR550VE) equipped with night vision during one hour. Because of the low contrast between the whitish *C. albinus* and *P. hoffmannseggii* and the white plaster, we studied behaviour towards dead ant workers and ant prey for *C. albinus* and *P. hoffmannseggii* directly during one hour under red light instead of using the camera. Food was accepted if the myrmecophile was seen licking, dragging or biting the maggot or dead worker for at least 30 s. Trophallaxis was tested as described above. For the potential myrmecophilous prey, RWA eggs, larvae and pupae, we checked after 24 h if they were eaten. In each trial, RWA eggs and *C. albinus* individuals were offered per five, all other food items were given individually.

For most myrmecophiles, we also tested egg predation in presence of five workers. We added five nurses (workers that transported brood when opening the nest) and five eggs in similar vials that we used in the other tests. Workers readily started to lick and transport the eggs.

During the tests, only one dead intact myrmecophile prey was encountered, and therefore we eliminated this observation for further interpretation to avoid the chance that this prey just died naturally during the test. Some myrmecophiles (e.g. the isopod *P. hoffmannseggii*) were given no living prey, due to their obvious life style as detritus feeder or scavenger.

The acceptance of a food source was tested with different individuals for each species. The number of replicates and the proportion of replicates accepted are given in Table 4.1. Some individuals were used again to test acceptance for a different food source, but trials for a particular food source were never repeated with the same individual. Myrmecophiles were starved for one day prior to the tests. Myrmecophiles were recorded in RWA mounds throughout all seasons, except for *P. humeralis* that was only recorded in winter and Aleocharine larvae that were not found in winter. Ant brood can be found most of the year (even in winter we observed eggs), but the amount of brood peaks in spring and summer. Given that most sources and consumers are present throughout the year, we expect that most trophic interactions described here take place throughout the year, except for winter when most species are hibernating. Nonetheless, the strength of such interactions, will vary depending on the availability of food sources and the needs of consumers throughout the year. The tested myrmecophiles have not only a temporal overlap in the mound, but also a overlap in their distribution within the nest. We recently found that the tested myrmecophiles have some preference for particular parts of the nest, but they also occur in the other parts in somewhat lower densities (chapter 5). Therefore all myrmecophiles could occur together and interact with each other at some time and place in the nest.

Inference of trophic interactions through stable isotope analysis

The combined analysis of ratios of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ is a widely used tool in food web studies (Ponsard and Arditi 2000, Post 2002). It gives a rapid characterization of food web relationships and is able to constrain sources supporting food webs. It integrates unknown food sources and allows to estimate the importance of a food source in the diet of an animal (Phillips et al. 2014). In contrast with direct feeding tests, direct trophic interactions between two species are hard to estimate in complex food webs. The isotope ratios are expressed as δ units and give the deviation in parts per thousand from international standards:

$$\delta\,^{13}C \text{ or } \delta\,^{15}N = (R_{sample}/R_{standard} - 1) \times 1000\ [‰]$$

R = $^{13}C/^{12}C$ for $\delta^{13}C$ and R= $^{15}N/^{14}N$ for $\delta^{15}N$. Depending on the system and the tissue, a consumer tends to be enriched in ^{15}N relative to its diet, leading to a stepwise increase in $\delta^{15}N$ across trophic levels, with a reported average increase of 3.4 ‰ (Post 2002). Therefore, $\delta^{15}N$ can be used to estimate relative trophic positions or food chain lengths. The ratio of $^{13}C/^{12}C$ propagates through food web with little enrichment, but can vary substantially between different primary producers (e.g. in terrestrial sytems between C3 vs. C4 plants) (DeNiro and Epstein 1978, Post 2002). $\delta^{13}C$ can thus be used to infer primary sources supporting food webs.

As described above, most interactions, tested in the direct feeding tests, are expected to take place most of the year, but their strength can vary temporally which would be reflected in stable isotope analysis. To avoid this seasonal bias, we took samples for isotope analysis only in summer (2013-2014), when most consumers and sources are at their peak of abundance.

Individuals for isotope analysis had not been used previously in the direct tests. After collection, samples were directly stored in the freezer until isotope analysis. Stable isotope signatures of all species used in the direct preference tests were analysed, except for *P. humeralis* which was only found in winter. In addition, we sampled three additional obligate myrmecophiles (*Emphylus glaber*, *Hypoaspis oophila*, *Monotoma conicicollis*), the facultative myrmecophilous isopod *Porcellio scaber*, the host ant (*F. rufa*: workers, eggs, larvae) and organic nest material of the mound. *E. glaber* and *H. oophila* were only analysed in this experiment, because too few individuals were found to run direct tests in parallel. *M. conicicollis* was not used in the direct tests, because it is very similar with *M. angusticollis*. Identical direct, trophic interactions are hence expected. *H. oophila* is a mite species that lives among the egg piles of RWAs. They do no puncture eggs, but appear to live from secretions on the eggs (Donisthorpe 1927). Because of their small size, 20 eggs, 10 *C. albinus*, 10 *H. oophila* and 5 *A.* individuals were pooled per sample. The number of replicates per species can be found in the legend of Fig. 4.2. In contrast with the samples for the direct tests that were collected in several nests in five RWA populations, we took the majority of samples for isotope analysis in a single *F. rufa* colony consisting of 3 adjacent mounds in the West-Vleteren population (nest A, description see chapter 2: Parmentier et al. 2015a). *M.*

arietina was collected in a RWA nest in *F. polyctena* colonies in De Haan and Beernem (nest B, C). *E. glaber* was collected in a *F. rufa* colony in Boeschepe, France (nest D). Eggs and *H. oophila* were collected in nest A and D.

We weighed 0.1 to 1 mg of dry, homogenized material per sample into Sn cups and analysed for δ^{13}C and δ^{15}N on a Thermo Flash HT/EA elemental analyzer coupled to a Thermo Delta V Advantage IRMS with a Conflo IV interface, and data were corrected using an in-house calibrated Leucine standard and the certified IAEA-600 (caffeine). Reproducibility of standards within each batch were better than 0.1 ‰ for both δ^{13}C and δ^{15}N.

Tissue composition can bias δ^{13}C values, as lipids are generally depleted in ^{13}C compared to proteins and carbohydrates. We therefore applied the lipid-correction model proposed by McConnaughey and McRoy (1979) to normalize our δ^{13}C data. First the lipid content L of the sample is calculated from the sample C:N ratio (mg:mg)($R_{C:N}$):

$$L = 93/[1 + (0.246(R_{C:N}) - 0.775)^{-1}]$$

The lipid-normalized $\delta^{13}C'$ is calculated from the measured value of the sample ($\delta^{13}C$) and L:

$$\delta^{13}C' = \delta^{13}C + D[I + 3.90/(1 + 287/L)]$$

D refers to the isotopic difference between protein and lipid (assumed to be 6‰) and I is a constant (I = -0.207).

Stable isotope ratios of soil and litter and associated food webs can vary on small spatial scales (Ponsard and Arditi 2000). As a result, values of myrmecophiles from nest B, C and D are not comparable with those of nest A. Nest material of the four nests was used as a baseline of the respective food webs. Signatures of the myrmecophiles of nest B, C and D were rescaled to values relative to nest material of nest A by adding the difference between their signatures and the nest material of their nest to the values of the nest material of nest A.

Statistical analyses

All analyses were carried out in R, version 3.0.1 (R Core Team 2014).

In order to reconstruct the food web graphically based on our direct feeding tests, we used package *sandwich*. Food web parameters were also calculated with this package.

We tested with an ANOVA whether species differed in their $\delta^{15}N$ signature. Preliminary data analyses and Levene's test indicated that species were characterized by unequal variances. Therefore, we used the White-correction which implements a correction for heteroscedasticity (White 1980). Reported standard errors are robust and corrected for this heteroscedasticity. Then, we compared species pair wise using Games Howell Post Hoc Tests which can deal with unequal variances (Games and Howell 1976). Similar analyses were performed for $\delta^{13}C$ data. These analyses were carried out using packages *car* and *lmtest*.

Trophic levels calculated from the direct tests were correlated with $\delta^{15}N$-values using a Pearson's product-moment correlation.

RESULTS

Inference of trophic interactions via direct feeding tests

Table 4.1 summarizes the results of the direct preference tests. Species were broadly categorized in two trophic niches: scavengers that prey on ant brood and consume other ant-associated food sources and active hunters that prey on other living myrmecophiles. Brood predation was widespread (Table 4.1). With the exception of the beetle *S. aterrimus* and the springtail *C. albinus*, all myrmecophiles were found to prey on the host ant eggs. Ants were not efficient in deterring egg predators. Species that preyed on eggs without ants preyed at the same or somewhat lower (*T. angulata*) rate on eggs in presence of ants. However, we should need much more replicates to test whether there is a statistical difference in egg predation. With few exceptions (*L. anceps*, *S. aterrimus*, *C. albinus* and *P. hoffmannseggii*), a large fraction of the community accepted ant larvae. Pupae were not attacked, except for one replicate of *Q. brevis*. Almost all myrmecophile species acted as kleptoparasites by preying on ant prey. A large part of the myrmecophiles also fed on corpses of ant workers. The beetle *D. maerkelii* was shown to be the only specialist that engaged in trophallaxis (Table 4.1).

The two spiders, *T. biovatus* and *M. arietina*, were specialist predators of other small myrmecophile prey (*C. albinus,* mites, beetle larvae*,* spiderlings, isopod, Ptiliidae*)*. *T. biovatus* preyed cannibalistically on small conspecific spiderlings. *S. aterrimus* was a specialist hunter of the springtail *C. albinus*. This genus is known to have a specialized labium that can be projected to catch springtails (Scmitz 1943). *S. aterrimus* also

Table 4.1. Matrix with trophic interactions in RWA mounds. Myrmecophile species in rows indicate consumers. Trophic sources directly associated with ants and myrmecophile prey offered are represented in the columns. Fraction in a cell corresponds to the number of trials where the food source was accepted out of total trials. Different individuals were used for the trials of a particular food source. Cells with consumption are grey coloured. The myrmecophiles in the groups “other beetles”, “Collembola” and “Isopoda” were given no living, myrmecophilous prey, due to their obvious life style as detritus feeder or scavenger. Third column indicates trophic niche based on this table: a scavenger (S) mainly feeds on ant-associated food, an active hunter (A) preys on living myrmecophiles. A category is set in brackets when evidence is poor or when the other trophic niche is likely more important.

			ANT ASSOCIATED FOOD							MYRMECOPHILOUS PREY							
		trophic niche	ant eggs	ant eggs + workers	ant larva	ant pupa	ant prey	dead ant	trophallaxis	*C. albinus*	*P. hoffmannseggii*	*A. talpa*	*M. angusticollis*	Ptiliidae	*Aleocharine larva* (*)	*T. biovatus spiderling*	*mite*
ROVE BEETLES	*T. angulata*	S (+ A)	40/41	7/10	8/8	0/5	4/9	4/5	0/15	0/7	0/6	0/5	0/6	0/5	1/6	0/5	0/6
	N. flavipes	S	22/23	1/2	5/5	0/5	5/5	5/8	0/6	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	L. formicetorum	S + A	5/8		3/5	0/5	4/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	4/5	0/5	0/6
	L. anceps	S (+ A)	18/35		0/5	0/5	3/7	3/6	0/7	0/6	0/6	0/5	0/5	0/5	2/5	0/5	0/5
	A. talpa	S	4/22	1/3	4/5	0/5	3/8	4/5	0/9	0/5	0/5	0/5	0/5	0/5	0/5	0/8	0/13
	D. maerkelii	S	11/21	8/21	2/5	0/5	4/7	4/5	6/6	0/5	0/5	0/5	0/5	0/5	0/6	0/5	0/5
	P. humeralis	S (+ A)					4/4	5/6		0/5	1/5	0/5	0/4			0/5	0/6
	Q. brevis	S + A	13/14	13/15	5/5	1/5	6/6	0/5	0/5	0/7	2/7	0/6	1/7	4/5	4/5	5/7	4/5
SPIDERS	*S. aterrimus*	A	0/22	0/7	0/5	0/5	1/7	0/7	0/5	5/6	0/5	0/5	0/5	0/5	0/8	0/5	2/6
	T. biovatus	A + (S)	8/21	10/20	2/5	0/6	1/9	0/5	0/5	6/7	0/5	0/8	0/5	0/8	6/7	3/5	3/5
	M. arietina	A + (S)	1/9		1/3	0/5	0/8	0/5	0/5	4/5	2/4	0/6	0/5	3/6	4/6	2/6	4/6
OTHER BEETLES	*C. quadripunctata*	S	16/24	6/9	3/7	0/5	1/5	1/5	0/5								
	M. paykulli	S	14/21	7/12	3/5	0/5	3/8	6/8	0/9								
	M. angusticollis	S	17/25	2/4	1/6	0/5	5/5	1/6	0/8								
COLLEMBOLA	*C. albinus*	S	0/12	0/10	0/5	0/5	3/10	0/7	0/5								
ISOPODA	*P. hoffmannseggii*	S	12/20	2/7	0/7	0/5	0/5	0/5	0/12								

(*) We regularly observed cannibalism among Aleocharinae larvae

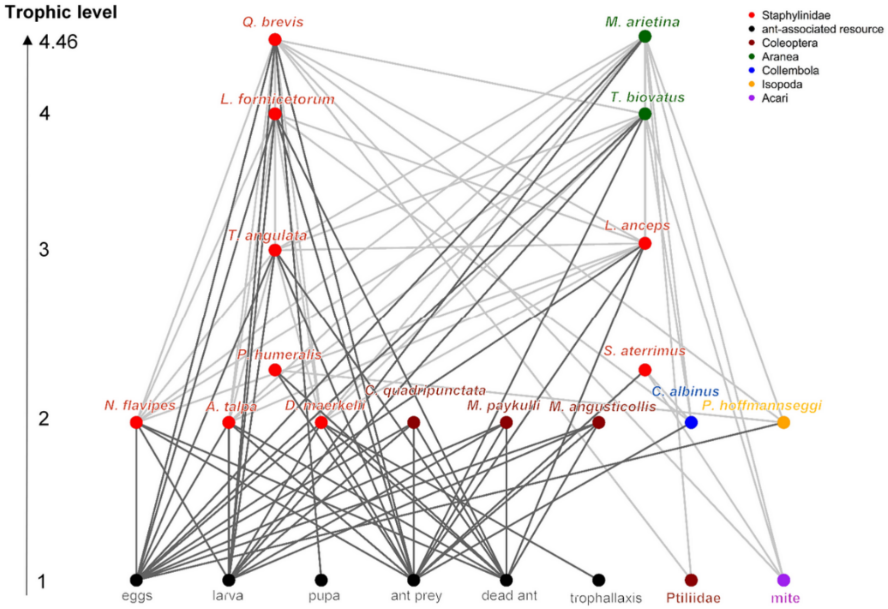

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Figure 4.1. Graphical representation of the trophic interactions in the RWA myrmecophile community (based on Table 4.1). Trophic level is based on averaged chain length, which is 1 plus the average chain length of all paths from each node to a basal species). Black links refer to trophic pathways were the source was associated with the host ants. Grey links refer to predator - prey interactions between myrmecophile species.

preyed on mites. Adult beetles (*M. angusticollis* and *A. talpa*) were not eaten, except for one registered attack of *Q. brevis* on *M. angusticollis*. *Q. brevis* is a very generalist predator, feeding on most other myrmecophiles excluding the quick springtail *C. albinus*. Rove beetle larvae were also preyed on by adult rove beetle of three species. Additionally we regularly observed that Aleocharine larvae preyed cannibalistically on each other (Table 4.1). The trophic interactions described above also occur in more natural conditions in presence of ants and nest material. Rove beetles were often observed feeding on maggots in lab ant nests. Initially we stored myrmecophiles, a high number of ants and some nest material, that we had collected in the field, in 1 L pots. Then we repeatedly observed that the initial large numbers of *C. albinus* and Aleocharine larvae were dramatically reduced the following day. Most of the prey-predator interactions were also observed in lab nests with ants. The trophic interactions reported in Table 4.1 are graphically presented by a food web using the cheddar package in R in Fig. 4.1. Trophic levels of the nodes are based on averaged chain length, which is 1 plus the average chain length of all paths from each node to a basal species. The food web consisted of 96 links connecting 24 nodes, resulting in a link density of 4 and a connectance of 0.17. The largest chain length connected five nodes, the mean chain length was 3.26.

Inference of trophic interactions through stable isotope analysis

Components of the myrmecophile food web associated with RWA differed significantly in $\delta^{15}N$ values (ANOVA, $F_{23,177} = 52.06$, $P < 0.0001$) which ranged from -1.7‰ ± 0.4‰ SE in nest material to 6.7‰ ± 0.3‰ SE in the rove beetle *L. formicetorum* (Fig. 4.2). We found a continuum in $\delta^{15}N$ values across groups of myrmecophiles, rather than consistent stepwise increases, which woul have corresponded to distinct trophic levels. The baseline $\delta^{15}N$ value of this study was found in nest material (-1.7‰ ± 0.4‰ SE). Different compartments of the food web had significant differences in $\delta^{13}C$ (ANOVA, $F_{23,177} = 21.83$, $P < 0.0001$), the latter being lowest for *H. oophila* (-26.4‰ ± 0.5‰ SE) and highest for *M. conicicollis* (-22.7‰ ± 0.2‰ SE) (Fig. 4.2). Some species have significantly lower or higher $\delta^{13}C$ values than organic nest material (-25.2‰ ± 0.1‰ SE), which was the presumed basal resource of the food web. The relatively high variance in $\delta^{13}C$ hence indicates the presence of multiple basal resources (Fig. 4.2, Table 4.2), There were clear differences (cf. Post Hoc Tests Table 4.2) among several myrmecophiles, even with similar $\delta^{15}N$ values. A good example of this trophic niche separation can be found in the congeneric species *M. angusticollis* and *M. conicicollis* (Fig. 4.2, Table 4.2).

The facultative myrmecophilous isopod *P. scaber* was the least enriched in ^{15}N, with an average $\delta^{15}N$ of 1.6‰ ± 0.3‰ SE. Interestingly, the obligate myrmecophilous isopod *P. hoffmannseggii* was considerably more enriched in ^{15}N compared with *P. scaber* ($\delta^{15}N$ of 5.4‰ ± 0.1‰ SE, Games Howell Post Hoc Test $P < 0.0001$). $\delta^{15}N$ values of ant workers (mean ± SE = 2.2‰ ± 0.1‰), larvae (mean ± SE = 2.5‰ ± 0.1‰) and eggs (mean ± SE = 2.0‰ ± 0.2‰) were relatively low compared to most myrmecophiles. Rove beetles' $\delta^{15}N$ values spanned a gradient from 2.6‰ to 6.7‰. Some species (*N. flavipes*, *A. talpa*, *L. anceps*, *D. maerkelii*) which preyed on ant brood in the direct tests, showed $\delta^{15}N$ values (2.6 to 3.2 ‰) only slightly higher than ant eggs and larvae.

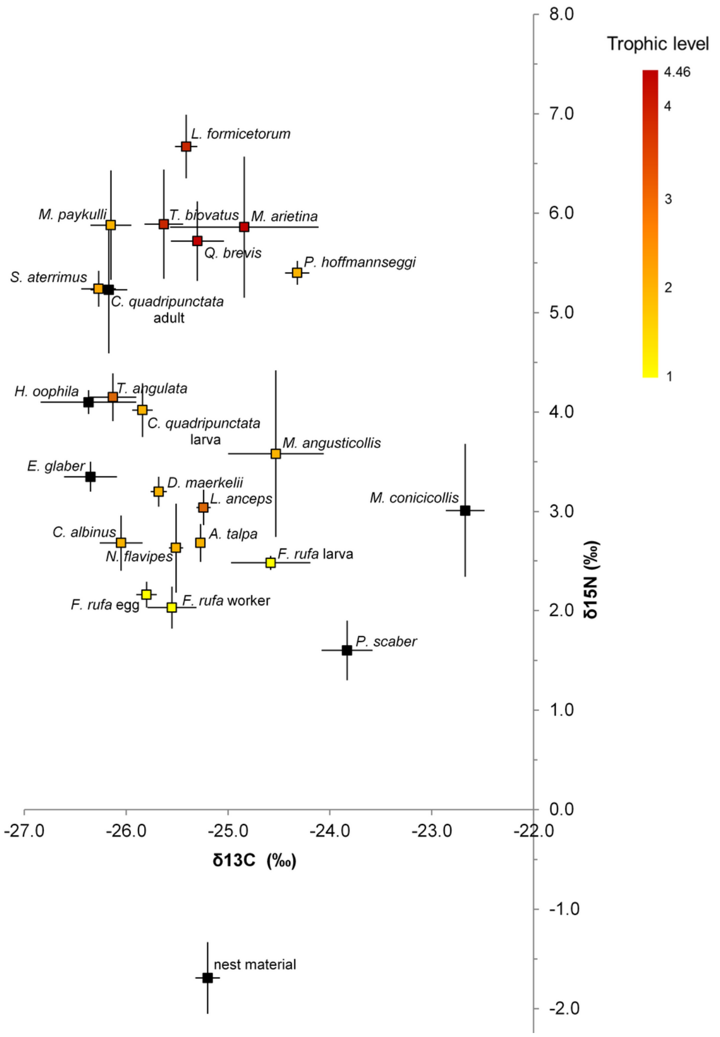

Figure 4.2. Isotopic signatures for myrmecophiles associated with RWAs. Means and SEs (corrected for heteroscedasticity) for δ^{13}C and δ^{15}N (‰) are displayed for *L. formicetorum* (n = 10), *M. paykulli* (n = 6), *T. biovatus* (n = 11), *M. arietina* (n = 5), *Q. brevis* (n = 6), *P. hoff mannseggi* (n = 18), *S. aterrimus* (n = 12), *C. quadripunctata* adult (n = 3), *T. angulata* (n = 12), *H. oophila* (n_{pooled} = 3), *C. quadripunctata* larva (n = 9), *M. angusticollis* (n = 11), *E. glaber* (n = 4), *D. maerkelii* (n = 9), *L. anceps* (n = 12), *M. conicicollis* (n = 13), *A. talpa* (n_{pooled} = 10), *C. albinus* (n_{pooled} = 4), *N. flavipes* (n = 10), *F. rufa* larva (n = 3), *F. rufa* worker (n = 10), *F. rufa* egg (n_{pooled} = 4),*P. scaber* (n = 8), nest material (n = 8). Symbols of species that were tested in the direct feeding tests are filled following a trophic level colour gradient as calculated in Fig. 4.1.

Myrmecophiles that preyed on other myrmecophiles in the direct tests showed, as expected, relatively high δ^{15}N signatures (*L. formicetorum* 6.7‰, *T. biovatus* 5.9‰, *M. arietina* 5.9‰, *Q. brevis* 5.7‰, *S. aterrimus* 5.2‰). The histerid beetle *M. paykulli* was also considerably enriched in ^{15}N (δ^{15}N: 5.9‰). Species with relatively low δ^{15}N values (*N. flavipes* 2.6‰, *C. albinus* 2.7‰, *A. talpa* 2.7‰, *M. conicicollis* 3.0‰, *L. anceps* 3.0‰ *E. glaber* 3.4‰, *M. angusticollis* 3.6‰, *C. quadripunctata* 4.0‰, *T. angulata*

4.2‰) still have higher ^{15}N-enrichments than expected for detrivores feeding only on nest material (cfr *P. scaber* with δ^{15}N = 1.6‰ and an enrichment of 3.3‰ relative to nest material*)*.The mite *H. oophila* is reported to feed on ant egg secretions (Donisthorpe 1927). It is considerably enriched in ^{15}N (δ^{15}N: 4.1‰) by 2.1‰ compared with ant eggs (Games Howell Post Hoc, $P = 0.006$) and showed similar 13C values to ant eggs. The δ^{15}N-values of the different species within our community were highly correlated with the trophic level (average chain length) calculated from our direct tests (Table 4.1, Fig. 4.1) (Pearson's product-moment correlation = 0.75, $P < 0.001$). Data points in the stable isotope plot (Fig. 4.2) are colored in accordance with trophic level of the direct tests.

Table 4.2. Species means of δ^{15}N and δ^{13}C values (‰) and corresponding SEs (corrected for heteroscedasticity). Species with no letters in common are significant different at the α = 0.05 level (Games-Howell Post Hoc Test).

	δ^{15}N				δ^{13}C		
	Species	Mean	SE		Species	Mean	SE
a	*Leptacinus formicetorum*	6.7	0.3	f	*Monotoma conicocollis*	-22.7	0.2
abcd	*Thyreosthenius biovatus*	5.9	0.6	ef	*Porcellio scaber*	-23.2	0.3
abcdef	*Myrmetes paykulli*	5.9	0.6	de	*Platyarthrus hoffmannseggii*	-24.3	0.1
abcdefghijkl	*Mastigusa arietina*	5.9	0.7	abcdef	*Monotoma angusticollis*	-24.5	0.5
abcd	*Quedius brevis*	5.7	0.4	abcdef	*Formica rufa* larva	-24.6	0.4
ab	*Platyarthrus hoffmannseggii*	5.4	0.1	abcdef	*Mastigusa arietina*	-24.8	0.7
abc	*Stenus aterrimus*	5.2	0.2	ab	*nest material*	-25.2	0.1
abcdefghijklmn	*Clytra quadripunctata* adult	5.2	0.6	b	*Lyprocorrhe anceps*	-25.2	0.1
cdefgh	*Thiasophila angulata*	4.2	0.2	b	*Amidobia talpa*	-25.3	0.1
de g	*Hypoaspis oophila*	4.1	0.1	abcde	*Quedius brevis*	-25.3	0.3
cdefghi	*Clytra quadripunctata* larva	4.0	0.3	ab	*Leptacinus formicetorum*	-25.4	0.1
abcdefghijklmn	*Monotoma angusticollis*	3.6	0.8	abc	*Notothecta flavipes*	-25.5	0.1
efghij	*Emphylus glaber*	3.4	0.2	abcd	*Formica rufa* egg	-25.6	0.2
f hij	*Dinarda maerkelii*	3.2	0.2	abc	*Thyreosthenius biovatus*	-25.6	0.2
f hijk m	*Lyprocorrhe anceps*	3.0	0.2	a c	*Dinarda maerkelii*	-25.7	0.1
bcdefghijklmn	*Monotoma conicocollis*	3.0	0.7	a c	*Formica rufa* worker	-25.8	0.1
ijklmn	*Amidobia talpa*	2.7	0.2	a c	*Clytra quadripunctata* larva	-25.8	0.1
ghijklmn	*Cyphoderus albinus*	2.7	0.3	abc	*Cyphoderus albinus*	-26.1	0.2
ghijklmn	*Notothecta flavipes*	2.6	0.5	abc	*Thiasophila angulata*	-26.1	0.2
jklmn	*Formica rufa larva*	2.5	0.1	abc	*Myrmetes paykulli*	-26.2	0.2
l n	*Formica rufa* worker	2.2	0.1	abc	*Clytra quadripunctata* adult	-26.2	0.2
klmn	*Formica rufa* egg	2.0	0.2	c	*Stenus aterrimus*	-26.3	0.2
mn	*Porcellio scaber*	1.6	0.3	abc	*Emphylus glaber*	-26.4	0.3
o	nest material	-1.7	0.4	abcde	*Hypoaspis oophila*	-26.4	0.5

DISCUSSION

We found in the myrmecophile community associated with red wood ants (RWAs) multiple trophic interactions with myrmecophiles feeding on ant-associated food sources or preying on other myrmecophiles. This results in a complex food web spanning different trophic levels. Interestingly, the trophic levels of our direct preference tests were highly correlated with δ^{15}N-values, indicating that species with a higher trophic level have higher δ^{15}N values.

Social insects are exposed to a diverse group of parasites ranging from bacteria and viruses to arthropods that threaten colony functioning (Schmid-Hempel 1998). They can attack all stages of their host, i.e. eggs, larvae, pupae and adult workers. There is a growing body of literature which shows the prevalence of parasites that feed on brood in social insect colonies (Hölldobler and Wilson 1990, Witte et al. 2008, von Beeren et al. 2010) and there are even indications that this results in an alteration of host life strategies (Hovestadt et al. 2012). Our study demonstrates that brood predation is a very widespread strategy in communities of social insect associates: except for two species, all symbionts in this study were found to prey on ant eggs and/or larvae. Even species previously described as commensals, such as the isopod *P. hoffmannseggii* and the larvae of *C. quadripunctata*, readily accepted this food source. The displayed trophic interactions could be affected by the presence of ants. However ants were not efficient in protecting eggs against most brood predators. The strongest deterring effect of ants on myrmecophilous consumers should be expected here, because of the high value of brood for the colony (Hölldobler and Wilson 1990). Therefore we can assume that they will also have a rather limited effect on other trophic interactions in the nest. This is further confirmed by observations in lab nests with large numbers of ants, where most interactions were observed. Interestingly, the obligate myrmecophilous isopod *P. hoffmannseggii* was considerably more enriched in ^{15}N compared with the facultative, myrmecophilous isopod *P. scaber.* Assuming an average enrichment of 3.4‰ per trophic level, it appears that *P. scaber* mainly feeds on nest material, whereas *P. hoffmannseggii* might specialize in brood parasitism. We only integrated one facultative myrmecophile in this study. But we could expect that also for other myrmecophiles, the obligate counterpart likely has got more adaptations and gets easier access to richer food sources compared with the facultative counterpart. We found, however, that many of the brood parasitic symbionts were only slightly enriched in ^{15}N compared with ant brood, suggesting that ant brood may not form the bulk of their diet. This finding was confirmed by the wide variation in $\delta^{13}C$ signatures in different myrmecophiles (Fig. 4.2). Some species might be deterred by the ants and lower their brood predation in the presence of ants (cf. chapter 3: Parmentier et al. 2015b). A potential preference for other food sources or the variability in ^{15}N enrichment could also explain the relative low enrichment in ^{15}N compared with ant brood for those species. Pupae and adult workers were not attacked. However, there are records of myrmecophiles living outside the nest that specialize in preying on RWA workers (chapter 1: Parmentier et al. 2014).

In addition to parasitic brood predators, kleptoparasites also impose costs on insect colonies by stealing food collected by foragers outside the nest (Breed et al. 2012). RWA carry a constant supply of proteinaceous invertebrate prey to their nest which are mainly allocated to brood development (Punttila et al. 2004, Mooney and Tillberg 2005). We found that all beetles as well as the springtail *C. albinus* fed on ant prey. This might be a preferred food source for rove beetles, which often instantly ate the prey and in some cases dragged it around. An exception was the rove beetle *S. aterrimus* which only fed on the ant prey in one trial. Two myrmecophilous spiders and the ant isopod *P. hoffmannseggii* were never attracted to dead prey. The main food source for RWA colonies, however, is not prey but honeydew collected from aphids around the nest (Skinner 1980). These sugar-rich excretions are used by the colony as the principal energy source. Foragers returning from aphid colonies, regurgitate this honeydew to other workers in the nest in a conditioned behaviour known as trophallaxis (Hölldobler and Wilson 1990). The large contribution of honeydew in their diet is expressed in the relative low $\delta^{15}N$ values akin to what was found in other studies (Fiedler et al. 2007, Skinner 2008). A number of myrmecophiles, but also symbionts of other social insects, were reported to mimic the behaviour of a begging worker or to steal indirectly a droplet of workers in trophallaxis (Hölldobler and Wilson 1990, Ellis et al. 2002). This behaviour was also reported for the beetle *D. maerkelii* (Hölldobler and Wilson 1990), one of the focal species in this study. Our results suggest that this behaviour is probably restricted to very specialized species, as we recorded it only in *D. maerkelii*. Interestingly, in spite of its relatively large size, this beetle was characterized by relative low $\delta^{15}N$ values, possibly mirroring the importance of honeydew in its diet.

Symbionts can also act as mutualists when they provide benefits for their partner. For example, some symbionts in social insect colonies are known to perform cleaning services in the colony and lower fungal infestations (Biani et al. 2009). The large amount of organic material and dead ant workers or other cadavers in a warm humid RWA nest are potential sources for parasitic fungus infestations. We show that a large group of intranidal beetles (the same group that feed on living prey except for *S. aterrimus* and *Q. brevis*) have the potential to speed up the decomposition of ant corpses by feeding on them. Especially the histerid beetle *M. piceus* was often observed licking and biting dead ant workers and was also considerably enriched in ^{15}N. A particularly important role in this early corpse decomposition and in controlling fungi infestations can also be expected from mites (Eickwort 1990), which are the most numerous group of myrmecophiles in RWA mounds (Kielczewski and Wisniewski

1962). Unfortunately, little is known about their taxonomy, biology and degree of association with RWAs (facultative or obligate).

Ant nests and the surrounding territory are heavily defended and are avoided by predators or parasitoids (Hölldobler and Wilson 1990). Myrmecophiles which live in association with ants are therefore subjected to lower predation or parasitization risk (Kronauer and Pierce 2011), and ant nests and the surrounding territory have thus been considered as an enemy-free space, *sensu* Jeffries and Lawton (1984). It has often been postulated that the association with protective ants in and around their nest is a key factor in the evolutionary transition to a myrmecophilous life style (Atsatt 1981, Pierce and Braby 2002, Kronauer and Pierce 2011). This protective role of ants was experimentally demonstrated in honeydew producing homopterans and lycaenid caterpillars tended by ants (Pierce et al. 1987, Völkl 1992, Bishop and Bristow 2003). In our study, however, we observed a multitude of predator-prey links among the myrmecophiles. This contradicts clearly with the classical view of social insect nests as an enemy free space from the perspective of the associates (Kronauer and Pierce 2011). In systems with multiple inquilines, predator-prey interactions among inquilines might be as prevalent as in other soil ecosystems, with the key difference being that predation pressure is not imposed by regular predators (which would be deterred by the presence of ants), but by specialized inquilines that also have integrated in the nests of the host. We found that particularly the younger stages (e.g. nymphs, larvae) of brood parasites were highly vulnerable to predation by other brood parasites. This intra-guild predation of brood parasites is an unexpected benefit for RWAs. Populations of those parasites, but also of other inquilines, might be predominantly controlled by other inquilines rather than by their host. Indeed, during hours of observation, none of the myrmecophiles were killed by their ant host and live unharmed in the deepest brood chambers (chapter 3: Parmentier et al. 2015b). This suggests that RWAs have little direct control on inquiline populations in contrast with the army ant *Leptogenys* as suggested in Witte et al. (2008).

The food web in RWA colonies was found to be surprisingly diverse with all species data jointly taking up a relatively wide ‘isotope space’ (cf. Layman et al. 2007) considering the ecosystem is dominated by terrestrial C3 vegetation. While overlap in stable isotope signatures between myrmecophile species occurred, many species were found to have distinctly different $\delta^{13}C$ and $\delta^{15}N$ signatures (Fig. 4.2), suggesting they are sufficiently specialized in their dietary habits to represent distinct trophic niches. The wide range of $\delta^{13}C$ does not support organic nest material as the sole basal food source and indicates that some species at the base of the food web consume unsampled resources found in RWA mounds such as bacteria, protozoa bark, fungi, algae (Laakso and Setälä 1998, Korganova 2009). However, the interpretation of $\delta^{13}C$ signatures can be complicated due to variability in trophic fractionation or due to selective assimilation of certain components of litter (e.g. cellulose, lignin, and starch) characterized by different $\delta^{13}C$ values (Pollierer et al. 2009, Maraun et al. 2011, Klarner et al. 2013). An extra complication in interpreting these isotope data, is the fact that the ant mound microcosm is not closed. Indeed, food and organic material of different sources is constantly brought to the nests by the ants. While a number of isotope mixing models have recently been developed and refined to estimate the contribution of different basal food sources to the diet of consumers within a food web (cf. Phillips et al. 2014) we did not perform such an analysis on our dataset as we feel that adequate sources signatures of potential food items which we have missed in our sampling approach are lacking.

Based on the direct tests we can broadly categorize myrmecophiles as active hunters, scavengers or a combination of both. However stable isotopes indicate that trophic niches are much more compartmentalized. A striking example is offered by the two very similar congeneric species *M. angusticollis* and *M. conicicollis*, which have similar $\delta^{15}N$ values, but are clearly different in $\delta^{13}C$ (Table 4.2, Fig. 4.2), suggesting a specialization on different food sources (e.g. different types of fungi). A similar isotopic niche partitioning was found in several congeneric Mesostigmatid mites (Klarner et al. 2013). Active hunters, such as the spiders *T. biovatus* and *M. arietina*, but also the specialized rove beetle *S. aterrimus* that mainly prey on other living myrmecophiles in the direct observation tests, were characterized by high $\delta^{15}N$ values. Ant brood, dead workers or ant prey is not or only poorly accepted by this group. Secondly, a diverse group of species was found to scavenge mainly on ant prey, dead ant workers and ant brood. Their $\delta^{15}N$ vary from low values comparable to the ant host to relative high values. Finally, species such as *Q. brevis* were found to both scavenge and hunt and show intermediate to high $\delta^{15}N$ values. A unique feeding niche can be found with the mite *H. oophila*, which lives among wood ant eggs, and for which our stable isotope data support the hypothesis that this mite predominantly feeds on egg secretions.

It should be noted that many soil organisms (e.g. mites, isopods, Collembola, earthworms) live facultatively in nests of social insects (Laakso and Setälä 1998, chapter 1: Parmentier et al. 2014) and in contrast with true or obligate inquilines, do not display any morphological, chemical or behavioural adaptations to their host.

Laakso and Setälä (1998) showed that the food web of those facultative associates in RWA mounds was highly different compared with the surrounding soil, consisting of less predators but with a higher biomass at the base. This facultative associate food web is probably highly interwoven at all trophic levels with the inquiline food web.

In this study we combined direct feeding tests and stable isotope analysis. Direct feeding tests have the advantage that trophic interactions between different groups can directly be detected and trophic levels easily reconstructed afterwards. However this technique is time-consuming and food sources can easily be overlooked or difficult to isolate and provide to consumers. Stable isotope analysis, on the other hand, is nowadays a widely used tool in terrestrial and aquatic ecosytems to study food web relationships. It gives a rapid and time-integrated characterization of your food web in which trophic levels and the proportion of different food sources to the diet of a consumer can be assessed (Post 2002, Hood-Nowotny and Knols 2007, Boecklen et al. 2011). In addition to traditional food web studies based on natural variation in stable isotopes, stable isotope tracers can be added deliberately and tracked from detritrus to consumers in the food web. This allows us to study movement of energy within and across ecosystems and to identify key players in a food web. This aspect of stable isotope analysis was applied in other microcosm systems such as pitcher plants (Butler et al. 2008) and could be interesting to use in our ant microcosm system as well. Stable isotope analysis have also limitations, including multiple sources of variation in isotopic signatures, limited taxonomic resolution of sources and reliance on literature values for key parameters (Boecklen et al. 2011). Both techniques give different insights in the food web and should be considered when characterizing food webs in-depth. However, the congruence in trophic levels in both techniques found in this study stresses the power of isotope analysis as a faster tool for identifying trophic levels than direct preference tests.

Overall, this study demonstrates the complex trophic interactions in an inquiline community associated with RWAs. It provides us a new and broader perspective on the dynamics in small inquiline microcosms. Inquilines in this study have different trophic niches spanning from active hunting to scavenging and detrivory. Most inquilines impose costs on their host directly by preying on the brood or indirectly by stealing food. However, multiple predator-prey interactions among inquiline parasites might lower the costs of the inquiline community on their host.

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CHAPTER 5

Do well-integrated species of an inquiline community have a lower brood predation tendency? A test using red wood ant myrmecophiles

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ABSTRACT

A host infected with multiple parasitic species provides a unique system to test evolutionary and ecological hypotheses. Different parasitic species associated with a single host are expected to occupy different niches. This niche specialization can evolve from intraguild competition among parasites. However, niche specialization can also be structured directly by the host when its defence strategy depends on the parasite's potential impact. Then it can be expected that species with low or no tendency to prey on host brood will elicit less aggression than severe brood parasitic species and will be able to integrate better in the host system.

We examined this hypothesis in a large community of symbionts associated with European red wood ants (*Formica rufa* group) by testing the association between 1) level of symbiont integration (i.e. presence in dense brood chambers vs. less populated chambers without brood) 2) level of ant aggression towards the symbiont 3) brood predation tendency of the symbiont.

Symbionts differed vastly in integration level and we demonstrated for the first time that relatively unspecialized ant symbionts or myrmecophiles occur preferentially in brood chambers. Based on their integration level, we categorize the tested myrmecophiles into three categories: 1) species attracted to the dense brood chambers 2) species rarely or never present in the brood chambers 3) species randomly distributed throughout the nest. The associates varied greatly in brood predation tendency and in aggression elicited. However, we did not find a correlation for the whole myrmecophile community between a) brood predation tendency and host's aggression b) integration level and host's aggression c) integration level and brood predation tendency.

Our results indicate that red wood ants (RWAs) did not act more hostile towards species that have a high tendency to prey on brood compared to species that are less likely or do not prey on brood. We show that potentially harmful parasites can penetrate into the deepest parts of a social insect fortress. We discuss these seemingly paradoxical findings in relation to models on coevolution and evolutionary arms races and list factors which can make the presence of potentially harmful parasites within the brood chambers evolutionary stable.

INTRODUCTION

Parasitism or the exploitation of one species by another species, is one of the most successful strategies in natural ecosystems (Combes 2005). The interactions between host and parasite often result in an evolutionary arms race where both partners develop adaptations and counter-adaptations against each other (Dawkins and Krebs 1979). Most studies focus on the interaction between a single parasite and its host and address the adaptations and counter-adaptations. However, hosts are typically parasitized by an assemblage of species (Petney and Andrews 1998). In such polyparasitism systems, the parasite's potential impact can vary substantially. Furthermore, parasites in such systems tend to specialize in different temporal and spatial niches associated with their host. For example, non-pollinating parasitic fig wasps present clear contrasts in oviposition timing, which promotes parasite co-existence (Proffit et al. 2007) and trematodes avoid competition by parasitizing different parts of their snail host (Hechinger et al. 2009). As an adequate defence response against parasites involves costs (Sheldon and Verhulst 1996), it could be beneficial for the host if its level of aggression is hierarchically adjusted to the harmfulness of the symbiont. Such plastic defence has been demonstrated in studies with a small number of parasites associated with mammals, pine weevils and social insects (Moore 2002, Mburu et al. 2009, Ennis et al. 2010, von Beeren et al. 2010), where hosts maximize the investment of costly defence strategies towards potential more harmful parasites, while potential less detrimental symbionts are tolerated.

A diverse group of organisms, ranging from commensals to true parasites, succeeded to penetrate into the well-defended nests of social insects (Kistner 1979, Hölldobler and Wilson 1990). Those fortresses provide a unique environment with different microhabitats and abundant food resources. David Kistner categorized social insect symbionts in two major categories based on their behaviour: integrated species "which by their behaviour and their hosts' behaviour can be seen as incorporated into their hosts' social life", and non-integrated species, "which are not integrated into the social life of their hosts but which are adapted to the nest as an ecological niche (Kistner 1979)." Here we use the same nomenclature, but categorize symbionts rather on nest location than on their behaviour or host behaviour. In our definition, integrated species are able to penetrate into the dense brood chambers, whereas non-integrated species occur in sparsely populated nest chambers without brood at the periphery of the nest. There are indications that intraguild competition among social insect parasites can cause temporal niche specialization (Witek et al. 2013). Alternatively, niche specialization can develop by a differential degree of tolerance of the host towards the symbionts. In that context, it is hypothesized that symbionts with lower potential costs are more integrated in the host's colony and incite less aggression (Hughes et al. 2008). These predictions were supported in a study with the army ant *Leptogenys* (von Beeren et al. 2010). Rove beetles preying on the host larvae elicited a strong aggression response. They were poorly integrated because they occur only at the edges of the colony. Rove beetles that do not prey on brood were better integrated in the colony. They did not receive aggression and were found in the central part of the nest. Some highly specialized myrmecophiles, however, do not follow these predictions. These species, such as larvae of the *Maculinea* butterflies, *Microdon* syrphid flies and *Lomechusa* rove beetles can integrate in the inner brood chambers of particular ant species without eliciting aggression (Hölldobler and Wilson 1990, Hovestadt et al. 2012). Those parasites have developed advanced chemical and behavioural adaptations to deceive their host (Hölldobler and Wilson 1990, Elmes et al. 1999). Those hosts and parasites are involved in a complex evolutionary arms race and their association may be stable due to frequency-dependent selection and geographic mosaic coevolution (Pierce et al. 2002, Nash et al. 2008). However, in associations with less specialized species, which are the scope of this study, hosts could detect those intruders and adjust their aggression to the potential fitness costs that the parasite could incur on the host (von Beeren et al. 2010).

Our knowledge on life history strategies of social insect symbionts in species-rich host-macroparasite communities is weak and is mainly based on army ant host systems (Akre and Rettenmeyer 1966, Kistner 1979, 1982, Hölldobler and Wilson 1990, Gotwald 1995). In parallel to the rich myrmecophile communities of tropical army ants (Rettenmeyer et al. 2010), nests of European red wood ants (RWAs) are also hotspots for myrmecophile diversity (chapter 1: Parmentier et al. 2014). However the organization of army ants and RWAs is totally different. Army ants have an atypical life style: they do not construct permanent nests and regularly migrate to new temporal bivouacs. This organization also affect the symbionts as they have to coordinate their life cycle intimately with the host's migrations (Akre and Rettenmeyer 1968, von Beeren et al. 2015). RWAs, on the other hand, construct a permanent, central nest. The aboveground part of their nest is a heap of organic thatch material, which provides plenty of hiding places for associated species and parasites throughout the mound. Because of these differences in the organization of their host, it is particularly interesting to compare the myrmecophile communities of army ants with those of nest-inhabiting RWAs.

In this study, our ultimate aim was to test whether RWA myrmecophiles with a lower or no tendency to prey on brood are better integrated in the host ant colony. We tested the adaptive defence response of the host with a very large number of symbionts. We first determined three parameters for the different symbionts: (1) their level of integration in the colony (2) the level of host aggression elicited (3) their tendency to prey on ant brood. Linking these parameters allowed us to test the following hypotheses:

a) Species with a lower level of brood predation elicit less aggression

   Some studies showed that ants are able to detect potential more harmful enemies and adjust their level of aggression concordantly (von Beeren et al. 2010, Pamminger et al. 2011). They argue that this hierarchy of aggression responses might promote colony fitness.

b) Well-integrated species that reside in the dense brood chambers elicit lower level of aggression

   Better integrated symbionts are expected to elicit less aggression and are therefore able to stay in the dense brood chambers.

c) Well-integrated species that live among the brood have a lower or no tendency to prey on brood

   From the perspective of the host, it is beneficial that it only tolerates species with low or no tendency to prey on brood, while severe brood parasites are only tolerated at the periphery of the nest or colony.

Consequently, species with low or no tendency to exhibit brood predation are tolerated and can integrate well into the colony, while species with a high brood parasite tendency are deterred to the edges of the colony by an elevated aggression response of the host.

MATERIAL AND METHODS

Study system

A strikingly large number of obligate myrmecophiles can be found with the mound building European RWAs (*Formica rufa* group) (chapter 1: Parmentier et al. 2014). This myrmecophile community completely consists of rather unspecialized symbionts, except for the specialized, but rare myrmecophile *Lomechusa pubicollis* (Donisthorpe

1927). Specialized myrmecophiles (symphiles or true guests sensu Erich Wasmann (Wasmann 1894)) are treated by the ants as members (fed and groomed) of the colony as a result of special glands (e.g. appeasement gland) and morphological (e.g. modified antennae) and behavioural adaptations (e.g. food soliciting). Unspecialized myrmecophiles (synechthrans and synoeketes sensu Erich Wasmann (Wasmann 1894)), however, often look very similar to non-myrmecophile relatives and are ignored or treated with hostility (Wasmann 1894, Donisthorpe 1927, Hölldobler and Wilson 1990). Apart from obligate myrmecophiles, RWA mound also host many facultative or occasional myrmecophiles, These arthropods mostly live away from ants, but can often be found in RWA mounds as well (chapter 1: Parmentier et al. 2014). RWA nests are heterogenic in worker distribution, with the largest abundances found in the inner brood chambers (Rosengren et al. 1987). One could expect that more detrimental species would be recognized by the RWA hosts and are only tolerated at the outer edges of the nest away from the brood. However, it is not clear in what way other factors (e.g. abundance of hiding places, behavioural and chemical adaptations of symbionts) could affect this relation. To test our hypothesis for the RWA myrmecophiles community, we quantified three parameters: 1) level of integration 2) level of host ant aggression and (3) brood predation tendency, and examined whether they were linked. Hypothesis testing was done by using eight staphylinid beetle species (*Quedius brevis*, *Dinarda maerkelii*, *Thiasophila angulata*, *Notothecta flavipes*, *Lyprocorrhe anceps, Amidobia talpa*, *Leptacinus formicetorum*, *Stenus aterrimus*), two spiders (*Thyreosthenius biovatus*, *Mastigusa arietina*), one isopod (*Platyarthrus hoffmannseggii*), one springtail (*Cyphoderus albinus*), and five non-staphylinid beetle species: *Clytra quadripunctata* (Coleoptera: Chrysomelidae), *Monotoma angusticollis* (Coleoptera: Monotomidae), *Monotoma conicicollis* (Coleoptera: Monotomidae), *Dendrophilus pygmaeus* (Coleoptera: Histeridae) *Myrmetes paykulli* (Coleoptera: Histeridae). In addition, we collected *Porcellio scaber* in the mounds, which lives facultatively associated with RWAs. All tested myrmecophiles are relatively unspecialized following the definition given above (Table 5.1). Myrmecophiles were caught by spreading nest material onto a large white tray in the field. We used the adult stage for all species, except for *C. quadripunctata* where we tested the larvae. Those larvae live in the nest and have a case in which they can hide. The adults of this species live on plants around the nests. After collecting myrmecophiles in the field, ants and their brood were gently placed back in the nest. Myrmecophiles were collected in seven RWA populations (chapter 2: Parmentier et al. 2015a) across Western Flanders, Belgium and in one population in Boeschepe, France. RWA populations consisted of *Formica rufa* and/or *Formica* *polyctena* mounds. Those closely related species have a very analogous colonial organization in the study area. Their myrmecophile community is likewise analogous (chapter 2: Parmentier et al. 2015a).

Experiments

The experiments were performed between December 2012 and June 2015.

Experiment I: Level of integration

In this experiment, we wanted to test whether myrmecophiles occupied different niches in RWA nests. More specifically we were interested whether myrmecophiles preferred to stay in densely populated chambers with ant brood or in less densely populated areas. Following our definition given above, integrated myrmecophiles penetrate into the densely populated chambers with brood, whereas poorly integrated species prefer sparsely populated chambers without brood. We constructed laboratory nests consisting of six round plastic pots (diameter 8 cm, height 5 cm) which were connected with plastic tubes (length 2 cm, inner diameter 1.1 cm). The pots and connections were arranged in such a way that every pot was connected with two other pots (Fig. 5.1). The bottom of the pots and connection tubes were filled with plaster of Paris (pots ca. 1 cm, tubes ca. 0.3 cm). We coated the inner walls of the pots with fluon to prevent ants and myrmecophiles from climbing up. In every pot (hereafter called chamber) we spread 10 g nest material (fine organic material) of a deserted *F. rufa* nest, to approach natural nest conditions and enabling myrmecophiles to hide. Transport and exchange of this nest material between the chambers was limited. All pots were sealed with a lid to prevent desiccation. We started each replicate by adding 360 *F. rufa* workers, 100 larvae of different sizes, 50 pupae and an egg pile (ca. 50 eggs/larvae) to the nest. Ants and their brood were collected in a supercolony in Boeschepe, France. After one day, myrmecophiles were apportioned randomly to the six chambers. The nest was placed in complete darkness to mimic natural conditions. Two days later, chamber openings were gently sealed with moist cotton plug and the

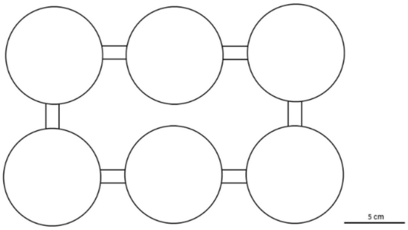


Figure 5.1. Schematic overview of the test nest. The nest consists of six chambers, in which each is connected with two other chambers.

nest was taken out of the darkness. The number of workers, brood and myrmecophiles were counted by spreading out the content of each chamber onto a large plastic tray with fluon coated walls. To distinguish *M. angusticollis* from *M. conicicollis*, we used a magnifier (4X, Eschenbach). Workers, brood and myrmecophiles that were found in the connection tubes were not considered. We replicated this experiment 16 times in total. We used different individuals for all myrmecophile species in each replicate, except for *D. pygmaeus*. For this species we found only three individuals and the same individuals were re-used in successive trials. The number of individuals per species recorded in each replicate at the beginning and at the end of the experiment is listed in Table A-5.2 of Appendix 5-2. Myrmecophiles for this experiment were collected in the Boeschepe population, but also in other RWA populations (*F. rufa* and *F. polyctena*) to increase our sample size. Aggression experiments for several myrmecophile species indicated that *F. rufa* workers did not act more aggressively towards myrmecophiles collected in *F. polyctena* colonies than towards myrmecophiles found in their own colony (Appendix 5-1). Chemical analyses of the

cuticular hydrocarbons confirm this lack of colony-specific and even RWA host-specific (i.e. individuals found in *F. rufa* do not differ from those found in *F. polyctena*) adaptation in all myrmecophiles tested in this paper (chapter 6). Therefore behaviour of myrmecophiles is expected not to be affected by the RWA colony of origin. Ant workers and brood were placed back in the host supercolony after the experiment.

Experiment II: Level of aggression elicited

We tested ant aggression toward myrmecophiles to study whether myrmecophiles elicited different aggression responses. Myrmecophiles and ants were collected in the same *F. rufa* supercolony in Westvleteren, except for *D. pygmaeus* and *M. arietina.* Those species were only found in *F. polyctena* populations. Based on the lack of RWA host-specific adaptation (Appendix 5-1, chapter 6), we assume that these aggression tests of *D. pygmaeus* and *M. arietina* are comparable with those of the other myrmecophiles collected in the *F. rufa* colony (West-Vleteren). This was further confirmed with the high aggression of *F. polyctena* workers towards *M. arietina* found in the same colony, which was similar to the aggression of *F. rufa* towards those spiders (Appendix 5-1). We used a small rectangular plastic arena (8 cm x 5.5 cm), filled with ca. 1 cm plaster of Paris and coated with fluon. Forty *F. rufa* workers were acclimatized for one hour to the arena. Then a myrmecophile was added and after ten seconds, the first twenty interactions (i.e. antennae of ant crossed the myrmecophile) with the ants were scored. Trials were performed in darkness under red light and were recorded with a video camera (SONY HDR-XR550VE). Videos were subsequently analysed in VirtualDub which allows to watch videos frame by frame. Ant aggression was scored by the proportion of aggressive interactions (acid spraying, chasing, biting, opening mandibles) out of the first 20 interactions. We used different myrmecophile individuals for each replicate, workers were re-used for several trials.

Experiment III: Brood predation tendency

Brood predation tendency of a myrmecophile species was quantified as the proportion of individuals that preyed on RWA eggs. We filled small plastic vials (diameter 4.5 cm) with ca. 1 cm of moistened plaster of Paris. Subsequently, we piled five RWA eggs in the centre and introduced a myrmecophile. Myrmecophiles were collected in different RWA populations in the study region described above. Eggs were collected in *F. rufa* colonies (Boeschepe and West-Vleteren). After one day, we checked whether the myrmecophile preyed on the eggs. For each myrmecophile species, we used different individuals in all replicates. We used acceptance of ant eggs (at least one egg eaten), rather than proportion of eggs eaten as the latter might be affected by the size of the myrmecophilous species. Individuals were starved for one day prior to the experiment. This index allowed us to classify myrmecophiles according to their tendency of brood predation. In the presence of ants, the success rate for the parasite might be lower. For the species that were attracted to the brood chamber in *Experiment I*, we also ran replicates with workers (five eggs and five workers in the same vial), to see if they still have a tendency to prey on ant brood.

Data analysis

Experiment I: Level of integration

In all trials, ants stored the brood in one chamber (hereafter called the brood chamber). Chambers gradually spanned a large gradient in worker density with the brood chamber containing always the largest number of workers with an overall mean ± SD of 46.7% ± 14.1 (Table A-5.3 in Appendix 5-2), reflecting the heterogeneity of worker density in natural wood ant nests (Gösswald 1989a), pers. observations TP).

Analyses were performed in R 3.2.1 (R Core Team 2014). Differences in association with the brood chambers in the myrmecophile community were compared using a generalized linear mixed model in a Bayesian setting with function blmer in R package ‘blme’ version 1.0.4 (Chung et al. 2013). In contrast with generalized linear mixed models, this type of models can handle complete separation in a dataset by using a weak prior (Bolker 2015). A part of our dataset was completely separated as some species were never observed in any of the brood chambers. The full model included the fixed factor ‘species’ and the random factor ‘replicate’. In addition, we incorporated an observation random factor to account for overdispersion (Browne et al. 2005). A Type II Wald chisquare test was conducted with the Anova function in package ‘car’ version 2.1.0 (Fox and Weisberg 2011) to assess whether species differed in level of integration (i.e. found in or outside the brood chamber). Post-hoc differences were tested by the glht function provided by package ‘multcomp’ version 1.4.1 (Hothorn et al. 2008). We controlled the false discovery rate (multiple testing problem) by adjusting the *P*-values with the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

To test attraction or repulsion towards the brood chamber of a single species, we directly tested for each species whether the observed proportion of individuals in the brood chambers (pooled over the 16 replicates) deviated from a proportion of 1/6 with an exact binomial test. Indeed, in a six-chamber nest, we expect that a species with attraction to the brood chamber will have significant more occurrences than 1/6 in the brood chamber. In contrast, the occurrence probability in the brood chambers will be lower than 1/6 for species that avoid those chambers. We controlled the false discovery rate (multiple testing problem) of the multiple exact binomial tests by adjusting the P-values with the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

Experiment II and III: Level of aggression elicited and brood predation tendency

We ran a quasibinomial GLM with “species” as single explanatory factor and elicited aggression as dependent variable. Similarly, we tested with a quasibinomial GLM whether proportion of individuals preying on brood was significantly different. Significance was tested with a Likelihood Ratio chisquare test implemented in package *car*. Confidence intervals of aggression response and proportion individuals preying on eggs were calculated by the function confint (Table 5.2).

Do well-integrated species of an inquiline community have a lower brood predation tendency?

We subdivided our main hypothesis in three parts: a) Do species with a lower tendency of brood predation elicit lower level of aggression? b) Do species that reside in the dense brood chambers elicit lower level of aggression? c) Do species that live among the brood have a lower tendency of brood predation? The three subhypotheses were tested by running both a Pearson product-moment and Spearman Rank correlation between a) brood predation tendency and level of aggression elicited b) level of integration and level of aggression elicited c) level of integration and brood predation tendency. We did not possess data on brood predation for *P. scaber* nor data on level of integration for *M. arietina* (all individuals were killed before the end of the experiment). Therefore, correlation between brood predation tendency and aggression elicited was run without *P. scaber* (N_{species} = 17), correlation between level of integration and aggression elicited was run without *M. arietina* (N_{species} = 17) and *c*orrelation between level of integration and brood predation tendency was done wihthout *M. arietina* and *P. scaber* (N_{species} = 16). In addition, we calculated the same correlations, but only focusing on the eight rove beetles (Staphylinidae) instead of all myrmecophiles. Analyses were performed in R 3.2.1.

RESULTS

Level of integration

Myrmecophiles differed significantly in preference for RWA brood chambers (BGLME, Chisq = 112.76, DF = 17, P < 0.001). Results of Benjamini-Hochberg Post-hoc tests are given with a letter code in Fig. 5.2. Myrmecophiles could be classified into three categories based on their association with the brood chambers: 1) attraction to the dense brood chambers 2) avoidance of the brood chambers and 3) random distribution (Fig. 5.2, Table 5.1). *Clytra quadripunctata* (mean proportion in brood chamber = 0.45, 95 % CI: 0.30-0.61, P < 0.001)*, T. angulata* (mean proportion in brood chamber = 0.37, 95 % CI: 0.27-0.48, P < 0.001) and *M. conicicollis* (mean proportion in brood chamber = 0.33, 95 % CI: 0.21-0.47, P = 0.011) were significantly attracted to the brood chambers (proportions in brood chamber significantly more than random 1/6 = 0.167 distribution). The highest attraction was found in the case-larvae of *C. quadripunctata*. The high attraction of this species to the dense brood parts of the nest was also directly observed in the field (sometimes they were also observed crawling on the mound). In the deep, central part of the nest, we also regularly found empty pupal cases which suggests that pupation also takes place in the heart of the nest. In contrast *Q. brevis* (mean proportion in brood chamber = 0.00, 95 % CI: 0.00-0.13, P = 0.043), *D. pygmaeus* (mean proportion in brood chamber = 0.00, 95 % CI: 0.00-0.10, P = 0.011) and the facultative associate *P. scaber* (mean proportion in brood chamber = 0.03, 95 % CI: 0.00-0.12, P = 0.011) significantly avoided the dense brood chambers (proportions in brood chambers significantly lower than random 1/6 = 0.167 distribution) (Table 5.1). *Q. brevis* and *D. pygmaeus* were even never observed in the brood chambers (Table 5.1). The spider *M. arietina* was always (15 individuals) killed before the end of the experiment (see Fig. cover page chapter 5), which might indicate that this species is not able to survive in a high density of workers without much hiding places. Field observations supported this apparent weak integration of the spider. It was never found in material with brood, but it was mainly found under pieces of bark in the nest. When disturbed, they ran rapidly away and hided in crevices and holes in the bark. Many distinct egg packets of this species (cf. Donisthorpe 1927) could be found on the bark. Finally a group of myrmecophiles was rather randomly distributed in the nest, i.e. they were neither significantly attracted nor repelled from the brood chambers (Table 5.1).

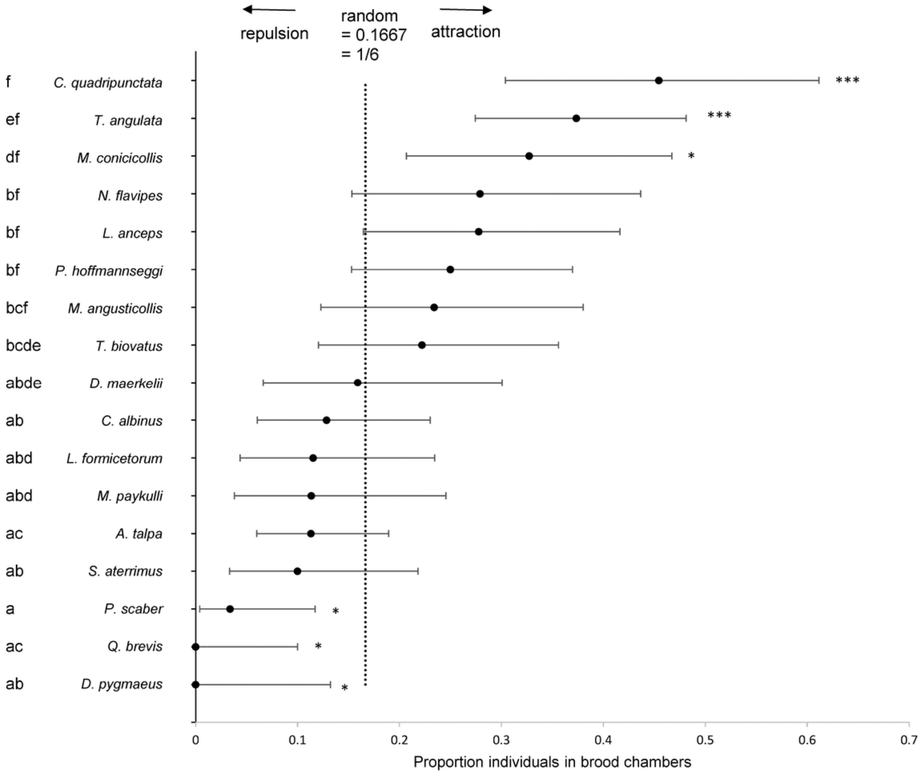


Figure 5.2. Level of integration of myrmecophiles. Proportion of individuals for different myrmecophilous species that were found in the brood chamber in the 6-chamber nest are given. Species attracted to the brood chambers (well-integrated) have proportions significant greater than 1/6, species that avoided the brood chambers (poorly integrated) have proportions significant lower than 1/6. Species without neither attraction nor repulsion, have a more random distribution and the proportions in the brood chamber are not significantly different from 1/6. The observed proportion for a given myrmecophilous species was tested with an exact binomial two-sided test. P-values were corrected for multiple testing by the Benjamini-Hochberg method (false discovery rate), $*P < 0.05$, $***P < 0.001$. Species with no letters in common are significant different at the $\alpha = 0.05$ level (Bayesian generalized linear mixed model followed by Benjamini-Hochberg Post Hoc Tests).

Table 5.1. Proportion of individuals in brood chamber for the tested myrmecophiles. Attraction to or repulsion from the brood chamber was tested with an exact binomial two-sided test (deviation from a random distribution of 1/6 was tested). Reported P-values (P_{corr}) were adjusted for multiple testing by the Benjamini-Hochberg method (false discovery rate). N = number of individuals tested, for D. pygmaeus three individuals were re-used in different replicates. For M. arietina, all individuals were killed during the experiment and therefore no testing was done. 95% CI: 95% confidence. Host specifity based on Table A-1 in chapter 1: Parmentier et al. (2014) (strict specialist: only records with RWAs, specialist: some records with non RWAs, but RWAs are the main host, moderate: records with RWAs, but distribution in non-RWAs probably important as well, generalist: myrmecophiles have no preference for a particular ant species, but are always found in presence of ants). Graphical representation of brood chamber association is given in Fig. 5.2.

Species	Taxon	Myrmecophily	Host specifity	N	Prop. brood chamber	95 %CI	P_{corr}	Brood chamber
Clytra quadripunctata	Coleoptera (Chrysomelidae)	obligate	specialist	44	0.45	0.30-0.61	<0.001	attraction
Thiasophila angulata	Coleoptera (Staphilinidae)	obligate	specialist	91	0.37	0.27-0.48	<0.001	attraction
Monotoma conicicollis	Coleoptera (Monotomidae)	obligate	strict specialist	55	0.33	0.21-0.47	0.011	attraction
Notothecta flavipes	Coleoptera (Staphilinidae)	obligate	specialist	43	0.28	0.15-0.44	0.133	random
Lyprocorrhe anceps	Coleoptera (Staphilinidae)	obligate	specialist	54	0.28	0.16-0.42	0.102	random
Platyarthrus hoffmannseggii	Isopoda (Platyarthridae)	obligate	generalist	68	0.25	0.15-0.37	0.138	random
Monotoma angusticollis	Coleoptera (Monotomidae)	obligate	strict specialist	47	0.23	0.12-0.38	0.357	random
Thyreosthenius biovatus	Araneae (Linyphiidae)	obligate	specialist	54	0.22	0.12-0.36	0.357	random
Dinarda maerkelii	Coleoptera (Staphilinidae)	obligate	specialist	44	0.16	0.07-0.30	1.000	random
Cyphoderus. albinus	Collembola (Cyphoderidae)	obligate	generalist	70	0.13	0.06-0.23	0.553	random
Leptacinus formicetorum	Coleoptera (Staphilinidae)	obligate	specialist	52	0.12	0.04-0.23	0.516	random
Myrmetes paykulli	Coleoptera (Histeridae)	obligate	specialist	44	0.11	0.04-0.25	0.514	random
Amidobia talpa	Coleoptera (Staphilinidae)	obligate	specialist	106	0.11	0.06-0.19	0.260	random
Stenus aterrimus	Coleoptera (Staphilinidae)	obligate	strict specialist	50	0.10	0.03-0.22	0.357	random
Porcellio scaber	Isopoda (Porcellionidae)	facultative	facultative	59	0.03	0.00-0.12	0.011	repulsion
Dendrophilus pygmaeus	Coleoptera (Histeridae)	obligate	specialist	26	0.00	0.00-0.13	0.043	repulsion
Quedius brevis	Coleoptera (Staphilinidae)	obligate	moderate	35	0.00	0.00-0.10	0.011	repulsion
Mastigusa arietina	Araneae (Dictynidae)	obligate	moderate	15	NA			

Table 5.2. Proportion aggressive interactions of ant workers towards myrmecophiles and proportion myrmecophile individuals preying on ant brood (= brood predation tendency) for different myrmecophile species. N = number of individuals tested, 95% CI: 95% confidence interval, NA = not available.

Species	Proportion aggressive interactions	N	95% CI	Proportion individuals preyed on brood	N	95% CI
Amidobia talpa	0.12	22	0.08-0.17	0.18	22	0.06-0.36
Cyphoderus albinus	0.00	15	0.00-0.02	0.00	15	0.00-NA
Clytra quadripunctata	0.01	10	0.00-0.03	0.67	24	0.48-0.83
Dinarda maerkelii	0.27	22	0.21-0.33	0.52	21	0.33-0.72
Dendrophilus pygmaeus	0.19	6	0.10-0.31	1.00	9	NA-1.00
Lyprocorrhe anceps	0.25	21	0.19-0.31	0.51	35	0.36-0.67
Leptacinus formicetorum	0.42	11	0.32-0.51	0.81	16	0.59-0.95
Monotoma angusticollis	0.03	20	0.01-0.06	0.68	25	0.49-0.83
Mastigusa arietina	0.73	12	0.64-0.81	0.10	10	0.01-0.36
Monotoma conicicollis	0.05	20	0.02-0.08	0.50	18	0.29-0.71
Myrmetes paykulli	0.23	18	0.13-0.25	0.67	21	0.46-0.83
Notothecta flavipes	0.63	21	0.56-0.70	0.96	23	0.83-1.00
Platyarthrus hoffmannseggii	0.05	20	0.03-0.09	0.60	20	0.39-0.79
Porcellio scaber	0.07	10	0.03-0.13	NA	NA.	NA
Quedius brevis	0.82	12	0.74-0.88	0.93	14	0.73-0.99
Stenus aterrimus	0.13	20	0.08-0.18	0.00	22	0.00-NA
Thiasophila angulata	0.45	35	0.40-0.50	0.98	41	0.90-1.00
Thyreosthenius biovatus	0.24	26	0.19-0.29	0.38	21	0.20-0.58

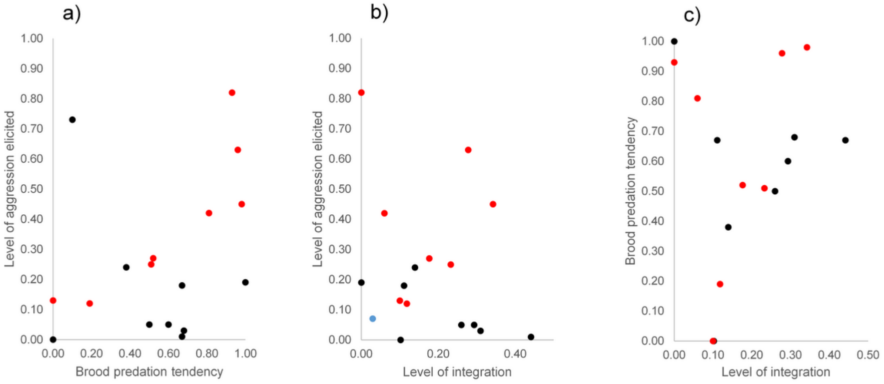
Level of aggression elicited and brood predation tendency

Ant aggression ranged vastly depending on the myrmecophile species (quasibinomial GLM, LR Chisq = 1563.5, *P* < 0.001) (Table 5.2). Some species such as *C. albinus, M. angusticollis* and *C. quadripunctata* were not or only very rarely attacked, while others such as *Q. brevis* and *M. arietina* were heavily attacked. The proportion of individuals that preyed on ant eggs varied greatly among myrmecophile species (quasibinomial GLM, LR Chisq = 199.72, *P* < 0.001) (Table 5.2). *Cyphoderus albinus* and *S. aterrimus* never preyed on ant eggs. In contrast, more than 90% of the individuals of *N. flavipes*, *D. maerkelii*, *T. angulata*, *Q. brevis* and *D*. *pygmaeus* preyed on the ant eggs (Table 5.2). In the presence of ants, a similar (*C. quadripunctata N* = 9*,* proportion individuals preying on eggs = 0.67, *M. conicicollis, N* = 8, proportion individuals preying on eggs = 0.50) or lower proportion of egg predation (*T. angulata, N* = 10, proportion individuals preying on eggs = 0.70) was recorded for the three species that were attracted to the brood chambers compared with the tests without ants.

Do well-integrated species of an inquiline community have a lower brood predation tendency?

Ants did not respond more aggressively towards myrmecophiles that have a higher brood predation tendency (Spearman's rank correlation: r = 0.36, *P* = 0.153, Pearson's product-moment correlation: r = 0.32, *P* = 0.206) (Fig. 5.3a). For example the severe brood parasite *C. quadripunctata* elicited hardly any aggression, whereas the low virulent spider *M. arietina* provoked a strong aggression response (Table 5.2). We did not find a correlation between the level of integration of the myrmecophiles and the aggression response of the ants (Spearman's rank correlation: r = -0.22, *P* = 0.399, Pearson's product-moment correlation: r = -0.22 *P* = 0.404), Those factors were also not linked, when we excluded the observation of the only facultative myrmecophile *P. scaber* (Spearman's rank correlation: r = -0.22, *P* = 0.422, Pearson's product-moment correlation: r = -0.25 *P* = 0.341) ((Fig. 5.3b). Illustrative for this lack of association is the high level of ant aggression towards some species (e.g. *T. angulata*) with a preference for the brood chambers. Finally, nest location preference was also not associated with the brood predation tendency of the myrmecophiles (Spearman's rank correlation: r = 0.08, *P* = 0.761, Pearson's product-moment correlation: r = 0.13, *P* = 0.624) (Fig. 5.3c). Here, some species with a high brood predation tendency (*C. quadripunctata, T. angulata*) preferred the dense brood chambers, whereas other species ranging from no to high brood predation tendency preferentially occurred away from the brood chambers or had no nest location preference.

Fig. 5.3 Relationship between brood predation tendency - level of elicited aggression - level of integration. a Relationship between level of elicited aggression and brood predation tendency (b) relationship between level of integration and level of elicited aggression and (c) relationship between level of integration and brood predation tendency. Level of aggression is the mean proportion of aggressive interactions out of 20 interactions with *F. rufa* workers (Exp.2). Brood predation tendency is the proportion of individuals that preyed on *F. rufa* eggs (Exp.3). Level of integration is the proportion of individuals integrated in the densely populated brood chamber (Exp. 1). Red points refer to staphylinid myrmecophiles, black points to non-staphylinid myrmecophiles, the blue point to the facultative myrmecophile *P. scaber*.



When we only focused on the eight rove beetles, we found a strong positive correlation between worker aggression and brood predation tendency (Spearman's rank correlation: r = 0.88, *P* = 0.007, Pearson's product-moment correlation: r = 0.86, *P* = 0.007) (Fig. 5.3a red points). However, level of integration of rove beetles was not correlated with aggression response (Spearman's rank correlation: r = 0.02, *P* = 0.977, Pearson's product-moment correlation: r = - 0.09, *P* = 0.831) and not with brood predation tendency (Spearman's rank correlation: r = 0.38, *P* = 0.360, Pearson's product-moment correlation: r = 0.27 *P* = 0.513). This means that ants responded more aggressively to rove beetles that are potentially more harmful, but they were not able to deter some harmful species (e.g. *N. flavipes* and *T. angulata*) from the brood chambers. In addition both rove beetles (*Q. brevis* and *L. formicetorum*) with a high (e.g. *Q. brevis*) and a low tendency (*S. aterrimus*) of brood predation had a relatively low integration.

DISCUSSION

In several multi-symbiont systems, it has been reported that symbionts are not homogenously distributed within the host system but occupy different spatial and temporal niches (Friggens and Brown 2005, von Beeren et al. 2010, Witek et al. 2013).

This is further supported by our data on RWA symbionts. We showed that those symbiont species are indeed heterogeneously distributed across their host nests. More specifically, some species were attracted to the densely populated brood chambers, whereas rather poorly integrated species clearly avoided those dense brood chambers. Another group did not appear to be attracted or repulsed by the dense brood chambers. We showed here for the first time the attraction of relatively unspecialized (synechthrans and synoeketes sensu Wasmann (Wasmann 1894)) species towards the brood chambers in social insects. Generally it is assumed that only specialized (symphiles sensu Wasmann (Wasmann 1894)) species are able to settle among the brood in ant colonies (Hölldobler and Wilson 1990).

Niche selection in multiple symbiont systems can result from avoiding competition with other symbionts (described as niche partitioning) (Proffit et al. 2007, Witek et al. 2013). However, in several host-multiparasite systems, it has been reported that the host adjusts its defence to the potential negative impact of the symbiont (Moore 2002, Mburu et al. 2009, Ennis et al. 2010, von Beeren et al. 2010). Niche selection of symbionts can then be an outcome of differential host-symbiont interaction rather than resulting from competition among symbionts. In this case, niche occupation or level of integration results from a varying tolerance of the host for different symbionts. For example, the army ant *Leptogenys* behaves more aggressively towards some associated rove beetles than to others. Therefore the less aggressed species can thrive in the centre of the colony, whereas the other species are only tolerated at the margins of the colony. From an evolutionary point of view, it is a good strategy to be more aggressive to symbionts with a high brood predation tendency and chase them away from the brood chambers. This was hypothesized in Hughes et al. (2008) and supported in von Beeren et al. (2010). In our experiments, ants did act more aggressively towards rove beetles with a higher potential for brood predation and more peaceful to species with no or low brood predation tendency. However, this association was absent, when we look at the entire myrmecophile community, including non-staphylinid myrmecophiles. For example, the spider *M. arietina* had a very low tendency for brood predation, but was heavily persecuted in the aggression experiments and bitten to death in all nest location preference trials. Moreover, our results did not show a correlation between nest location and brood predation tendency for staphilinids and the myrmecophile community as a whole. Species with a preference for the brood chambers were even characterized by a relatively high brood predation tendency. They are not only potentially dangerous, but incur real costs, as the presence of ant workers did not stop them from parasitizing on the brood. Species that avoided brood chambers ranged from non-brood predators to species with a high brood predation tendency. There was also no correlation between nest location and ant’s aggression response for staphylinids and the myrmecophile community as a whole. In contrary to the expectations that species in the brood chambers will provoke less aggression, we found that some species that hardly elicited an aggressive response stayed away from the inner brood chambers or had a more random distribution. Some species (e.g. *T. angulata*), on the other hand, elicited a strong response, but still preferred the dense brood chambers and managed to cope with this highly stressful conditions.

It is puzzling how symbionts with a high brood predation tendency succeed to live within the dense brood chambers without being repulsed. At the proximate level, the tested myrmecophiles employ different strategies to overcome ant defence. In contrast with army ants, wood ant mound architecture provide a plethora of hiding places. Small and slender myrmecophiles, especially rove beetles can quickly squeeze in small holes and cracks when aggressed. Severe brood parasitic rove beetles could therefore, in spite of being recognized as potential harmful, integrate well in the colonies. *Clytra quadripunctata*, the myrmecophile with the highest preference for the brood chamber, on the contrary, relies on a morphological adaptation. When attacked, they withdraw in their protective case and seal the opening with their well armoured head (Donisthorpe 1927). *Monotoma* beetles are slow-moving small beetles and retract their legs when attacked which render them difficult to detect. Future research will also reveal whether chemical strategies such as chemical insignificance are involved in the integration of brood predators (Dettner and Liepert 1994, Lenoir et al. 2001a, van Zweden and d’Ettorre 2010).

At the ultimate level, the lack of rejection of brood predators in the brood chambers can be explained by two theoretical models that are not mutually exclusive. “The evolutionary lag hypothesis” states that no genetic variation in defence strategies emerged in the host at this point. But once available, efficient defence will spread and become fixed. This hypothesis assumes that parasite repulsion is beneficial from the host’s perspective. Here the parasite is currently the winning partner in an ongoing evolutionary arms race and it only takes time before the host evolves counter-adaptations (Rothstein 1975, 1990). However, when a host is infected by multiple parasites, as in our ant-myrmecophile study system, defence strategies can be a compromise to different parasites and clear co-evolutionary traits are consequently harder to identify (Rothstein 1990). Alternatively, the evolutionary equilibrium hypothesis predicts that owing to the costs involved with parasite repellence, parasite acceptance or tolerance counter-intuitively can become beneficial. The arms-race comes here to a standstill in a stable equilibrium and the observed defence strategy is than determined by a balance of parasite load and the costs to defend against those parasites (Zahavi 1979, Rohwer and Spaw 1988, Lotem et al. 1992). For example, the Jacobin cuckoo (*Clamator jacobinus*) lay a non-mimetic egg in the nest of its host. The host cannot eject or puncture the egg because it is too large (double size of host egg) and has a thick shell. The host can still avoid brood parasitism by abandoning the nest, but this entails high costs due to an elevated predation and parasitism risk later in the season which exceed the costs for accepting the cuckoo egg. Therefore a non-mimetic cuckoo egg and the lack of a host defence response will here be a stable equilibrium (Krüger 2011). Defence against parasitic myrmecophiles could also be costly for ants. First, regular task switching to defensive roles involve costs for workers due to time needed to perform defensive behaviour and energy costs owing to shifts in behavioural state (Duarte et al. 2011, Goldsby et al. 2012). Second, myrmecophiles and especially rove beetles may emit repellent, toxic, or alarm inducing chemicals when aggressed (Huth and Dettner 1990, Stoeffler et al. 2011) and might interfere as such normal colony routine and organization.

The presence of brood predators among the brood can dramatically affect colony fitness (Thomas and Wardlaw 1992, Sammataro et al. 2000). However, different mechanisms can lower the cost of the parasites on their RWA host. First, wood ant nests provide a multitude of food resources. We demonstrated that most myrmecophiles only facultatively feed on ant brood (chapter 4: Parmentier et al. in press). Second, RWA parasites control each other by intraguild predation (chapter 4: Parmentier et al. in press). Brood predation can also be lower for some species in presence of ants implying that ants partly deter some brood predators (chapter 3: (Parmentier et al. 2015b). Finally, RWAs nests regularly abandon their nest and construct new mounds on another location. However untested yet, it is argued that nest displacement can be an effective tool to control parasite infection (von Beeren et al. 2010).

CONCLUSIONS

This study provides a unique insight in the different strategies of social insect symbionts and the interactions with their host. We demonstrated that symbionts associated with ants differ greatly in the level of integration in the host nest. We showed that unspecialized species can thrive in the densely populated brood chambers, whereas others are poorly integrated and prefer scarcely populated chambers. Moreover we demonstrated that myrmecophiles have a varying degree of brood predation tendency. Remarkably, a myrmecophile's level of integration in the colony or its brood predation tendency is not linked with the intensity of the aggression response of the host. We found that some potential brood predators are poorly integrated, but others manage to live and are attracted to the brood chambers. Some brood predators appear thus to be in the lead in an evolutionary arms race with their host, as the host does not recognize them as a dreadful foe or do not manage to repel them from the brood chambers. Further investigations will lead to a better understanding in the dynamics between host and parasite and will explore mechanisms which make the presence of brood predators among the brood evolutionary stable.

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APPENDIX CHAPTER 5

Appendix 5-1: Is the aggression response of RWA workers towards myrmecophiles elevated when these myrmecophiles are collected in other RWA nests?

In this behavioural experiment, we tested whether the aggression response of *F. rufa* (West-Vleteren colony) workers towards myrmecophiles collected in the same West-Vleteren colony ("local *rufa* treatment") was different from the aggression response of those *F. rufa* workers (of the same West-Vleteren colony) towards myrmecophiles collected in *F. polyctena* colonies ("*polyctena* treatment"). Experiments followed the protocol outlined in the material and method section "Experiment II: Level of aggression elicited". For every replicate different myrmecophile individuals were introduced in the test arena. In total, we compared aggression in the "local *rufa*" treatment with the "*polyctena*" treatment for 12 out of 18 myrmecophiles that were tested in the main manuscript. Data on aggression in the local rufa treatment can also be found in Table A-5.1. For every tested myrmecophile, we ran a quasibinomial GLM to test whether the proportion of aggressive interactions of F. *rufa* workers in the "local rufa treatment" differed from the "*polyctena* treatment". Significance was tested with a Likelihood Ratio chisquare test implemented in the R package car. P-values are adjusted for multiple testing (Benjamini and Hochberg, false discovery rate: Benjamini and Hochberg (1995)).

Aggression of *F. rufa* workers was similar in the "local *rufa*" and "*polyctena* treatment" for the 12 tested species (Table A-5.1). If there was local or RWA host-specific adaptation, you would expect that *F. rufa* workers would act more aggressively toward myrmecophiles found in *F. polyctena* mounds than toward inquilines found in their own colony. *M. paykulli* has the highest chemical similarity (see chapter 6) with its host out of the 18 myrmecophiles tested in the main document. Nevertheless, these behaviour data also suggest that this species lack RWA host-specific chemical adaptation (Table A-5.1).

Table A-5.1. Proportion aggressive interactions of *F. rufa* workers (West-Vleteren) towards myrmecophiles found in *F. polyctena* colonies (“polyctena” treatment) compared with aggression of *F. rufa* workers (West-Vleteren) towards myrmecophiles found in the same *F. rufa* colony. *N* = number of individuals tested, 95% CI: 95% confidence interval. P = uncorrected P-values, P_{cor} = P-values controlled with the Benjamini-Hochberg method (multiple testing problem). Note that the CI for myrmecophiles in the local treatment can be slightly different from those given in 5.2 in the main file. This is because the CI are estimated in different models. Here we used per species a quasibinomial model with treatment as factor, in Table 5.2 of the main file we used one quasibinomial model with species as factor.

Species	“*polyctena*” treatment			“Local *rufa*” treatment			P	P_{cor}
	Proportion aggressive interactions	*N*	95% CI	Proportion aggressive interactions	*N*	95% CI		
Amidobia talpa	0.08	21	0.05-0.12	0.12	22	0.08-0.16	0.141	0.524
Cyphoderus albinus	0.01	20	0.00-0.02	0.00	15	0.00-0.01	0.257	0.524
Lyprocorrhe anceps	0.28	14	0.21-0.35	0.25	21	0.19-0.30	0.470	0.564
Monotoma angusticollis	0.05	25	0.03-0.07	0.03	20	0.02-0.05	0.09	0.524
Mastigusa arietina	0.73	12	0.64-0.80	(*)				
Monotoma conicicollis	0.05	17	0.03-0.08	0.05	20	0.03-0.07	0.736	0.803
Myrmetes paykulli	0.14	16	0.10-0.20	0.18	18	0.13-0.24	0.302	0.524
Notothecta flavipes	0.52	24	0.45-0.59	0.63	21	0.56-0.71	0.035	0.420
Platyarthrus hoffmannseggii	0.04	20	0.02-0.07	0.05	20	0.03-0.09	0.452	0.564
Porcellio scaber	0.07	15	0.03-0.13	0.07	10	0.02-0.15	0.994	0.994
Quedius brevis	0.74	8	0.60-0.85	0.82	12	0.71-0.90	0.318	0.524
Thiasophila angulata	0.50	31	0.43-0.56	0.45	35	0.39-0.51	0.328	0.524
Thyreosthenius biovatus	0.28	26	0.22-0.34	0.24	26	0.18-0.29	0.349	0.524

(*) The high aggression response of *F. rufa* towards *M. arietina* (cf. Exp. I: all 15 individuals were killed before the end of the experiment, see Fig. cover page chapter 5, Exp II: proportion aggressive interactions = 0.73) was very striking. Here, there could also be an effect of host or colony specific chemical adaptation. Unfortunately this species was only found in *F. polyctena* colonies, so we were unable to test whether *F. rufa* provoked higher aggression towards this species when found in the same colony or originating from *F. polyctena*. But there was no chemical similarity at all with *F. polyctena* (chapter 6) for this species. Moreover we also tested aggression of *F. polyctena* workers following the protocol of Exp. II towards two *M. arietina* individuals found in the same *F. polyctena* colony. Aggression was likewise very high:

- *M. arietina* ind. 1: proportion aggressive interactions = 0.65: (of which 4 biting interactions)
- *M. arietina* ind. 2: proportion aggressive interactions = 0.80: (of which 6 biting interactions)

Individual 1 was even deadly wounded during the aggression tests and died shortly after. Given these data, we assume that *F. rufa* workers would behave in a similar way when *M. arietina* was found in the same colony.

Appendix 5-2: Experimental set-up and distribution of RWA workers and brood at the end of the experiments.

Table A-5.2. Number of individuals recorded at the end of the experiment in the different replicates is given per myrmecophile species. Number of individuals at the beginning of the experiment is given in brackets below. All individuals of *M. arietina* were killed before the end of the experiment. Predation of other myrmecophiles on *C. albinus* explains its low survival.

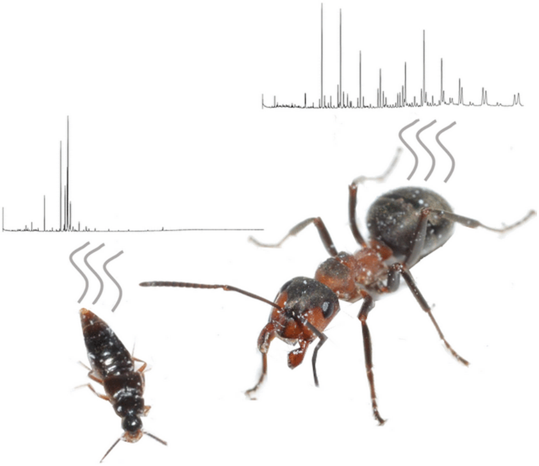
	A. talpa	*C.quadripunct ata*	*C. albinus*	*D.pygmaeus*	*D.maerkelii*	*L. formicetorum*	*L. anceps*	*M. angusticollis*	*M. conicicollis*	*M.paykulli*	*M. arietina*	*N. flavipes*	*P.hoffmannse ggii*	*P. scaber*	*Q. brevis*	*S. aterrimus*	*T. angulata*	*T. biovatus*
replicate 1	10 (11)		5 (20)	1 (1)	2 (2)	4 (4)	4 (4)	7 (7)	4 (4)	4 (4)	0 (5)	5 (5)	2 (5)	5 (5)	3 (3)	3 (3)	8 (8)	3 (3)
replicate 2	6 (7)		7 (20)	1 (1)	1 (1)	2 (2)	2 (3)	4 (4)	4 (4)	4 (4)	0 (3)	4 (5)	5 (5)	4 (5)	3 (3)	2 (3)	6 (6)	2 (4)
replicate 3	9 (9)		8 (20)	1 (1)	1 (1)	2 (2)	4 (4)	3 (4)	3 (3)	3 (3)	0 (4)	4 (5)	5 (5)	4 (5)	3 (3)	3 (3)	5 (5)	4 (4)
replicate 4	13 (13)		9 (20)	1 (1)	2 (3)		2 (3)	4 (5)	4 (5)	3 (4)	0 (2)	3 (3)	2 (5)	4 (5)	3 (3)	4 (4)	3 (5)	6 (6)
replicate 5	10 (11)		6 (20)	1 (1)	2 (2)	8 (8)	4 (4)	3 (5)	3 (5)	2 (4)		2 (3)	6 (6)	5 (5)	3 (3)	3 (3)	2 (5)	3 (6)
replicate 6	8 (10)		3 (20)	1 (1)	2 (2)	1 (1)	3 (3)	2 (4)	6 (6)	3 (4)		3 (3)	3 (5)	3 (5)	3 (3)	2 (3)	3 (5)	6 (6)
replicate 7	3 (10)		5 (20)	3 (3)	3 (3)	5 (6)	6 (6)	2 (3)	5 (5)	6 (4)		4 (3)	4 (4)	4 (5)	2 (3)	4 (4)	10 (10)	4 (5)
replicate 8	9 (9)	6 (6)	3 (20)	1 (1)	5 (5)	2 (2)	3 (4)	2 (3)	3 (3)	3 (3)		3 (3)	5 (5)	3 (5)	3 (3)	3 (3)	7 (7)	2 (3)
replicate 9	9 (10)	7 (7)	3 (19)	1 (1)	6 (6)	1 (2)	3 (3)	4 (5)	2 (2)	3 (3)		4 (4)	5 (5)	2 (4)	3 (3)	3 (3)	5 (5)	3 (3)
replicate 10	9 (10)	7 (7)	0 (15)	3 (3)	4 (5)	5 (5)	3 (3)	2 (2)	2 (2)	2 (2)			4 (5)	4 (4)	3 (3)	4 (4)	6 (6)	3 (3)
replicate 11	1 (5)	5 (5)		1 (1)	2 (2)	1 (3)	4 (4)	2 (2)	2 (2)	1 (1)		1 (2)	5 (5)	4 (4)	0 (1)	4 (4)	6 (6)	2 (3)
replicate 12	3 (5)	4 (5)		1 (1)	2 (2)	2 (3)	2 (2)	1 (1)	2 (2)	1 (1)		2 (2)	7 (7)	3 (4)	1 (1)	1 (3)	10 (10)	3 (4)
replicate 13	3 (5)	5 (5)		1 (1)	2 (2)	0 (3)	3 (3)		2 (2)	1 (1)		1 (2)	5 (5)	5 (5)	1 (1)	4 (4)	5 (5)	2 (3)
replicate 14	6 (6)	3 (5)	21 (30)	3 (3)	4 (5)	9 (11)	4 (5)		6 (6)	3 (5)		3 (5)	7 (7)	3 (5)	2 (2)	4 (5)	3 (5)	5 (6)
replicate 15	4 (4)	2 (3)		3 (3)	3 (3)	4 (8)	3 (3)	2 (4)	2 (2)	2 (3)		2 (3)	3 (5)	3 (5)	1 (2)	3 (3)	7 (7)	4 (6)
replicate 16	3 (7)	5 (5)		3 (3)	3 (3)	6 (7)	4 (5)	9 (9)	5 (6)	3 (3)	0 (1)	2 (3)		3 (4)	1 (1)	3 (3)	5 (5)	2 (4)
Total *N* individuals	**106** (132)	**44** (48)	**70** (224)	**26** (26)	**44** (47)	**52** (67)	**54** (59)	**47** (56)	**55** (59)	**44** (49)	**0** (15)	**43** (51)	**68** (79)	**59** (75)	**35** (38)	**50** (55)	**91** (100)	**54** (69)

Table A-5.3. Distribution of workers in the test nest chambers over the different replicates. Brood chambers always supported the largest number of workers and are marked in grey. Total workers at the end of the experiment is function of the number of workers (=360) at the start of the experiment, dead workers and workers emerged from pupae during the experiment.

	Chamber 1	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**Total Workers**
replicate 1	7	**252**	32	12	24	41	368
replicate 2	13	**137**	125	17	7	38	337
replicate 3	6	13	4	**249**	19	19	310
replicate 4	110	**130**	28	15	9	8	300
replicate 5	9	44	67	32	32	**136**	320
replicate 6	39	26	36	94	**115**	24	334
replicate 7	33	**141**	61	40	37	49	361
replicate 8	73	**145**	40	41	26	51	376
replicate 9	27	66	38	41	**105**	32	309
replicate 10	20	20	**207**	16	23	33	319
replicate 11	**154**	152	28	7	16	17	374
replicate 12	33	12	19	74	74	**135**	347
replicate 13	**137**	81	12	66	23	7	326
replicate 14	**140**	37	31	70	18	23	319
replicate 15	62	69	14	71	**161**	9	386
replicate 16	88	18	32	59	42	**100**	339

CHAPTER 6

Arthropods associate with their red wood ant host without matching the nestmate recognition cues



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ABSTRACT

Social insect colonies provide a valuable resource that attracts and offers shelter to a large community of arthropods. Previous research has suggested that many specialist parasites of social insects chemically mimic their host in order to evade aggression. In the present study, we carry out a systematic study to test how common such chemical deception is across a group of 22 arthropods that are associated with red wood ants (*Formica rufa* group). In contrast to the examples of chemical mimicry documented in some highly specialized parasites in previous studies, we find that most of the rather unspecialized RWA associates surveyed did not use chemical mimicry to evade host detection. Instead, some species employed a strategy known as "chemical insignificance" to evade aggression. Rather than trying to match the chemical hydrocarbon profile of their host, these species avoided detection by a suppression in the production of hydrocarbon cues. Others showed no disguise at all and were rapidly detected by the host, but relied on general defense and flight tactics to evade aggression. These results offer key insight into the early steps in which free-living arthropods have evolved into specialist social insect parasites.

INTRODUCTION

Organisms throughout the animal and plant kingdom use a variety of chemical strategies to deceive other species (Wyatt 2012). They produce signals that mask their true nature from the target species, thereby tricking them to believe they are mating partners, nestmates, harmless or even mutualistic. Spectacular examples can be found in *Mastophora* bolas spiders that lure male moth prey by imitating the female moth sex pheromone (Eberhard 1977) and in the pitchers of carnivorous plants that spread the odor of flowers to trap insects (Joel 1988). Chemical deception, however, has been most thoroughly explored in parasites of social insects (Kistner 1982, Hölldobler and Wilson 1990, van Zweden and d'Ettorre 2010). Previous studies have shown that many arthropods succeed in penetrating the social fortresses of their hosts and evade aggression by matching the chemical profile of their social insect host (Nash and Boomsma 2008, van Zweden and d'Ettorre 2010). Such deception can occur by passively acquiring the host's cuticular hydrocarbons that are used in nestmate recognition ("chemical camouflage") or in some cases even by actively producing them ("chemical mimicry") (Nash and Boomsma 2008, van Zweden and d'Ettorre 2010). We will refer to both types of matching as chemical mimicry throughout the rest of the document. In a few cases, the secretion of compounds that appease, repel or manipulate the host have also been reported (Hölldobler and Wilson 1990, Thomas et al. 2002, Akino 2008), whereas a small number of studies have also documented a strategy of "chemical insignificance", whereby arthropods suppress the production of hydrocarbons used in nestmate recognition to escape detection (Nash and Boomsma 2008, van Zweden and d'Ettorre 2010).

Animals living inside the nest of social insects are known as inquilines. The group of arthropod inquilines associated with social insects comprise parasitic social insects (here referred to as "social inquilines") and a wide variety of non-social arthropods (Kistner 1982, Hölldobler and Wilson 1990). Current data indicate that chemical mimicry is especially common among social inquilines that are phylogenetically related to their host (Nash and Boomsma 2008, Buschinger 2009), which is to be expected, since their shared ancestry means that the host recognition cues can be imitated without requiring specific, entirely novel adaptations (Nash and Boomsma 2008). Surprisingly, however, complete or partial chemical mimicry or camouflage has also been reported as an integration mechanism in the large majority (45 out of 56 studies, Appendix 6-1: Table A-6.1) of the very diverse group of inquiline arthropods that live in the nest of a non-related social insect host. Nevertheless, most of these studies are biased in the sense that they have focused mainly on highly specialized parasites, which interact very closely with their host. Such species engage in regular grooming interactions with their host, solicit food from them, and are often treated as regular colony members (Hölldobler and Wilson 1990, Kronauer and Pierce 2011, Parker 2016). In many cases, these specialized arthropods also combine complex chemical adaptations with other advanced strategies such as morphological structures and acoustical mimicry (Hölldobler and Wilson 1990, Di Giulio et al. 2015, Parker 2016). The true incidence of chemical mimicry in these systems, however, may well be lower than presumed, as there likely is a strong publication bias towards studies where such adaptations were found. In addition, most studies typically compare only a single or a few associates with their host and there have been no studies that systematically surveyed and compared mechanisms of chemical integration in a large community of arthropods associated with a single host.

The aim of the present study was to carry out a systematic study of cuticular chemical similarity across a group of less specialized, ant-associated arthropods. Such species are not accepted in the colony by being groomed, fed or transported in contrast with specialized symbionts (Kistner 1982, Hölldobler and Wilson 1990). They are attacked or ignored, exhibit limited interaction with their host and elicit aggression to a varying degree (Parmentier et al. 2016b). Currently, there is very little information on what chemical integration mechanisms or strategies such species use to evade host aggression, even though they can provide us with key insight into the early steps of a host-parasite co-evolutionary arms race and the way in which free-living arthropods may have evolved into specialist parasites. Hence, we here analyzed the chemical profiles of a large community of arthropods associated with red wood ants and compared them to those of their host workers. Subsequently, we link the chemical data with other functional traits of the community.

MATERIALS AND METHODS

Study system

As a study system we used red wood ants (*Formica rufa* group) and their community of associated arthropods. In our study region (Northern Belgium and Northern France), three of the six species of the *F. rufa* group can be found: *Formica rufa* Linnaeus, 1761, *Formica polyctena* Förster,1850 and *Formica pratensis* Retzius, 1783 (Dekoninck et al. 2010). They tend to differ in ecological preferences and in colonial organization (Seifert 2007), but the sampled colonies of all species were all polygynous (containing multiple queens) and polydomous (having a colony that spreads out over multiple mounds) and all occurred along forest edges.

Red wood ants (RWAs) support a very diverse community of arthropods. Most RWA myrmecophiles live as inquilines inside the nest, whereas other myrmecophiles live extranidally in the close vicinity of the nest (Parmentier et al. 2014). Besides obligate myrmecophiles, RWA nests also host a wide array of facultative myrmecophiles. These species are not strictly associated with ants, but can occur in RWA mounds (Parmentier et al. 2014). A large group of myrmecophiles associated with RWAs can also be found in nests of other ant species (Parmentier et al. 2014). RWAs also interact with aphids outside the nest. These provide sugary honeydew which is a major food source for RWAs (Skinner 1980). Aphids can also be considered as myrmecophiles, but the focus of this study is on commensalic and parasitic arthropods (Parmentier et al. 2016a), so-called synechtrans and synoeketes (Wasmann 1894). However, the exact nature of the association of RWA myrmecophiles with their host is poorly known. For example, many RWA myrmecophiles also provide mutualistic services to their host by preying on other parasitic myrmecophiles in the nest (Parmentier et al. 2016a).

Sample collection

Myrmecophiles were collected in three different *F. rufa* populations (R1: Boeschepe, R2: Vladslo, R3: West-Vleteren), six *F. polyctena* populations (O1: De Haan, O2: Beisbroek, O3: Beernem, O4: Aartrijke, O5: Roksem and O6: Herentals) and one *F. pratensis* population (P1: Veltem-Beisem) (Fig. 6.1). In every population, we collected samples of a single polydomous colony. Nest material was taken at different locations in the nest and was gently spread onto a white tray in the field. All myrmecophiles and ants were then collected by using an aspirator, which was regularly cleaned with hexane to minimize contamination. An overview of the 18 collected inquiline (intranidal) myrmecophiles with some life history traits is given in Table 6.1. In addition to these 18 RWA inquilines, three RWA myrmecophiles that live extranidally were collected: *Coccinella magnifica,* which is a ladybird (Coccinellidae) closely related to *Coccinella septempunctata* (Sloggett et al. 1998) and adults of the leaf beetle (Chrysomelidae) *Clytra quadripunctata* (larvae live intranidally) were captured on plants around RWA mounds, whereas *Pella humeralis*, a rove beetle (Staphylinidae) that mostly scavenges in the neighbourhood of ant trails (Donisthorpe 1927), was found at the periphery of a RWA nest. Finally, two facultative myrmecophiles were collected: *Porcellio scaber* (the common rough woodlouse) and the rove beetle *Xantholinus linearis*. In contrast to the myrmecophiles mentioned before, those species can be found away from ants, but are occasionally found in RWA mounds (Parmentier et al. 2014). Finally, we collected individuals of the ladybird *C. septempunctata,* the free-living relative of *C. magnifica*. Myrmecophiles and ants were kept together with some nest material and transferred with a clean forceps to 2 ml glass vials (Sigma-Aldrich) in the lab. Animals were stored in the freezer at -18 °C until extraction.

Chemical analyses

CHCs from small myrmecophiles and ant workers were extracted for 10 minutes in 30 µL of hexane (HPLC, Sigma-Aldrich) in 2 ml vials capped with a PTFE septum (Sigma-Aldrich). Large myrmecophiles (the leaf beetle *C. quadripunctata* adult + larva, the ladybird *C. magnifica* adult + larva), the ladybird *C. septempunctata* and the isopod *P. scaber* were extracted in 200 µL of hexane for 10 minutes. Samples were evaporated to dryness at room temperature in a laminar flow hood and stored at -18 °C. Prior to analysis, samples were redissolved in either 6 µL, 30 µL or 200 µL hexane depending on the concentration of cuticular compounds that was present. For small myrmecophiles or species with low hydrocarbon concentrations, CHCs of multiple individuals were extracted per sample. 2 µL of each hexane extract was injected into a SHIMADZU QP 2010 ULTRA coupled gas chromatograph/mass spectrometer coupled with a DB-5ms capillary column (30 m x 0.25 mm x 0.25 µm). Samples diluted in 6 µL were manually injected, whereas samples diluted in 30 µL and 200 µL were injected with an autosampler. The method had an initial temperature profile consisting of 1 minute at 70 °C, two temperature ramps from 70 °C to 150 °C at 20 °C min^{-1} and from 150 °C to 320 °C at 3 °C min^{-1}, after which the final temperature of 320 °C was held for 15 minutes. We used helium as a carrier gas at a flow rate of 1 mL min^{-1}, splitless injection, an inlet temperature of 280 °C, and a final pressure of 75 kPa. The electron ionization voltage was auto-tuned to enhance the acquisition performance according to the molecular weight of the compounds, and the ion source temperature was set to 300 °C. In each batch we ran a linear C7 to C40 linear alkane ladder standard (49452-U, Supelco) at two different concentrations (0.001 µg/mL and 0.01 µg/mL). Retention indices were calculated using cubic spline interpolation (Messadi et al. 1990) based on the elution times of the external alkane ladder and compound quantities (ng) in the samples were estimated based on the compound peak areas and those of the closest eluting alkane in the alkane ladder standard. These calculations were done using an in-house developed R script (available from the authors on request).

Peak identifications were restricted to CHCs with chain lengths between C20 and C40, which encompasses the range of CHCs previously described in RWAs (Martin et al. 2008). The identification and quantification of larger chain length CHCs, which are present in *Formica* ants (Martin et al. 2008), was not possible given the limitations of the used column and GCMS system. All detectable CHC peaks in the samples were identified and used in the analyses. Hydrocarbons were identified on the basis of their retention index, mass spectra and expected fragmentation patterns and diagnostic ions. Double bond positions of alkenes were not determined. CHC peak quantities (ng) were square root transformed and standardized by the total CHC amount (ng).

Characteristics of the chemical profile

Chemical similarity was estimated by the Bray-Curtis (BC) distances between host workers and myrmecophiles in terms of their CHC profile based on square-root transformed relative quantities (ng). The amount of CHCs produced per unit of cuticular surface area (“CHC concentration”, in ng/mm^2, i.e. corrected for the variation in body size) were based on measured absolute CHC quantities (ng) divided by total cuticular surface area in a sample. Surface areas were calculated by subdividing the bodies of the animals into geometric shapes (detailed methodology, mean species surface and number of samples in Appendix 6-2 and Table A-6.2 therein) of which the dimensions were determined using a Wild M3 binocular stereomicroscope with a measuring eyepiece.

The proportion of CHC in the chemical profile was measured by dividing the total quantities of CHC (all detectable hydrocarbons) by the quantities of CHCs and non-CHCs (quantities larger than 0.1% of total quantities). This cut-off value was used to prevent that noise or contamination would be considered.

Associations between the different traits of the myrmecophile community

Here we assessed whether BC distance and CHC concentration (ng/mm^2) on the cuticle were correlated with other functional traits of the RWA myrmecophile community explored in previous studies in order to find general patterns in chemical integration mechanisms. These functional traits were explored in previous studies (Parmentier et al. 2016a, b) and include the trophic position of myrmecophiles using δ^{15}N-values, host specificity (categories are here given a rank order: strict specialist = 4: only records with RWAs; specialist = 3: some records with non RWAs, but RWAs are the main host; moderate = 2: records with RWAs, but distribution in non-RWAs probably important as well; generalist = 1: myrmecophiles have no preference for a particular ant species, but are always found in presence of ants), level of aggression elicited, brood predation tendency and level of nest integration (proportion individuals present in brood chambers) (Table 6.1).

Statistical analyses

For visualisation of the chemical similarities of the complete CHC profile, we applied non-metric multidimensional scaling (NMDS) on the BC distance matrix of the relative square root transformed CHC quantities (ng). We selected the square root transformation as it minimizes the effect of very large peaks, but still preserves quantitative information very well. It can also deal with zero values in contrast with for example the widely used Aitchinson transformation (Aitchison 1986). Nevertheless, preliminary calculations using untransformed square root transformed and fourth root transformed data showed that our data were robust to different types of transformations. Apart from the similarity of the total set of hydrocarbons, we examined the pattern of similarity with a subset of different classes of hydrocarbons (n-alkanes, alkenes, methyl-branched alkanes and dimethyl-branched alkanes) separately. Indeed, there is some evidence that ants only use a subset of peaks to recognize nestmates (Martin et al. 2008, Guerrieri et al. 2009) and myrmecophiles could therefore deceive the host by matching a part of the bouquet. Peaks of a particular class were square root transformed and divided by the total (after square root transformation) amount of compounds belonging to that class in the profile. For each myrmecophile species CHC similarity with workers of the host ant species was tested by an ANOSIM permutation (Primer software version 7.0.11, 9999 permutations) test based on the BC distance of the standardized CHC abundances. Most myrmecophiles were collected in two or even three RWA host species. To account for possible species-specific chemical adaptations to their RWA host ant species, we used for these species a two-way crossed design in which we included a factor that grouped RWA workers and samples of a particular myrmecophile species and a factor which grouped RWA workers and myrmecophile individuals collected in nests of the same RWA species. A more detailed grouping of workers and myrmecophiles per nest dramatically reduced the maximum number of permutations in many species. Therefore we preferred to test the differences between RWA workers and myrmecophiles across nests of the same RWA host species rather than across individual nests. Number of ants and myrmecophiles used for these tests are listed in Table 6.2. For some myrmecophiles, there were too few samples to run 9999 permutations and then the maximum possible number of permutations was tested (see Table 6.2). The clown beetles (Histeridae) *M. paykulli* and *D. pygmaeus* had many compounds in common with their hosts, but lacked some compounds which might be present in trace quantities. To avoid that the absence of compounds could affect our analysis, we ran similar NMDS ordinations for the complete and different subsets of the CHC profile shared by the three RWA species, *M. paykulli* and *D. pygmaeus*. For this shared CHC dataset, we found that a NMDSs ordination based on the Aitchinson transformation (Aitchison 1986) and Euclidean distance matrix had lower STRESS (= better representation of the dissimilarities across the samples) than a NMDS ordination based on square root transformed data and a BC distance matrix (STRESS: 11.8 vs. 14.5). Therefore we selected for the NMDS and ANOSIM of the shared data set this transformation and distance matrix. Because of the high similarity in the profile of RWA workers and beetles, these myrmecophiles might rely not only on species-specific but also on colony-specific adaptations to the chemical profile of the supporting colony. As a result, differences between workers and either *M. paykulli* or *D. pygmaeus* were tested with a two-way crossed ANOSIM in which we included a factor that grouped workers and *M. paykulli* or *D. pygmaeus* and a factor that grouped the workers and beetles collected in the same nests.

A non-parametric Kruskal-Wallis test was performed to test overall difference in CHC concentration and proportion of CHCs. Per myrmecophile species CHC concentrations (ng/mm^2) differences with CHC concentrations (ng/mm^2) of RWAs were tested using a Wilcoxon rank sum test with continuity correction.

The association of the Bray-Curtis distance to the host workers (based on the mean BC distance between an individual and workers found in the same nest, see Table 6.1) and CHC concentration (ng/mm^2) with other functional traits of RWA inquilines were tested with Spearman correlations. Extranidal myrmecophiles and larvae of the leaf beetle *C. quadripunctata* were not included in the correlation analyses. This latter lives enclosed in a case made of ant nest material and ants do not directly detect the chemical composition of the larvae. The same analyses were also performed focusing on only the group of 8 inquiline Staphylinidae beetles.

Confidence intervals of BC distance to the host workers, CHC concentrations and proportion CHC found in Table 6.1 were estimated by bootstrapping using package boot. Confidence intervals or standard errors of the other parameters of Table 6.1 were taken from earlier studies.

All the analyses were done in R version 3.2.1 (R Core Team 2014). P-values in analyses with multiple tests, i.e. ANOSIM permutation tests, Wilcoxon rank sum tests, Spearman correlation tests, were corrected for multiple testing using the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995).

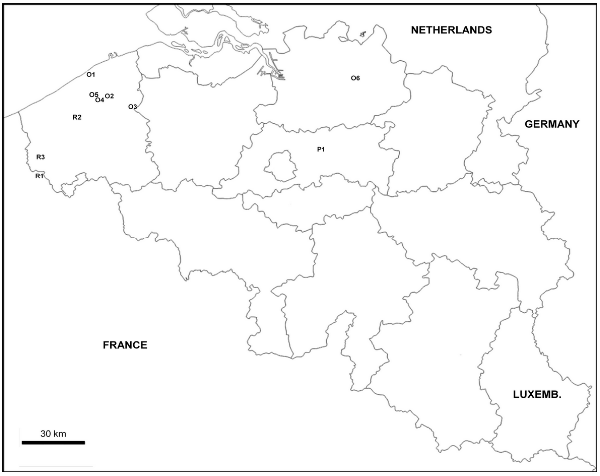

Figure 6.1. Map of the sampled RWA populations in Belgium and Northern France**.**

RESULTS

Characteristics of the chemical profile

120 different CHC peaks were found in total across all our samples (Appendix 6-3: Table A-6.3). Some peaks contained different CHCs that could not be separated with the described GCMS's settings. Red wood ants (RWAs) possessed most CHC peaks (*Formica rufa* = 86, *Formica polyctena* = 87, *Formica pratensis* = 83) together with the clown beetles *Myrmetes paykulli* (*N* = 87) and *Dendrophilus pygmaeus* (*N* = 78) (Table A-6.3). *Myrmetes paykulli* had 83 out of 87 compounds in common with RWAs, *D. pygmaeus* 76 out of 78. The tested organisms (larvae of two myrmecophiles were considered as distinct organisms) differed significantly in the proportion of hydrocarbons in their profile (Kruskal-Wallis rank sum test, Chi-squared = 158.86, df = 24, P < 0.001) (Table 6.1). As expected, the profile of RWA workers comprised almost uniquely CHCs (e.g. *F. polyctena* 0.97, CI: 0.97-0.98). Myrmecophiles, however, varied vastly in the proportion of CHC in their chemical cuticular profile. Some species' profile contained akin to their ant host mainly CHC (e.g. *M. paykulli* 0.95, CI: 0.93-0.96). In other species non-CHC ranged from an important part to almost complete domination of the profile (e.g. the rove beetle *Quedius brevis* 0.03, CI: 0.00-0.04). Characteristic chemical chromatograms and figures of the RWA hosts and associated myrmecophiles are given in Appendix 6-4.

The NMDS of the standardized CHC quantities separated the RWA workers clearly from most myrmecophiles (Fig. 6.2A). This clear distinction in profile between myrmecophiles and their host is confirmed by the ANOSIM permutation tests (Table 6.2). Whenever the sample size was high enough, myrmecophiles were highly significantly different from their host (Table 6.2). A similar distinction between RWA workers and myrmecophiles was observed in the analyses with subsets of the CHC profile (Fig 6.2B for dimethyl-alkanes, but other subsets generated similar NMDS plots). Only the clown beetles (Histeridae) *M. paykulli* and *D. pygmaeus* aggregated within the RWA cluster (Fig. 6.2A, 6.2B) and showed high similarity in their chemical profile with RWAs (Appendix 6-3). More detailed NMDS analyses focusing on the CHC compounds that RWA workers and these beetles had in common were also performed. The RWA workers clustered in distinct nest-specific clusters. *Dendrophilus pygmaeus* and *M. paykulli* were not found within the cluster of the host nest, although the latter tend to plot closer to their host nest than to other RWA nests (Fig. 6.3A, 6.3B). Similar patterns were found for all subsets of the CHC profile (Fig. 6.3B, only plot for dimethyl-alkanes is provided). Permutation tests showed that *M. paykulli* (4 separate ANOSIM tests for all CHC compounds, alkanes, methyl- and dimethylalkanes, for all tests $R > 0.8$, $P < 0.001$, permutations = 9999) and *D. pygmaeus* (4 separate ANOSIM tests for all CHC compounds, alkanes, methyl- and dimethylalkanes, for all tests $R = 1$, $P = 0.067$, lowest value possible as the max. number of permutations was 15) were chemically different from host nest workers: In spite of their similarity in CHCs, they also elicited a significant aggression response (Table 6.1). CHC concentration per mm² body surface was significantly different across all tested organisms (Kruskal-Wallis rank sum test, Chi-squared = 124.85, df = 23, $P < 0.001$). RWAs were characterized, except for one species, by the highest CHC concentration per mm² body surface (mean concentration ± SE: 228.6 ng/mm² ± 25.7, Table 6.1, Fig. 6.4).

Seventeen out of 21 myrmecophiles (for the ladybird *Coccinella magnifica* and the leaf beetle *Clytra quadripunctata* only the larvae had lower concentrations) had significantly lower CHC concentration than RWA workers (Appendix 6-5: Table A-6.4). The lowest concentrations were found in the facultative isopod *Porcellio scaber* (mean concentration ± SE: 0.19 ng/mm² ± 0.02, Table 6.1), but also 10 obligate myrmecophiles (Table 6.1) had concentrations 10 to 1000-fold lower than RWAs. Some species (*P. scaber*, the rove beetle *Stenus aterrimus,* the root-eating beetles (Monotomidae) *Monotoma angusticollis* and *Monotoma conicicollis*, the isopod *Platyarthrus hoffmannseggii*, the spider *Thyreosthenius biovatus* and the springtail *Cyphoderus albinus*) with very low concentrations of hydrocarbons per mm² of cuticle were mostly ignored in aggression trials and are therefore expected to be chemically insignificant (Table 6.1). However, other species with very low hydrocarbon concentrations (and other compounds as well) were immediately detected and heavily persecuted (e.g. the spider *M. arietina* and the beetle *Q. brevis*).

Table 6.1. Functional traits of arthropods associated with RWAs. BC dissimilarity, CHC concentration and CHC proportion were determined in this study. Trophic niche: S = scavenger, A = active hunter. Means and confidence interval in brackets are provided, for traits with data with unequal variances, ± SE is given.

Species	Taxon	Host specificity	BC dissimilarity	CHC concentration (ng/mm²)	CHC proportion	Brood predation tendency	Trophic level (δ^{15}N) / niche	Prop. in brood chamber	Prop. aggressive interactions
OBLIGATE INQUILINE									
Amidobia talpa	Coleoptera (Staphylinidae)	specialist	0.55 [0.50-0.59]	36.1 ± 15.9	0.75 [0.56-0.88]	0.18 [0.06-0.36]	2.7 ± 0.2 / S	0.11 [0.06-0.19]	0.12 [0.08-0.17]
Dinarda maerkelii	Coleoptera (Staphylinidae)	specialist	0.63 [0.59-0.66]	78.0 ± 14.4	0.37 [0.15-0.52]	0.52 [0.33-0.72]	3.2 ± 0.2 / S	0.16 [0.07-0.30]	0.27 [0.21-0.33]
Leptacinus formicetorum	Coleoptera (Staphylinidae)	specialist	0.67 [0.61-0.72]	6.7 ± 7.7	0.20 [0.07-0.47]	0.81 [0.59-0.95]	6.7 ± 0.3 / A+S	0.12 [0.04-0.23]	0.42 [0.32-0.51]
Lyprocorrhe anceps	Coleoptera (Staphylinidae)	specialist	0.64 [0.60-0.68]	34.9 ± 8.4	0.34 [0.17-0.55]	0.51 [0.36-0.67]	3.0 ± 0.2 / S	0.28 [0.16-0.42]	0.25 [0.19-0.31]
Notothecta flavipes	Coleoptera (Staphylinidae)	specialist	0.70 [0.67-0.73]	144.9 ± 24.2	0.86 [0.76-0.92]	0.96 [0.83-1.00]	2.6 ± 0.5 / S	0.28 [0.15-0.44]	0.63 [0.56-0.70]
Quedius brevis	Coleoptera (Staphylinidae)	moderate	0.93 [0.92-0.94]	11.2 ± 3.0	0.01 [0.01-0.03]	0.93 [0.73-0.99]	5.7 ± 0.4 / A+S	0.00 [0.00-0.10]	0.82 [0.74-0.88]
Stenus aterrimus	Coleoptera (Staphylinidae)	strict specialist	0.87 [0.86-0.89]	0.4 ± 0.1	0.01 [0.01-0.02]	0.00 [0.00-NA]	5.2 ± 0.2 / A	0.10 [0.03-0.22]	0.13 [0.08-0.18]
Thiasophila angulata	Coleoptera (Staphylinidae)	specialist	0.49 [0.47-0.52]	80.7 ± 14.5	0.41 [0.29-0.54]	0.98 [0.90-1.00]	4.2 ± 0.2 / S	0.37 [0.27-0.48]	0.45 [0.40-0.50]
Emphylus glaber	Coleoptera (Cryptophagidae)	specialist	0.73 [0.69-0.77]		0.03 [0.01-0.09]		3.4 ± 0.2		0.30 [0.16-0.50]
Monotoma angusticollis	Coleoptera (Monotomidae)	strict specialist	0.69 [0.66-0.72]	1.5 ± 0.3	0.10 [0.06-0.17]	0.68 [0.49-0.83]	3.6 ± 0.8 / S	0.23 [0.12-0.38]	0.03 [0.01-0.06]
Monotoma conicicollis	Coleoptera (Monotomidae)	strict specialist	0.30 [0.23-0.38]	4.1 ± 2.8	0.46 [0.20-0.75]	0.50 [0.29-0.71]	3.0 ± 0.7 / S	0.33 [0.21-0.47]	0.05 [0.02-0.08]
Dendrophilus pygmaeus	Coleoptera (Histeridae)	specialist	0.16 [0.13-0.20]	91.8 ± 26.4	0.95 [0.84-0.99]	1.00 [NA-1.00]		0.00 [0.00-0.13]	0.19 [0.10-0.31]
Myrmetes paykulli	Coleoptera (Histeridae)	specialist	0.14 [0.13-0.16]	107.6 ± 15.2	0.95 [0.92-0.97]	0.67 [0.46-0.83]	5.9 ± 0.6 / S	0.11 [0.04-0.25]	0.23 [0.13-0.25]
Mastigusa arietina	Araneae (Dictynidae)	moderate	0.71 [0.68-0.75]	1.3 ± 0.4	0.15 [0.07-0.28]	0.10 [0.01-0.36]	5.9 ± 0.7 / A		0.73 [0.64-0.81]
Thyreosthenius biovatus	Araneae (Linyphiidae)	specialist	0.83 [0.81-0.85]	3.5 ± 2.3	0.02 [0.01-0.03]	0.38 [0.20-0.58]	5.9 ± 0.6 / A	0.22 [0.12-0.36]	0.24 [0.19-0.29]
Cyphoderus. albinus	Collembola (Cyphoderidae)	generalist	0.35 [0.30-0.40]	26.2 ± 7.1	0.62 [0.37-0.82]	0.00 [0.00-NA]	2.7 ± 0.3 / S	0.13 [0.06-0.23]	0.00 [0.00-0.02]
Platyarthrus hoffmannseggii	Isopoda (Platyarthridae)	generalist	0.59 [0.56-0.62]	7.3 ± 0.1	0.20 [0.12-0.32]	0.60 [0.39-0.79]	5.4 ± 0.1 / S	0.25 [0.15-0.37]	0.05 [0.03-0.09]
Clytra quadripunctata (larva)	Coleoptera (Chrysomelidae)	specialist	0.56 [0.49-0.63]	0.6 ± 0.01	0.16 [0.05-0.40]	0.67 [0.48-0.83]	4.0 ± 0.3 / S	0.45 [0.30-0.61]	0.01 [0.00-0.03] (with case) 0.87 [0.70-0.96] (naked larva)
OBLIGATE, EXTRANIDAL MYRMECOPHILE									
Clytra quadripunctata (adult)	Coleoptera (Chrysomelidae)	specialist	0.40 [0.36-0.43]	289.0 ± 15.8	0.97 [0.93-0.99]		5.2 ± 0.6		
Coccinella magnifica adult	Coleoptera (Coccinellidae)	strict specialist	0.51 [0.48-0.53]	204.2 ± 21.6	0.91 [0.86-0.94]				
Coccinella magnifica larva	Coleoptera (Coccinellidae)	strict specialist	0.45 [0.49-0.63]	74.4 ±19.6	0.62 [0.46-0.75]				
Pella humeralis	Coleoptera (Staphylinidae)	moderate	0.22 [0.16-0.30]	115.4 ± NA	0.99				0.13 [0.06-0.24]
FACULTATIVE INQUILINE									
Porcellio scaber	Isopoda (Porcellionidae)	facultative	0.80 [0.75-0.84]	0.2 ± NA	0.02 [0.01-0.08]		1.6 ± 0.3 / S	0.03 [0.00-0.12]	0.07 [0.03-0.13]
Xantholinus linearis	Coleoptera (Staphylinidae)	facultative	0.33 [0.26-0.41]	79.5 ± NA	0.76				

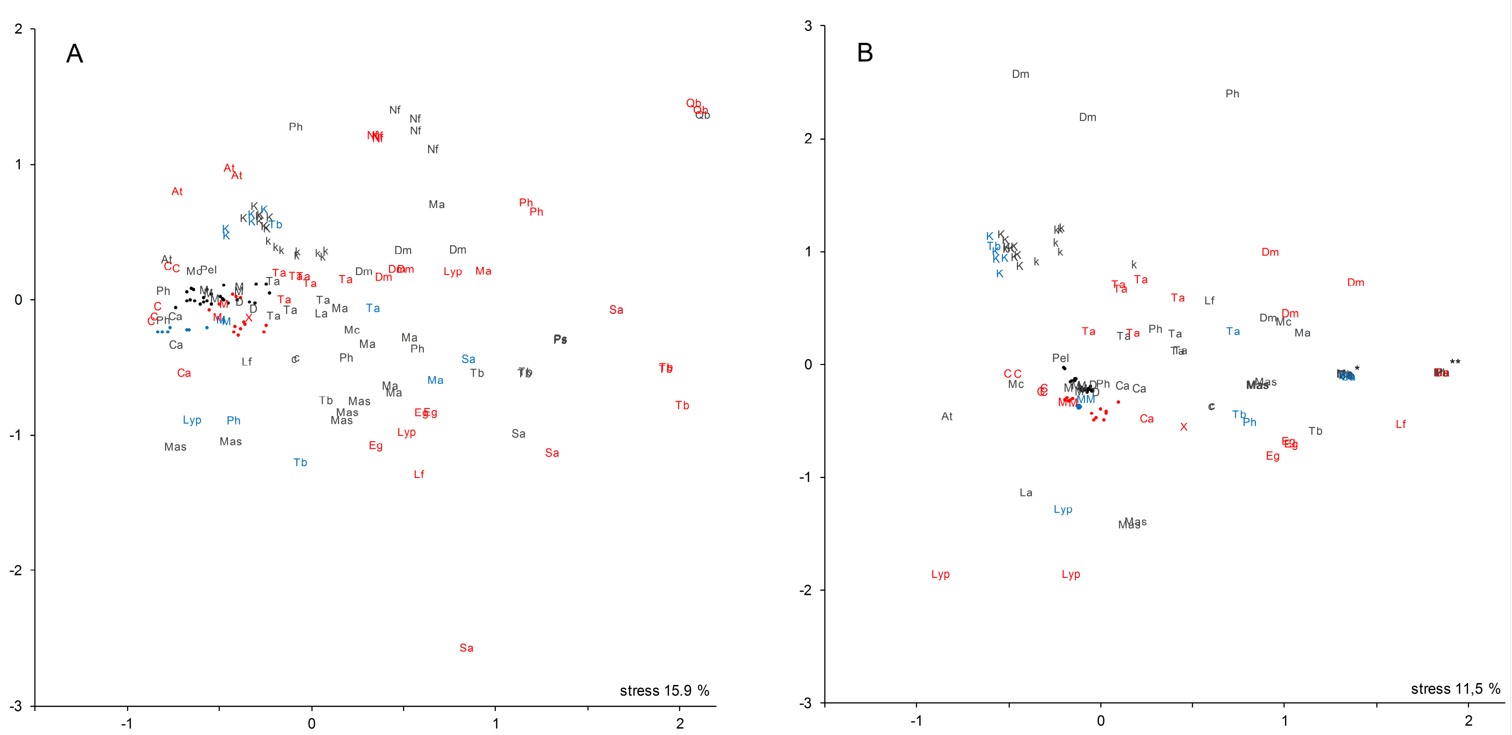

Figure 6.2. Chemical similarity among myrmecophiles and RWA hosts. NMDS plot displays the Bray-Curtis distances among myrmecophiles and RWA hosts: A) for all detected CHCs, B) for all detected dimethyl-alkanes. RWA workers are represented by colored dots: *Formica polyctena* (black), *F. rufa* (red), *F.pratensis* (blue). Myrmecophiles are indicated with codes of which the color correspond with the color of the host species dots. Codes: *Amidobia talpa* (At), *Coccinella magnifica* adult (K), C. *magnifica* larva (k), *Clytra quadripunctata* adult (C), *C quadripunctata* larva (c), *Cyphoderus albinus* (Ca), *Dendrophilus pygmaeus* (D), *Dinarda maerkelii* (Dm), *Emphylus glaber* (Eg), *Leptacinus formicetorum* (Lf), *Lyprocorrhe anceps* (La), *Mastigusa arietina* (Mas), *Monotoma angusticollis* (Ma), *Monotoma conicicollis* (Mc), *Myrmets paykulli* (M), *Notothecta flavipes* (Nf), *Pella humeralis* (Pel), *Platyarthrus hoffmannseggii* (Ph), *Porcellio scaber* (Ps), *Quedius brevis* (Qb), *Stenus aterrimus* (Sa), *Thiasophila angulata* (Ta), *Thyreosthenius biovatus* (Tb), *Xantholinus linearis* (X). Overlapping codes in B) (*): *M. angusticollis* (3 black, 1 blue), *T. biovatus* (black), *S. aterrimus* (blue and black) and (**): *S. aterrimus* (2 red, 1 black), *P. scaber* (2 black), *M. angusticollis* (black), *T. biovatus* (black) and *P. hoffmannseggii* (black).

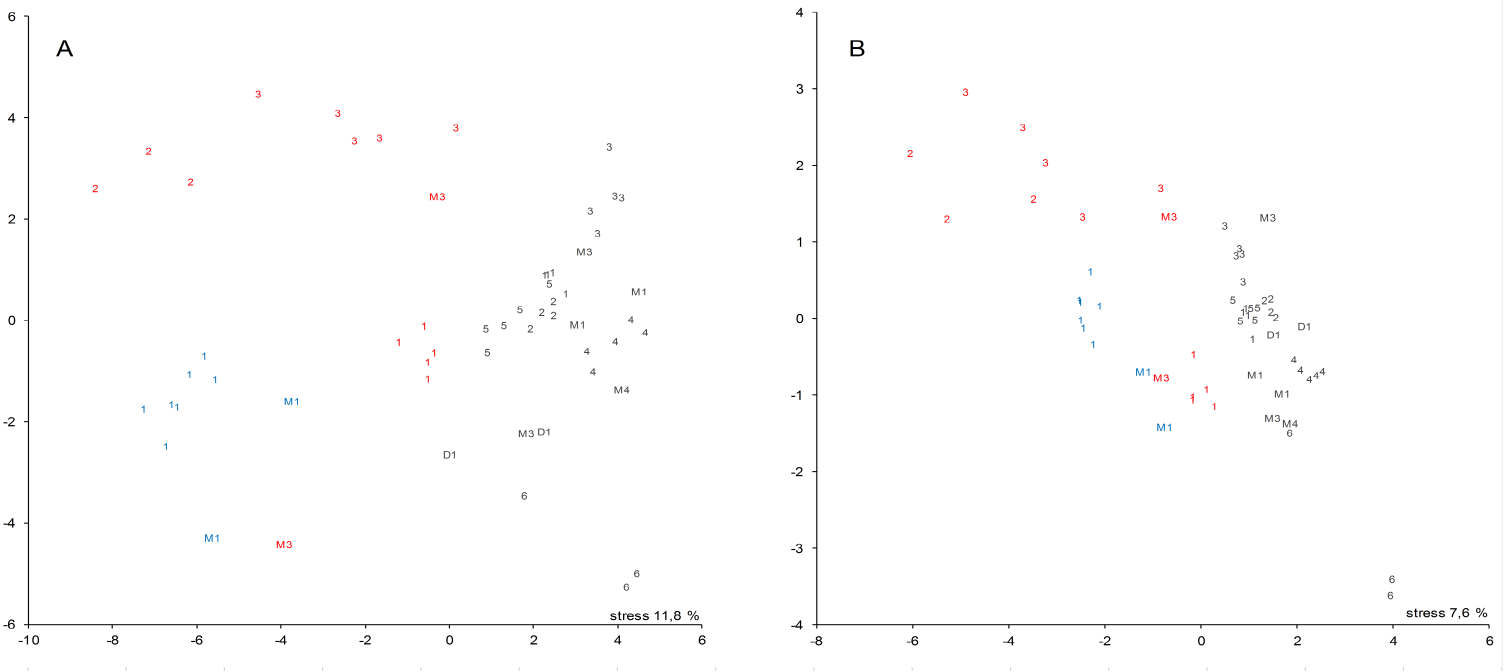

Figure 6.3. Chemical similarity between the clown beetles *Myrmetes paykulli*, *Dendrophilus pygmaeus* and RWA hosts. NMDS plot displays the Euclidean distances among *M. paykulli*, *D. pygmaeus* and RWA hosts: A) for all shared CHCs, B) for all shared dimethylalkanes. RWA workers are represented with a colored number which refers to nest origin and host species. Black numbers refer to the 6 *F. polyctena* nests, red to the 3 *F. rufa* nests, and blue numbers to the single *F. pratensis* nest. The code numbers correspond with the numbers in the nest codes in Fig.6.1. The first letter of the codes of *M. paykulli* (9 individuals) and *D. pygmaeus* (2 individuals) is respectively “M” and “D” followed by the colored number code of the nest in which the beetles were collected.

Table 6.2. Results of ANOSIM permutation tests in which Bray-Curtis distances between myrmecophiles and workers of the RWA host species that supported them were compared with the Bray-Curtis distances between different workers of the RWA host species. *N* = number of individuals for each myrmecophile species used in the ANOSIM tests, N_{workers} = number of RWA workers across the 3 RWA host species which were compared with the myrmecophile species in the ANOSIM tests. In total we sampled in *Formica rufa*: 13 workers, in *F. polyctena*: 26 workers and in *F. pratensis* 7 workers.

Species	**R**	**Permutations**	***N***		N_{workers}		***P***	**BH *P***
				F. rufa	*F. polyctena*	*F. pratensis*		
Amidobia talpa	1.00	9999	4	13	26		0.0001	< 0.001
Clytra quadripunctata adult	1.00	8568	5	13			0.0001	< 0.001
Clytra quadripunctata larva	1.00	378	2		26		0.0030	0.004
Coccinella magnifica adult	1.00	9999	13		26	7	0.0001	< 0.001
Coccinella magnifica larva	1.00	9999	8		26	26	0.0001	< 0.001
Cyphoderus albinus	0.88	5292	3	13	26		0.0003	< 0.001
Dendrophilus pygmaeus	0.51	378	2		26		0.0240	0.026
Dinarda maerkelii	1.00	9999	6	13	26		0.0001	< 0.001
Emphylus glaber	1.00	560	3	13			0.0020	0.003
Leptacinus formicetorum	1.00	378	2	13	26		0.0003	0.004
Lyprocorrhe anceps	1.00	9999	4	13	26	7	0.0001	< 0.001
Mastigusa arietina	1.00	9999	5		26		0.0001	< 0.001
Monotoma angusticollis	0.99	9999	8	13	26	7	0.0001	< 0.001
Monotoma conicicollis	0.99	378	2		26		0.0030	0.004
Myrmetes paykulli	0.22	9999	9	13	26	7	0.037	0.039
Notothecta flavipes	1.00	9999	7	13	26		0.0001	< 0.001
Pella humeralis	0.94	27	1		26		0.0037	0.004
Platyarthrus hoffmannseggii	0.94	9999	8	13	26	7	0.0001	< 0.001
Porcellio scaber	1.00	378	2		26		0.0030	0.004
Quedius brevis	1.00	9999	4	13	26		0.0002	< 0.001
Stenus aterrimus	0.99	9999	7	13	26	7	0.0001	< 0.001
Thiasophila angulata	1.00	9999	11	13	26	7	0.0001	< 0.001
Thyreosthenius biovatus	0.99	9999	10	13	26	7	0.0001	< 0.001
Xantholinus linearis	1.00	14	1	13			0.0710	0.071

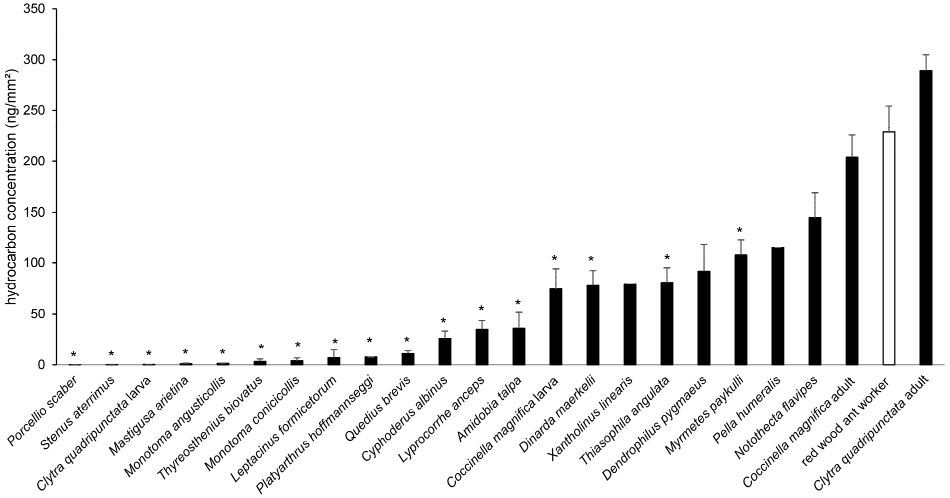

Figure 6.4. CHC concentration of RWA workers, inquiline and extranidal myrmecophiles. CHC concentration significant different (Benjamini-Hochberg corrected P-values smaller than 0.05) from RWA CHC concentration are indicated with an asterisk (*).

Association between different traits in myrmecophile community

Neither CHC BC distance nor CHC concentration (ng/mm²) was significantly correlated with other traits in the community of inquiline myrmecophiles. RWAs did not show higher aggression towards species with higher CHC concentration or lower CHC similarity (Table 6.1). In addition, no evidence was found that species that preferred densely populated brood chambers were more similar in hydrocarbon concentration or had lower proportions of CHC than species living at the edges of the nest. Similarly when focusing only on the inquiline rove beetles, neither CHC BC distance nor CHC concentration was significantly associated with other functional traits. However, robustness of the correlations between traits of these small datasets was low as indicated by large bootstrap CIs. Ideally, a large number of related myrmecophiles should be compared to study general patterns in myrmecophile strategies.

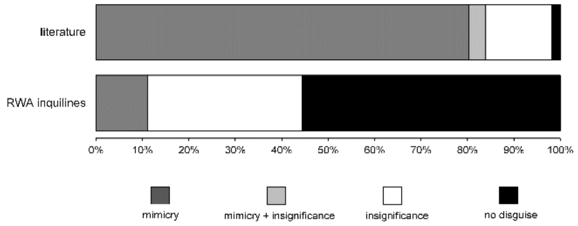

Figure 6.5. Comparison between cuticular chemical strategies. Upper bar ("literature") depicts the distribution of strategies of inquiline arthropods associated with social insects found in literature, lower bar represents the distribution of strategies found in arthropods that live in RWA nests (= "RWA inquilines").

DISCUSSION

The present study sheds light on the chemical similarities of the cuticular hydrocarbon (CHC) profile of arthropods associated with red wood ants (RWAs). Most arthropods studied so far make use of chemical mimicry and camouflage to integrate in the nest of the host (Fig. 6.5, Table A-6.1). However we demonstrate that only two arthropods associated with RWAs exploit these strategies, whereas the majority seems to rely on chemical insignificance or does not show any disguise at all.

The cuticular hydrocarbon (CHC) profile of the three tested RWA species was analogous with previous studies on RWAs (Martin et al. 2008, Włodarczyk 2011). The profiles were dominated by odd-chain alkanes, methyl-alkanes, dimethyl-alkanes and alkenes and are characterized by relatively heavy CHCs. Martin et al. (2008) suggested that chemical species identity in RWAs is mainly based on dimethyl-alkanes, which are particularly varied in this group. The clown beetles *Dendrophilus pygmaeus* and *Myrmetes paykulli* had almost all components in common with the RWA workers. However, chemical mimicry was not perfect which is echoed by the fact that the beetles are regularly detected and even bitten by the ants (pers. observations TP), but their compact tank-like morphology protects them from fatal bites. Moreover, *Formica rufa* aggression towards *M. paykulli* individuals found in the same nest was not lower than towards individuals transferred from a *F. polyctena* colony (Parmentier et al. 2016b). The chemical cuticular profile of the 18 other obligate RWA myrmecophile species (inquiline + extranidal) was clearly different from their RWA host (Appendix 6-4, Fig. 6.2). In contrast with RWAs, non-hydrocarbon compounds, such as alcohols and esters contributed significantly and in some cases even dominated the profile. These species did not show CHC adaptations specific to their RWA host nest or to their RWA host species (Fig. 6.2), which was confirmed by aggression transfer experiments performed in 11 species of this group (Parmentier et al. 2016b). For all these myrmecophiles, we found that the aggression response of *F. rufa* workers towards individuals found in their nest and towards individuals transferred from *F. polyctena* nests was not significantly different (Parmentier et al. 2016b). Interestingly, the majority of these non-mimicking species was characterized by significantly lower concentrations of hydrocarbons than their host, which could indicate that they deceive their host by adopting a chemical insignificance strategy. The group with low CHC concentrations, i.e. the rove beetle *Stenus aterrimus,* the root-eating beetles *Monotoma angusticollis* and *Monotoma conicicollis*, the isopod *Platyarthrus hoffmannseggii*, the spider *Thyreosthenius biovatus* and the springtail *Cyphoderus albinus* provoked hardly any aggression and were mostly ignored. All these species, and especially the two *Monotoma* species, typically walk very slowly, which makes their cryptic biology even more efficient. Based on their behavior, their low hydrocarbon concentrations and the lack of ant aggression, we assume that these species deceive their host by being chemically insignificant. Workers did also not pay attention to the facultative myrmecophile *Porcellio scaber* (common rough isopod). This species had extremely low CHC concentrations which could make it preadapted to a cryptic life in ant colonies and even in bee hives (Kärcher and Ratnieks 2010). In spite of very low CHC-concentrations, some species did not show disguise: the spider *Mastigusa arietina* and the rove beetles *Leptacinus formicetorum* and *Quedius brevis* were heavily aggressed, bitten and even chased. High aggression towards these species could be caused by non-detected compounds (e.g. heavier than C40) or by non-hydrocarbon compounds that can cause strong aggression response in low concentrations. Naked larvae of the leaf beetle *Clytra quadripunctata* were fiercely attacked, but normally they are protected by a case made of excrements and nest material (Donisthorpe 1927) which does not attract the attention of ant workers (Table 6.1). In case of detection, they can withdraw into the case and block the opening with their horny head (Donisthorpe 1927). Rather than matching the profile of the worker caste, myrmecophiles might target the sexual castes (Hojo et al. 2009), ant brood (Nash et al. 2008) or even nest material. However, the CHC composition of brood, sexuals and nest material, is typically only slightly different (Elmes et al. 2002, Hojo et al. 2009, Bos et al. 2011, Van Oystaeyen et al. 2014) and could not explain the vast differences we observed in the majority of the myrmecophiles. In contrast to many parasites that want to mask their identity in the nest (cf. Table A-6.1), mutualists can produce distinct compounds or profiles to attract their partner ant species (Richard et al. 2007, Hojo et al. 2014). Some of the RWA myrmecophiles might provide some indirect mutualistic services (Parmentier et al. 2016a) or they might even mimic the distinct profile of true mutualists to mask their identity (cf. aphid predators in (Liepert and Dettner 1996, Lohman et al. 2006)). However, none of the RWA myrmecophiles were treated (grooming, transporting, antennae tapping) as mutualists by the ants (Parmentier et al. 2016b). Therefore we argue that the tested myrmecophiles do not carry or imitate a distinct “mutualist” chemical profile. We also did not find evidence that RWA myrmecophiles only match a part of the CHC-profile, as the chemical differences were similar across different subsets of the CHC profile.

There were no general patterns in myrmecophilous strategies used by invertebrates found in the RWA community. Slight chemical distances in groups of conspecific mound-building *Formica* ants already lead to overt aggression (Sorvari et al. 2008, Martin et al. 2012). Therefore it can be expected that the degree of ant aggression towards species with distinct cuticular profiles, as observed in our community, is not linearly linked with CHC distance. Rather the absence or presence of specific compounds might lead to a different degree of ant aggression. CHC distance and CHC concentration were also not related to location in the nest. Counterintuitively, the CHC mimicking clown beetle *Dendrophilus pygmaeus* was always found at the periphery of the nest, while species with distinct CHC profiles (e.g. the rove beetle *Thiasophila angulata*) preferred the densely populated brood chambers (Parmentier et al. 2016b).

It is surprising that in our study system only 2 out of 18 inquiline arthropods closely match the CHC profiles of their host, whereas this strategy is found in most arthropods living in social insect nests studied up till now (Table A-6.1). This discrepancy could be explained by the specific structure of a RWA nest of which the aboveground part is a dome-shaped mound constructed with organic material, needles, twigs and other plant material (Gösswald 1989a). This haystack-like structure provides many more hiding places for myrmecophiles than classic earth nests found in most temperate ant species (Seifert 2007). Detection of myrmecophiles could further be hampered by the relative large size of RWAs (Parmentier et al. 2016c). Hence, RWA myrmecophiles might not require chemical mimicry as they could easily run away or hide when detected. However, the underground part of a RWA nest is very similar to a classic underground ant nest and most of the inquilines used in this study were also found there. Moreover, the majority of RWA inquilines can easily live in chambers with high-densities of workers (Parmentier et al. 2016b). We believe that the aforementioned discrepancy can mainly be explained by a biased focus on chemical strategies of rather specialized arthropod inquilines up to now. These species are by their behavior and the host's behavior well integrated into the host colony (Hölldobler and Wilson 1990) and elicit little or no aggression. Species that mimic their host are for example the caterpillars of *Maculinea* which are treated as true larvae of the colony, *Thorictus* beetles which cling on the antennae of their host (Lenoir et al. 2013) and *Varroa* mites, which are phoretic ectoparasites of bees (Le Conte et al. 2015). The intense interaction of these symbionts with their host is likely only possible by chemically matching the host, whether or not combined with advanced adaptations at the behavioral and morphological level (Hölldobler and Wilson 1990, Barbero et al. 2009b, Di Giulio et al. 2015). Similarly to these specialized arthropods, parasitic social insects ("social inquilines") intensely interact with their social insect host (Buschinger 2009) and a permanent integration is likely only possible by mimicking the host. In contrast with these two groups of specialized parasites, associates of the RWA community do not interact with their host and do not exhibit behavioral and morphological adaptations very different from their non-ant associated relatives (Donisthorpe 1927). They are ignored or provoke aggression to different degrees (Donisthorpe 1927, Freude et al. 1974) and are consequently classified as synechtrans and synoeketes sensu (Wasmann 1894). In spite of their weak integration, these species can impose costs on their host by preying on brood and stealing food (Parmentier et al. 2016a). Although these unspecialized species might outnumber the group of specialized associates (Wasmann 1894, Kistner 1979, Parmentier et al. 2014), little is known on their chemical integration strategies. The CHC profile of three myrmecophilous beetles that live in the vicinity of the nests of *Lasius fuliginosus*, showed no apparent similarity in CHC composition with their host (Stoeffler et al. 2011). The authors suggested that these extranidal beetles show no disguise as they have plenty of hiding places outside the nest and hardly interact with their host compared to inquilines found inside nests. Our results indicate that unspecialized associates can also survive as inquilines inside densely populated nests without mimicking the CHC profile. Some are chemical insignificant or protected by a case, but a large group show no cuticular disguise. These species might have a similar profile compared to free-living relatives. This is suggested by the slight difference in CHC that we observed between the obligate myrmecophilous ladybird *Coccinella magnifica* and its free-living sister species *C. septempunctata* and lower chemical distance of the facultatively myrmecophilous rove beetle *Xantholinus linearis* compared with most other obligately myrmecophilous rove beetles (Table 6.1). Species without disguise can survive by rapid, swift movements, hiding, death feigning (e.g. the rove beetle *Q. brevis*), a hard exoskeleton (e.g. the clown beetle *D. pygmaeus*) and possibly by secreting repellent volatiles (Stoeffler et al. 2011).

Our study stresses that the initial transition towards a myrmecophilous life does not require advanced chemical strategies. Species might rely on traits or tactics already present in their free-living relatives such as chemical insignificance, larval cases and tergal glands. These tactics are sufficient to penetrate and exploit a colony and might be the onset of the evolution towards advanced chemical (special glands, chemical mimicry), morphological and behavioral strategies needed for a complete assimilation into colony life as seen in the most specialized myrmecophiles (Parker 2016).

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APPENDIX CHAPTER 6

Appendix 6-1: Literature study on the integration strategies and behaviour of inquiline arthropods associated with social insects.

Table A-6.1. Integration strategies and behaviour of inquiline arthropods associated with social insects.

Species	Family	Strategy	Behaviour	Host(s)	Ref.
TERMITOPHILE					
Coleoptera					
Philotermes howardi	Staphylinidae	mimicry	Beetle solicits and receives proctodeal and stomodeal fluids from their host and engages in allogrooming with them.	*Reticulitermes virginicus*	[1] [2]
Trichopsenius depressus	Staphylinidae	mimicry	Beetle solicits and receives proctodeal and stomodeal fluids from their host and engages in allogrooming with them.	*Reticulitermes virginicus*	[1] [2]
Trichopsenius frosti	Staphylinidae	mimicry	Termite host grooms beetles, phoresy.	*Reticulitermes flavipes*	[3] [4]
Xenistusa hexagonalis	Staphylinidae	mimicry	Beetle solicits and receives proctodeal and stomodeal fluids from their host and engages in allogrooming with them.	*Reticulitermes virginicus*	[1] [2]
MYRMECOPHILE					
Acari					
Unknown	Acari	insignificance	phoretic on pupae and larvae	*Leptogenys distinguenda*	[5]
Araneae					
Cosmophasis bitaeniata	Salticidae	mimicry	Spider removes larva from the mandibles of a minor worker, spider prefers to feed on ant larvae.	*Oecophylla smaragdina*	[6] [7] ([8]
Gamasomorpha maschwitzi	Oonopidae	mimicry (only part of explanation	Spiders were typically observed crawling on top of adult workers or callows, trail following.	*Leptogenys distinguenda*	[9][10]
Coleoptera					
Diomus thoracicus	Coccinellidae	mimicry	The larva feeds uniquely on ant brood, most of the time they were located in the brood piles.	*Wasmannia auropunctata*	[11]
Thorictus buigasi	Dermestidae	mimicry + insignificance	phoretic,, cling the ant antennae	*Cataglyphis viatica*	[12]
Thorictus martinezi	Dermestidae	mimicry	phoretic,, cling to ant antennae	*Caraglyphis lenoiri*	[12]
Thorictus sulcicollis	Dermestidae	mimicry	phoretic,, cling to ant antennae	*Cataglyphis hispanica*	[12]
Sternocoelis hispanus	Histeridae	mimicry	Beetle is found frequently near larvae, licked by ants, climb on larvae and lick larvae, phoretic.	*Aphaenogaster senilis*	[13]
Diaritiger fossulatus	Pselaphidae	mimicry	food begging	*Lasius fuliginosus*	[14]
Unknown	Ptiliidae	mimicry	phoretic on larvae	*Leptogenys distinguenda*	[5]
Myrmecaphodius excavaticollis	Scarabaeidae	mimicry	grooming, trophallaxis	*Solenopsis*	[15]
Aenictobia fergusoni	Staphylinidae	mimicry	follow ant column, ignored by ants	*Aenictus* sp. 18a of SKY	[16]
Aenictobia thoi	Staphylinidae	mimicry	Beetle follows ant column, ignored by ants.	*Aenictus laeviceps*	[16]
Aenictoteras malayensis	Staphylinidae	mimicry	follow ant column, palpated, myrmecomorph	*Aenictus gracilis*	[16]
Aenictoxenus sp. (undescribed)	Staphylinidae	mimicry	phoretic on ant's abdomen	*Aenictus* sp. 18a of SKY	[16]
Chitosa nigrita	Staphylinidae	mimicry	few interactions	*Aphaenogaster senilis*	[13]
Mimaenictus wilsoni	Staphylinidae	mimicry	Beetle follows ant column, transported and palpated by ants, myrmecomorph.	*Aenictus laeviceps*	[16]
Rosciszewskia gracilis	Staphylinidae	mimicry	follow ant column, myrmecomorph	*Aenictus gracilis*	[16]
Trachydonia leptogenophila	Staphylinidae	mimicry	trail following	*Leptogenys distinguenda*	[5]
Trichotobia gracilis	Staphylinidae	trichomes/ 2 peaks similar to larvae	transported by ants	*Aenictus gracilis*	[16]
Weissflogia rhopalogaster	Staphylinidae	mimicry	myrmecomorph, transported by ants	*Aenictus* sp. 18a of SKY	[16]
Zyras comes	Staphylinidae	mimicry	food begging, antennal communication, trail-following	*Lasius fuliginosus*	[14]
Diptera					
Dohrniphora kistneri	Phoridae	different profile, some similarity with ant larvae	follow ant column, quickly running, not palpated	*Aenictus laeviceps*	[16]
Dohrniphora sp. 1	Phoridae	no mimicry	follow ant column, quickly running, not palpated	*Aenictus gracilis*	[16]
Dohrniphora sp. 2 (undescribed)	Phoridae	mimicry	follow ant column, quickly running, not palpated	*Aenictus* sp. 18a of SKY	[16]

Puliciphora rosei	Phoridae	insignificance	trail following	*Leptogenys distinguenda*	[5]
Rhynchomicropteron necaphidiforme	Phoridae	insignificance	trail following	*Leptogenys distinguenda*	[5]
Vestigipoda maschwitzi	Phoridae	some similarity with ant larva	mimicking of morphology larva	*Aenictus gracilis*	[16]
Microdon albicomatus	Syrphidae	mimicry	Larva and pupae have unique dome-shaped morphology, specialist brood predator	*Myrmica incompleta*	[17]
Microdon myrmicae	Syrphidae	insignificance			[18]
Gastropoda					
Allopeas myrmekophilos	Subulinidae	insignificance	carried by workers	*Leptogenys distinguenda*	[5]
Hemiptera					
Paracletus cimiciformis	Aphididae	mimicry	Specialized morph transported to brood pile, where it sucks larva haemolymph.	*Tetramorium*	[19]
Hymenoptera					
Dilocantha lachaudii	Eucharitidae	partial mimicry	Wasps after emergence are transported outside nest.	*Ectatomma tuberculatum*	[20]
Isomerala coronata	Eucharitidae	partial mimicry	Wasps after emergence are transported outside nest.	*Ectatomma tuberculatum*	[20]
Kapala sulcifacies	Eucharitidae	mimicry	Larva attaches to foraging workers, larva parasitizes larva. Workers Workers assist the hatching wasps, and exhibit considerable interest (antennation, grooming) toward the young parasites	*Ectatomma ruidum*	[21]
Palaripsis eikoae	Aphidiidae	mimicry	The wasp often mounted and rubbed against the worker ants and sometimes teased them to regurgitate food to itself.	*Lasius sakagamii*	[22]
Isopoda					
Exalloniscus maschwitzi	Oniscidae	insignificance	phoretic on pupae	*Leptogenys distinguenda*	[5]
Lepidoptera					
Maculinea alcon	Lycaenidae	mimicry	Larva is transported to the brood chamber, tended and fed.	*Myrmica rubra*, *Myrmica scabrinodis*	[18, 23]
Maculinea teleius	Lycaenidae	?		*Myrmica*	[18]
Maculinea rebeli	Lycaenidae	mimicry	Larva is transported to the brood chamber, tended and fed.	*Myrmica schencki*	[24], [25][26]
Maculinea nausithous	Lycaenidae	?		*Myrmica rubra*	[18]
Niphanda fusca	Lycaenidae	mimicry	brought by foraging workers to nest, trophallaxis	*Camponotus japonicus*	[27]
Orthoptera					
Myrmecophilus sp.	Myrmecophilidae	mimicry	This species licks the body surfaces of ants, disrupts the trophallaxis between ants, or is fed liquid food from ants by direct mouth-to-mouth contact.	Several ants	[28]
Thysanura					
Malayatelura ponerophila	Ateluridae	mimicry	Silverfishes were frequently observed moving their body surface directly over the cuticle of adult and callow worker ants.	*Leptogenys distinguenda*	[29][10]
unknown		insignificance	few interactions	*Aphaenogaster senilis*	[13]
Thysanura gen. sp.		insignificance	follow ant column, phoretic on ant abdomen	*Aenictus* sp. 18a of SKY	[16]
BEE ASSOCIATES					
Acari					
Varroa jacobsoni	Varroidae	mimicry	specialized ectoparasite	*Apis mellifera*	[30]
Varroa destructor	Varroidae	mimicry	specialized ectoparasite	*Apis mellifera & Apis cerana*	[31][32]
Diptera					
Braula coeca	Braulidae	mimicry	Specialized cleptoparasites that lives on the head and thorax of bees	*Apis mellifera*	[33]
Hymenoptera					
Mutilla europaea	Mutillidae	pre-integration: insignificance post-integration: mimicry	Female velvet ants enter wasp nests, lay eggs on host pupae and leave the nests.	*Polistes biglumis*	[34][35]
Lepidoptera					
Acherontia atropos	Sphingidae	mimicry	Cleptoparasite of nectar and honey, no intense interaction with host	*Apis mellifera*	[36]
WASP ASSOCIATES					
Coleoptera					
Metoecus paradoxus	Rhipiphoridae	mimicry	Larva attaches to a foraging worker and is brought to cell where it feeds on larva.	*Vespula vulgaris*	[37]

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Appendix 6-2: Surface estimation ants and myrmecophiles

Ant bodies were subdivided in multiple ellipsoids (head, alitrunk, petiole, gaster, coxa, femur, tibia and tarsus). The leg parts were only measured in one front leg and the respective areas were multiplied by six (six legs). For the spiders *T. biovatus* and *M. arietina*, the body plan was simplified to two ellipsoids (prosoma and opisthosoma). In addition, the area of a front leg was measured by subdividing it in ellipsoids and multiplying its total area with eight (eight legs). Surface areas of all rove beetles, *C. albinus*, C*. magnifica* (larva)*, C. quadripunctata* (adult) *D. pygmaeus*, *E. glaber*, *M. conicicollis*, *M. angusticollis* and *M. paykulli* were calculated based on a single ellipsoid approximation for the whole body. Surface area of *C. quadripunctata* larvae was estimated by the area of two ellipsoids. The dorsal area of *P. hoffmannseggii*, *C. magnifica* (adult), *C. septempunctata*, *P. scaber* (adult) was calculated by the half of an ellipsoid's area, whereas the flat, ventral area was estimated by the surface of an ellipse.

The approximate surface of the ellipsoids was calculated by using the Knud Thomsen formula:

$$\text{surface}_{\text{ellipsoid}} = 4\pi\cdot((a^{1.6075}\, b^{1.6075} + a^{1.6075}c^{1.6075} + b^{1.6075}c^{1.6075})/3)^{1/1.6075}$$

where a refers to the length, b to the width and c to the depth of the ellipsoid.

Table A-6.2. Mean cuticle surface of myrmecophiles and RWA and corresponding concentration of CHC (ng/mm²). $N_{samples}$ refers to the number of samples of which the CHC concentration was calculated. The surface of individuals of those samples ($N_{individuals}$) was first estimated by the method described above. In a pooled sample $N_{samples} < N_{individuals}$, the total sample CHC-quantity was divided by the sum of the surfaces of all individuals in that sample.

Species	$N_{individuals}$	Surface ± SD (mm²)	$N_{samples}$	Concentration CHC (ng/mm²)
Porcellio scaber	6	34.07 ± 3.47	2	0.19
Stenus aterrimus	7	9.42 ± 1.19	7	0.42
Clytra quadripunctata larva	2	87.45 ± 43.38	2	0.56
Mastigusa arietina	5	19.86 ± 1.93	5	1.26
Monotoma angusticollis	46	4.47 ± 0.38	8	1.49
Thyreosthenius biovatus	38	5.98 ± 1.28	9	3.51
Monotoma conicicollis	11	4.81± 0.27	2	4.11
Leptacinus formicetorum	7	3.46 ± 0.94	2	7.28
Platyarthrus hoffmannseggii	35	9.90 ± 1.60	8	7.68
Quedius brevis	3	23.21 ± 023	3	11.19
Cyphoderus albinus	60	0.73 ± 0.13	3	26.23
Lyprocorrhe anceps	21	3.05 ± 0.47	4	34.90
Amidobia talpa	40	1.34 ± 0.13	4	36.05
Coccinella magnifica larva	8	36.29 ± 11.71	8	74.44
Dinarda maerkelii	5	10.59 ± 0.09	5	78.03
Xantholinus linearis	1	17.10	1	79.47
Thiasophila angulata	39	5.33 ± 0.67	8	80.69
Dendrophilus pygmaeus	2	10.22 ± 0.00	2	91.84
Myrmetes paykulli	9	7.34 ± 0.40	9	107.56
Pella humeralis	1	16.47	1	115.40
Notothecta flavipes	7	6.96 ± 0.85	7	144.85
Coccinella magnifica adult	13	65.19 ± 14.69	13	204.16
RWA worker	36	48.72 ± 8.10	36	228.61
Clytra quadripunctata adult	5	91.12 ± 12.81	5	289.03
Emphylus glaber	1	5.46		

Appendix 6-3: Overview of cuticular hydrocarbon composition.

Table A-6.3. Relative proportions of cuticular hydrocarbons of RWAs and associates, t = traces < 0.0001. Grey row represents the mean, white row the SD of a CHC peak.

		F. polyctena		*F. pratensis*	*F. rufa*	*A. talpa*	*C. quadripunctata* adult	*C. quadripunctata* larva	*C. magnifica* adult	*C. magnifica* larva	*C. septempunctata*	*C. albinus*	*D. pygmaeus*	*D. maerkelii*	*E. glaber*	*L. formicetorum*	*L. anceps*	*M. arietina*	*M. angusticollis*	*M. conicicollis*	*M. paykulli*	*N. flavipes*	*P. humeralis*	*P. hoffmannseggii*	*P. scaber*	*Q. brevis*	*S. aterrimus*	*T. angulata*	*T. biovatus*	*x. linearis*
1	C_{22}	0.0002		0.0002	t	0.0009	-	-	-	-	-	-	0.0002	0.0004	-	-	-	-	-	-	0.0011	0.0036	-	-	-	-	-	-	-	-
		0.0002		0.0001	0.0001	0.0019	-	-	-	-	-	-	0.0001	0.0011	-	-	-	-	-	-	0.0012	0.0011	NA	-	-	-	-	-	-	NA
2	$C_{23:1}$	0.0003		0.0009	t	-	-	-	-	-	-	-	0.0001	-	-	-	-	-	-	-	0.0002	-	-	-	-	-	-	-	-	-
		0.0003		0.0003	t	-	-	-	-	-	-	-	0.0001	-	-	-	-	-	-	-	0.0002	-	NA	-	-	-	-	-	-	NA
3	C_{23}	0.0103		0.0004	0.0037	-	-	-	0.0226	0.0402	0.0172	-	0.0004	0.0033	-	-	0.0024	-	0.0040	-	0.0019	-	0.0094	0.0009	0.0604	-	-	0.0053	-	0.0065
		0.0045		0.0003	0.0035	-	-	-	0.0091	0.0354	0.0068	-	0.0001	0.0051	-	-	0.0029	-	0.0074	-	0.0013	-	NA	0.0018	0.0022	-	-	0.0053	-	NA
4	11,9-MeC_{23}	0.0006		t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0003	-	-	0.0019	-	-	-	-	-	-
		0.0005		t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0004	-	NA	0.0055	-	-	-	-	-	NA
5	5-MeC_{23}	0.0016		t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0004	-	0.0011	0.0003	-	-	-	-	-	-
		0.0014		t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0006	-	NA	0.0007	-	-	-	-	-	NA
6	3-MeC_{23}	0.0005		-	0.0004	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	0.0050	-	0.0011	0.0006	0.0013	0.0053	-	-	-	-	-	-
		0.0005		-	0.0005	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	0.0140	-	0.0008	0.0011	NA	0.0149	-	-	-	-	-	NA
7	x,y-diMeC_{23}	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
8	C_{24}	0.0026		0.0003	0.0016	0.0007	-	-	0.0024	0.0010	0.0018	0.0025	0.0011	0.0051	-	-	0.0017	-	0.0145	0.0016	0.0032	0.0042	0.0027	0.0156	0.0716	-	-	0.0024	-	0.0030
		0.0014		0.0001	0.0015	0.0014	-	-	0.0006	0.0015	0.0006	0.0044	0.0002	0.0014	-	-	0.0034	-	0.0367	0.0023	0.0020	0.0019	NA	0.0430	0.0003	-	-	0.0020	-	NA
9	x-MeC_{24}	0.0003		-	0.0005	-	0.0023	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		0.0007		-	0.0009	-	0.0027	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
10	4-MeC_{24}	-		-	-	-	0.0043	-	-	0.0052	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	0.0027	-	-	0.0061	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
11	x-$C_{25:1}$	0.0182		0.0018	0.0055	-	-	-	-	-	-	0.0142	0.0018	-	-	-	-	-	0.0029	-	0.0089	-	0.0012	0.0363	-	0.1508	-	-	0.0025	-
		0.0186		0.0012	0.0093	-	-	-	-	-	-	0.0057	0.0011	-	-	-	-	-	0.0082	-	0.0104	-	NA	0.0360	-	0.1012	-	-	0.0075	NA
12	y-$C_{25:1}$	0.0005		0.0003	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0001	-	-	-	-	0.5727	-	-	-	-
		0.0004		0.0001	0.0001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0002	-	NA	-	-	0.2297	-	-	-	NA
13	C_{25}	0.1179		0.0212	0.0998	0.0136	0.0055	0.0359	0.0829	0.0991	0.0249	0.0092	0.1307	0.1647	0.0226	0.0035	0.0239	0.0379	0.0564	0.0369	0.1343	0.2086	0.1046	0.0525	0.1570	0.2765	0.1465	0.1570	0.0600	0.1492
		0.0667		0.0088	0.0546	0.0081	0.0035	0.0006	0.0148	0.0694	0.0086	0.0023	0.0004	0.0738	0.0040	0.0050	0.0254	0.0132	0.0154	0.0220	0.0604	0.0481	NA	0.0384	0.0115	0.3308	0.1688	0.0438	0.0789	NA
14	13,11,9-MeC_{25}	0.0118		0.0013	0.0066	0.0011	0.0861	-	0.0011	0.0008	0.0030	0.0144	0.0040	0.0006	-	-	-	-	0.0033	0.0121	0.0077	0.0091	0.0059	0.0022	-	-	-	0.0014	-	0.0014

		0.0061		0.0002	0.0048	0.0012	0.1051	-	0.0008	0.0016	0.0016	0.0133	0.0030	0.0016	-	-	-	-	0.0095	0.0172	0.0057	0.0040	NA	0.0041	-	-	-	0.0019	-	NA
15	7-MeC$_{25}$	0.0042		0.0001	0.0004	0.0005	-	-	0.0030	0.0005	-	0.0014	0.0015	-	-	-	-	-	-	0.0023	0.0025	0.0202	0.0049	0.0059	-	-	-	-	-	-
		0.0023		t	0.0004	0.0010	-	-	0.0009	0.0010	-	0.0024	0.0010	-	-	-	-	-	-	0.0032	0.0022	0.0085	NA	0.0120	-	-	-	-	-	NA
16	5-MeC$_{25}$	0.0032		0.0001	0.0004	0.0012	-	-	0.0001	0.0014	-	0.0033	0.0010	0.0088	-	-	-	-	-	0.0015	0.0022	0.0805	0.0030	0.0013	-	-	-	0.0007	-	-
		0.0019		t	0.0004	0.0025	-	-	0.0004	0.0016	-	0.0030	0.0005	0.0051	-	-	-	-	-	0.0022	0.0019	0.0197	NA	0.0025	-	-	-	0.0013	-	NA
17	x,y-diMeC$_{25}$	0.0003		0.0003	T	0.0106	0.0033	-	0.0011	0.0007	0.0027	-	0.0009	-	-	-	-	-	-	0.0025	0.0006	0.0053	0.0016	-	-	-	-	-	-	-
		0.0002		0.0001	T	0.0063	0.0045	-	0.0011	0.0015	0.0020	-	0.0009	-	-	-	-	-	-	0.0035	0.0003	0.0017	NA	-	-	-	-	-	-	NA
18	3-MeC$_{25}$	0.0067		0.0005	0.0027	0.0597	-	-	0.0059	0.0067	0.0052	0.0023	0.0053	0.0005	-	-	0.0048	-	0.0019	0.0137	0.0045	0.1391	0.0233	0.0036	-	-	-	0.0021	-	0.0014
		0.0032		0.0002	0.0028	0.0275	-	-	0.0023	0.0046	0.0035	0.0020	0.0031	0.0011	-	-	0.0069	-	0.0055	0.0194	0.0029	0.0165	NA	0.0056	-	-	-	0.0026	-	NA
19	5,y-diMeC$_{25}$	0.0013		0.0001	0.0009	-	0.0062	-	-	-	-	-	0.0005	-	-	-	-	-	0.0036	0.0026	0.0010	0.2405	-	0.0010	-	-	-	0.0013	-	-
		0.0008		t	0.0010	-	0.0076	-	-	-	-	-	0.0003	-	-	-	-	-	0.0101	0.0037	0.0008	0.0668	NA	0.0019	-	-	-	0.0023	-	NA
20	C$_{26}$	0.0066		0.0026	0.0052	0.0089	0.0021	-	0.0042	0.0060	0.0033	0.0042	0.0086	0.0096	-	0.0039	0.0071	0.0090	0.0243	0.0229	0.0080	0.0149	0.0056	0.0439	0.0825	-	-	0.0095	0.1249	0.0053
		0.0029		0.0017	0.0048	0.0034	0.0016	-	0.0010	0.0015	0.0006	0.0073	0.0016	0.0027	-	0.0055	0.0056	0.0133	0.0412	0.0163	0.0029	0.0047	NA	0.0600	0.0026	-	-	0.0015	0.1239	NA
21	3,y-diMeC$_{25}$	0.0012		0.0002	0.0003	0.0133	-	-	-	-	-	0.0044	-	-	-	-	-	-	0.0032	0.0020	0.0013	0.1308	0.0014	0.0003	-	-	-	-	-	-
		0.0007		t	0.0003	0.0062	-	-	-	-	-	0.0058	-	-	-	-	-	-	0.0090	0.0028	0.0005	0.0514	NA	0.0009	-	-	-	-	-	NA
22	x-MeC$_{26}$	0.0025		0.0006	0.0017	0.0240	0.0138	-	0.0027	-	0.0056	0.0060	0.0009	-	-	-	-	-	0.0090	0.0080	0.0022	0.0331	0.0023	0.0297	0.1165	-	-	-	-	-
		0.0011		0.0002	0.0013	0.0151	0.0152	-	0.0007	-	0.0031	0.0054	0.0006	-	-	-	-	-	0.0255	0.0113	0.0018	0.0341	NA	0.0548	0.0061	-	-	-	-	NA
23	4-MeC$_{26}$	-		-	-	0.0095	0.0314	-	-	0.0225	-	-	0.0005	-	-	-	-	-	-	-	0.0006	0.0114	-	-	-	-	-	-	-	-
		-		-	-	0.0095	0.0205	-	-	0.0127	-	-	0.0003	-	-	-	-	-	-	-	0.0003	0.0136	NA	-	-	-	-	-	-	NA
24	2-MeC$_{26}$	-		-	-	0.0075	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0039	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
25	w-C$_{27:1}$	0.0380		0.0207	0.0156	0.0094	0.0046	-	-	0.0417	-	0.0216	0.0130	-	0.2228	0.0019	-	-	-	-	0.0277	-	0.1398	0.0335	-	-	-	0.0057	0.0099	0.0052
		0.0167		0.0069	0.0099	0.0077	0.0043	-	-	0.0517	-	0.0190	0.0083	-	0.0779	0.0026	-	-	-	-	0.0356	-	NA	0.0349	-	-	-	0.0047	0.0251	NA
26	x-C$_{27:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0027	0.0002	-	-	-	-	-	0.0034
		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	0.0006	-	-	-	-	-	NA
27	y-C$_{27:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0109	-	-	-	-	-	-	-	-	-	-	-	0.0016	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0154	-	-	-	-	-	-	NA	-	-	-	-	0.0036	-	NA
28	z-C$_{27:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0076	-	0.0379	-	-	-	-	-	-	-	-	-	0.1386	0.0187	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0108	-	0.0132	-	-	-	-	NA	-	-	-	-	0.0410	0.0562	NA
29	C$_{27}$	0.0997		0.0587	0.1203	0.0815	0.0269	0.0607	0.1010	0.2299	0.0470	0.0089	0.1777	0.1689	0.1136	0.0921	0.0851	0.0573	0.0722	0.0719	0.1142	0.0530	0.0442	0.0284	-	-	0.1915	0.1743	0.2273	0.0774
		0.0622		0.0353	0.0792	0.0269	0.0144	0.0029	0.0223	0.0757	0.0116	0.0010	0.0522	0.0391	0.0744	0.0097	0.0417	0.0142	0.0412	0.0210	0.0463	0.0467	NA	0.0324	-	-	0.1952	0.0488	0.1711	NA
30	x,y-diMeC$_{26}$	-		-	-	0.3059	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0242	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
31	13,11,9-MeC$_{27}$	0.0252		0.0048	0.0130	0.0035	0.0591	0.0348	0.2023	0.0679	0.0970	0.0297	0.0135	-	-	0.0070	-	0.0309	0.0192	0.0671	0.0131	0.0020	0.0802	0.0380	-	-	0.0309	0.0030	-	0.0074
		0.0130		0.0010	0.0065	0.0044	0.0635	0.0045	0.0258	0.0533	0.0532	0.0148	0.0078	-	-	0.0099	-	0.0306	0.0309	0.0174	0.0083	0.0025	NA	0.0505	-	-	0.0818	0.0030	-	NA
32	7-MeC$_{27}$	0.0047		0.0008	0.0017	0.0089	0.0008	0.0622	0.1092	0.0236	0.0140	0.0059	0.0038	-	-	0.0025	-	-	0.0104	-	0.0036	-	0.0074	0.0110	-	-	-	0.0025	-	0.0013
		0.0051		0.0002	0.0014	0.0034	0.0011	0.0309	0.0377	0.0196	0.0131	0.0029	0.0006	-	-	0.0036	-	-	0.0152	-	0.0030	-	NA	0.0152	-	-	-	0.0047	-	NA

33	5-MeC$_{27}$	0.0016		0.0003	0.0009	0.0160	-	-	0.0018	0.0016	-	0.0007	0.0026	0.0950	-	-	-	-	-	-	0.0013	0.0085	0.0041	0.0020	-	-	-	0.0289	-	-
		0.0013		0.0001	0.0008	0.0060	-	-	0.0017	0.0017	-	0.0013	0.0001	0.0402	-	-	-	-	-	-	0.0011	0.0075	NA	0.0058	-	-	-	0.0180	-	NA
34	11,15-diMeC$_{27}$+9,y-diMeC$_{27}$	0.0018		0.0009	0.0010	0.0017	0.0065	-	0.0623	0.0329	0.0464	-	0.0020	0.0101	-	-	-	-	0.0042	-	0.0014	-	0.0015	0.0022	-	-	-	0.0018	-	-
		0.0018		0.0002	0.0015	0.0035	0.0055	-	0.0140	0.0261	0.0228	-	0.0003	0.0110	-	-	-	-	0.0120	-	0.0004	-	NA	0.0045	-	-	-	0.0052	-	NA
35	4-MeC$_{27}$	-		-	-	0.0050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0011	-	-	-	-	-	-	-
		-		-	-	0.0037	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
36	7,y-diMeC$_{27}$	-		-	-	-	-	-	0.0988	0.0237	0.0469	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	0.0193	0.0153	0.0301	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
37	3-MeC$_{27}$	0.0122		0.0107	0.0055	0.0956	0.0032	-	-	0.0120	-	0.0273	0.0110	0.0041	-	0.0115	0.0610	-	-	0.0632	0.0164	0.0235	0.0275	0.0014	-	-	-	0.0175	0.0080	0.0106
		0.0074		0.0015	0.0016	0.0313	0.0005	-	-	0.0076	-	0.0257	0.0067	0.0036	-	0.0162	0.1020	-	-	0.0179	0.0124	0.0285	NA	0.0039	-	-	-	0.0170	0.0240	NA
38	5,y-diMeC$_{27}$	0.0034		0.0009	0.0013	-	0.0061	-	0.0110	0.0038	-	0.0018	0.0036	-	-	-	-	-	-	-	0.0023	-	0.0062	0.0018	-	-	-	0.0136	-	-
		0.0017		0.0001	0.0012	-	0.0074	-	0.0026	0.0013	-	0.0017	0.0010	-	-	-	-	-	-	-	0.0005	-	NA	0.0036	-	-	-	0.0084	-	NA
39	7,y-diMeC$_{27}$+x,y,z-triMeC$_{29}$	0.0010		0.0002	t	-	-	-	0.0293	0.0055	0.0156	0.0010	0.0002	-	-	-	-	-	0.0031	-	0.0007	-	0.0015	0.0012	-	-	-	-	-	-
		0.0012		t	t	-	-	-	0.0152	0.0071	0.0104	0.0017	0.0002	-	-	-	-	-	0.0088	-	0.0005	-	NA	0.0027	-	-	-	-	-	NA
40	C$_{28}$	0.0038		0.0030	0.0030	0.0097	0.0066	0.0254	0.0057	0.0085	0.0067	0.0054	0.0035	0.0093	-	0.0093	0.0130	0.0100	0.0223	0.0209	0.0051	0.0068	-	0.0382	-	-	-	0.0069	-	0.0021
		0.0015		0.0018	0.0026	0.0047	0.0020	0.0093	0.0015	0.0055	0.0014	0.0078	0.0009	0.0016	-	0.0132	0.0079	0.0140	0.0421	0.0133	0.0027	0.0026	NA	0.0529	-	-	-	0.0019	-	NA
41	3,y-diMeC$_{27}$	0.0009		-	-	-	-	-	0.0072	-	-	-	-	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	-	-	-	-
		0.0006		-	-	-	-	-	0.0040	-	-	-	-	-	-	-	-	-	-	-	0.0006	-	NA	-	-	-	-	-	-	NA
42	x-MeC$_{28}$	0.0033		0.0004	0.0017	0.0400	0.0063	-	-	0.0035	0.0023	0.0016	-	-	-	0.0012	-	-	-	-	0.0016	-	0.0077	0.0002	-	-	-	-	-	-
		0.0022		0.0001	0.0016	0.0176	0.0044	-	-	0.0021	0.0011	0.0014	-	-	-	0.0017	-	-	-	-	0.0009	-	NA	0.0006	-	-	-	-	-	NA
43	x,y-diMeC$_{28}$	-		-	-	0.0073	-	-	0.0054	0.0018	0.0073	-	-	-	-	-	-	-	-	-	-	-	0.0019	-	-	-	-	-	-	-
		-		-	-	0.0077	-	-	0.0073	0.0023	0.0036	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
44	4-MeC$_{28}$	-		-	-	-	0.0695	-	-	0.0053	-	-	-	-	-	0.0065	-	-	-	-	0.0012	-	-	-	-	-	-	-	-	-
		-		-	-	-	0.0101	-	-	0.0035	-	-	-	-	-	0.0093	-	-	-	-	0.0006	-	NA	-	-	-	-	-	-	NA
45	w-C$_{29:1}$	-		-	-	-	-	-	-	0.0280	-	-	-	-	-	0.0052	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	0.0359	-	-	-	-	-	0.0073	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
46	x-C$_{29:1}$	0.0110		0.0061	0.0073	0.0007	-	-	-	0.1218	-	0.0089	0.0016	-	-	0.0059	0.0050	-	-	-	0.0072	-	0.0469	0.0094	-	-	-	0.0117	0.0039	0.1247
		0.0054		0.0035	0.0062	0.0013	-	-	-	0.1380	-	0.0080	0.0006	-	-	0.0084	0.0099	-	-	-	0.0086	-	NA	0.0155	-	-	-	0.0053	0.0116	NA
47	y-C$_{29:1}$	0.0008		-	0.0001	-	-	-	-	-	-	-	-	-	-	0.0632	-	-	-	-	0.0006	-	-	-	-	-	-	0.1102	-	0.0115
		0.0005		-	0.0001	-	-	-	-	-	-	-	-	-	-	0.0894	-	-	-	-	0.0012	-	NA	-	-	-	-	0.0357	-	NA
48	z-C$_{29:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0058	-	-	-	-	-	-	-	-	-	-	-	0.0245	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0082	-	-	-	-	-	-	NA	-	-	-	-	0.0101	-	NA
49	C$_{29}$	0.0431		0.0386	0.0431	0.0272	0.0529	0.0787	0.0084	0.0579	0.0240	0.0047	0.0495	0.0821	0.1257	0.0906	0.0738	0.0207	0.0677	0.0424	0.0474	0.0014	0.0059	0.0422	0.2280	-	0.1268	0.0386	0.2140	0.0215
		0.0250		0.0251	0.0283	0.0132	0.0052	0.0110	0.0027	0.0307	0.0074	0.0023	0.0165	0.0106	0.0657	0.0304	0.0415	0.0191	0.0332	0.0290	0.0438	0.0018	NA	0.0357	0.0155	-	0.0879	0.0174	0.2428	NA
50	x,y-diMeC$_{28}$	-		-	-	0.0056	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA

51	15,13,11,9-MeC$_{29}$	0.0239		0.0057	0.0098	0.0097	0.0302	0.0488	0.0167	0.0131	0.0398	0.0269	0.0174	0.0004	-	0.0099	-	-	0.0510	0.0562	0.0132	-	0.0929	0.0410	-	-	-	0.0137	0.0045	0.0129
		0.0136		0.0011	0.0060	0.0067	0.0043	0.0141	0.0027	0.0047	0.0186	0.0043	0.0042	0.0010	-	0.0140	-	-	0.0302	0.0054	0.0094	-	NA	0.0475	-	-	-	0.0113	0.0135	NA
52	7-MeC$_{29}$	0.0041		0.0036	0.0012	0.0246	0.0019	-	0.0721	0.0182	0.0113	0.0020	0.0038	-	-	0.0020	-	-	0.0017	0.0033	0.0029	-	0.0088	0.0028	-	-	-	0.0169	-	-
		0.0042		0.0010	0.0008	0.0203	0.0012	-	0.0198	0.0159	0.0065	0.0017	0.0006	-	-	0.0028	-	-	0.0047	0.0047	0.0020	-	NA	0.0048	-	-	-	0.0362	-	NA
53	5-MeC$_{29}$	0.0007		0.0004	0.0008	0.0051	0.0023	-	0.0029	0.0030	-	-	0.0007	0.0767	-	-	-	-	-	-	0.0013	-	0.0011	-	-	-	-	0.0031	-	0.0020
		0.0004		0.0002	0.0011	0.0025	0.0005	-	0.0006	0.0035	-	-	0.0001	0.0369	-	-	-	-	-	-	0.0009	-	NA	-	-	-	-	0.0026	-	NA
54	4-MeC$_{29}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0465	0.0008	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0254	0.0015	-	-	-	-	-	NA	-	-	-	-	-	-	NA
55	x,y-diMeC$_{29}$+7,y-diMeC$_{29}$	0.0052		0.0038	0.0018	0.0023	0.0098	-	0.0768	0.0229	0.0459	0.0035	0.0021	0.0023	-	0.0200	-	-	-	0.0460	0.0036	-	0.0381	0.0038	-	-	-	0.0167	-	-
		0.0038		0.0009	0.0016	0.0045	0.0025	-	0.0044	0.0191	0.0227	0.0031	0.0011	0.0035	-	0.0283	-	-	-	0.0053	0.0022	-	NA	0.0057	-	-	-	0.0097	-	NA
56	3-MeC$_{29}$+5,y-diMeC$_{29}$	0.0043		0.0008	0.0047	0.0181	0.0077	-	-	-	-	-	0.0055	-	-	0.0433	0.2911	-	-	0.0107	0.0047	-	0.0098	0.0003	-	-	-	-	-	0.0018
		0.0018		0.0002	0.0021	0.0120	0.0026	-	-	-	-	-	0.0003	-	-	0.0246	0.0738	-	-	0.0151	0.0038	-	NA	0.0010	-	-	-	-	-	NA
57	x,y,z-triMeC$_{29}$	0.0005		0.0003	t	-	-	-	0.0213	0.0020	0.0106	-	0.0001	-	-	-	-	-	-	-	0.0002	-	-	-	-	-	-	-	-	-
		0.0010		0.0001	t	-	-	-	0.0078	0.0026	0.0052	-	0.0001	-	-	-	-	-	-	-	0.0001	-	NA	-	-	-	-	-	-	NA
58	C$_{30}$	0.0028		0.0015	0.0023	0.0022	0.0089	0.0254	0.0006	0.0022	0.0026	0.0055	-	0.0058	-	0.0063	0.0083	-	0.0207	0.0032	0.0077	-	0.0045	0.0399	-	-	-	0.0022	-	0.0021
		0.0010		0.0008	0.0016	0.0027	0.0027	0.0056	0.0009	0.0017	0.0003	0.0079	-	0.0076	-	0.0089	0.0056	-	0.0380	0.0045	0.0113	-	NA	0.0533	-	-	-	0.0019	-	NA
59	3,y-diMeC$_{29}$	-		-	-	0.0152	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0016	-	-
		-		-	-	0.0133	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	0.0038	-	NA
60	x-MeC$_{30}$	0.0024		0.0007	0.0015	0.0246	0.0097	-	0.0014	-	0.0029	0.0014	0.0018	-	-	-	-	-	-	0.0069	0.0016	-	0.0054	0.0002	-	-	-	-	-	0.0015
		0.0017		0.0001	0.0012	0.0492	0.0036	-	0.0001	-	0.0006	0.0012	0.0003	-	-	-	-	-	-	0.0097	0.0009	-	NA	0.0006	-	-	-	-	-	NA
61	x,y-diMeC$_{30}$	-		-	-	-	-	-	-	-	-	-	-	0.0014	-	-	-	-	-	-	-	-	0.0015	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	0.0030	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
62	4-MeC$_{30}$	-		-	-	-	0.0513	-	-	0.0096	-	-	-	-	-	0.0073	0.0136	-	-	-	0.0010	-	-	-	-	-	-	-	-	-
		-		-	-	-	0.0260	-	-	0.0097	-	-	-	-	-	0.0104	0.0098	-	-	-	0.0008	-	NA	-	-	-	-	-	-	NA
63	v-C$_{31:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0013	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
64	6,10-diMeC$_{30}$	-		-	-	0.0029	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0035	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
65	3-MeC$_{30}$	-		-	-	-	-	-	-	-	-	-	-	-	-	-	0.0337	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	-	0.0107	-	-	-	-	-	NA	-	-	-	-	-	-	NA
66	w-C$_{31:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0072	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0119
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0102	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
67	x-C$_{31:1}$	0.0194		0.0205	0.0181	0.0011	0.0123	-	-	-	-	0.0104	0.0078	0.0332	-	0.0849	0.0025	-	0.2037	0.0527	0.0130	-	0.0294	0.0182	-	-	-	0.0142	-	0.1921
		0.0071		0.0044	0.0140	0.0023	0.0100	-	-	-	-	0.0092	0.0021	0.0365	-	0.0771	0.0051	-	0.1009	0.0328	0.0109	-	NA	0.0204	-	-	-	0.0048	-	NA
68	y-C$_{31:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0814	-	-	-	-	-	-	-	-	-	-	-	0.0018	-	0.0027
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0782	-	-	-	-	-	-	NA	-	-	-	-	0.0046	-	NA

69	z-C$_{31:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0330	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0467	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
70	4,8-diMeC$_{30}$	-		-	-	0.0033	0.0044	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0067	0.0018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
71	C$_{31}$	0.0171		0.0234	0.0255	0.0072	0.0347	0.0598	0.0013	0.0205	0.0033	-	0.0099	0.0255	-	0.0151	0.0423	-	0.0278	0.0342	0.0219	-	0.0016	-	-	-	-	0.0079	0.0031	0.0100
		0.0088		0.0130	0.0156	0.0034	0.0136	0.0284	0.0011	0.0166	0.0011	-	0.0021	0.0162	-	0.0213	0.0493	-	0.0498	0.0361	0.0194	-	NA	-	-	-	-	0.0169	0.0094	NA
72	2,6-diMeC$_{30}$	-		-	-	0.0059	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0097	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
73	15,13,11,9-MeC$_{31}$	0.0260		0.0109	0.0167	0.0100	0.0488	0.0388	0.0043	0.0075	0.0110	0.0329	0.0268	0.0104	-	0.0390	0.0010	-	0.0362	0.0567	0.0178	-	0.0422	0.0126	-	-	-	0.0133	0.0074	0.0242
		0.0101		0.0020	0.0076	0.0080	0.0300	0.0168	0.0023	0.0026	0.0024	0.0057	0.0040	0.0069	-	0.0278	0.0020	-	0.0335	0.0177	0.0076	-	NA	0.0146	-	-	-	0.0168	0.0172	NA
74	7-MeC$_{31}$	0.0015		0.0036	0.0011	0.0157	0.0026	-	-	-	0.0022	0.0015	0.0025	-	-	-	0.0034	-	-	-	0.0026	-	0.0017	0.0003	-	-	-	-	-	0.0017
		0.0006		0.0006	0.0008	0.0128	0.0004	-	-	-	0.0011	0.0013	0.0008	-	-	-	0.0067	-	-	-	0.0015	-	NA	0.0008	-	-	-	-	-	NA
75	5-MeC$_{31}$	0.0004		0.0037	0.0013	-	-	-	0.0013	-	-	-	0.0005	0.2222	-	-	-	-	-	-	0.0021	-	-	-	-	-	-	-	-	0.0021
		0.0006		0.0005	0.0016	-	-	-	0.0012	-	-	-	0.0002	0.0950	-	-	-	-	-	-	0.0015	-	NA	-	-	-	-	-	-	NA
76	9,y,11,y,13,y-diMeC$_{31}$	0.0123		0.0056	0.0053	0.0011	0.0246	-	0.0007	0.0027	0.0111	0.0142	0.0073	-	-	-	-	0.0689	-	0.0175	0.0066	-	0.0276	0.0116	-	-	-	0.0025	-	-
		0.0064		0.0014	0.0057	0.0022	0.0183	-	0.0011	0.0025	0.0037	0.0120	0.0018	-	-	-	-	0.0325	-	0.0248	0.0046	-	NA	0.0166	-	-	-	0.0036	-	NA
77	7,y-diMeC$_{31}$	0.0016		0.0023	0.0004	-	-	-	-	-	-	-	0.0011	-	-	-	-	-	-	0.0030	0.0006	-	-	-	-	-	-	-	-	-
		0.0019		0.0006	0.0006	-	-	-	-	-	-	-	0.0005	-	-	-	-	-	-	0.0043	0.0007	-	NA	-	-	-	-	-	-	NA
78	3-MeC31+5,y-diMeC$_{31}$	0.0039		0.0029	0.0025	0.0023	0.0088	-	-	0.0016	0.0032	0.0019	0.0052	-	-	0.0075	0.1801	-	-	0.0102	0.0048	-	0.0064	-	-	-	-	0.0022	-	-
		0.0013		0.0005	0.0017	0.0046	0.0062	-	-	0.0014	0.0010	0.0016	0.0003	-	-	0.0105	0.0726	-	-	0.0144	0.0049	-	NA	-	-	-	-	0.0032	-	NA
79	C$_{32}$	0.0029		0.0021	0.0023	-	0.0053	-	-	0.0031	0.0021	0.0055	0.0001	0.0004	-	-	0.0009	-	0.0111	0.0022	0.0029	0.0030	0.0059	0.1053	-	-	-	0.0020	-	0.0030
		0.0011		0.0006	0.0013	-	0.0028	-	-	0.0022	0.0003	0.0068	t	0.0009	-	-	0.0018	-	0.0314	0.0031	0.0022	0.0025	NA	0.1180	-	-	-	0.0026	-	NA
80	3,y-diMeC$_{31}$	-		-	-	-	-	-	-	-	-	-	0.0017	-	-	-	-	-	-	-	0.0005	-	-	-	-	-	-	-	-	-
		-		-	-	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	-	-	0.0008	-	NA	-	-	-	-	-	-	NA
81	x-MeC$_{32}$	0.0036		0.0018	0.0027	0.0140	0.0125	-	-	0.0043	0.0034	0.0061	0.0038	-	-	-	-	-	-	0.0059	0.0041	-	0.0036	0.0018	-	-	-	-	-	0.0043
		0.0016		0.0004	0.0017	0.0044	0.0062	-	-	0.0047	0.0007	0.0035	0.0011	-	-	-	-	-	-	0.0084	0.0030	-	NA	0.0037	-	-	-	-	-	NA
82	12,16-diMeC$_{32}$	0.0062		0.0017	0.0033	0.0011	0.0087	-	-	-	0.0047	0.0046	0.0026	-	-	-	-	-	-	0.0101	0.0046	-	0.0063	0.0036	-	-	-	-	-	-
		0.0026		0.0003	0.0020	0.0022	0.0058	-	-	-	0.0011	0.0040	0.0006	-	-	-	-	-	-	0.0143	0.0027	-	NA	0.0069	-	-	-	-	-	NA
83	4-MeC$_{32}$	-		-	-	-	0.0061	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	-	0.0024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
84	x-C$_{33:1}$	-		-	-	-	-	-	-	-	-	-	-	-	-	0.0625	-	-	0.0068	-	-	-	-	0.0048	-	-	-	-	0.0021	0.0045
		-		-	-	-	-	-	-	-	-	-	-	-	-	0.0442	-	-	0.0193	-	-	-	NA	0.0079	-	-	-	-	0.0062	NA
85	y-C$_{33:1}$	0.0066		0.0330	0.0149	-	-	-	0.0005	-	-	0.0039	0.0058	-	-	0.0683	0.0091	-	-	-	0.0162	-	0.0021	-	-	-	-	0.0023	-	0.0418
		0.0028		0.0047	0.0118	-	-	-	0.0010	-	-	0.0035	0.0023	-	-	0.0094	0.0166	-	-	-	0.0137	-	NA	-	-	-	-	0.0030	-	NA
86	4,y-diMeC$_{32}$	-		-	-	0.0014	0.0043	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	-	0.0027	0.0021	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA

87	C_{33}	0.0036		0.0127	0.0067	0.0054	0.0061	0.0382	0.0007	0.0121	0.0039	-	0.0012	0.0077	-	-	0.0021	0.0053	0.0311	-	0.0061	-	-	0.0013	-	-	-	-	-	0.0037
		0.0021		0.0087	0.0052	0.0070	0.0017	0.0023	0.0008	0.0156	0.0020	-	t	0.0071	-	-	0.0041	0.0074	0.0461	-	0.0117	-	NA	0.0038	-	-	-	-	-	NA
88	17,15,13,11,9-MeC_{33}	0.0341		0.0253	0.0238	0.0028	0.0274	0.0215	0.0038	0.0095	0.0236	0.0409	0.0490	0.0063	-	0.0146	0.0008	0.0224	-	0.0162	0.0295	-	0.0171	0.0113	-	-	-	0.0070	0.0262	0.0251
		0.0075		0.0038	0.0081	0.0044	0.0146	0.0109	0.0009	0.0024	0.0065	0.0078	0.0191	0.0034	-	0.0206	0.0017	0.0316	-	0.0229	0.0084	-	NA	0.0159	-	-	-	0.0073	0.0406	NA
89	7-MeC_{33}	0.0002		0.0006	t	0.0017	-	-	0.0018	-	0.0010	-	0.0004	-	-	-	-	-	-	0.0182	0.0011	-	-	-	-	-	-	-	-	0.0066
		0.0001		0.0002	0.0001	0.0033	-	-	0.0007	-	0.0007	-	0.0001	-	-	-	-	-	-	0.0196	0.0007	-	NA	-	-	-	-	-	-	NA
90	5-MeC_{33}	0.0002		-	0.0021	-	-	-	-	-	-	-	-	0.0281	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		0.0005		-	0.0035	-	-	-	-	-	-	-	-	0.0153	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
91	15,19-13,17-,11,21-diMeC_{33}	0.0644		0.0448	0.0526	0.0151	0.0452	0.0655	0.0046	0.0095	0.0568	0.0938	0.0644	0.0027	-	0.0082	0.0224	0.2524	0.0876	0.0669	0.0520	-	0.0388	0.0522	-	-	0.0319	0.0109	0.0194	0.0217
		0.0229		0.0083	0.0258	0.0130	0.0297	0.0092	0.0009	0.0034	0.0151	0.0107	0.0134	0.0045	-	0.0116	0.0372	0.0653	0.0946	0.0532	0.0188	-	NA	0.0564	-	-	0.0675	0.0148	0.0392	NA
92	5,y-diMeC_{33}+3-MeC_{33}	0.0033		0.0076	0.0033	0.0006	0.0113	-	-	-	0.0299	0.0028	0.0054	0.0010	-	-	-	-	-	0.0037	0.0046	-	0.0034	0.0007	-	-	-	-	-	-
		0.0011		0.0014	0.0018	0.0012	0.0075	-	-	-	0.0099	0.0024	0.0021	0.0025	-	-	-	-	-	0.0053	0.0032	-	NA	0.0019	-	-	-	-	-	NA
93	C_{34}	0.0039		0.0035	0.0013	0.0018	0.0045	-	0.0002	-	0.0137	0.0058	0.0028	0.0005	-	-	0.0127	-	0.0078	-	0.0126	-	0.0028	0.0202	-	-	-	0.0013	-	0.0059
		0.0017		0.0004	0.0012	0.0020	0.0022	-	0.0006	-	0.0062	0.0058	0.0003	0.0011	-	-	0.0060	-	0.0220	-	0.0251	-	NA	0.0293	-	-	-	0.0018	-	NA
94	3,y-diMeC_{33}	-		-	0.0013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	0.0019	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
95	x-MeC_{34}	0.0043		0.0039	0.0033	0.0024	0.0071	-	-	-	0.0036	0.0037	0.0045	0.0036	-	-	-	-	-	0.0169	0.0049	-	0.0020	0.0058	-	-	-	-	-	0.0050
		0.0016		0.0006	0.0018	0.0033	0.0026	-	-	-	0.0011	0.0032	0.0015	0.0089	-	-	-	-	-	0.0076	0.0024	-	NA	0.0091	-	-	-	-	-	NA
96	x,y-diMeC_{34}	0.0157		0.0130	0.0106	0.0020	0.0137	-	-	-	0.0161	0.0153	0.0117	-	-	-	-	-	-	0.0058	0.0110	-	0.0069	0.0114	-	-	-	0.0010	-	-
		0.0063		0.0024	0.0046	0.0026	0.0073	-	-	-	0.0063	0.0133	0.0017	-	-	-	-	-	-	0.0081	0.0045	-	NA	0.0162	-	-	-	0.0032	-	NA
97	$C_{35:1}$	0.0007		0.0222	0.0037	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	-	-	0.0106	-	0.0016	-	-	-	-	0.0007	-	0.0040
		0.0009		0.0032	0.0040	-	-	-	-	-	-	-	0.0005	-	-	-	-	-	-	-	0.0165	-	NA	-	-	-	-	0.0023	-	NA
98	C_{35}	0.0002		0.0058	0.0007	-	-	-	-	-	-	-	0.0001	-	-	-	0.0015	-	-	-	0.0026	-	-	-	-	-	-	-	-	-
		0.0004		0.0042	0.0011	-	-	-	-	-	-	-	t	-	-	-	0.0031	-	-	-	0.0061	-	NA	-	-	-	-	-	-	NA
99	17,15,13,11,9-MeC_{35}	0.0195		0.0353	0.0154	0.0012	0.0141	-	0.0036	-	0.0167	0.0261	0.0263	-	-	-	-	0.0263	-	0.0190	0.0217	-	0.0032	0.0102	-	-	-	0.0079	-	0.0198
		0.0036		0.0023	0.0053	0.0024	0.0055	-	0.0013	-	0.0056	0.0104	0.0121	-	-	-	-	0.0370	-	0.0268	0.0072	-	NA	0.0158	-	-	-	0.0056	-	NA
100	11,19-,11,23-,11,21-diMeC_{35}	0.1231		0.1229	0.1439	0.0090	0.0480	0.1466	0.0075	0.0043	0.0986	0.2176	0.1337	0.0075	0.2249	0.1142	0.0103	0.1127	0.1873	0.0998	0.1015	-	0.0524	0.1405	0.2839	-	0.4723	0.0300	0.2060	0.0531
		0.0369		0.0182	0.0535	0.0180	0.0205	0.0141	0.0031	0.0038	0.0395	0.0218	0.0013	0.0063	0.0126	0.1259	0.0132	0.1089	0.1584	0.0769	0.0225	-	NA	0.1206	0.0151	-	0.4206	0.0228	0.2157	NA
101	7,y-,5,y-diMeC_{35}	0.0039		0.0137	0.0270	-	0.0206	-	0.0023	-	0.1321	0.0154	0.0023	-	-	-	-	-	-	-	0.0071	-	0.0017	0.0008	-	-	-	-	-	0.0068
		0.0014		0.0028	0.0159	-	0.0078	-	0.0028	-	0.0672	0.0267	0.0003	-	-	-	-	-	-	-	0.0065	-	NA	0.0022	-	-	-	-	-	NA
102	x,y,z-triMeC_{35}	-		0.0070	0.0063	-	-	-	-	-	-	-	0.0007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-		0.0021	0.0131	-	-	-	-	-	-	-	0.0002	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
103	C_{36}	0.0042		0.0044	0.0031	0.0101	-	-	-	-	0.0140	0.0083	0.0022	-	-	-	0.0254	-	-	-	0.0042	-	0.0021	0.0230	-	-	-	-	-	0.0025
		0.0018		0.0009	0.0017	0.0088	-	-	-	-	0.0071	0.0075	0.0002	-	-	-	0.0176	-	-	-	0.0014	-	NA	0.0292	-	-	-	-	-	NA
104	x-MeC_{36}	0.0034		0.0043	0.0031	0.0022	-	-	-	-	-	0.0041	0.0036	-	-	-	-	-	-	0.0051	0.0033	-	-	0.0055	-	-	-	-	-	-
		0.0012		0.0006	0.0018	0.0031	-	-	-	-	-	0.0035	0.0001	-	-	-	-	-	-	0.0072	0.0009	-	NA	0.0131	-	-	-	-	-	NA

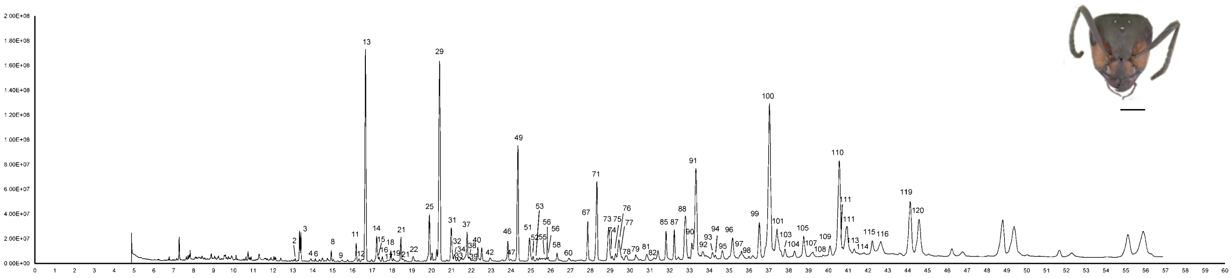
105	x,y-diMeC$_{36}$	0.0093		0.0153	0.0105	0.0009	0.0121	-	-	-	0.0091	0.0115	0.0095	-	-	-	-	0.0444	-	0.0076	0.0096	-	0.0037	0.0100	-	-	-	0.0024	-	0.0091
		0.0034		0.0027	0.0036	0.0018	0.0046	-	-	-	0.0043	0.0100	0.0013	-	-	-	-	0.0619	-	0.0107	0.0024	-	NA	0.0144	-	-	-	0.0038	-	NA
106	x,y-diMeC$_{36}$	t		0.0016	-	-	-	-	-	-	-	-	t	-	-	-	-	0.0825	-	-	-	-	-	-	-	-	-	-	-	-
		0.0001		0.0005	-	-	-	-	-	-	-	-	t	-	-	-	-	0.1162	-	-	-	-	NA	-	-	-	-	-	-	NA
107	C$_{37:1}$	0.0003		0.0045	0.0033	-	-	-	-	-	-	-	0.0001	-	-	-	-	-	-	-	0.0019	-	0.0013	-	-	-	-	0.0050	-	-
		0.0004		0.0006	0.0023	-	-	-	-	-	-	-	t	-	-	-	-	-	-	-	0.0031	-	NA	-	-	-	-	0.0073	-	NA
108	C$_{37}$	0.0003		0.0012	0.0002	-	-	-	-	-	0.0020	-	0.0001	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	-	0.0009	-	-
		0.0002		0.0003	0.0004	-	-	-	-	-	0.0010	-	t	-	-	-	-	-	-	-	0.0008	-	NA	-	-	-	-	0.0024	-	NA
109	19,17,15,13,11,9-MeC$_{37}$	0.0049		0.0167	0.0046	-	-	-	-	-	-	0.0072	0.0078	-	-	-	-	0.0812	-	-	0.0089	-	0.0016	0.0005	-	-	-	0.0127	-	-
		0.0013		0.0011	0.0022	-	-	-	-	-	-	0.0063	0.0007	-	-	-	-	0.0196	-	-	0.0059	-	NA	0.0013	-	-	-	0.0091	-	NA
110	11,y,13,y,15,y,17,y-diMeC$_{37}$	0.0560		0.1264	0.0734	0.0045	0.0400	0.1002	0.0044	-	0.0257	0.1019	0.0815	-	-	-	-	0.0999	-	0.0268	0.0688	-	0.0017	0.0131	-	-	-	0.0042	-	-
		0.0155		0.0165	0.0222	0.0090	0.0168	0.0146	0.0024	-	0.0096	0.0075	0.0160	-	-	-	-	0.0383	-	0.0379	0.0206	-	NA	0.0372	-	-	-	0.0071	-	NA
111	x,y-diMeC$_{37}$	0.0046		0.0365	0.0264	-	0.0012	-	-	-	0.0283	0.0235	0.0022	-	0.1156	-	0.0095	-	-	0.0056	0.0093	-	-	0.0169	-	-	-	-	0.0094	0.0335
		0.0025		0.0082	0.0150	-	0.0012	-	-	-	0.0159	0.0407	0.0002	-	0.1171	-	0.0190	-	-	0.0080	0.0073	-	NA	0.0456	-	-	-	-	0.0281	NA
112	x,y,z-triMeC$_{37}$	0.0011		0.0021	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0002	-	-	-	-	-	-	-	-	-
		0.0009		0.0008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0005	-	NA	-	-	-	-	-	-	NA
113	C$_{38}$	0.0023		0.0018	0.0030	-	-	-	-	-	-	-	0.0012	0.0020	-	-	0.0086	-	-	-	0.0022	-	-	0.0048	-	-	-	-	-	-
		0.0012		0.0005	0.0027	-	-	-	-	-	-	-	0.0002	0.0032	-	-	0.0099	-	-	-	0.0015	-	NA	0.0137	-	-	-	-	-	NA
114	x-MeC$_{38}$	0.0008		0.0031	0.0023	-	-	-	-	-	-	-	0.0009	-	-	-	-	-	-	-	0.0014	-	-	-	-	-	-	-	-	-
		0.0003		0.0017	0.0010	-	-	-	-	-	-	-	0.0001	-	-	-	-	-	-	-	0.0014	-	NA	-	-	-	-	-	-	NA
115	x,y-diMeC$_{38}$	0.0048		0.0158	0.0073	0.0006	0.0082	-	-	-	-	-	0.0044	-	-	-	-	-	-	-	0.0064	-	0.0026	0.0011	-	-	-	-	-	-
		0.0017		0.0056	0.0025	0.0012	0.0030	-	-	-	-	-	0.0013	-	-	-	-	-	-	-	0.0021	-	NA	0.0030	-	-	-	-	-	NA
116	w,z-diMeC$_{38}$	0.0015		0.0048	0.0130	-	-	-	-	-	-	-	0.0003	-	-	-	-	-	-	-	0.0029	-	-	-	-	-	-	-	-	-
		0.0014		0.0010	0.0227	-	-	-	-	-	-	-	t	-	-	-	-	-	-	-	0.0033	-	NA	-	-	-	-	-	-	NA
117	19,17,15,13,11,9-MeC$_{39}$	0.0009		0.0075	0.0011	-	-	-	-	-	-	-	0.0010	-	-	-	-	-	-	0.0034	0.0034	-	-	-	-	-	-	0.0157	-	-
		0.0004		0.0010	0.0006	-	-	-	-	-	-	-	0.0001	-	-	-	-	-	-	0.0048	0.0038	-	NA	-	-	-	-	0.0084	-	NA
118	7-MeC$_{39}$	0.0004		-	0.0004	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		0.0006		-	0.0007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-	-	-	NA
119	11,15- 15,19- 17,21- 13,17-diMeC$_{39}$	0.0327		0.0827	0.0450	0.0020	0.0294	0.1057	0.0025	-	-	0.0733	0.0304	-	0.0621	-	0.0318	-	-	0.0144	0.0362	-	0.0143	0.0116	-	-	-	0.0260	0.0327	0.0307
		0.0093		0.0116	0.0118	0.0040	0.0091	0.0143	0.0017	-	-	0.0091	0.0044	-	0.0503	-	0.0560	-	-	0.0203	0.0089	-	NA	0.0328	-	-	-	0.0161	0.0708	NA
120	5,y-7,y-,9,y-diMeC39	0.0087		0.0478	0.0409	0.0005	0.0186	0.0518	0.0004	-	-	0.0490	0.0050	-	0.1126	-	0.0104	-	-	-	0.0155	-	0.0057	0.0024	-	-	-	0.0146	0.0201	0.0216
		0.0045		0.0086	0.0443	0.0010	0.0021	0.0132	0.0010	-	-	0.0356	0.0011	-	0.0096	-	0.0179	-	-	-	0.0099	-	NA	0.0067	-	-	-	0.0125	0.0480	NA

Appendix 6-4: Chromatograms red wood ants and associated myrmecophiles

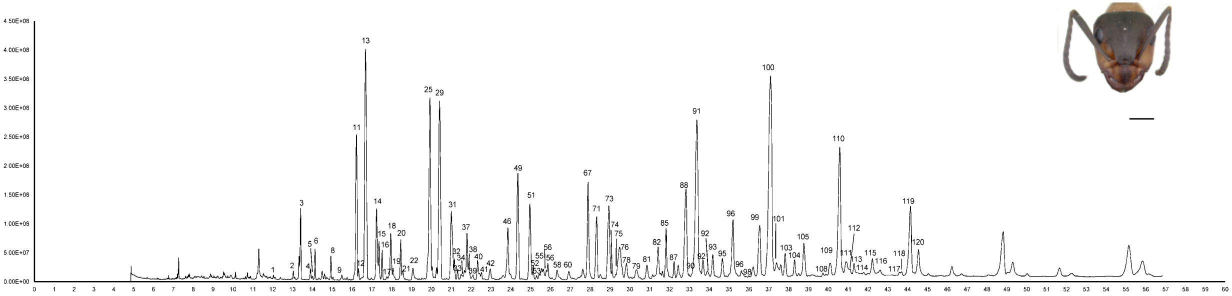
1	**C$_{22}$**	**31**	13,11,9-MeC$_{27}$	**61**	x,y-diMeC$_{30}$	**91**	15,19-13,17-,11,21-diMeC$_{33}$
2	C$_{23:1}$	**32**	7-MeC$_{27}$	**62**	4-MeC$_{30}$	**92**	5,y-diMeC$_{33}$+3-MeC$_{33}$
3	C$_{23}$	**33**	5-MeC$_{27}$	**63**	v-C$_{31:1}$	**93**	C$_{34}$
4	11,9-MeC$_{23}$	**34**	11,15-diMeC$_{27}$+9,y-diMeC$_{27}$	**64**	6,10-diMeC$_{30}$	**94**	3,y-diMeC$_{33}$
5	5-MeC$_{23}$	**35**	4-MeC$_{27}$	**65**	3-MeC$_{30}$	**95**	x-MeC$_{34}$
6	3-MeC$_{23}$	**36**	7,y-diMeC$_{27}$	**66**	w-C$_{31:1}$	**96**	x,y-diMeC$_{34}$
7	x,y-diMeC$_{23}$	**37**	3-MeC$_{27}$	**67**	x-C$_{31:1}$	**97**	C$_{35:1}$
8	C$_{24}$	**38**	5,y-diMeC$_{27}$	**68**	y-C$_{31:1}$	**98**	C$_{35}$
9	x-MeC$_{24}$	**39**	7,y-diMeC$_{27}$+x,y,z-triMeC$_{29}$	**69**	z-C$_{31:1}$	**99**	17,15,13,11,9-MeC$_{35}$
10	4-MeC$_{24}$	**40**	C$_{28}$	**70**	4,8-diMeC$_{30}$	**100**	11,19-,11,23-,11,21-diMeC$_{35}$
11	x-C$_{25:1}$	**41**	3,y-diMeC$_{27}$	**71**	C$_{31}$	**101**	7,y-,5,y-diMeC$_{35}$
12	y-C$_{25:1}$	**42**	x-MeC$_{28}$	**72**	2,6-diMeC$_{30}$	**102**	x,y,z-triMeC$_{35}$
13	C$_{25}$	**43**	x,y-diMeC$_{28}$	**73**	15,13,11,9-MeC$_{31}$	**103**	C$_{36}$
14	13,11,9-MeC$_{25}$	**44**	4-MeC$_{28}$	**74**	7-MeC$_{31}$	**104**	x-MeC$_{36}$
15	7-MeC$_{25}$	**45**	w-C$_{29:1}$	**75**	5-MeC$_{31}$	**105**	x,y-diMeC$_{36}$
16	5-MeC$_{25}$	**46**	x-C$_{29:1}$	**76**	9,y,11,y,13,y-diMeC$_{31}$	**106**	x,y-diMeC$_{36}$
17	x,y-diMeC$_{25}$	**47**	y-C$_{29:1}$	**77**	7,y-diMeC$_{31}$	**107**	C$_{37:1}$
18	3-MeC$_{25}$	**48**	z-C$_{29:1}$	**78**	3-MeC31+5,y-diMeC$_{31}$	**108**	C$_{37}$
19	5,y-diMeC$_{25}$	**49**	C$_{29}$	**79**	C$_{32}$	**109**	19,17,15,13,11,9-MeC$_{37}$
20	C$_{26}$	**50**	x,y-diMeC$_{28}$	**80**	3,y-diMeC$_{31}$	**110**	11,y,13,y,15,y,17,y-diMeC$_{37}$
21	3,y-diMeC$_{25}$	**51**	15,13,11,9-MeC$_{29}$	**81**	x-MeC$_{32}$	**111**	x,y-diMeC$_{37}$
22	x-MeC$_{26}$	**52**	7-MeC$_{29}$	**82**	12,16-diMeC$_{32}$	**112**	x,y,z-triMeC$_{37}$
23	4-MeC$_{26}$	**53**	5-MeC$_{29}$	**83**	4-MeC$_{32}$	**113**	C$_{38}$
24	2-MeC$_{26}$	**54**	4-MeC$_{29}$	**84**	x-C$_{33:1}$	**114**	x-MeC$_{38}$
25	w-C$_{27:1}$	**55**	x,y-diMeC$_{29}$+7,y-diMeC$_{29}$	**85**	y-C$_{33:1}$	**115**	x,y-diMeC$_{38}$
26	x-C$_{27:1}$	**56**	3-MeC$_{29}$+5,y-diMeC$_{29}$	**86**	4,y-diMeC$_{32}$	**116**	w,z-diMeC$_{38}$
27	y-C$_{27:1}$	**57**	x,y,z-triMeC$_{29}$	**87**	C$_{33}$	**117**	19,17,15,13,11,9-MeC$_{39}$
28	z-C$_{27:1}$	**58**	C$_{30}$	**88**	17,15,13,11,9-MeC$_{33}$	**118**	7-MeC$_{39}$
29	C$_{27}$	**59**	3,y-diMeC$_{29}$	**89**	7-MeC$_{33}$	**119**	11,15- 15,19- 17,21- 13,17-diMeC$_{39}$
30	x,y-diMeC$_{26}$	**60**	x-MeC$_{30}$	**90**	5-MeC$_{33}$	**120**	5,y-7,y-,9,y-diMeC39

Figures: Thomas Parmentier and kindly provided by Lech Borowiec
scale bar below figures corresponds with 1 mm.

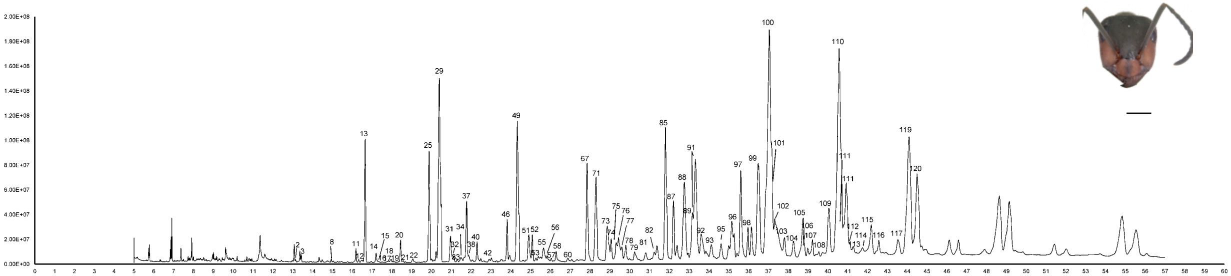
Formica rufa



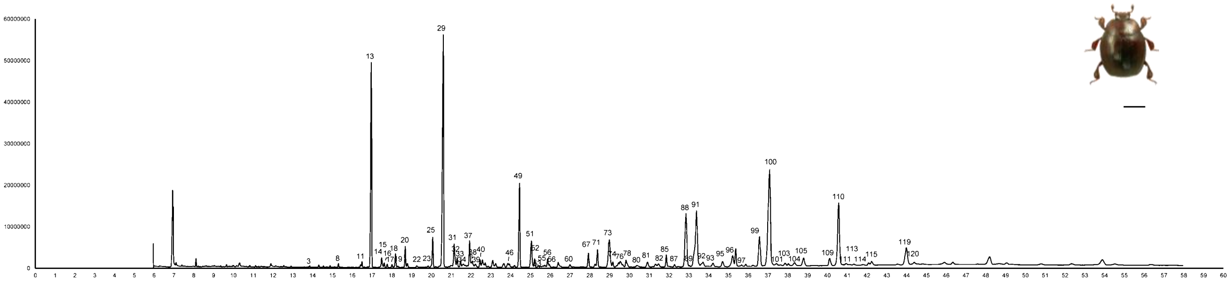
Formica polyctena



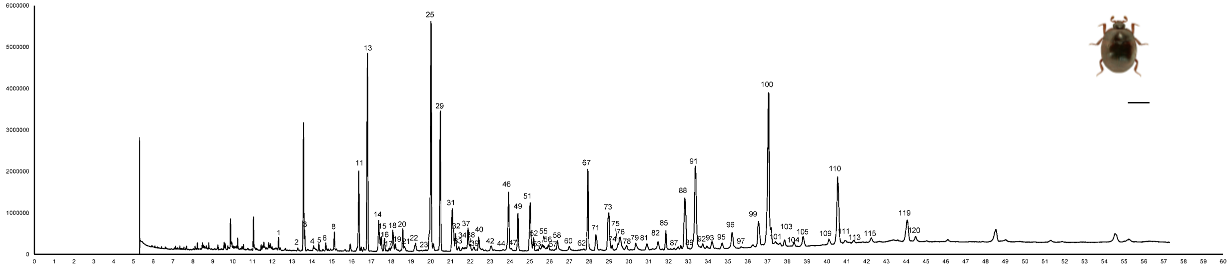
Formica pratensis



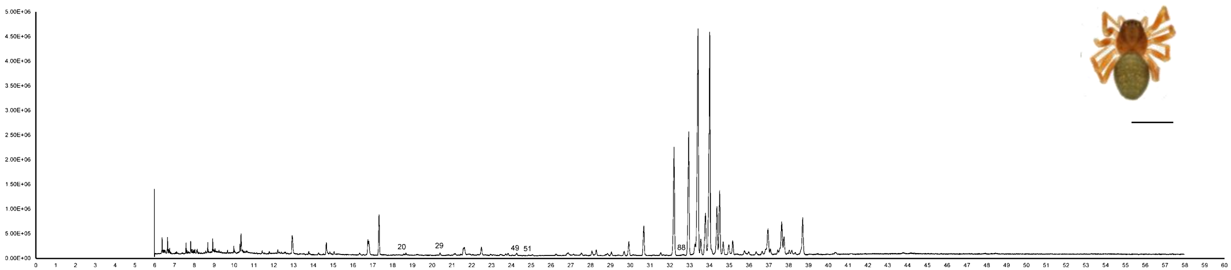
Dendrophilus pygmaeus



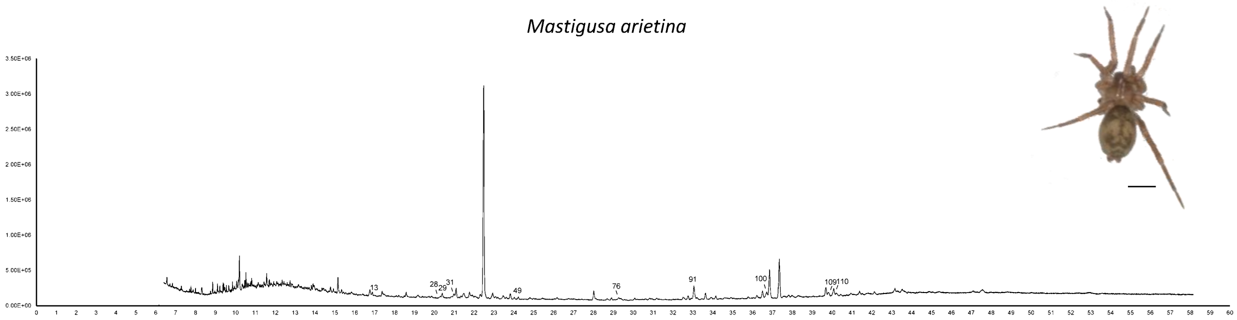
Myrmetes paykulli



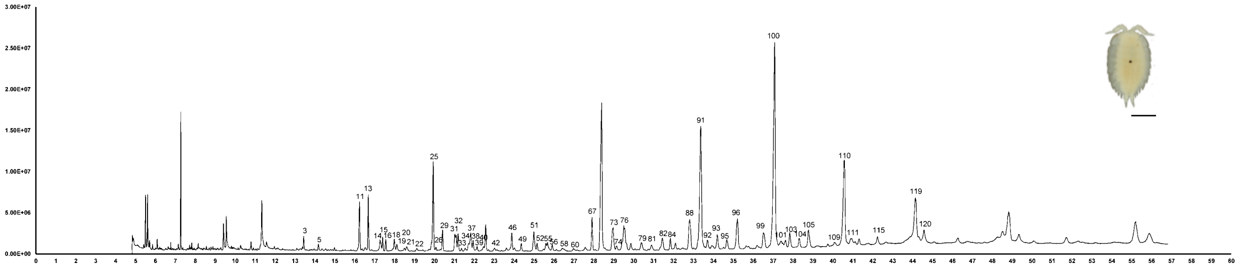
Thyreosthenius biovatus



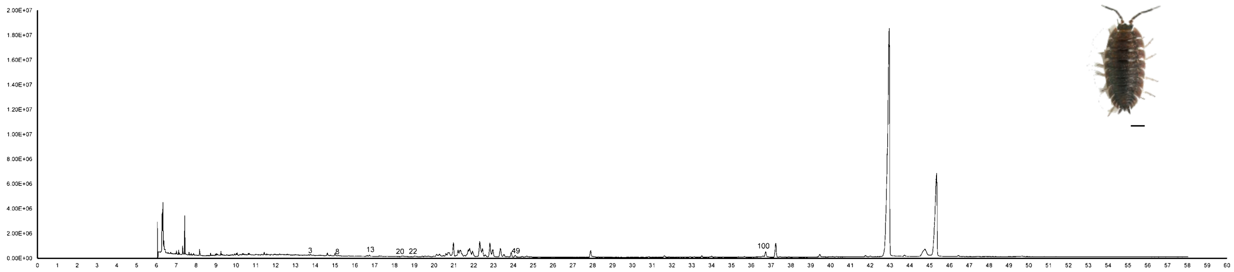
Mastigusa arietina



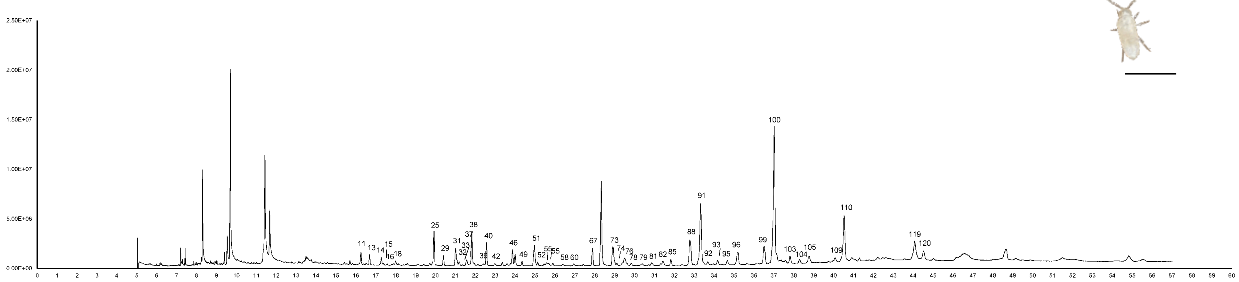
Platyarthrus hoffmannseggi



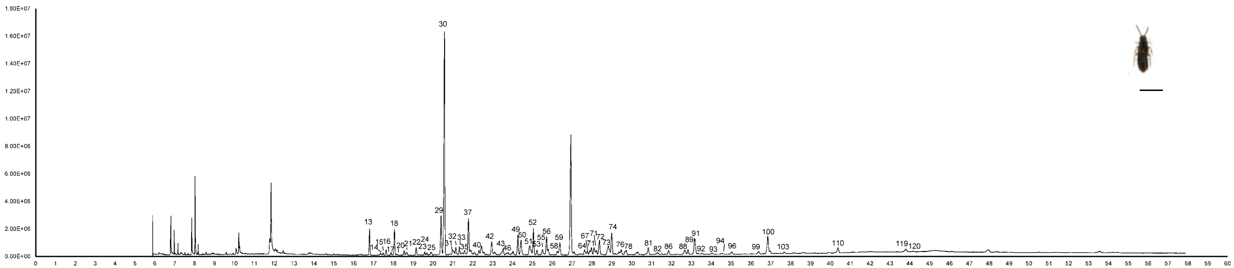
Porcellio scaber



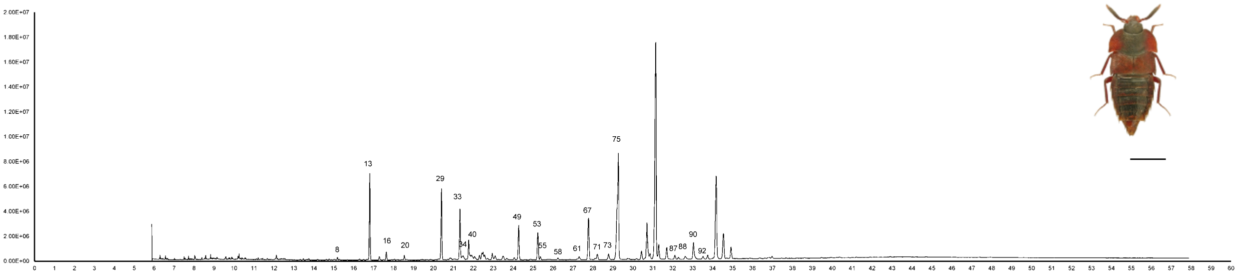
Cyphoderus albinus



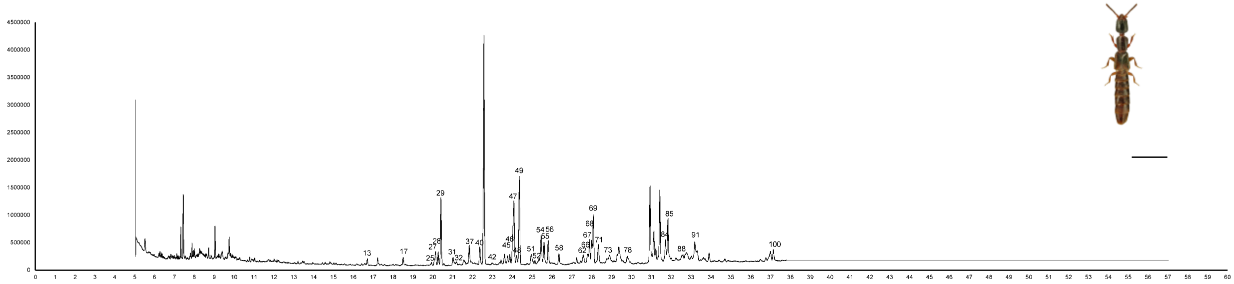
Amidobia talpa



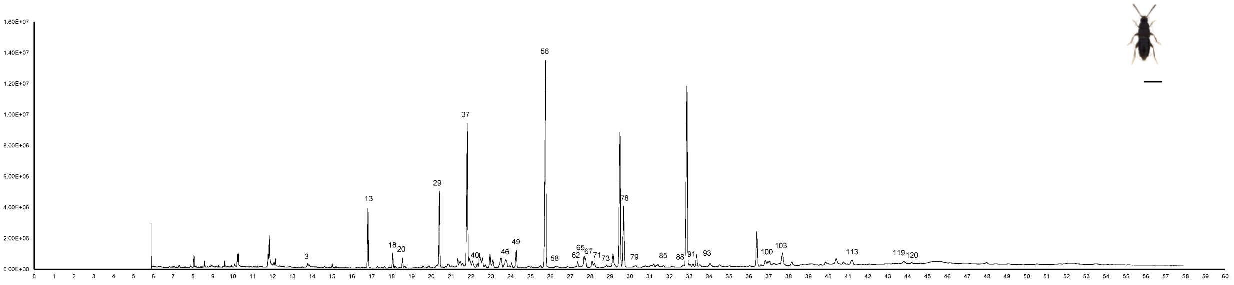
Dinarda maerkelii



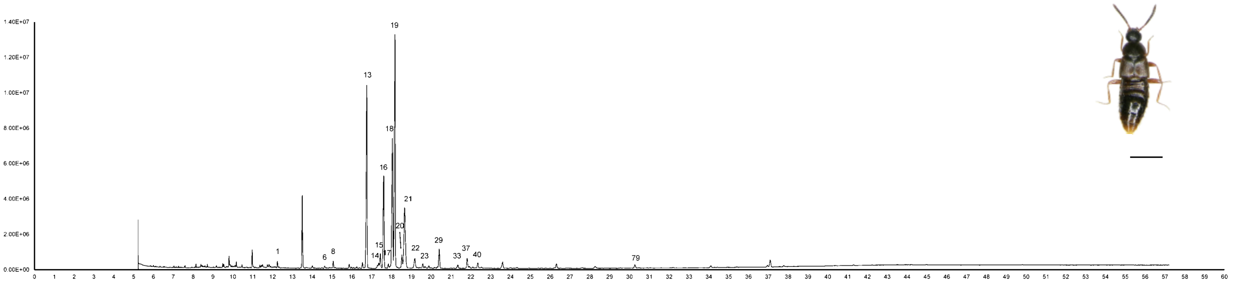
Leptacinus formicetorum



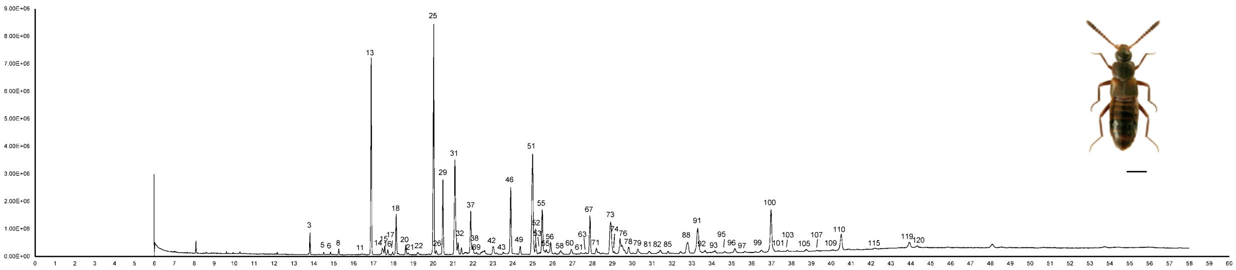
Lyprocorrhe anceps



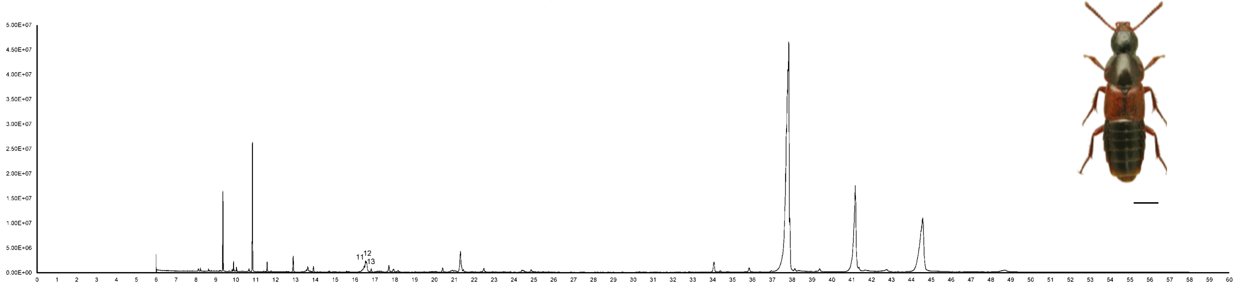
Notothecta flavipes



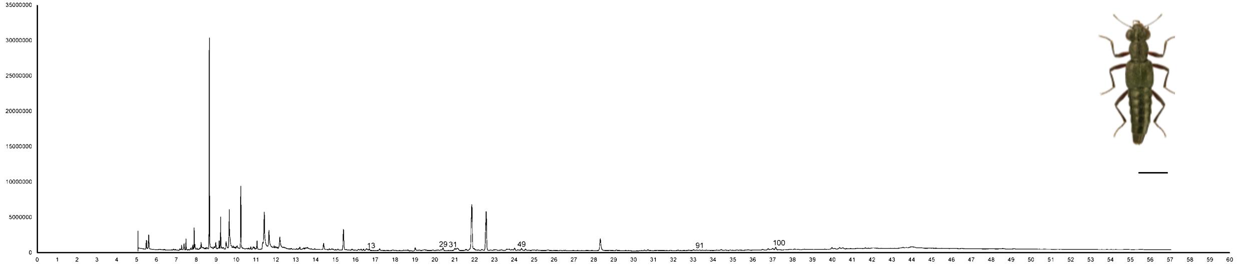
Pella humeralis



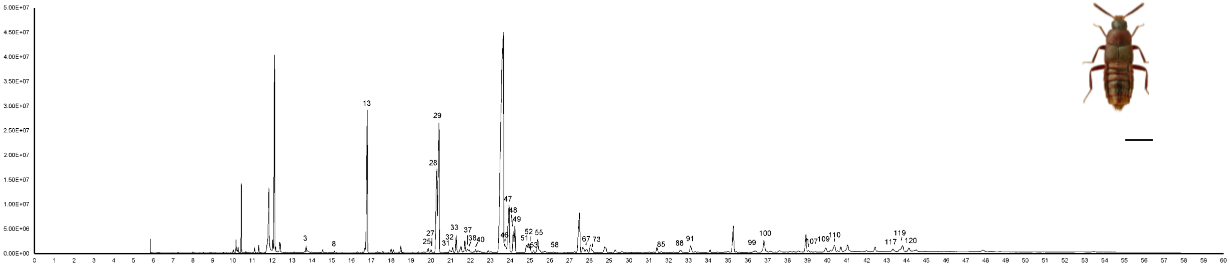
Quedius brevis



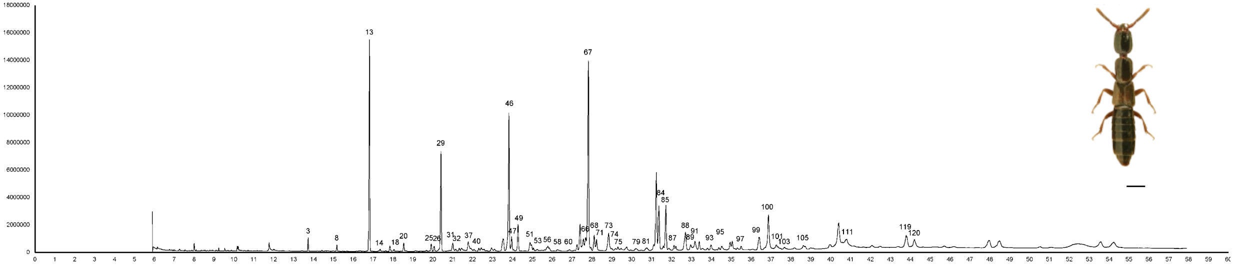
Stenus aterrimus



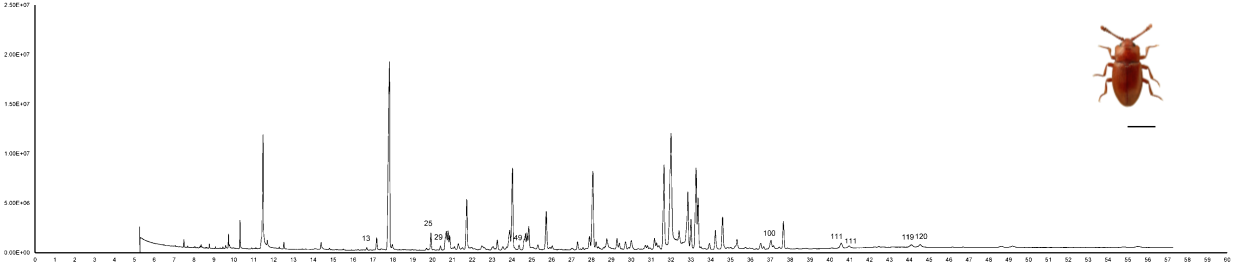
Thiasophila angulata



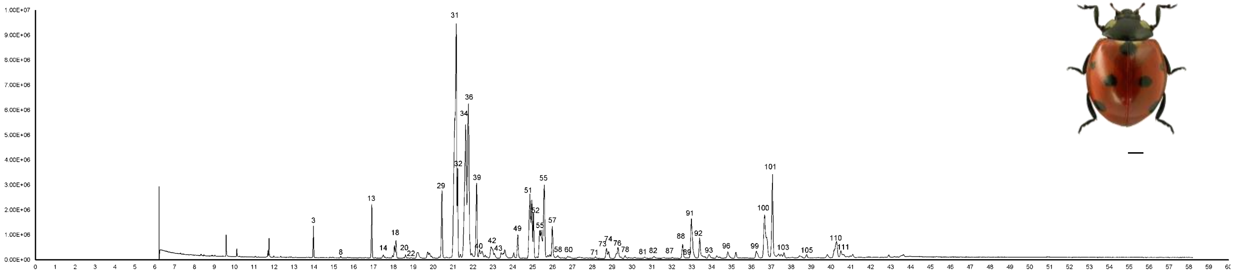
Xantholinus linearis



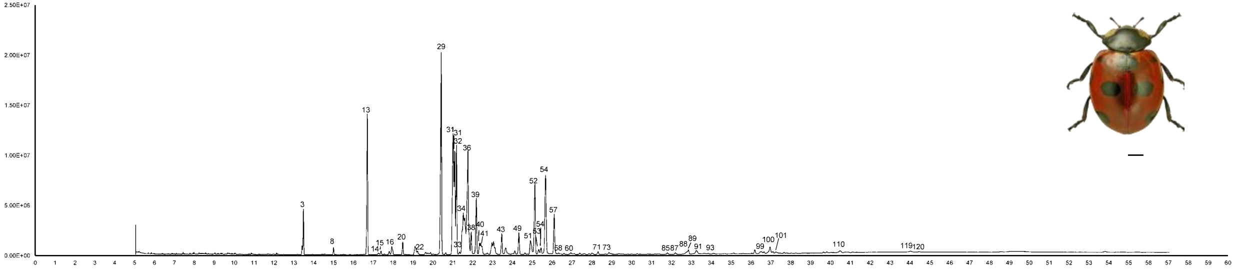
Emphylus glaber



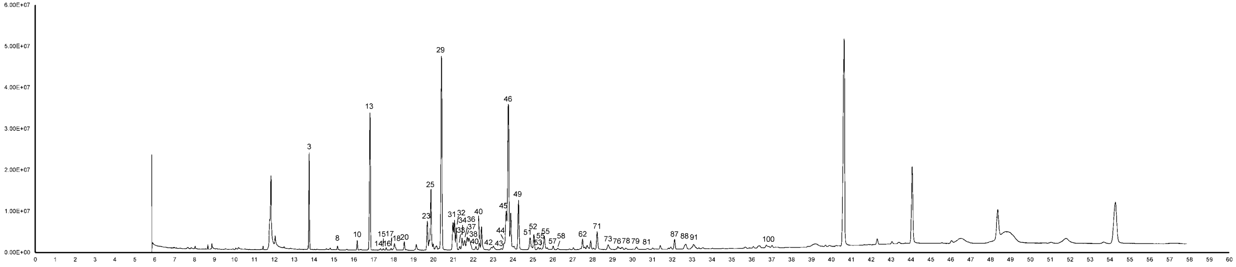
Coccinella septempunctata adult

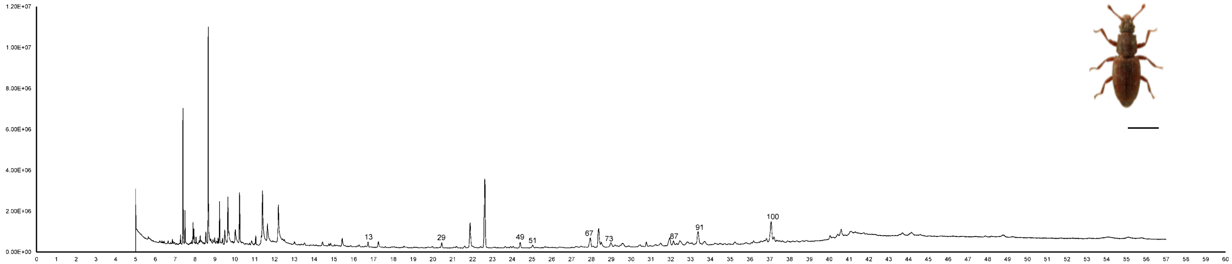


Coccinella magnifica adult

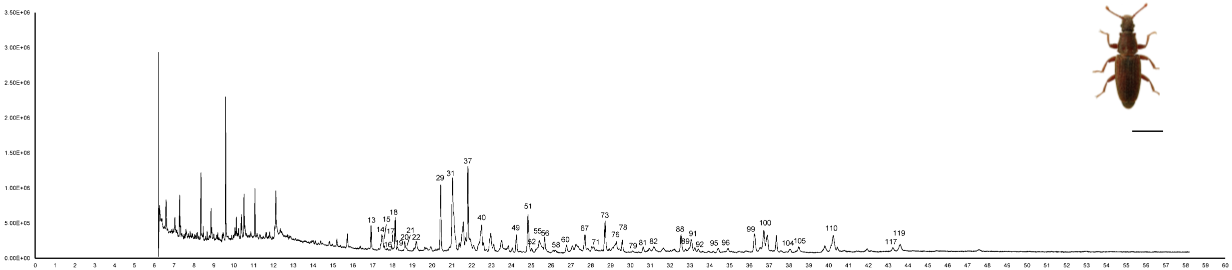


Coccinella magnifica larva



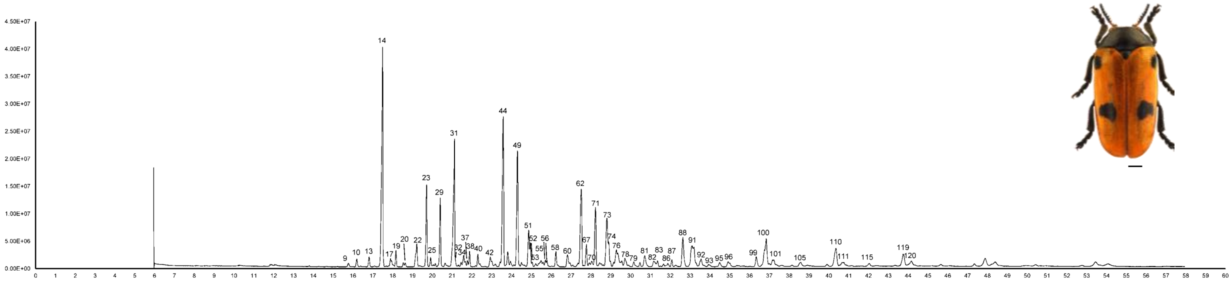


Monotoma angusticollis

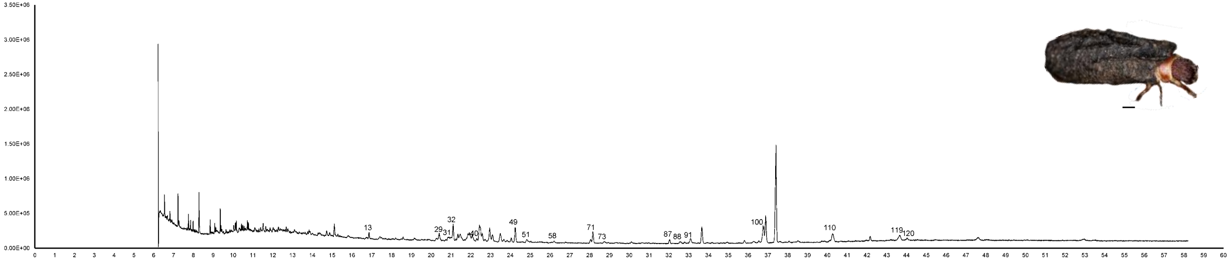


Monotoma conicicollis

Clytra quadripuncta adult



Clytra quadripunctata larva



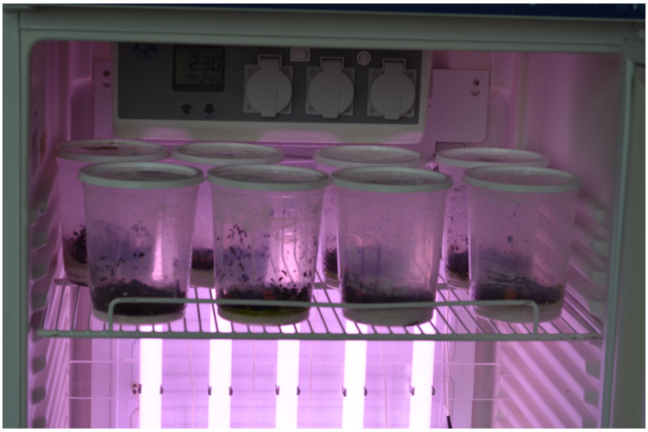
Appendix 6-5: Table Post hoc statistical test chapter 6

Table A-6.4. Wilcoxon rank sum tests to compare the hydrocarbon concentration between different myrmecophile species and RWA workers.

Species	***N***	**Hydrocarbons (ng/mm²)**	**SE**	**W**	P_{corr}
RWA workers	36	228.6	25.7		
Amidobia talpa	4	36.1	15.9	6	0.002
Clytra quadripunctata adult	5	289.0	15.8	118	0.325
Clytra quadripunctata larva	2	0.6	0.01	0	0.004
Coccinella magnifica adult	13	204.2	21.6	242	0.867
Coccinella magnifica larva	8	74.4	19.6	46	0.004
Cyphoderus albinus	3	26.2	7.1	0	0.001
Dendrophilus pygmaeus	2	91.8	26.4	16	0.294
Dinarda maerkelii	5	78.0	14.4	29	0.018
Leptacinus formicetorum	2	6.7	7.7	0	0.004
Lyprocorrhe anceps	4	34.9	8.4	2	0.000
Mastigusa arietina	5	1.3	0.4	0	0.000
Monotoma angusticollis	8	1.5	0.3	0	0.000
Monotoma conicicollis	2	4.1	2.8	0	0.004
Myrmetes paykulli	9	107.6	15.2	88	0.048
Notothecta flavipes	7	144.9	24.2	90	0.301
Pella humeralis	1	115.4	NA	11	0.678
Platyarthrus hoffmannseggii	8	7.3	0.1	0	0.000
Porcellio scaber	2	0.2	0.02	0	0.004
Quedius brevis	3	11.2	3.0	0	0.001
Stenus aterrimus	7	0.4	0.1	0	0.000
Thiasophila angulata	8	80.7	14.5	46	0.004
Thyreosthenius biovatus	9	3.5	2.3	0	0.000
Xantholinus linearis	1	79.5	NA	31	0.355

CHAPTER 7

Survival of persecuted myrmecophiles in laboratory nests of different ant species can explain patterns of host use in the field



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ABSTRACT

Myrmecophiles or ant associates are able to penetrate and survive inside the heavily defended nests of various ant species. With the exception of some highly specialized species, many of these myrmecophiles elicit a highly aggressive response and are frequently wounded or even killed by their hosts. Many myrmecophiles also appear to strongly prefer particular host species. The factors that allow the myrmecophiles to survive in these hostile environments and cause myrmecophiles to prefer particular host species are largely unknown. The aim of the present study was to examine the impact of the presence or absence of either the preferred host *Formica rufa* or one of several nonpreferred ant species on the long-term survival of three obligate, unspecialized beetle myrmecophiles, *Thiasophila angulata* (Erichson, 1837), *Lyprocorrhe anceps* (Erichson, 1837) and *Amidobia talpa* (Heer, 1841) and one facultative myrmecophile, the woodlouse *Porcellio scaber* Latreille, 1804. In addition, we tested whether host specificity was driven by the size of the ant host workers, because host specificity has previously been demonstrated to be inversely related to aggression towards macroparasites. Our results show that despite regular aggressive host interactions, survival of the obligate myrmecophilous beetles over a period of 20 days was no different from a control set-up without ants. By contrast, the facultative ant associate *P. scaber* hardly provoked any aggressive host response but its survival was lower in presence of *F. rufa* workers compared to a control set-up without ants. Furthermore, the data on survival in presence of 9 different ant host species show that the three obligate myrmecophile beetles survived better in presence of larger-bodied ant species, and was highest in presence of its preferred host *F. rufa*, which also has relatively large workers. The only exception to this trend was the low survival observed in presence of the large-bodied ant *Camponotus vagus* (Scopoli, 1763). Finally, species that were less successful in killing the beetles in our tests are also shown to support more myrmecophilous rove beetles in nature. Overall, our results shed new light on the interaction between ants and various associated macroparasites and on the factors that drive observed host preferences.

INTRODUCTION

Parasites have an intricate relationship with their host on which they can impose substantial costs (Poulin 2011). However, hosts have evolved an array of defence strategies at the behavioural, immunological and chemical level to counter parasites (Hart 1990, Clayton and Moore 1997, Schmid-Hempel 2011). A particularly useful system to test host-parasite interactions can be found in the nests of social insects. Social insect nests harbour a rich diversity of strictly associated symbionts including mutualists, commensals, and parasites (Kistner 1979, Hölldobler and Wilson 1990, Rettenmeyer et al. 2010). The parasites can have a dramatic effect on host fitness by consuming brood and host resources and inducing queen and worker mortality (Hölldobler and Wilson 1990, Schmid-Hempel 1998, Geiselhardt et al. 2007, Buschinger 2009, Hovestadt et al. 2012). The main defence response of social insects to macroparasites is aggression, in which,they exhibit biting, stinging, spraying defensive chemicals and chasing of the intruders (Hölldobler and Wilson 1990, Ayasse and Paxton 2002). Some ant associates or myrmecophiles evolved a specialized biology (symphiles or true guests sensu Wasmann 1894) and employ a plethora of strategies, including advanced behaviours, morphological adaptations, special defensive or appeasement glands and chemical mimicry. Such adaptations might lower ant aggression and enable the myrmecophiles to successfully integrate in ant colonies. What is more, they are treated as true colony members as they are fed, groomed and transported by the ants (Hölldobler and Wilson 1990, Akino 2008, van Zweden and d'Ettorre 2010, Kronauer and Pierce 2011). However, some myrmecophiles are seemingly unspecialized (synechthrans, i.e. indifferently tolerated guests, and synoeketes, i.e. hostile persecuted guests, sensu Wasmann 1894): they are very similar to their non-myrmecophilous counterparts and lack the aforementioned variety of adaptations (Donisthorpe 1927, Hölldobler and Wilson 1990). These myrmecophiles might be exposed to frequent ant aggression (Donisthorpe 1927, chapter 5: Parmentier et al. 2016b), which can lead to an elevated stress response in the myrmecophiles, injuries and ultimately death (Hölldobler et al. 1981, Nelson and Jackson 2009, pers. observations TP).

It is surprising how these myrmecophiles succeed to live in association with their host in such a hostile and stressful environment. The long-term effects of the host's defence response for those unspecialized myrmecophiles are unknown. Therefore, the effect of the association with host ants on the survival of three unspecialized, parasitic and myrmecophilous rove beetles associated with European red wood ants (RWAs) (*F.*

rufa group) was examined. First, 20-day survival of those myrmecophiles with the preferred host against survival in a control set-up without host workers was tested. The same tests were also done for a facultative myrmecophile (a species that is regularly found in ant nests, but is mainly found not to be associated with ants) to look whether the effect of host ants is similar on them compared with unspecialized myrmecophiles.

Surprisingly, many unspecialized myrmecophiles are associated with only a small group of ants (Donisthorpe 1927, Päivinen et al. 2002, chapter 1: Parmentier et al. 2014). The myrmecophiles of this study are restricted to mound building *Formica* ants. It is unclear why these relatively unspecialized myrmecophiles are only associated with mound building *Formica* species. Hitherto, it is unknown which mechanisms constrain the distribution of these species. A recent study showed that smaller workers in polymorphic RWA colonies are more aggressive and more successful in deterring intranidal myrmecophiles (chapter 3: Parmentier et al. 2015b). Consequently, the hypothesis under investigation is that ant species with on average smaller workers are more efficient in deterring unspecialized myrmecophiles. Interestingly, the mound building *Formica* ants have on average relatively large workers compared with other ant species in Europe (Seifert 2007) and support many unspecialized myrmecophiles (chapter 1: Parmentier et al. 2014). The relatively large mean worker size of these ants compared with other ant species in Europe could play a role in the strict association of many of those *Formica* associates. For that reason, we also assessed the survival of the three myrmecophilous beetle species in nests of eight other ant species spanning a gradient from one of the smallest to the largest ant species in the study area. We hypothesized that the survival rate of the unspecialized myrmecophiles would be highest in species with relatively large workers and would decrease in colonies of smaller ant species.

MATERIALS AND METHODS

Study species

Figure 7.1. Overview of the three myrmecophile beetles with their RWA host: (a) *Thiasophila angulata* with *Formica polyctena*, (b) *Lyprocorrhe anceps* with *Formica rufa*, and (c) *Amidobia talpa* with *Formica polyctena*. The myrmecophilous spider *Thyreosthenius biovatu*s can also be observed in the centre of b. Photo courtesy: T. Parmentier.

We collected adults of three myrmecophilous rove beetles (Staphylinidae, Aleocharinae): *Thiasophila angulata* (Erichson, 1837), *Lyprocorrhe anceps* (Erichson, 1837) and *Amidobia talpa* (Heer, 1841) in European RWA (*Formica rufa* group) nests in populations in Northern Belgium and in Northern France during the summer and autumn of 2014 and spring and summer of 2015 (Fig. 7.1). Two populations (West-Vleteren, Boeschepe) consisted of *Formica rufa* Linnaeus 1761 mounds, three of *Formica polyctena* Förster, 1850 (Beernem, Roksem, Aartrijke) mounds and both species occur sympatrically in the two remaining populations (De Haan and Beisbroek) (map see chapter 2: Parmentier et al. 2015a). Beetles were identified following Freude et al. (1974). We isolated the myrmecophiles by spreading nest material of *Formica rufa* or *Formica polyctena* nests on a large tray in the field. Ants and their brood were gently put back in the nests afterwards. Donisthorpe (1927) categorized the three beetle species following the classification of Erich Wasmann as synoeketes, which means that the beetles are rather unspecialized in morphology and behaviour compared with advanced myrmecophiles (symphiles) (Wasmann 1894). Synoeketes are not treated as colony members, but mostly ignored by the ants due to their small size and behaviour (Wasmann 1894). However, we found that the three species are detected by the ants and elicited aggression (chapter 5: Parmentier et al. 2016b, supplementary videos "*Amidobia talpa*", "*Lyprocorrhe anceps*" and "*Thiasophila angulata*"). Therefore they should rather be categorized in the group of synechtrans (unspecialized associates which provoke aggression). The complete life cycle of the beetles probably takes place inside the wood ant mounds (Donisthorpe 1927). This was supported by the occurrence of the adults in all seasons and the recording of larvae of different stages of the beetles inside the mound from spring to autumn (and raised in the lab to adults for identification). The larvae are free-living scavengers and are not nursed or carried by the workers (personal observations) in contrast with specialized beetle larvae such as *Lomechusa* and *Lomechusoides* (Hölldobler and Wilson 1990). The larvae of *T. angulata* are very similar to non-myrmecophilous larvae of the Aleocharinae and can be reared in absence of ants (Zagaja et al. 2014, pers. communication M. Zagaja). The adults are both brood predators and kleptoparasites as they prey on ant brood and food brought to the nest (chapter 3, 4: Parmentier et al. in press, 2015b). The three beetle species can be found throughout the nest mound (edge and centre of the nest). *Lyprocorrhe anceps* and *A. talpa* have no nest location preference, whereas *T. angulata* is attracted to the densely crowded brood chambers (chapter 5: Parmentier et al. 2016b). Morphological adaptations found in specialized myrmecophiles such as appeasement glands with trichomes are lacking in the three beetles (Freude et al. 1974). Freude et al. (1974) only report that the segments of the antennae of *T. angulata* are slightly compressed which could make it more difficult for ants to grab them. Their behaviour is also very similar to non-ant associated rove beetles. They escape from ant aggression by fleeing, hiding or bending their abdomen (Donisthorpe 1927, chapter 3: Parmentier et al. 2015b, supplementary videos "*Amidobia talpa*", "*Lyprocorrhe anceps*" and "*Thiasophila angulata*"). They probably excrete chemicals from their bent abdomen, which is a general defence strategy of rove beetles (Huth and Dettner 1990). The three beetles have a similar aleocharine morphology, but differ in size (*T. angulata* mean length 10 individuals ± SD = 2.85 mm ± 0.32, *L. anceps* mean length 10 individuals ± SD = 2.16 mm ± 0.20, *A. talpa* mean length 10 individuals ± SD = 1.53 mm ± 0.10, Fig. 7.1). In spite of their unspecialized myrmecophilous biology, they are very specialized in their host use. Their distribution is mainly restricted to European RWAs (*F. rufa* group) (Donisthorpe 1927, Freude et al. 1974, chapter 1: Parmentier et al. 2014) There are also some records for all three species in related mound building *Formica* species. The three species were occasionally observed in nests of *Lasius fuliginosus* (Latreille, 1798) and there is a single record of *T. angulata* in *Lasius brunneus* (Latreille, 1798) (see references in chapter 1: Parmentier et al. 2014), but these are probably infrequently used hosts (chapter 1: Parmentier et al. 2014, pers. observations TP). The three beetles are obligate myrmecophiles, as they cannot be found away from ants. However, a large number of species can occasionally be associated with ants (Donisthorpe 1927, Robinson and Robinson 2013, chapter 1: Parmentier et al. 2014). The widespread isopod *Porcellio scaber* Latreille, 1804 (adult size: 9 - 13.5mm, Berg and Wijnhoven 1997, identified following Berg and Wijnhoven (1997)) lives in a wide variety of habitats without ants (Berg and Wijnhoven 1997), but can also be very abundant in RWA mounds throughout the whole year (Robinson and Robinson 2013, chapter 1: Parmentier et al. 2014). Gravid females and juveniles were regularly observed in the mounds, which indicates that *P. scaber* is able to reproduce in the mounds. Isopods were collected in the same way as myrmecophilous beetles in RWA nests during spring 2015.

Ant aggression towards tested species

First, the interaction of *F. rufa* with the three myrmecophilous beetle species and *P. scaber* was examined. Therefore, a small rectangular plastic arena (length: 8 cm, width: 5.5 cm, height: 5 cm) was filled with ca. 1 cm plaster of Paris and coated with fluon. Forty *F. rufa* workers (West-Vleteren population) were acclimatized for one hour to the arena and then a myrmecophile found in the same colony was added. Ten seconds after the myrmecophile was introduced, the first twenty interactions with the ants were scored. In spite of these relatively short settling times, ants and myrmecophiles interacted similarly as in conditions where myrmecophiles were already integrated for days in lab nests (pers. observations TP). We also provide data of the effect of longer settling time (one hour) on ant aggression towards seven *T. angulata* beetles and compare these with the 10 s settling times (Appendix 7-1). These data confirm that longer settling times had no significant effect on the interaction between ants and myrmecophiles. Following interactions were observed from the perspective of the ant: ignoring (a worker's behaviour did not change when her antenna crossed the myrmecophile), showing interest (a worker started to antennate, turned her head or stopped walking or grooming when her antenna crossed the myrmecophile), opening mandibles (a worker aggressively opened her mandibles when her antennae crosses the myrmecophile), biting (a worker snapped with its mandibles and tried to grasp a myrmecophile) and acid spraying (a worker bent her gaster and sprayed acid after her antenna crossed the myrmecophile). Biting and acid spraying often followed directly after opening mandibles. In these cases only the last interaction was recorded. Ant aggression was scored by the proportion of aggressive interactions (acid spraying, biting, opening mandibles) out of the first 20 interactions. From the perspective of the myrmecophiles, the number out of 20 interactions that were directly preceded or followed by abdomen bending were counted. Trials were performed in darkness under red light and were recorded with a video camera (SONY HDR-XR550VE). Videos were subsequently analysed in VirtualDub 1.10.4 (http://www.virtualdub.org) which allowed to watch videos frame by frame.

Survival Experiment

In this experiment, 20-day survival of the three beetle species in *F. rufa* nests were compared with their survival in a control set-up without ants. *Formica rufa* workers were collected in a highly polydomous population in Boeschepe, Northern France. In addition survival of the three beetle species in nests of other ant species, ranging from one of the smallest to the largest ant species in the study region, were tested. Therefore colony fragments of *Solenopsis fugax* (Latreille, 1798) (Eastern bank river Meuse, Dinant), *Tetramorium caespitum* (Linnaeus, 1758) (Duinbossen, Lombardsijde), *Lasius niger* (Linnaeus, 1758) (urban region, Oostende), *Myrmica ruginodis* Nylander, 1846 (St-Sixtusbossen, West-Vleteren), *Formica cunicularia* Latreille 1798 (Duinbossen, Lombardsijde), *Lasius fuliginosus* (Provinciedomein, Raversijde, Aartrijksesteenweg, Aartrijke) were collected in different sites across Belgium during the summer and autumn of 2014 and spring and summer of 2015. Survival was also tested with *Monomorium pharaonis* (Linnaeus, 1758) and *Camponotus vagus* (Scopoli, 1763) of which we already had established lab colonies. *Monomorium pharaonis* is an indoor pest in Belgium and does not occur outside buildings (Dekoninck et al. 2003). Several colonies of *C. vagus* have only recently established in Belgium and are able to persist outdoors (Dekoninck and Pauly 2002, new records WD). Ants were identified using the key provided in Seifert 2007.

Within one day after collecting the myrmecophiles, between 9 and 13 individuals of each rove beetle species were placed together in 1 L plastic, cylindrical containers (diameter: 8.5 cm, height: 13.5 cm) with a 1.5-2 cm bottom of plaster of Paris. The top 5 cm inner wall of the containers were coated with fluon to prevent ants and myrmecophiles from escaping through 20 ventilation pin holes made in the container's lid. Myrmecophiles were collected in different RWA populations (*F. polyctena* and *F. rufa*) across West Flanders, Belgium and in Boeschepe, France to obtain sufficient numbers of individuals (Appendix 7-2). The tested myrmecophiles do not closely resemble the cuticular hydrocarbon profile of their RWA host colony (chapter 6). Moreover, conspecific beetles associated with *F. polyctena or F. rufa* do not substantially differ in their cuticular chemical profile (chapter 6). This lack of chemical adaptation to their host is further confirmed by aggression tests (chapter 5: Parmentier et al. 2016b Additional file: Table S3 or chapter 5: Table A-5.1). In these tests, we compared aggression of *F. rufa* workers of one colony (West-Vleteren, description see chapter 2: Parmentier et al. 2015a) towards myrmecophiles collected in the same colony with their aggression towards myrmecophiles found in *F. polyctena* colonies. Interestingly, the aggression response of the *F. rufa* workers was not significantly different towards beetles collected in *F. rufa* or *F. polyctena* colonies. Based on these chemical and behavioural data, we argue that the myrmecophile's colony of origin did not significantly affect the results of the survival experiments. Another confounding factor that might influence myrmecophile survival in our experiments is intra- and interspecific competition. However, no aggression between the beetles was observed. By providing food ad libitum, negative competition effects on survival were minimalized. Depending on the treatment, 100 workers of either *F. rufa, F. cunicularia*, *L. fuliginosus*, *L. niger*, *M. ruginodis*, *T. caespitum*, *M. pharaonis*, *S. fugax* were added. Because of their large size, only 50 workers of *C. vagus* were used (cf. Fig. 7.3). For polymorphic species workers of all worker subcastes were used (Fig. 7.3). Workers were randomly picked from nests, hence we assume that all worker subcastes (or size cohorts) are represented in numbers similar to their natural distribution. In addition, between 9 and 13 individuals per beetle species were added to containers described as above, but without adding ants. These containers served as controls. Survival of myrmecophiles was monitored every two days for a total period of 20 days. Two cut maggots (larvae of *Phaenicia sericata*), an Eppendorf tube (1.5 mL) filled with water and one with honey water were provided. The same food sources were offered in the same quantities in the control containers. Eppendorf tubes were sealed with a cotton plug soaked in either water or honey water. Maggots were replaced every two days, honey water every four days. Dead ant workers were replaced by new workers of the corresponding stock colonies every two days. Corpses of myrmecophiles were also removed to prevent contamination. The containers were kept in constant dark and at room temperature (20°C ± 2°C). Every treatment was replicated between eight and ten times (Appendix 7-2 Table A-7.2) with workers of another colony, except for *S. fugax*, *M. pharaonis* and *C. vagus* where we only had one colony at our disposal. For these species, different workers per replicate were used, but from the same (super)colony. Nest material of ant nests was not added to the containers. However myrmecophiles were able to hide under dead ants, prey, Eppendorf tubes and cotton made loose by the ants.

Similarly, survival of the facultative nest-inhabitant *P. scaber* was evaluated in *F. rufa* nests and in a control set-up. Thirty specimens were monitored for 20 days in the plastic containers described above. Individuals were counted every four days. The treatment group with 100 *F. rufa* workers and the control were compared and replicated eight times (in total 8 x 30 = 240 individuals were tested per treatment). Water and honey water as well as two slices of carrot were provided. The latter were replaced every four days. Dead isopods were removed and dead workers were replaced every four days. For this facultative myrmecophile we also compared survival in containers with addition of 25 mL nest material in an additional experiment. We were interested whether we would observe the same effect of the ants on the isopods with much more hiding places in the nests. Nest material was taken of a deserted *F. rufa* nest and was replaced after 10 days. Here, we only counted survivors after 20 days in a treatment with 100 *F. rufa* workers and a in control treatment without workers (in total 9 x 30 = 270 individuals were tested with *F. rufa* and 8 x 30 = 240 were tested in the control treatment).

Worker size

Maximum head width of the ant species used in the survival experiment was measured. This allows us to link the mean worker size of ant species with their efficiency in killing the myrmecophilous beetles. For each ant species, maximum head width from a random set of workers was measured. More workers were measured for a given species when it showed a high degree of polymorphism ($N = 30$ for *S. fugax*, *T. caespitum*, *M. pharaonis* and *M. ruginodis*, $N = 50$ for *F. cunicularia* and *L. niger,* $N = 100$ for *F. rufa* and *C. vagus*.)

Data analysis

The proportion of aggressive interactions towards the four associates were modelled with a quasibinomial GLM (family = quasibinomial in function glm) to account for overdispersion and tested with a likelihood ratio test. Subsequently, a set of quasibinomial GLMs were conducted to compare post hoc the proportion of aggressive interactions between the four associates. P-values of these six pairwise tests were Bonferroni corrected.

In the survival analyses, survival of the three obligate myrmecophilous beetles subjected to 10 different treatments was evaluated. In particular, the survival per beetle species in nests of *F. rufa,* in nests of eight other ant species and in a control set-up were tested against each other. Survival data per myrmecophile species were fitted with a mixed-effects Cox proportional-hazards model (Therneau 2015) by using the coxme function implemented in R version 3.2.1 (R Core Team 2014). This package allows the incorporation of random factors (Therneau 2015). In the Cox proportional-hazards model, we test whether the hazard ratio of a treatment is significantly different from 1 (Cox 1972). The hazard ratio can be interpreted in our experiment as the mortality rate in a particular treatment relative to the mortality rate of a reference treatment. Treatment (i.e ant species and control) was used as a fixed factor, replicate was modelled as a random factor. In a series of pairwise tests, survival of myrmecophiles in treatments with different ant species and the control set-up against survival in nests of *F. rufa* (reference level) was compared. P-values were estimated with a likelihood ratio test (Anova function in car package) and Bonferroni corrected. Second, survival of myrmecophiles in nests of different ant species, including *F. rufa*, against the control set-up without ants (reference level) was tested. P-values were again estimated with a likelihood ratio test and Bonferroni corrected.

For the facultative associate *P. scaber,* survival data of the experiment without nest material (control vs. treatment with 100 *F. rufa* workers), were similarly fitted with a mixed-effects Cox proportional-hazards model and significance tested with a likelihood ratio test. As we did not count *P. scaber* individuals at regular time intervals in the extra experiment with nest material, we could not do a survival analysis here. In this experiment, we only compared the proportion of surviving isopods (out of 30) after 20 days in a control set-up versus a treatment with 100 *F. rufa* workers with a quasibinomial GLM. Significance was tested with a likelihood ratio test.

All tests were two-tailed and a significance level of $\alpha = 0.05$ was used.

RESULTS

Aggression of *F. rufa* towards beetles and *P. scaber*

Ants exhibited frequent aggressive behaviour, such as biting, opening mandibles and acid spraying (proportions can be found in Table 7.1, supplementary videos "*Amidobia talpa*", "*Lyprocorrhe anceps*" and "*Thiasophila angulata*"). The proportion of aggressive interactions of *F. rufa* towards the four myrmecophiles was significantly different (quasibinomial GLM, LR Chisq= 262.37, df = 4, P < 0.001). Bonferroni corrected pairwise tests can be found in Appendix 7-2 Table A-7.3. *Thiasophila angulata* elicited most aggression (proportion aggressive interactions = 0.45, CI: 0.40-0.51), followed by *L. anceps* (proportion aggressive interactions = 0.25, CI: 0.19-0.31) and *A. talpa* (proportion aggressive interactions = 0.12, CI: 0.08-0.17). When interacting with ants, beetles accelerated, turned and avoided contact. They also regularly bent their abdomen (proportion interactions in which beetles bent their abdomen: *T. angulata* = 0.13, CI: 0.09-0.18, *L. anceps* = 0.15, CI: 0.10-0.22 *A. talpa* = 0.03, CI: 0.01-0.06) (Table 7.1). When beetles where clamped between the ant mandibles, they always succeeded to escape. In spite of its large size, *P. scaber* was largely ignored (proportion aggressive interactions = 0.07, CI: 0.03-0.13) and was not bitten or sprayed with formic acid during the 20 interactions of the aggression experiment (Table 7.1).

Survival of beetles and *P. scaber* in nests with *F. rufa* versus control

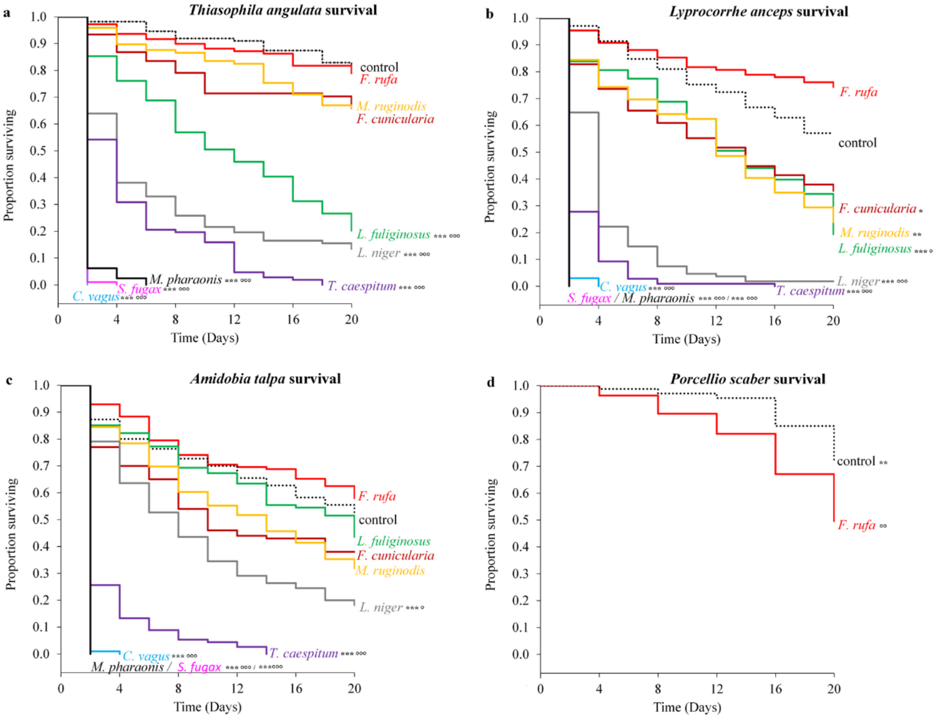
Formica rufa workers did not reduce survival in the long term for the three obligately myrmecophilous beetles compared with the control set-up (Bonferroni corrected pairwise test, *T. angulata*: *P* = 1.000, *L. anceps: P* = 0.286*, A. talpa P* = 1.000, Fig. 7.2 a,b,c, Appendix 7-2: Table A-7.2). Conversely, *F. rufa* workers induced a significant mortality of the facultative associate *P. scaber* compared with the control set-up (Likelihood ratio test, Chisq= 7.87, P = 0.005, Fig. 7.2 d). In an additional 20-day experiment with nest material, the proportion of surviving isopods per replicate was also significantly reduced (quasibinomial GLM, Likelihood ratio test, Chisq = 39.307, P < 0.001) in presence of *F. rufa* workers (mean = 0.85, CI: 0.78-0.90) compared with a control set-up without workers (mean = 0.51, CI: 0.43-0.59).

Table 7.1. Interactions between ant and associates are categorized in different categories. Mean proportions of a particular category out of a total of 20 interactions are given. 95% confidence intervals were calculated by running quasibinomial models for every interaction and with the function confint in R. They are listed in brackets under the means. Aggressive interactions are opening mandibles, biting and acid spraying. Species with a different letter code elicit a significant different proportion of aggressive interactions (Bonferroni corrected pairwise tests) The category "Abdomen bending" refers to the proportions of the 20 interactions that were directly preceded or followed by abdomen bending.

	N	Ignoring	Showing interest	Opening mandibles	Biting	Acid spraying	Proportion aggressive interactions		Abdomen bending
T. angulata	35	0.40 [0.34-0.46]	0.15 [0.11-0.18]	0.32 [0.27-0.37]	0.12 [0.08-0.16]	0.01 [0.01-0.02]	0.45 [0.40-0.51]	a	0.13 [0.09-0.18]
L. anceps	21	0.65 [0.58-0.72]	0.10 [0.07-0.15]	0.19 [0.14-0.24]	0.06 [0.03-0.10]	0.00	0.25 [0.19-0.31]	b	0.15 [0.10-0.22]
A.talpa	22	0.79 [0.72-0.84]	0.09 [0.06-0.13]	0.09 [0.06-0.13]	0.03 [0.01-0.06]	0.00	0.12 [0.08-0.17]	c	0.03 [0.01-0.06]
P. scaber	10	0.84 [0.75-0.91]	0.10 [0.05-0.16]	0.07 [0.03-0.12]	0.00	0.00	0.07 [0.03-0.13]	c	-

Survival of beetles in nests with other ant species

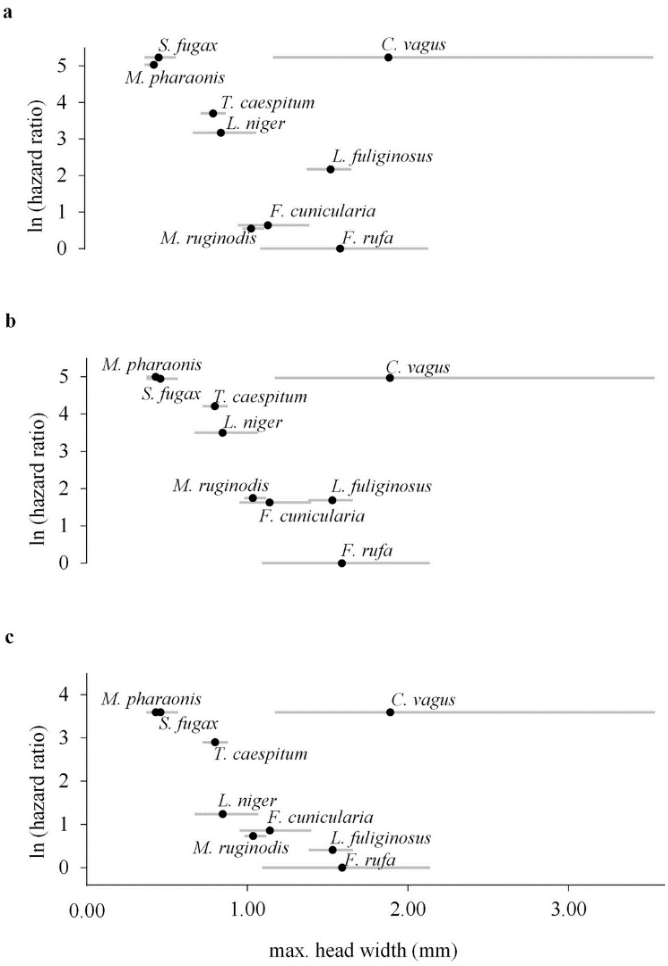
There was a large variation in survival of the three beetles when associated with other ant species (Fig. 7.2 a,b,c, Appendix 7-2: Table A-7.2). In general, the survival ratio of the three beetle species was very similar in nests of the different ant species. Survival of the beetles was highest when associated with *F. rufa* workers compared with other ant species (Bonferroni corrected pairwise tests listed in Appendix 7-2: Table A-7.2). *Monomorium pharaonis*, *S. fugax* and *C. vagus* killed all rove beetles (*T. angulata*, *L. anceps*, *A. talpa*) within the first six days, most of which did not survive the first hours. *Tetramorium caespitum* and *L. niger* also significantly reduced survival of all rove beetles compared with survival in *F. rufa* nests (Bonferroni corrected pairwise tests listed in Appendix 7-2: Table A-7.2). *Myrmica ruginodis*, *L. fuliginosus* and *F. cunicularia* caused reduced survival in one or two beetles species compared with *F. rufa*. While there are records for the three beetle species with *L. fuliginosus* (chapter 1: Parmentier et al. 2014), survival of *L. anceps* and *T. angulata* was significantly lower when associated with this ant species compared with their preferred *F. rufa* host.



Figuur 7.2. Twenty day survival curves of (a) *Thiasophila angulata*, (b) *Lyprocorrhe anceps*, (c) *Amidobia talpa* and (d) *Porcellio scaber* in a treatment with the normal host *Formica rufa* and a control treatment without ants. Survival curves with other ant species are also given in a, b, and c. Significances of Bonferroni corrected pairwise tests (cf. Appendix 7-2 Table A-7.2) of a treatment compared with a treatment with *F. rufa* (reference) are represented by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significances of Bonferroni corrected pairwise tests of a treatment compared with the control treatment are represented by hollow circles: ° $P < 0.05$, °° $P < 0.01$, °°° $P < 0.001$.

Relationship of worker size and myrmecophile survival

In Fig. 7.3 the ln(relative mortality rate) with a particular ant species for the three beetle species vs. the maximum head width size of the ant species was plotted. The ln(relative mortality rate rate) of the three beetle species initially decreased linearly with larger ant species and reaches its minimum with the large *F. rufa* species (Fig. 7.3). However, the extreme efficiency of the largest species *C. vagus* to kill the beetles, deviates from the larger worker-higher survival pattern observed in the other eight ant species.



Figuur 7.3. Relationship between ant species size (maximum head width) and the ln (relative mortality rate) of (a) *Thiasophila angulata*, (b*) Lyprocorrhe anceps*, (c) *Amidobia talpa*. Dots show the mean of the maximum head width and grey bars the range of max head widths. Here, the reference level of the relative mortality rate is the treatment with *F. rufa*. Therefore the ln (relative mortality rate) in nests of *F. rufa* is 0 (ln (mortality rate *F. rufa* / mortality rate *F. rufa* = ln (1). = 0)).

DISCUSSION

RWAs acted aggressively towards three associated rove beetles. These obligate myrmecophiles reacted agitated, often bent their abdomen and fled away. Nevertheless, these short-term antagonistic interactions did not harm the myrmecophiles over a period of 20 days. Interestingly, survival of the common soil-dwelling isopod *P. scaber*, which can be highly abundant in RWA mounds, did decrease due to RWA association. Ants mostly ignored these isopods and were not observed to bite or chase them. However, isopods are reported to have a reduced life time or a lowered reproductive investment when exposed to both abiotic and biotic stress (Hornung and Warburg 1994, Kight and Nevo 2004, Castillo and Kight 2005). The numerous interactions with ants in the experiments might indeed represent an elevated biotic stress level which ultimately led to lower survival ratios. RWA mounds can still be sources rather than sinks for *P. scaber* as well as for other facultative myrmecophiles, when the benefits of a thermoregulated, moist environment with ample of food sources (Rosengren et al. 1987, Kronauer and Pierce 2011) outweigh the stress costs associated with the ants. The three beetles have no specialized morphological (Donisthorpe 1927, Freude et al. 1974), chemical (chapter 6) or behavioural adaptations (Donisthorpe 1927, supplementary videos “*Amidobia talpa*”, “*Lyprocorrhe anceps*” and “*Thiasophila angulata*”) compared with more advanced myrmecophiles. We observed in all three species in varying degree, the bending of the abdomen, which stopped ants from attacking. Emitting chemicals from glands in their bent abdomen is a general defence strategy of non-ant associated and ant-associated rove beetles (Huth and Dettner 1990). However, it cannot be excluded that the beetles have evolved gland contents specifically adapted to deter wood ants. Possibly, the beetles have, akin to other parasitic social insect associates (Fisher and Sampson 1992, Kilner and Langmore 2011), a thicker cuticle to better resist ant bites and stings. Another possibility is that the rove beetles are difficult to catch by their small size and agility for the relatively large wood ants. The negative effects caused by ant aggression could also be compensated by indirect positive hygienic effects of the ants on the beetles. Ants possess glands which contain fungicidal and antimicrobial chemicals and these are important in suppressing pathogens in the moist and warm nests (Poulsen et al. 2002, Yek and Mueller 2011).

In this study, we show that the general traits (fleeing, hiding, abdomen bending) of these beetles are insufficient for association with most non-host ant species. The impact of different ant species on the myrmecophiles differed dramatically and some ant species even immediately killed the beetles. It is rather surprising that RWAs, which are commonly assumed as extremely dominant and aggressive towards other ants and arthropods (Mabelis 1978, 1984, Skinner and Whittaker 1981, Batchelor and Briffa 2010), are unsuccessful in killing or harming these beetles. Moreover it is remarkable that the unspecialized beetles of this study only have a narrow preferred host range, i.e. mound building *Formica* ants. The relatively large size of RWAs (Seifert 2007) might hamper them to successively detect, attack and/or handle small myrmecophiles and might be more suited to attack larger species, including conspecific competitors.

Small animals are harder to detect and are more agile (Blanckenhorn 2000) and size constraints can be important in explaining interactions between species. For example, large aerial insectivorous bats either cannot detect small insects, or they detect them too late to allow manoeuvring for capture (Barclay and Brigham 1991). Therefore their diet is constrained to large and less agile insects, whereas small bats effectively detect and hunt small insects (Barclay and Brigham 1991). Small workers in a polymorphic ant colony could have more antennal glomeruli to process olfactory cues as shown in some carpenter ants (Mysore et al. 2009, 2010). Small workers could also be ergonomically more efficient in catching, stinging and biting myrmecophiles that match their size. Moreover we reported recently that within a RWA colony, smaller workers were more aggressive than large workers towards myrmecophiles (chapter 3: Parmentier et al. 2015b). Therefore, we hypothesized that the same size-based aggression response could operate at the species level, whereby species with small workers detect and/or attack these myrmecophiles more easily and efficiently. Interestingly, survival of all three beetles indeed gradually increased with larger ant species and reached its maximum in the relatively large RWAs (Fig. 7.3). However, a linear association was violated with the extreme low survival in nests of *C. vagus*, the largest ant species known for the study region. Other factors than worker size could affect the efficiency of ants to kill myrmecophiles. For example, polymorphic ant species could have size classes which are more efficient in deterring (chapter 3: Parmentier et al. 2015b) and killing myrmecophiles. In addition, the defence mechanism (acid spraying vs stinging), the composition of defence chemicals and behaviour of ant taxa (Hölldobler and Wilson 1990) could affect the mortality rate of myrmecophiles. The observed effect on myrmecophile survival of different ant species in our tests are in line with the known diversity of rove beetle myrmecophiles associated with those ant species/taxa in Northern Europe (Päivinen et al. 2002). RWAs have most associated myrmecophilous rove beetles (N = 26) followed by *L. fuliginosus* (N = 21). The subgenus *Serviformica* (includes *F. cunicularia*) (N = 10), *Lasius* (except *L. fuliginosus*) (N = 16), and *Myrmica* species have a moderate number (N = 6). Finally, *Tetramorium* (N = 2), *Camponotus* (N = 1), *Solenopsis* (N = 0) and *Monomorium* (N = 0) have a very small or no records of associated rove beetles in Northern Europe (numbers based on Table 1 in Päivinen et al. 2002). There are also no records of myrmecophilous rove beetles associated with *Solenopsis* and *Monomorium* in the European myrmecophile list of Wasmann (1894) and in the British list of Donisthorpe (1927). It is postulated that colony size of ants (and corresponding number of niches in nests) is an important factor in predicting myrmecophile diversity (Kronauer and

Pierce 2011). In addition to this rule, we suggest that some ant species are more successful in expelling or killing myrmecophiles, which could constrain myrmecophile distribution and host range patterns.

SUPPLEMENTARY VIDEOS

The videos “*Amidobia talpa*”, “*Lyprocorrhe anceps*” and “*Thiasophila angulata*” may be consulted at the following link: https://www.youtube.com/channel/UCbdIeMJM-oO6AHqCgnH_bcQ

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APPENDIX CHAPTER 7

Appendix 7-1. Effect of longer myrmecophile settling time (one hour) on ant aggression.

Some myrmecophiles need longer settling times than the 10 s used in our experiments before normal interactions between ants and myrmecophiles can be observed (pers. communication C. von Beeren). In this experiment, we tried to assess whether longer settling times of myrmecophiles could affect the aggression response of the ants in our study system. *Thiasophila angulata* beetles were collected in a *F. rufa* colony (West-Vleteren) in October 2015 and were kept with workers and nest material in a plastic 1 L container until the aggression tests on 30/11/2015. These tests were similar to those described in the material and method section “Ant aggression towards tested species“. The only difference was that the first 20 interactions were now scored after one hour instead of 10 s after the introduction of the myrmecophile. We replicated these aggression tests seven times with different beetle specimens.

The proportion of aggressive interactions in both treatments were modelled with a quasibinomial GLM and differences were tested with a likelihood ratio test. Confidence intervals of all interactions are overlapping in both treatments (Table A-7.1). We did not find significant differences in the proportion of aggressive interactions between the two treatments (quasibinomial GLM, Chisq LR 2.5729, *P* = 0.2762). This suggests that the settling time of 10 s used in our experiment is sufficient to reflect the interactions between host ants and the unspecialized myrmecophiles studied here.

Table A-7.1. Interactions between *F. rufa* and *T. angulata* for a settling time of 1 h and 10 s (data from Table 7.1). Mean proportions of a particular category out of a total of 20 interactions are given. 95% confidence intervals were calculated by running quasibinomial models with the function confint in R. They are listed in brackets under the means. Aggressive interactions are opening mandibles, biting and acid spraying. The category “Abdomen bending” gives the proportion of interactions in which the beetle bent its abdomen.

Settling time	*N*	Ignoring	Showing interest	Opening mandibles	Biting	Acid spraying	Proportion aggressive interactions	Abdomen bending
1 h	7	0.39 [0.27-0.53]	0.07 [0.03-0.15]	0.48 [0.34-0.62]	0.06 [0.02-0.14]	0.00	0.54 [0.39-0.67]	0.26 [0.13-0.42]
10 s	35	0.40 [0.34-0.46]	0.15 [0.12-0.19]	0.32 [0.26-0.38]	0.12 [0.08-0.16]	0.01 [0.01-0.02]	0.45 [0.39-0.51]	0.13 [0.08-0.19]

Appendix 7-2. Statistical tests chapter 7.

Table A-7.2. A series of quasibinomial GLMs compare survival of three beetles introduced in colony fragments of different ant species and in a control set-up without ants, with survival of the beetles introduced in a *F. rufa* colony fragment (*P*-values indicated as P_{rufa}). P-values are Bonferroni corrected. Bonferroni corrected *P*-values of a series of quasibinomial GLMs, which compared survival of three beetles in colony fragments of different ant species with a control set-up without ants, are also reported (*P*-values indicated as P_{control}). Number of replicates and total number of myrmecophile individuals per treatment are given. In each replicate survival of 9-13 individuals of the three myrmecophile species were tested.

			T. angulata			*L. anceps*			*A. talpa*	
Ant species	$N_{\text{replicates}}$	$N_{\text{individuals}}$	P_{rufa}	$P_{control}$	$N_{\text{individuals}}$	P_{rufa}	$P_{control}$	$N_{\text{individuals}}$	P_{rufa}	$P_{control}$
F. rufa	10	109	reference	1.000	109	reference	0.286	112	reference	1.000
control	10	111	1.000	reference	105	0.286	reference	110	1.000	reference
F. cunicularia	9	91	1.000	0.731	87	**0.025**	0.333	100	**0**.368	1.000
M. ruginodis	10	97	1.000	1.000	109	**0.002**	0.065	116	1.000	1.000
L. fuliginosus	10	109	**<0.001**	**<0.001**	93	**<0.001**	**0.020**	101	0.417	1.000
L. niger	10	97	**<0.001**	**<0.001**	108	**<0.001**	**<0.001**	110	**<0.001**	**0.016**
T. caespitum	9	107	**<0.001**	**<0.001**	108	**<0.001**	**<0.001**	113	**<0.001**	**<0.001**
C. vagus	10	100	**<0.001**	**<0.001**	100	**<0.001**	**<0.001**	100	**<0.001**	**<0.001**
M. pharaonis	8	81	**<0.001**	**<0.001**	98	**<0.001**	**<0.001**	83	**<0.001**	**<0.001**
S. fugax	10	100	**<0.001**	**<0.001**	100	**<0.001**	**<0.001**	100	**<0.001**	**<0.001**
Total individuals		1002			1017			1045		

Table A-7.3. Bonferroni corrected *P*-values of Post hoc pairwise tests in which we compared the proportion of aggressive ant interactions between the four associates.

	T. angulata	*L. anceps*	*A.talpa*
T. angulata			
L. anceps	< 0.001		
A.talpa	< 0.001	0.003	
P. scaber	< 0.001	0.001	0.6168

CHAPTER 8

When David is Goliath's pitbull: task division in co-inhabiting ant species

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ABSTRACT

Nearly all social insects have a highly developed nestmate and species recognition system that is quite effective at keeping out any intruders. Rare cases of “parabiosis”, however, are known in some ants where two species apparently live peacefully alongside each other within the same nest. We here report on such an association between the tiny Afrotropical ant *Strumigenys maynei* and the large ant *Platythyrea conradti*. We demonstrate that both ants peacefully share the same arboreal nests in spite of the presence of clearly distinct nestmate recognition cues. Because of the large size differences, we hypothesized that each of the two species would benefit from specializing in carrying out particular tasks, in analogy to the size-related division of labor observed in species with size-polymorphic workers. In line with this theory, we find that the tiny ant *S. maynei* was highly efficient at nest defense against intranidal arthropods and alien ant intruders, whereas the large ant *P. conradti* was highly skilled in nest-engineering. We show that the described association formally qualifies as a mutualism, as *P. conradti* clearly benefited from the supreme defense capabilities of *S. maynei*, and that, conversely, *S. maynei* took advantage of small prey thriving in the organic nest material collected by *P. conradti*. Overall, our study suggest that ants can associate with a morphologically distinct ant partner as an alternative to developing distinct worker castes.

INTRODUCTION

Most social insects live inside well-defended “fortresses” from which any intruders are effectively excluded (Wilson 1971, Hölldobler & Wilson 1990,). Such nest defense is aided by a highly developed nestmate and species recognition system that relies on the presence of colony- or species-specific chemical cues (van Zweden & D’Ettorre 2010). Ants, like most other social insects, are typically very aggressive towards non-nestmates and alien intruders, yet rare examples of “parabiosis” are also known in which two distinct ant species peacefully share the same nest (Hölldobler and Wilson 1990, Menzel and Blüthgen 2010). These ants tolerate the presence of another ant species in the same nest, even if both ant species typically raise their brood in different nest chambers. Surprisingly, such species do not show any aggression towards each other, often exploit the same food sources and may even use the same pheromone trails (Menzel and Blüthgen 2010). The most widely accepted theory is that parabiotic associations are of a mutualistic nature and are beneficial for both ant partners. For example, in a parabiotic association between two South-East Asian ants, one of the species, *Crematogaster modiglianii*, was shown to benefit from the presence of the stronger and more aggressive *Camponotus rufifemur*, whilst the latter took advantage of the pheromone trails and nest construction capabilities of *Cr. modiglianii* (Menzel and Blüthgen 2010). A similar mutualistic association was also demonstrated in South-American parabiotic ants (Davidson 1988, Vantaux et al. 2007). Nevertheless, Menzel et al. (2014) also reported that some parabiotic partners seemed to be exploited, without receiving any return benefits from the partner.

Parabiosis can involve tight associations where both partners show colony-specific tolerance levels. In this case, only the partner colony is tolerated and conspecific and heterospecifc workers of other compound nests are aggressed by both partner colonies (Orivel and Dejean 1997, Emery and Tsutsui 2013). Other associations are less strict, as a partner might also tolerate other colonies of the partner (Menzel et al. 2008b). In contrast with most social insect parasites that mimic the odor of their host to get accepted, parabiotic ants succeed to associate even when they each have distinct chemical cuticular profiles (Orivel and Dejean 1997, Menzel et al. 2008a, 2009). It is suggested that parabiotic ants are able to recognize the chemical profile of the partner using a learning process which leads to colony or species-specific tolerance (Orivel and Dejean 1997). Nevertheless, there are also indications that a parabiotic life style imposes selection pressures on the chemical profile, as parabiotic ants frequently possess exceptionally long-chain hydrocarbons and higher amounts of methylbranched alkenes and alkadienes (Menzel and Schmitt 2012), or may carry cuticular compounds that are thought to appease the other partner (Menzel et al. 2013).

In the present study, we explored an apparent parabiotic association between the large Ponerinae ant *Plathythyrea conradti* and the tiny Myrmicinae ant *Strumigenys maynei* that was recently discovered in Ivory Coast (Yéo et al. 2006). The aim of our study was three-fold. First, we investigated the nature and specificity of the association by measuring the level of aggression between the two partners and analyzing whether they could discriminate conspecific and heterospecific workers of alien compound nests. Second, we carried out a chemical analysis of the cuticular hydrocarbon profiles of *P. conradti* and *S. maynei* of different compound nests to determine the chemical congruency between the associated ants across different nests. We then linked these chemical data with the behavioral assays and discuss these results with respect to the specificity of the ant association. Finally, we studied the potential benefits for both partners to engage in the association. In species with size-polymorphic workers, it is well documented that workers of particular size cohorts specialize on carrying out specific tasks inside the colony, such as nest defense (Hölldobler and Wilson 1990, Tian and Zhou 2014, Parmentier et al. 2015b). Analogously, we hypothesized that a similar specialization in nest defense between the two ant partners that vary greatly in size and morphology could bring distinct benefits to the association. Defense capabilities were tested for both ant partners towards intranidal intruders (myrmecophiles) and towards extranidal enemies (alien ant species).

MATERIAL AND METHODS

Study site and study organisms

Platythyrea conradti and *Strumigenys maynei* were found in a gallery forest along the Bandama river in the Lamto Ecological Station (6°13’ N, 5°01’ W), Ivory Coast in January 2016 (dry season) (Fig. 8.1A). The distribution of *S. maynei* (Myrmecinae) is restricted to the forest zones of West and central Africa, and Uganda (Bolton 2000). This tiny (ca. 2.5 mm long) ant is often found nesting in rotten wood in the leaf litter layer, but also in holes in trees (Bolton 2000). Most species of *Strumigenys* are specialized predators that capture small arthropods (Hölldobler and Wilson 1990, Bolton 1999). Colonies of *S. maynei* are headed by multiple queens (polygyne) and produce large numbers of workers. *Platythyrea conradti* (Ponerinae) is a large (ca. 15 mm long) Afrotropical arboreal ant that produces relatively small colonies (max. 500

workers). The workers hunt solitarily and prey on a wide variety of arthropods that they kill by a powerful sting (Dejean 2011). Both *P. conradti* and *S. maynei* are found in absence of each other across their overlapping distribution in the Afrotropical region (pers. observations KY, Bolton 2000). However, Yéo et al. (2006) reported that *S. maynei* colonies were present in 9 of 12 inspected *P. conradti* colonies in Lamto. These compound nests were typically found in hollow branches of living trees (usually *Pancovia bijuga*, Sapindaceae) 0.5-2 m above ground and of which the opening was stuffed with organic material (cf Fig. 8.1E). A number of associated arthropods or myrmecophiles were also detected in the compound nests (pers. observations KY, WD).

In this study, hollow branches of ten living trees, which were characteristically filled with organic material, were opened using a machete. Organic material was collected by hand, whereas ants and myrmecophiles were aspirated. The organic material was carefully sieved in the lab to find additional ants and myrmecophiles. Ants and myrmecophiles were housed per nest in plastic 1 L containers with a bottom layer of moist plaster and organic material of the original nest. Cotton plug soaked in sugar water was regularly provided.

The nature and specificity of the association.

In a series of aggression experiments, the behavior of *S. maynei* and *P. conradti* towards workers of the partner colony found in the same compound tree nest and towards workers of *S. maynei* and *P. conradti* found in other nests was tested. In all tests, the proportion of aggressive interactions (opening mandibles, biting and stinging) observed during a total of twenty interactions were scored as the dependent variable. We defined an interaction as the crossing of ant antennae with the introduced individual or one of its body parts. Indeed, the tiny *S. maynei* workers did not interact with the whole body of the much larger *P. conradti* workers, but mainly just with their body parts (legs, antennae) that contact the ground. Test arenas had a plaster bottom and fluon coated walls to prevent animals from escaping. Because of the large size differences between *S. maynei* and *P. conradti* (Fig. 8.1A,D), different test arenas and number of workers were used depending on the interaction tested. Number of trials for each interaction is listed in Table 8.1.

Aggression of *P. conradti* towards *P. conradti* workers of the same colony and alien colonies were done by introducing a *P. conradti* worker in an arena with one *P. conradti* worker. Both workers originated from either nest N1, N4 or N7, but tests were done blind to the origin of the introduced worker. Both workers were replaced in every trial.

Aggression of *P. conradti* towards co-inhabiting and alien workers of *S. maynei* was tested by introducing one *S. maynei* worker in an arena (diameter 8 cm) with 10 workers of *P. conradti*. Workers originated from nest N1 and N4, but tests were done again blind with respect to the origin of the *S. maynei* worker.

Aggression of *S. maynei* towards co-inhabiting and alien *P. conradti* workers was analyzed in an arena with a diameter of 3 cm. Here, the behavior of three individuals of *S. maynei* towards one *P. conradti* worker was followed. These tests were done with two colonies of *Strumigenys maynei* (nest N1 and nest N7) and workers were replaced in every trial. *Platythyrea conradti* workers also originated from nest N1 and nest N7, but tests were performed blind with respect to the origin of the *P. conradti* workers. Interactions were scored after the *P. conradti* worker calmed down and did no longer walk around, whereas in all other tests described below aggression scoring was recorded starting 10 s after introduction of an individual in the arena. Aggression of *S. maynei* towards workers of alien *S. maynei* colonies was tested by introducing a *S. maynei* individual in an arena (diameter 8 cm) with 40 *S. maynei* workers from either nest N1, N4 or N7. The introduced *S. maynei* individuals belonged to one of these colonies, but tests were performed blind with respect to the origin of these workers. Aggression tests with *S. maynei* as host were observed under a Leica MZ6 stereo-microscope.

The effect of nest origin, i.e. same nest or alien nest, of an introduced *S. maynei* worker on the proportion of aggressive interactions elicited in an arena with 40 *S. maynei* workers were analyzed using a generalized linear mixed model (GLMM) with a binomial error distribution using R package lme4. Significance was tested using a likelihood ratio test using R package car. The origin of the introduced worker was included as a fixed factor, whereas the 9 possible combinations of host colony and introducer colony (nest of host colony- nest of introducer colony: N1-N1, N1-N4, N1-N7, N4-N1, N4-N4, N4-N7, N7-N1, N7-N4, N7-N7) were included as a random intercept. In addition, an observation-level random intercept was incorporated to account for possible overdispersion (Browne et al. 2005). A similar model and analysis was run to assess the effect of nest origin of an introduced *P. conradti* worker on the proportion of aggressive interactions elicited in an arena with three *S. maynei* workers. As the two ant species originated from only two nests, the random factor that implemented the combination of acceptor and introducer colony had only 4 levels (nest of host colony- nest of introducer colony: N1-N1, N1-N7, N7-N1, N7-N7). Aggression of *P. conradti* towards other *P. conradti* workers and towards *S. maynei*, either from the same or an alien nest, was not modelled as no variation was observed within a treatment.

Cuticular hydrocarbon profiles of the ant partners.

Cuticular compounds of freeze-killed *S. maynei* workers (5 samples from nest N8, 3 samples from nest N10) were extracted in 30 µL of hexane (HPLC, Sigma-Aldrich) in 2 ml vials with PTFE septum (Sigma-Aldrich) for 10 minutes. Because of their small size, 5 *S. maynei* workers were pooled per sample. The large *P. conradti* workers (5 samples from nest N8, N9 and 10) were extracted in 200 µL of hexane for 10 minutes. Colonies of *S. maynei* and *P. conradti* were analyzed from compound nest N8 and N10, the samples of the S. maynei colony from N9 were contaminated and only the P. conradti colony of that nest was therefore analyzed.Samples were evaporated at room temperature to dryness and stored at -18 °C. Prior to analysis, *S. maynei* samples were diluted again in 30 µL and *P. conradti* samples in 200 µL, and 2 µL of these solutions were injected in a Thermo GC/MS (Trace 1300 ISQ) equipped with a Restek RXi-5sil MS column (20 m x 0.18 mm x 0.18 µm). The method had an initial temperature profile consisting of 1 minute at 40 °C, two temperature ramps from 40 °C to 200 °C at 20 °C min-1 and from 200 °C to 340 °C at 8 °C min-1, after which the final temperature of 340 °C was held for 4 minutes. We used helium as a carrier gas at a flow rate of 0.9 mL min-1, splitless injection and an inlet temperature of 290 °C. All samples and a linear C7 to C40 linear alkane ladder standard (49452-U, Supelco) at a concentration of 0.001 µg/mL and 0.01 µg/mL were run in the same batch. Retention indices were calculated using cubic spline interpolation based on the elution times of the external alkane ladder standard. These calculations were done using an in-house developed R script (available from the authors on request).

Per species, we only included peaks comprising at least 0.1 percent of the total profile area in each of the samples. Peaks were identified on the basis of their retention index and mass spectra. The analysis of the level of similarity among cuticular profiles was based on the hydrocarbon peaks that were shared by both ants, as it is generally assumed that this group of components is pivotal in nestmate recognition in ants (van Zweden and D'Ettorre 2010). The areas of the hydrocarbon peaks were first transformed by the Aitchison transformation (Aitchison 1986) and samples were then grouped by a hierarchical cluster analysis (Euclidean distances, Ward's method) using the R function hclust.

Potential benefits of the association.

Aggression tests of *P. conradti* and *S. maynei* towards myrmecophiles and alien ants were conducted similarly as described in the aggression trials above. Myrmecophiles were introduced in an arena (diameter 8 cm) with 10 workers of *P. conradti* found in the same nest (nest N2, N4, N6, N7 and N9), workers of alien ant species were introduced in an arena (diameter 8 cm) with 10 *P. conradti* workers of nest N10. Aggression of *S. maynei* towards myrmecophiles and alien ant workers was tested in the same way, but observations were done under a Leica MZ6 stereo-microscope. Myrmecophiles here were introduced in test arenas with 40 *S. maynei* workers collected in the same nest (nest N7, N9 and N10), alien ants were introduced in an arena with 40 *S. maynei* workers of Nest 10. Different myrmecophile and alien ant individuals were used per trial. The confidence intervals corresponding with the mean proportion of aggressive interactions of (1) *S. maynei* towards alien ants, (2) *S. maynei* towards myrmecophiles, (3) *P. conradti* towards alien ants and (4) *P. conradti* towards myrmecophiles were assessed by running four different quasibinomial general linear models.

RESULTS

The nature and specificity of the association

Colonies of *Platythyrea conradti* and *Strumigenys maynei* were always found together in the 10 inspected branches. Brood of both species was present in most of the inspected nests, but was clearly separated. The ten colonies of *S. maynei* were all polygynous and contained multiple breeding queens and winged male and female sexuals were also recorded (Fig. 8.1D). In one *P. conradti* colony, male sexuals were observed. *Platythyrea conradti* did not show any aggression towards workers of *S. maynei* living in the same compound nest or coming from an alien nest (Table 8.1, Suppl. video S1). In contrast, alien *P. conradti* workers were directly and fiercely attacked by biting and stinging (Fig. 8.1C). The workers involved in the fight could not be separated and fought until death. Therefore the proportion aggressive interactions in Table 8.1 was set to 1. Like *P. conradti*, *Strumigenys maynei* was very aggressive towards conspecific workers of an alien nest (GLMM, Likelihood ratio test, df = 1, Chisq = 32.56, $P < 0.001$) (Fig. 8.1B). They showed limited aggression towards *P. conradti* living in the same nest and aggression was not elevated when *P. conradti* originated from an alien nest (GLMM, Likelihood ratio test, df = 1, Chisq = 1.33, P = 0.248) (Table 8.1, Suppl. video S1). *Strumigenys maynei* was never observed soliciting for food and grooming behavior between heterospecific workers was also absent. No interspecific brood predation was observed between the ant partners in lab nests.

Figure 8.1. Overview of the compound nest microcosm of *P. conradti* and *S. maynei* and some interactions. A. Inhabitants of the compound nest: (1) *P. conradti*, (2) *S. maynei*, (3) Pselaphinae sp. (4) *Holotrochus* sp. (5) adults and nymphs of *Neoasterolepisma delamarei.* B. Aggression between workers of *S. maynei* originating from different nests. C. Aggression between workers of *P. conradti* originating from different nests. D. *P. conradti* and *S. maynei* queen, workers and alate queen. E. Typical compound nest with opening at a height between 1 and 2 m filled with organic material. The dashed line indicates the shape and depth of the nest in the hollow branch. Photo courtesy T. Parmentier.

Cuticular hydrocarbon profiles of the ant partners

A total of 78 different peaks were distinguished across both ant species. The majority of the peaks consisted of hydrocarbons (N = 59) (Table 8.2). There were also non-hydrocarbon compounds which we did not identify (N = 19). The profile of *Platythyrea conradti* and *S. maynei* was distinct with a much higher proportion of light hydrocarbons (between n-C21 and n-C27) (Table 8.2, Fig. 8.3). Nevertheless, both species shared 30 hydrocarbon peaks (Table 8.2, Fig. 8.3). *Platythyrea conradti* and *S. maynei* formed two distinct clusters in the hierarchical cluster analysis and the workers grouped per nest within both clusters (Fig. 8.4).

Table 8.1. Mean proportion of aggressive interactions of S. maynei and P. conradti towards inhabitants of the same or alien nests. Number of trials (N), 95% confidence intervals in brackets.

Introduced species		*N*	*Platythyrea conradti*	*N*	*Strumigenys maynei*
Ants					
Strumigenys maynei same nest		20	0.00	18	0.02 [0.01-0.05]
Strumigenys maynei alien nest		20	0.00	36	0.42 [0.26-0.59]
Platythyrea conradti same nest		20	0.00	30	0.07 [0.05-0.10]
Platythyrea conradti alien nest		15	1.00	30	0.05 [0.03-0.08]
Alien ants					
Monomorium pharaonis		10	0.00 [0.00-NA]	5	0.91 [0.82-0.97]
Monomorium bicolor		10	0.03 [0.01-0.06]	5	0.91 [0.82-0.97]
Crematogaster sp. 1		10	0.01 [0.00-0.03]	4	0.90 [0.79-0.96]
Crematogaster sp. 2		10	0.02 [0.00-0.04]	5	0.98 [0.92-1.00]
Oecophylla longinoda		10	0.08 [0.04-0.13]	5	0.90 [0.81-0.96]
Myrmecophiles					
COLEOPTERA					
Pselaphinae sp.	subtribe Batrisina	9	0.01 [0.00-0.05]	7	0.58 [0.49-0.66]
Scydmaeninae sp.	"*Napoconnus* complex" of genera	5	0.00 [0.00-NA]	5	0.42 [0.32-0.52]
Holotrochus sp.	Staphylinidae: Osoriinae	8	0.00 [0.00-NA]	5	0.56 [0.46-0.66]
COLLEMBOLA					
Cyphoderus subsimilis	Cyphoderidae	5	0.00 [0.00-NA]	5	0.45 [0.35-0.55]
THYSANURA					
Neoasterolepisma delamarei	Lepismatidae	9	0.25 [0.15-0.37]	4	0.83 [0.73-0.90]
Mesonychographis myrmecophila	Nicoletiidae: Atelurinae	3	0.00 [0.00-NA]	-	-

Potential benefits of the association

Myrmecophiles and alien ants elicited no or only limited aggression in *P. conradti*. Surprisingly, *P. conradti* avoided alien ants and regul tried to escape even when the introduced workers were much smaller (Suppl. video 2, 3). This can be demonstrated by the total number of escapes out of total number of interactions in trials with: *Monomorium bicolor* N = 8/200, *Crematogaster* sp. 1 N = 8/200, *Crematogaster* sp. 2 N = 20/200 and *O. longinoda* N = 8/200. *P. conradti* seldomly initiated a fight with an alien ant and showed in general merely aggression when it was bitten or stung by the introduced alien ant worker. Alien ants and myrmecophiles evoked a strong aggression response in *S. maynei*, in clear contrast to what we observed in *P. conradti*. *Strumigenys maynei* workers typically grabbed the legs of the intruder and tried to sting (Suppl. video 4, 5, 6, 7 and 8). An overview of the tested interactions between the inhabitants of the compound nests is schematically illustrated in Fig. 8.2.

We observed *Strumigenys maynei* with small prey (three times with Collembola: *Cyphoderus subsimilis*, two times with Pseudoscorpiones) living in the organic material holding between its mandibles in lab nests.

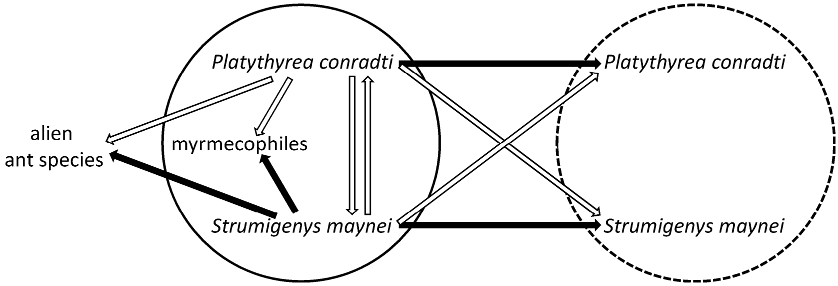

Figure 8.2. Schematic overview of aggressive interactions in *P. conradti* - *S. maynei* compound nests based on Table 8.1. The solid circle symbolizes the focal nest, the dashed circle an alien nest. Arrows refer to an interaction between *P. conradti* or *S. maynei* towards species at the end of the arrow. White arrows indicate none or minimal aggression, whereas black arrows designate overt aggression.

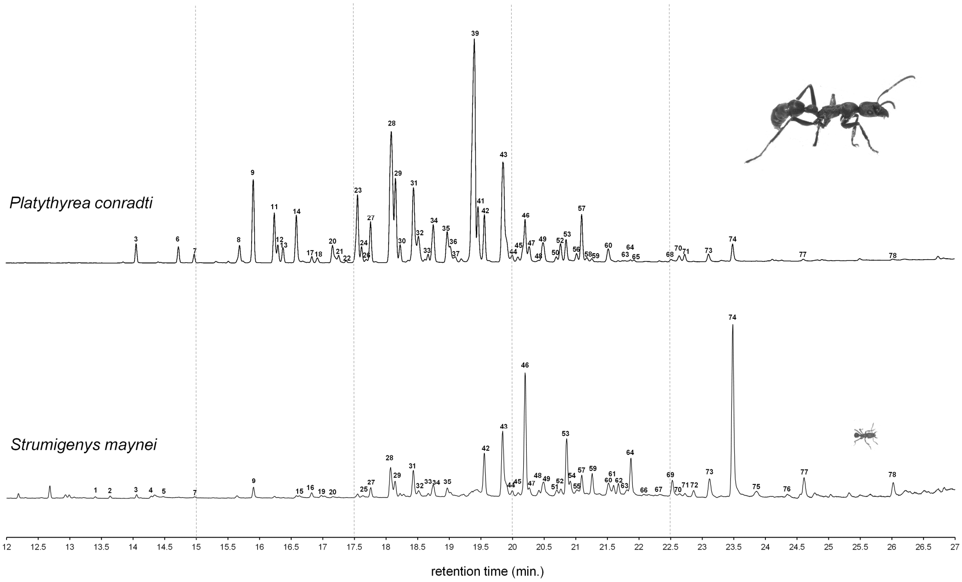

Figure 8.3. Representative gas chromatograms of the two co-inhabiting ant species with the relative intensity of peaks in function of retention time. The identity of the peaks corresponding with the peak numbers is given in Table 8.2.

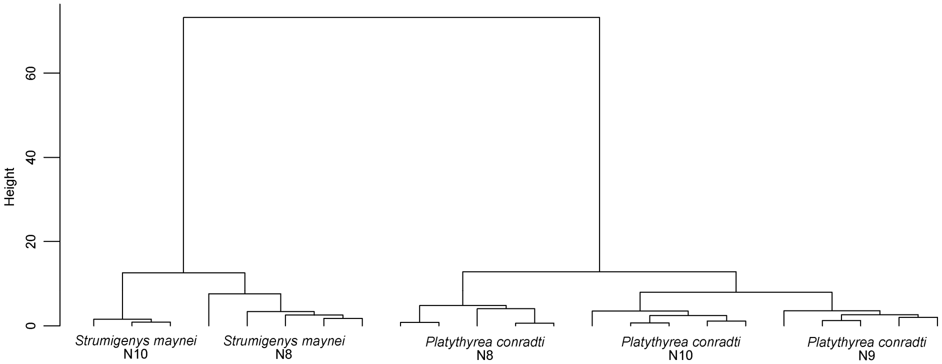

Figure 8.4. Hierarchical cluster analysis of the shared cuticular hydrocarbons of *Platythyrea conradti* and *Strumigenys maynei* (Euclidean distance, Ward's method). Colonies of *S. maynei* and *P. conradti* were analyzed from compound nest N8 and N10, the samples of the S. maynei colony from N9 were contaminated and only the *P. conradti* colony of that nest was therefore analyzed.

Table 8.2. Comparison of cuticular components (mean percentages ± SD) of *S. maynei* (N_{pooled} = 9) and *P. conradti* (*N* = 15). HC = hydrocarbon, non-HC = non-hydrocarbon component.

Peak no.	**retention index**		***S. maynei***	***P. conradti***	**Peak no.**	**retention index**		***S. maynei***	***P. conradti***
1	2026.78	non-HC	0.33 ± 0.13	-	**40**	2686.00	non-HC	0.27 ± 0.54	-
2	2053.23	non-HC	0.50 ± 0.54	-	**41**	2686.73	4,16-diMeC26	-	1.41 ± 0.71
3	2100.02	n-C21	0.22 ± 0.06	0.32 ± 0.25	**42**	2699.59	n-C27	7.37 ± 2.63	4.56 ± 1.98
4	2130.00	non-HC	0.28 ± 0.33	-	**43**	2732.58	13,11,9-MeC27	3.00 ± 1.96	8.45 ± 2.48
5	2149.00	non-HC	8.56 ± 13.68	-	**44**	2750.09	5-MeC27	0.21 ± 0.11	0.52 ± 0.15
6	2172.96	3-MeC21	-	0.67 ± 0.71	**45**	2760.52	non-HC	6.51 ± 14.07	0.41 ± 0.44
7	2199.84	n-C22	0.22 ± 0.11	0.18 ± 0.12	**46**	2773.96	3-MeC27	8.74 ± 2.90	4.98 ± 1.57
8	2276.74	C23:1	-	0.25 ± 0.27	**47**	2781.75	5,y-diMeC27	0.66 ± 0.19	0.77 ± 0.22
9	2299.58	n-C23	0.34 ± 0.17	2.94 ± 1.16	**48**	2799.17	n-C28	1.15 ± 0.36	0.13 ± 0.09
10	2303.00	non-HC	0.48 ± 0.34	-	**49**	2806.40	3,y-diMeC27	2.23 ± 1.42	1.90 ± 0.54
11	2335.71	11,9-MeC23	-	1.68 ± 0.50	**50**	2831.48	12-MeC28	-	0.38 ± 0.08
12	2341.78	7-MeC23	-	0.31 ± 0.22	**51**	2832.00	non-HC	0.68 ± 0.21	-
13	2350.62	5-MeC23	-	0.45 ± 0.17	**52**	2839.97	non-HC	0.58 ± 0.42	0.45 ± 0.25
14	2373.01	3-MeC23	-	2.33 ± 1.58	**53**	2850.61	non-HC	10.88 ± 10.60	0.82 ± 0.39
15	2377.00	non-HC	0.14 ± 0.09	-	**54**	2862.00	4-MeC28	1.58 ± 0.46	-
16	2398.00	non-HC	0.56 ± 0.36	-	**55**	2869.00	non-HC	1.13 ± 0.62	-
17	2399.56	n-C24	-	0.56 ± 0.27	**56**	2869.84	x-C29:1	-	0.64 ± 0.31
18	2408.97	3,13-diMeC23	-	0.71 ± 1.24	**57**	2879.34	y-C29:1	0.74 ± 0.47	1.65 ± 0.81
19	2415.00	non-HC	0.14 ± 0.06	-	**58**	2888.87	non-HC	-	0.15 ± 0.06
20	2434.72	x-MeC24	0.17 ± 0.03	1.11 ± 0.30	**59**	2899.04	n-C29	4.73 ± 1.80	0.27 ± 0.14
21	2445.26	6-MeC24	-	0.25 ± 0.13	**60**	2930.37	15,13,11,9-MeC29	2.19 ± 0.72	0.93 ± 0.28
22	2458.02	4-MeC24	-	0.21 ± 0.17	**61**	2940.00	7-MeC29	0.25 ± 0.20	-
23	2477.21	x-C25:1	-	1.88 ± 0.77	**62**	2950.00	5-MeC29	0.40 ± 0.22	-
24	2484.73	y-C25:1	-	0.46 ± 0.21	**63**	2962.05	x,y-diMeC29	1.31 ± 0.55	0.13 ± 0.05
25	2486.00	non-HC	0.98 ± 0.67	-	**64**	2973.11	3-MeC29	4.28 ± 1.14	0.19 ± 0.05
26	2492.54	4,14-diMeC24	-	0.21 ± 0.13	**65**	2980.09	5,y-diMeC29	-	0.10 ± 0.03
27	2499.80	n-C25	1.13 ± 0.55	5.36 ± 2.64	**66**	3000.00	n-C30	0.17 ± 0.07	-
28	2534.29	13,11,9-MeC25	0.68 ± 0.88	10.74 ± 1.52	**67**	3030.00	x-MeC30	0.67 ± 0.43	-
29	2541.89	7-MeC25	0.30 ± 0.45	2.01 ± 1.32	**68**	3052.34	non-HC	-	0.14 ± 0.09
30	2550.63	5-MeC25	-	1.04 ± 0.44	**69**	3056.00	non-HC	1.07 ± 0.45	-
31	2573.83	3-MeC25	1.58 ± 0.82	7.58 ± 2.10	**70**	3069.92	C31:1	0.18 ± 0.09	0.31 ± 0.21
32	2582.63	5,y-diMeC25	0.27 ± 0.23	1.56 ± 0.64	**71**	3080.70	C31:1	0.15 ± 0.09	0.23 ± 0.19
33	2599.71	n-C26	0.45 ± 0.25	1.19 ± 0.49	**72**	3100.00	n-C31	0.94 ± 0.77	-
34	2607.99	3,y-diMeC25	0.72 ± 0.46	3.42 ± 1.91	**73**	3129.40	15,13,11,9-MeC31	2.27 ± 1.54	0.62 ± 0.29
35	2633.19	x-MeC26	0.23 ± 0.22	2.64 ± 0.40	**74**	3178.38	non-HC	10.77 ± 5.32	1.94 ± 1.49
36	2644.41	6-MeC26	-	0.25 ± 0.09	**75**	3228.00	non-HC	1.11 ± 0.70	-
37	2657.98	4-MeC26	-	0.44 ± 0.21	**76**	3300.00	n-C33	0.21 ± 0.09	-
38	2662.00	unknown HC	0.64 ± 0.42	-	**77**	3328.47	x-MeC33	1.34 ± 0.81	0.22 ± 0.10
39	2678.57	C27:1	2.14 ± 5.07	16.81 ± 4.49	**78**	3527.26	x-MeC35	1.17 ± 0.44	0.13 ± 0.08

DISCUSSION

In this study, we identified a unique association between two Afrotropical ants. Colonies of the tiny ant *Strumigenys maynei* and the large ant *Platythyrea conradti* lived together in all inspected tree nests in the study area. There was little or no aggression between the two partners, but there were also no signs of intimate heterospecific interactions such as trophallaxis or grooming which are observed between ants and specialized myrmecophiles or social parasites (Hölldobler and

Wilson 1990, Buschinger 2009). Because of the strict association and the lack of aggression between the two partners, this association can be considered as a parabiosis.

Our results confirmed that parabiotic partners might associate with distinct nestmate recognition cues (Orivel and Dejean 1997, Menzel et al. 2008a, 2009, Emery and Tsutsui 2013) (Fig. 8.3, 8.4). Previous studies showed that parabiotic partners hardly shared any cuticular compounds (summarized in Table 1 in Emery and Tsutsui 2013). *Platythyrea conradti* and *S. maynei*, in contrast, had 51 percent of hydrocarbons in common. It is unclear, however, whether the parabiotic lifestyle of the ants of this study exerts selection on the presence and proportional composition of nestmate recognition cues to facilitate the recognition and/or acceptance in the association. The ants clearly perceive conspecific workers with a slightly different chemical profile in both species as they showed strong aggression against conspecific workers. Tolerance of the parabiotic species can be limited to a single heterospecific partner colony. In this case, there is no aggression between parabiotic partners of the same nest, but both species are aggressive towards allocolonial (= from another compound nest) workers of their partner species (Orivel and Dejean 1997, Emery and Tsutsui 2013). It is suggested that the partners learn to recognize the distinct chemical odor of their partner colony (Orivel and Dejean 1997). Other associations are less specific and are characterized by complete or a gradient of tolerance towards allocolonial workers from the partner species (Menzel et al. 2008b). The association between *S. maynei* and *P. conradti* is also not specific, as there is no elevated aggression towards allocolonial workers of the partner. Both species apparently accept all colonies from the partner species. This can be explained by the recognition of species-specific rather than colony-specific chemical cues or the detection of appeasing cues (Menzel et al. 2013). However, the tolerance of the parabiotic partner might also be caused by a merely mechanistic process. Possibly *S. maynei* is too small to be detected efficiently by *P. conradti*. However, it was reported that *P. conradti* detects *S. maynei* when it feeds on its prey and carries them away (Yéo et al. 2006). On the other hand, *P. conradti* workers might be too large to be attacked by *S. maynei* workers. It should be noted here that they successfully attacked *Paltothyreus tarsatus*, an ant which equals the size of *P. conradti* (pers. observations TP).

The ant partners of the compound nests of this study are peculiar because of the extreme size differences. Therefore we hypothesized that these distinct morphs in the compound nest could be an alternative strategy for worker polymorphism in a single colony of an ant species. Worker polymorphism is assumed to benefit colony fitness as some worker castes are more efficient in the performance of certain tasks (Oster and Wilson 1978). Rather than diversifying the morphology of their own worker caste, ants might form a mutualistic association with a morphologically distinct ant species which is more efficient in certain tasks. In particular, we demonstrated that *S. maynei* is much more efficient in nest defending. Typical threats for ant nests are competitor ants, that may rob and destroy the colony and associated myrmecophiles that can prey on the brood and steal prey (Hölldobler and Wilson 1990, Parmentier et al. 2016a). Remarkably, the large *P. conradti* workers ignored all myrmecophiles, except for *N. delamarei*. *Platythyrea conradti* also ignored competitor ants or even tried to escape. Fights were never initiated by this ant and aggressive behavior was only observed after it was attacked. In a previous study, it was described that these ants crouched with their mandibles open and folded their antennae backwards when they were confronted with competitor ants at a feeding site (Dejean 2011). It was hypothesized that *P. conradti* opened its mandibles to release repellent volatiles secreted by the mandibular glands (Dejean 2011). This peculiar crouching behavior was also observed in our behavioral trials, but rarely in combination with mandible opening. In clear contrast, *S. maynei*, displayed overt aggression towards myrmecophiles and towards alien ants. They typically clung to the legs of the enemies and folded their abdomen to sting. It can be expected that the rather passive *P. conradti* colonies highly benefit from the presence of a large legion of very aggressive *S. maynei* workers. This large worker force of tiny ants is particularly efficient to repel small intruders and competitors, which are largely overlooked by the large *P. conradti* workers. By analogy, small workers in polymorphic red wood ants were demonstrated to be supreme defenders against small, intranidal myrmecophilous parasites (Parmentier et al. 2015b). The large *Platythyrea conradti* workers might be more suited to repel large arthropods or vertebrates in parallel with the defense specialization of large workers in polymorphic ant colonies against large enemies (Lamon and Topoff 1981, Hölldobler and Wilson 1990, Batchelor et al. 2012). There is a vast amount of literature that stresses the specialization of morphologically distinct worker castes in nest defense, but here we argue that morphologically distinct ant species, can be analogously specialized in different tasks.

Strumigenys maynei colonies, in their turn, might also benefit from the parabiotic association. *Platythyrea conradti* workers fill the nest entrances with a plug of fine and coarse organic material (Fig. 8.1E) and create as such a microcosm for small arthropods. This can be demonstrated by the enormous abundance of mainly

Collembola (*Cyphoderus subsimilis*) that were regularly detected inside the nests (Suppl. Video 9). The genus *Strumigenys* is a group of small predators that capture living prey with their odd-shaped mandibles (Bolton 1999). In this study and in Yéo et al. (2006) it was demonstrated that *S. maynei* captured intranidal prey. It appears that *S. maynei* indirectly profits of the nest engineering skills of *P. conradti* to feed on prey living inside the compound nest. Previously, it was observed that *P. conradti* hunted actively several arthropods in the tree canopy in the rainy season, whereas *S. maynei* workers never foraged further than 10 cm away from the nest entrances (Yéo et al. 2006). This further suggests that *S. maynei* finds its food inside the nest.

Given the apparent benefits for both partners in this parabiotic association and the absence of potential costs, i.e. no food competition and brood predation, this parabiosis is expected to be mutualistic in nature. This is in line with previous studies on parabioses in the Neotropical and Oriental associations between *Camponotus* and *Crematogaster* species which gave evidence that the association was favorable for both parabiotic partners. *Crematogaster* takes advantage of *Camponotus'* ability to construct ant-garden nests and its supreme nest defending abilities (Davidson 1988, Vantaux et al. 2007, Menzel and Blüthgen 2010). *Camponotus* benefits from *Crematogaster* through following its pheromone trails to food sites (Vantaux et al. 2007, Menzel and Blüthgen 2010). However, parabioses between *Camponotus* and *Crematogaster* can also shift to commensalism and parasitism, when there is aggressive competition, exploitation and no apparent benefits for one partner (Menzel et al. 2014).

The parabiotic system of this study is an excellent model system to test interactions between symbiotic arthropods. Further behavioral, ecological and chemical studies that compare the strategies of *S. maynei* and *P. conradti* living in association compared to free-living colonies of both species could greatly contribute to our knowledge on the factors that promote the association and cooperation of two distinct species.

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SUPPLEMENTARY VIDEOS

The supporting videos may be consulted at the following link:
https://www.youtube.com/channel/UCbdIeMJM-oO6AHqCgnH_bcQ

- Video S1. *P. conradti* vs. *S. maynei*
- Video S2. *P. conradti* vs. *Crematogaster* sp. 1
- Video S3. *P. conradti* vs. *Tetramorium* sp.
- Video S4. *S. maynei* vs. *Crematogaster* sp. 2
- Video S5. *S. maynei* vs. *Monomorium pharaonis*
- Video S6. *S. maynei* vs. *Tetramorium* sp.
- Video S7. *S. maynei* vs. *Neoasterolepisma delamarei*
- Video S8. *S. maynei* and *P. conradti* vs. Pselaphinae sp.
- Video S9. Inside view of the branch nest after removal of organic material. Hundreds of *Cyphoderus subsimilis* and workers of *P. conradti* and *S. maynei* can be observed.

GENERAL DISCUSSION

This thesis explored the functioning of myrmecophile communities and their interaction with the host and environment. In this general discussion, I will first focus on what this thesis adds to the field of symbiont interactions in social insects. Detailed discussion of my findings can be found in the separate chapters. Here, I will highlight four general discoveries and patterns found throughout this thesis. Further, the advantages and limitations of the red wood ant (RWA) model system will be evaluated. Finally, I will look ahead to future research avenues in the field of social insect symbionts.

MAIN FINDINGS

The role of the environment in myrmecophile distribution and diversity

A major part of this thesis deals with biotic interactions occurring in ant microcosms. In **chapter 2**, we also examined whether abiotic factors affect the distribution and diversity of myrmecophiles associated with RWAs. We studied the ecology of social insect associates from a metapopulation/metacommunity perspective (Hanski and Gilpin 1991), where each RWA mound was considered as a distinct patch with a local myrmecophile community. Local abiotic conditions such as pH and moisture did not affect the total number of species in the mound (**chapter 2**) and the presence/absence of a particular myrmecophile species (unpub. results). Abiotic variables, nevertheless, were averages of the complete mound and the presence of species was determined from pooled samples taken from different locations in the mound. This made it impossible to detect within nest niche preferences of myrmecophiles. I expect, however, that micro-climatic conditions do play a role in the distribution of species within the mound. For example the largest number of myrmecophiles inside a mound was typically found near or in the central stem or under bark which provide relative stable localities in terms of moisture and temperature. Chapter 5 showed that also biotic interactions could affect location preference within a mound, with some species avoiding the dense brood chambers and others that were attracted to these chambers. An interplay of biotic and abiotic conditions might jointly shape the spatial preference of myrmecophiles inside the nest. The spatial organization of the mounds in a forest had a strong effect on myrmecophile diversity. Isolated nests supported clearly a less diverse community. This is in line with the results of studies in large forest complexes in Finland (Päivinen et al. 2004, Härkönen and Sorvari 2014). Another interesting result of this study is that a relative diverse myrmecophile community can persist in very small and impoverished forest fragments. Consequently, the protection of small RWA sites, typical for many parts of Flanders (Loones et al. 2008), could be very valuable.

Complex dynamics in a social insect microcosm

A brand-new paper published by Ivens et al. (2016) in Annual Review of Entomology invites researchers to study symbiont assemblies associated with social insects from a community ecology context using network analysis, rather than focusing on one-to-one interactions between a single symbiont and a host. The authors argue that "*this approach will provide new and complementary insights into the evolutionary and ecological dynamics between social insects and their associates, and will facilitate comparisons across different social insect-symbiont assemblages as well as across different types of ecological networks*". I can only agree with the authors based on the findings of the two systems that I investigated. The proposed approach of focusing on a whole community of myrmecophiles was already implemented in many parts of this thesis. As a result of this approach, it became clear that ant nests might be dynamic micro-ecosystems with a multitude of direct and indirect interactions between host-symbiont and symbiont-symbiont. In **chapter 1**, the diversity of RWA myrmecophiles was listed for the first time. This overview gives a very static image of the symbiont community as it merely lists 125 species, of which I found about one third during this thesis. However, it is a worthwhile baseline that reminds us that the interactions tested in this thesis were conducted with only a fraction of the total diversity known to be associated with RWAs. The presented interactions in this thesis are thus only the "top of the iceberg" of a complex interaction web occurring in RWA mounds. Note, however, that most social insect nests support many fewer symbionts.

An essential element to understand ecosystems is the characterization of the trophic relationships between its members. Therefore an extensive food web analysis was performed on the RWA myrmecophile community in **chapter 4**. It became clear in this analysis that many prey-predator interactions occur among the symbionts. In addition, it was demonstrated that most myrmecophiles were both brood parasites and kleptoparasites. In contrast, we showed in **chapter 8** that the myrmecophile community can also be a prey for the ant host. Here, the host *Strumigenys maynei* captures small symbionts that thrive in the ideal conditions of the parabiotic nest. Another important aspect in community dynamics is the characterization of agonistic behaviour between the members. There was no aggression (excluding predation) between myrmecophile species in both model systems (**chapter 5**, **chapter 8**). However, the aggression response of RWA workers towards myrmecophiles was highly variable (**chapter 5**). Some myrmecophile species were completely ignored, others provoked a moderate aggression response, yet others were fiercely attacked and chased. Moreover the symbionts occupied different niches inside the nest, with some penetrating in the central dense brood chambers, and others preferring chambers at the edge of the nests. Next, we analysed whether level of aggression of the RWA host, integration level (brood chamber vs. non-brood chamber) and brood predation tendency were correlated. No association was found between these parameters, which reflects that harmful parasites do not provoke a larger aggression response and can penetrate into the deepest parts of a social insect fortress. Interestingly, the presence of hostile RWA workers, did not affect survival of obligate RWA myrmecophiles, whereas a facultative myrmecophile's survival was decreased (**chapter 7**). In the parabiotic system of **chapter 8**, we found peculiar dynamics between the hosts and symbionts. *Platythyrea conradti* hardly attacked the symbionts, whereas *S. maynei* exhibited strong aggression towards all myrmecophiles.

The role of cuticular hydrocarbon recognition cues in social insect-symbiont interactions

Social insects developed an advanced nestmate recognition system based on a colony-specific composition of non-volatile cuticular compounds (van Zweden and d'Ettorre 2010). It is widely demonstrated that symbionts can break the "chemical code". They either deceive the host by the active production of the host's chemical profile (chemical mimicry sensu strictu), by the passive transfer of the host's chemical profile (chemical camouflage) and/or by carrying very low concentrations of chemical compounds (chemical insignificance) (Nash and Boomsma 2008, van Zweden and d'Ettorre 2010). These strategies were found in almost all tested social insect inquilines (symbionts living in the nest) (see Table A-6.1 in Appendix **chapter 6**). Consequently, it was surprising that the majority of the RWA myrmecophiles studied in this PhD thesis carried completely different chemical profiles (**chapter 6**). Some of them probably rely on chemical insignificance, as they had very low concentrations of cuticular compounds and were mostly ignored. Nevertheless, another group had normal concentrations of cuticular compounds and provoked a moderate to strong aggression response. So it appeared that these species did not invest in chemical deception, but rather rely on efficient escape behaviour and defence mechanisms with volatiles. Similarly, it was recorded that extranidal myrmecophilous beetles did not mimic their host (Stoeffler et al. 2011). Chemical mimicking of the host's chemical profile is likely to be a very specialized strategy. We argue that a historical focus on specialized symbionts, caused a distorted view on the chemical strategies applied by symbionts. Results of the RWA microcosm showed that unspecialized myrmecophiles can infiltrate in social insect nests without chemical deception, but with more primitive defence techniques such as swift movements, defence chemicals, death feigning and a hard, protective exoskeleton. One can argue that the non-mimicking strategy is typical of the RWA community, because of the typical nest structure of RWA mounds. As the thatch of a RWA mound generates an enormous amount of hiding places, the selection pressure on symbionts to mimic the host could be much lower than in nests of other social insect / ant species. However, it should be noted that the survival of three RWA myrmecophiles was not affected by RWA workers in lab nests without thatch (**chapter 7**). Moreover many RWA myrmecophiles can also associate with ants with simple earth nests which have less hiding places (**chapter 1**). More studies are needed to give an accurate view of the distribution of the different chemical integration systems. Based on the prevalence of related, unspecialized social insect symbionts who likely outnumber specialized symbionts (Donisthorpe 1927, Kistner 1982, Hölldolber and Wilson 1990, chapter 1: Parmentier et al. 2014), the absence of mimicry might be very common in social insect symbionts.

The effect of body size in social insect host-symbiont interactions

A recurrent theme in this thesis was the strong aggression response of small ant workers towards myrmecophiles. In **chapter 3**, we described that small RWA workers were more aggressive and more efficient in deterring associated myrmecophiles. In **chapter 7**, we found that the survival of three beetles associated with RWAs was relatively high in nests of species with large ant workers, and decreased when associated with smaller ant species. Survival in nests of the smallest ant species was in general less than a few hours, because they were directly attacked, bitten and/or stung and did not manage to escape. Finally, in **chapter 8**, we demonstrated that the tiny ant *S. maynei* attacked fiercely alien ants and myrmecophiles, whereas the large parabiotic partner *P. conradti* ignored myrmecophiles and alien ants and even avoided some alien ants. Body size is a key trait of organisms which is under strong evolutionary pressure (Blanckenhorn 2000). It is generally believed that selection for a higher fecundity and sexual selection will promote a larger body size in organisms over evolutionary time (Blanckenhorn 2000). However, these selective pressures are counterbalanced by a selection pressure that entails the costs of becoming too large. A major hurdle of becoming too large is a higher probability of detection by enemies and a lower agility and manoeuvrability to escape (Blanckenhorn 2000). This will lead to disproportionate killing or predation of larger individuals (Macchiusi and Baker 1991, Fincke et al. 1997, Blanckenhorn 2000). Similarly, it can be expected that small social insect symbionts benefit from their size to remain undetected or to escape successfully from aggression in the nest (Kistner 1982). Symbionts are in general smaller than their social insect host (Kistner 1979). Symbionts exceeding the size of their host are rare and are restricted to very specialized species, such as the myrmecophile *Paussus* (cf. Introduction Fig. I. 3.B) associated with *Pheidole*, that deceive the host chemically and/or acoustically (Geiselhardt et al. 2007, Barbero et al. 2009b). Non-integrated symbionts might rely on several mechanisms such as defensive glands, armoured protective structures, slow movement or death feigning, but also small size might thus be a major advantage, especially in species lacking the aforementioned defence mechanisms (Donisthorpe 1927, Hölldobler and Wilson 1990). There are some anecdotal indications that the presence of social insects exerts a selection pressure on the size of symbionts. Karl Hölldobler discovered two morphs of the ant cricket *Myrmecophilus acervorum* of different sizes (Hölldobler 1947). The larger "major" morph was found primarily in nests of ant species with larger workers, such as *Formica*, *Camponotus* and *Myrmica*, whereas the smaller "minor" morph was associated with species that have smaller workers, such as *Tetramorium* and *Lasius*. Analogously, we discovered that individuals of the ant isopod *P. hoffmannseggii* were much larger (max. head width female: 5.7 mm) when associated with *F. rufa* than individuals (max. head width female: 4.3 mm) found with the smaller ant *L. flavus* (unpub. results), but differences in abiotic conditions could also affect the observed size differences. There are also symbionts such as the myrmecophilous cockroaches *Attaphila* and crickets *Myrmecophilus* that are far below the average size of relatives which suggests an adaptive role of small size for symbionts in social insect nests (Wheeler 1900). The social insect host, in turn, will benefit from smaller workers (that match the size of the symbionts) to detect and aggress the small symbionts more efficiently. The presence of symbionts that try to stay unnoticed or sneak away could therefore exert selection on ants to become smaller or to develop/maintain small worker castes as was suggested in **chapter 2**. Alternatively, large ants could associate with small ants that are more efficient in deterring small symbionts or intruders as demonstrated in **chapter 8**. Symbionts are also prone to size constraints (Blanckenhorn 2000) which might hamper the evolution of a further decrease in size when trying to associate with small ant species. In **chapter 7** we clearly demonstrated that when the myrmecophilous beetles equalled the size of the workers of the ant species, they were rapidly killed. The symbionts could here not rely on swift escape behaviour effective in larger ant species. It is clear that size asymmetries between social insect hosts and symbionts (but also between social insects and competitors) is an unexplored domain and a meta-analysis on the size differences between host-symbiosis could be fruitful to gain insight in this process.

EVALUATION OF THE RWA MICROCOSM AS A MODEL SYSTEM

At the start of this thesis, my rather vague plan was to study myrmecophiles associated with temperate ants. My fascination for these organisms has been fuelled after reading the influential work “The Ants” of Hölldobler and Wilson during my master thesis. Unfortunately, little was known on the distribution of myrmecophiles in Belgium. Therefore I decided to start preliminary work on the very abundant “pan”myrmecophiles *Cyphoderus albinus* and *Platyarthrus hoffmannseggii* which can be found in every garden. In addition I focused on the conspicuous extranidal myrmecophiles *C. magnifica* and *C. quadripunctata* associated with RWAs, which I had observed during my master thesis on task division in RWAs and of which some populations were known. During one of the first collection trips, I detected a small rove beetle, which later proved to be *Stenus aterrimus*, walking unharmed among hundreds of ants on the surface of a RWA nest. This odd observation captured my attention and made my curious whether I could also find this beetle inside the nest. So I grabbed some nest material by hand and inspected for myrmecophiles. I was aware that some beetles could live in RWA mounds, but was convinced that they would not occur in the impoverished and highly fragmented study sites in Western Flanders. So I was really amazed when I found five different rove beetle species and a spider in that small sample. By inspecting more mounds I found quickly more and more associated species. Interestingly, the same myrmecophiles were also found in other fragmented RWA populations. The relatively large diversity of myrmecophiles found in RWAs provides a unique opportunity to compare different strategies and to test interactions between myrmecophiles living in the same nest. Moreover most species were found in large densities. Occasionally, more than 50 individuals of the same species could be present in a sample of 1 L. Thus, the main advantage of this study system is clearly the relative ease to collect large numbers of individuals of different myrmecophile species, which was an essential prerequisite for most experiments. Mounds are long-living, stable and very resilient to minor disturbances. Our method, where we sampled a minor fraction of the nest and put all the material gently back, did not severely harm the nests. They recovered quickly and therefore myrmecophiles could be “harvested” multiple times in the same nests during this thesis. Another advantage is that the conspicuous nests of RWAs are easy to find in the field in contrast to those of most other ant species. This allowed to map the distribution of all nests in a site, which was essential to conduct the metapopulation study of chapter 2.

However, it turned out that the RWA microcosm could be a challenging model system for several reasons. Firstly, sampling myrmecophiles in RWA mounds can be very painful. Wood ants are extremely aggressive and readily bite and spray formic acid when disturbed. Not only the mound surface, but also its direct perimeter, is crowded with thousands of ants leaving or returning to the nest. This makes it impossible to approach the nest without having aggressive ants crawling on and in your shoes and clothes. Additionally, the most efficient way to collect the largest number of myrmecophiles was simply grabbing nest material with bare hands. Other methods that I tried such as sampling with a shovel, with gloves and with pitfalls placed inside the nest were far less successful. With bare hands, it was much easier to reach the nest material near and in the cracks of the tree stump or a fallen branch around most mounds were constructed. Here the largest number of myrmecophiles could be found. So this thesis involved thousands of bites and regularly swollen arms, but after several years, however, you get used to this annoying part of sampling RWA mounds. A more essential limitation of the RWA microcosm system is the fact that red woods ants are very hard to keep in the lab for a long period of time. The life span of workers is strongly shortened, brood is poorly raised and queens lay few eggs in lab settings. This impairs the study of long term fitness costs or benefits of myrmecophiles on RWAs. The colony fragments (1000-2000 workers) that I collected also do not show typical RWA behaviour in the lab, such as nest construction or the formation of foraging trails. An initial idea to compare the capability of the myrmecophile community to follow trails of their RWA host could therefore not be tested. More natural behaviour could be mimicked by collecting large nest fragments or even whole colonies (cf (Gösswald 1989b). However, this strategy conflicts with a more ethical constraint of RWA microcosms. Because of factors such as habitat fragmentation, shading and closure of the tree canopy and agriculture, there has been a dramatic decline of RWAs all over Europe and consequently they gain legal protection in many countries (Gösswald 1989b, Dekoninck et al. 2010). A thoughtful and non-destructive sampling of RWAs should therefore be preferred. An ideal model system should support symbionts with different degrees of specialization. However, only two main categories could be distinguished in the species we found in the RWA microcosm: (1) facultative myrmecophiles and (2) obligate myrmecophiles that are all relatively unspecialized as they do not (except for *D. maerkelii* that engages in trophallaxis) integrate in colony life. Only *Lomechusa* and *Lomechusoides* beetles and to a lesser degree *Hetaerius ferrugineus* are specialized (“symphile”) species that can be found with RWAs (chapter 1: Parmentier et al. 2014). It would be ideal to compare the strategies (behaviour, chemical ecology, distribution, host specificity …) of these species with the group of less specialized myrmecophiles that were explored in this thesis. However, In spite of numerous samplings at different locations, these species were never found with RWAs (*Lomechusa emarginata* was found with *Formica fusca*). Probably they are very rare or have got a rather localized distribution. A last limitation of this study system is the difficulty to let the myrmecophiles reproduce in lab conditions. In contrast with ants, most myrmecophiles could be kept alive for months when placing them on moist plaster and providing dead maggots or springtails. However, few species produced a limited amount of larvae, of which only a handful reached the adult stage because of low fecundity in the lab and/or high cannibalism. The initial plan to compare myrmecophile fitness (measured by the number of offspring) in absence and presence of host ants was therefore not feasible. Consequently, **chapter 7** which evaluates the effect of an aggressive host on myrmecophiles was based on the survival of myrmecophiles rather than on myrmecophile fitness.

FUTURE PERSPECTIVES

During the course of my PhD thesis, many questions arose. Only a part could be addressed, because of the limitations of the model systems or time constraints. Here are some aspects on the topic of ant-symbiont interactions that I think are valuable to examine in the future:

Exploring in depth the role of myrmecophiles

The different types of symbiosis with ants were explained in the introduction and corresponding Fig. I.1. There it was stressed that mutualisms, commensalisms and parasitisms should be viewed as extremes of a spectra of possible interactions between symbionts and ants. Therefore, it is necessary to study all possible roles of myrmecophiles to have an accurate view of their impact on their host and to position them correctly along the gradient of mutualism to commensalism and to parasitism. We demonstrated that most species of the tested RWA community were brood predators or cleptoparasites. Some species, such as the springtail *C. albinus* appeared to have no or only a limited effect on their host. No direct positive effects of the myrmecophiles could be unravelled. Hence, the species of RWA myrmecophile community can be situated along the commensalism-parasitism gradient (cf Fig. I.1), but the exact position ("role") along this continuum is elusive. We tried to rank the severity of the parasites by comparing the proportion of specimens that prey on brood.

But it is clear that the severity of parasitism is expected to be much more complex and depends on factors such as the presence of other food sources, the efficiency of the RWA worker to deter the parasites, intra- and interspecific competition, temporal dynamics … The exact costs of the different myrmecophiles on their host and their relative ranking along the parasitism-commensalism gradient, can only be addressed by comparing the fitness costs of controlled RWA nests with and without myrmecophiles. However, as noted above, long-term (e.g. 6-12 months) monitoring of infected and non-infected RWA colonies or colony parts is not possible because of high mortality, limited fecundity and poor food acceptance of RWAs in the lab. This type of long-term fitness experiments could be conducted with less challenging ants such as *Lasius* ants, *Serviformica* ants or *F. sanguinea*. Unfortunately, these ants harbour a poorer diversity of myrmecophiles and it is harder to get a sufficient number of associated myrmecophile individuals. Long-term fitness studies can also highlight unexpected long-term effects of parasitism. Hovestadt et al. (2012) predicted by modelling that the presence of the myrmecophilous brood parasite *Microdon* in colonies of *Formica lemani* could have an unexpected benefit for the host as it would promote the production of gynes. The developmental switch of a larva to a worker or gyne is largely affected by the amount of received food. As in parasitized colonies the small number of remaining ant larvae can get access to a larger amount of food, a larger number of larvae develop to gynes. Modelling and long-term fitness experiments could reciprocally inform and constrain one another.

This thesis almost entirely focused on conflicts between ants and symbionts, and positive effects of a symbiosis were only reported in the last chapter on parabiotic defence specialization. The role of an ant associate in a mutualistic association with ants is typically the offering of food, such as honeydew in aphids and some lycaenid caterpillars, gongylidia or hyphal swellings of myrmecophilous fungi or food bodies and nectar secretions in myrmecophytes. In return the symbiont is protected against enemies (Hölldobler and Wilson 1990, Fig. I.1 Introduction). In the RWA microcosm system, no apparent positive effects are present as the myrmecophiles do not offer food rewards to the host. However, we showed one indirect positive effect in **chapter 4**: some brood parasitic parasites could help the host by preying on other brood parasites. Another type of positive effect of ant symbionts is the provision of hygienic cleaning services which was shown in mites associated with bees (Biani et al. 2009). Similarly, the mite *Hypoaspis oophila* which lives on the eggs of RWAs, can provide cleaning services. It is reported that this mite does not puncture the eggs but merely feeds on the secretions of the eggs. This was confirmed by detailed observation with the binocular. So, at first sight, this species appears to be a commensal. But the following observation suggests that this species could also have mutualistic characteristics: I placed some brood, ants and nest material in a box at room temperature. After two weeks I opened the box and all ants were dead ant the nest material was completely overgrown with fungi, except for an egg pile with some little mites running on it. This strongly suggests that these mites help to clean the eggs and keep fungi away. But also other RWA myrmecophiles could assist in cleaning the nest. The best candidates for this role are the springtail *C. albinus* and the isopod *P. hoffmannseggii*, which both can be very abundant in RWA mounds and other ant nests (cf. Collembola in the parabiotic system: suppl. video I) and whose relatives feed on fungi (Hanlon and Anderson 1979, Berg and Wijnhoven 1997). We hypothesized that these myrmecophiles help to maintain fungus infestation under control. The study of this putative positive side-effect was launched by a thesis student, but unfortunately not finished. A detailed study of these potential cleaning interactions could give us a more accurate view of the (variable) role of myrmecophiles along the parasitism-mutualism continuum.

Gradient of specialization

One of the most pertinent topics in evolutionary biology is the inference of the evolutionary trajectory of general traits to specialized traits (Futuyma and Moreno 1988). In the case of social insect symbionts, little is known how specialized myrmecophily could have arisen from free-living arthropods. The focus of most studies is on specialized symbionts and unspecialized symbionts were hitherto surprisingly neglected in behavioural and chemo-ecological studies. I hope that this work can contribute to our knowledge of less specialized myrmecophiles. As indicated above, myrmecophiles in RWA microcosms span only a limited degree of specialization. Therefore, other, but related myrmecophiles (for example in the group of Aleocharinae rove beetles) associated with different ants and showing different degrees of specialization should be studied to fully cover the characteristics of the evolutionary trajectory of myrmecophily of a myrmecophilous group found in RWAs. One ant-symbiont system where all gradients of specialization are present should be much more practical. Interestingly, the root aphid fauna associated with the yellow meadow ant *Lasius flavus* is such a system. These ants typically nest in open meadows and lawns (Seifert 2007). In special constructed aphid chambers, they provide shelter and protection to root aphids that feed on the grass roots. The ants do not forage above ground, but are completely dependent on the underground root aphids (Seifert 2007).

Some root aphid species are not associated with the ant, others only facultatively and some cannot survive without the ant. Within the group of specialized root aphids, some have lost the winged stage and/or get a privileged treatment such as transportation to feeding sites or nursing of their eggs (pers. observations TP, Pontin 1960). A detailed study of such a system where behavioural, morphological, chemical and distribution (e.g. generalist or host specific) data are combined could give us an unprecedented view on the evolutionary steps in myrmecophily.

How can the presence of a parasitic myrmecophile be evolutionary stable?

During this thesis I often wondered how these RWA mounds could persist with the presence of thousands of associated brood parasites and cleptoparasites. Nonetheless, we found two mechanisms that might lower parasite pressure. First, we demonstrated the existence of intraspecific (among size cohorts and tasks in RWAs) or interspecific (in the parabiotic system) specialization in defence against brood parasitic myrmecophiles. This implies that a particular group of workers will deter parasites more efficiently than an average worker. Probably a more efficient mechanism is the intra-guild predation of brood predators as demonstrated in **chapter 4**. Ants could also reduce parasite pressure by moving regularly to new locations as suggested by McGlynn (2012). Depending on the trail following capabilities of the myrmecophiles, it can be expected that relocated nests will contain significantly less parasites. Interestingly, RWAs often move to new locations or new mounds bud from the central mound (Gösswald 1989a, Ellis and Robinson 2014). Preliminary tests showed that the RWA myrmecophiles were unable to follow RWAs that were forced to move to a new nest in the lab. Additionally, it could be interesting to test whether parasite pressure is a driver for nest movement. In that case ants actively avoid the parasites by nest movement. This active avoidance mechanism is in contrast with parasite loss as a side-effect of nest moving initiated by other processes (e.g. worsening abiotic conditions, natural colony multiplication). However, the most effective way to get rid of parasites is independent colony founding by a dispersing RWA queen.

It is clear that this is a very challenging topic, as the costs of myrmecophiles on their host are mediated by many parameters. Ideally this topic should be tackled with a combination of experimental data and theoretical modelling.

The nature and specificity of chemical defence mechanisms

The secretion of volatile defensive chemicals is a widely applied strategy of social insect symbionts (especially in rove beetles), but this group of chemicals is poorly known. The focus of research was hitherto mainly on non-volatile cuticular hydrocarbons. A study on the volatiles of three myrmecophilous rove beetles by Stoeffler et al. (2011) showed that the composition of tergal gland secretion of the beetles appeared to be highly adaptive. One beetle mimicked the panic pheromone of its host, another beetle replaced an aggressive inducing component and the third beetle secreted an appeasement pheromone. Again, a study that compared the composition of volatile chemicals of myrmecophiles (along a gradient of myrmecophily) and this of related free living species could be very informative. Moreover, it should be tested whether the gland secretions of the myrmecophile cause an effect in all ants or only in the preferred host(s). In that case, myrmecophiles with a relatively unspecialized morphology could still employ a specialized defence system specifically targeted to their preferred host(s).

Spatial dynamics of myrmecophiles

During this thesis, I never observed RWA myrmecophiles running from one mound to another or leaving the nest by flying (except the extranidal beetle *C. quadripunctata*). Interestingly, most winged species did not fly when captured. In addition, RWA myrmecophiles are seldomly captured with traps (pers. communication T. Struyve). These observations suggest that dispersal is relatively limited. A detailed experimental approach with techniques such as mark-recapture, pitfalls around the nest and flight traps should give a first idea of the dispersal capabilities and frequencies of these myrmecophiles. Next, a population-genetic approach can give us insight in gene flow between different fragmented forest sites. Population genetic studies on *Phengaris* (= *Maculinea*) butterflies showed that they are rather good dispersers and can maintain fully functional metapopulations when the patches are no further apart than 10 km (Ugelvig et al. 2012). It is unclear whether there is gene flow between populations of RWA myrmecophiles that live in different forest fragments. The role of the nests of other, less preferred, ant species as potential stepping stones to different RWA mounds/sites might be vital for a large number of species and should be integrated in studies on (RWA) myrmecophile spatial dynamics.

CONCLUSION

In this study, we demonstrated that social insects, and especially ants, can support a rich diversity of associated symbionts. Their nests might form complex and dynamic micro-ecosystems inhabited by multiple symbionts characterized by different strategies. These microcosms are driven by abiotic interactions and biotic interactions between host-symbiont and between symbiont-symbiont. We argue that these microcosms are ideal model systems to test evolutionary and ecological hypotheses on symbiosis in all its facets. I hope that this thesis encourages further research on the underexplored, but rewarding topic of social insect symbiont ecosystems.

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CV AND BIBLIOGRAPHY

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Personalia

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DATE OF BIRTH: 18/06/1985
MARITAL STATUS: married, two daughters
NATIONALITY: Belgian
TEL: 0474/76.62.71
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Academic career

Oct 2011 - present	PhD in Biology *Conflict and cooperation between ants and ant-associated arthropods* Kuleuven, Lab of Socioecology and Socioevolution Supervisor: Prof. Tom Wenseleers, Co-supervisor: Dr. Wouter Dekoninck (RBINS) FWO aspirant (grant no.11D6414N) the original 4-year project was extended with one year till 30/09/2016 due to long-term illness between Oct 2011 - Feb 2011
Oct 2010 - Sept 2011	Research project *The role of social sanctions in the mutualism between ants and aphids* Kuleuven, Lab of Socioecology and Socioevolution Supervisor: Prof. Tom Wenseleers
2009 - 2010	Master of Science in Biology with the greatest distinction University of Ghent Thesis: Taakverdeling en voedselecologie bij de werksters van *Formica rufa* en *Formica polyctena* Supervisor: Prof. Frederik Hendrickx
2004 - 2008	Bachelor of Science in Biology with the greatest distinction University of Ghent

Supervision and teaching experience

Supervision of

MSc students

- 2011-2012: Joachim van Lommel
- 2014-2015: Thomas Vanmechelen

BSc students

- 2011-2012: Ellen De Sutter
- 2012-2013: Stef Rutten

High school students, geïntegreerd project

- 2013-2014: Gust Denteneer
- 2015-2016: Pjotr Rombout

Assisting general zoology practicals

Teaching and supervising Msc students at Ant and Bee Course, Lamto Ivory Coast: Jan 2016

Advising nature conservationists to optimally protect RWAs, advising municipal officials to eradicate invasive ants.

International talks

Parmentier, T. (2016). Interactions entre les fourmis de bois (*Formica rufa* group) et les myrmécophiles associés. Ant and Bee Course. Lamto, Ivory Coast, 21-30 January 2016.

Parmentier, T., Bouillon, S., Dekoninck, W., Wenseleers, T. (2015). Trophic interactions in the myrmecophile community associated with RWAs. Central European Meeting of IUSSI. Lichtenfels, Germany, 25-28 March 2015.

Research stays abroad

Lamto, Ivory Coast. Co-organiser of a GTI (Global Taxonomy Initiative) course on bees and ants for African Master students 21-30 January 2016.

Ant Course 2010, August 16-26, Danum Valley Field Centre, Sabah Borneo: international field course on the ecology, evolution and taxonomy of ants.

Awards

Prijs Francine Ronsse for an excellent Master thesis.

Skills and qualifications

- Statistical packages: R, S, Statistica

  Profound knowledge of multivariate and univariate statistics

- Techniques: microsatellite genotyping, HPLC, GCMS, stable isotopes and food web analysis, spatial ecology, behavioural and ecological biodiversity studies

- Expertise in rearing and culturing a wide variety of arthropods.

- Expertise in conducting experiments with different groups of arthropods: Collembola, Isopoda, Hymenoptera, Coleoptera, Araneae, Acari, Thysanura …

- Demonstrations for children and the public of social insects.

- Languages:
  - Dutch: mother tongue
  - English: full working proficiency
  - French: good
  - German: basic competence

HOBBIES

Basketball, bee keeping, gardening

BIBLIOGRAPHY

A1 Publications: included in ISI Web of Science

- **Parmentier Thomas**, Dekoninck Wouter, Wenseleers Tom (2016). Survival of persecuted myrmecophiles in laboratory nests of different ant species can explain patterns of host use in the field. *Myrmecological News* (IF: **2.9**). (status: accepted)

- **Parmentier Thomas**, Dekoninck Wouter, Wenseleers Tom (2016). Do well-integrated species of an inquiline community have a lower brood predation tendency? A test using RWA myrmecophiles. *BMC Evolutionary Biology*, art.nr. 8853245011917493 (IF: **3.37**).

- **Parmentier Thomas**, Bouillon Steven, Dekoninck Wouter, Wenseleers Tom (2015). Trophic interactions in an ant nest microcosm: a combined experimental and stable isotope (δ13C/δ15N) approach. *Oikos*, art.nr. 10.1111/oik.02991 (citations: 1) (IF: **3.44**). (status: in press)

- Vantaux Amelie, Schillewaert Sharon, **Parmentier Thomas**, Van den Ende Wim, Billen Johan, Wenseleers Tom (2015). The cost of ant-attendance and melezitose secretion in the black bean aphid *Aphis fabae*. *Ecological Entomology, 40*, 511-517 (citations: 1) (IF: **1.7**).

- **Parmentier Thomas**, Dekoninck Wouter, Wenseleers Tom (2015). Metapopulation processes affecting diversity and distribution of myrmecophiles associated with RWAs. *Basic and Applied Ecology, 16*, 553-562 (citations: 4) (IF: **1.94**).

- **Parmentier Thomas**, Dekoninck Wouter, Wenseleers Tom (2015). Context-dependent specialisation in colony defence in the RWA *Formica rufa*. *Animal Behaviour, 103*, 161-167 (citations: 3) (IF: **3.14**).

- **Parmentier Thomas**, Dekoninck Wouter, Wenseleers Tom (2014). A highly diverse microcosm in a hostile world: a review on the associates of red wood ants (*Formica rufa* group). *Insectes Sociaux, 61*, 229-237(citations: 12) (IF: **1.02**).

- **Parmentier Thomas**, Dekoninck W, Hendrickx F (2012). Site fidelity of *Formica rufa*: micro-scaled and persistent despite disturbance (Hymenoptera: Formicidae). *Sociobiology, 59*, 1413-1425 (citations: 1) (IF: **0.58**).

- Vantaux Amélie, **Parmentier Thomas**, Billen Johan, Wenseleers Tom (2012). Do *Lasius niger* ants punish low quality black bean aphid mutualists? *Animal Behaviour, 83* (1), 257-262 (citations: 9) (IF: **3.07**)

A3 publications: published in national peer-reviewed journals

- Dekoninck W., **Parmentier T**., Casteels J., Wegnez P., Vankerkhoven F. (2015). Recente waarnemingen van de glanzende gastmier *Formicoxenus nitidulus* (Nylander, 1846) in Vlaanderen (Hymenoptera: Formicidae).*Bulletin de la Société royale belge d'Entomologie/Bulletin van de Koninklijke Belgische Vereniging voor Entomologie, 151*, 15 17.

- Dekoninck W., **Parmentier T**., Seifert B. (2015). First records of a supercolonial species of the *Tapinoma nigerrimum* complex in Belgium (Hymenoptera: Formicidae). *Bulletin de la Société royale belge d'Entomologie/Bulletin van de Koninklijke Belgische Vereniging voor Entomologie, 151*, 206-209.

- **Parmentier T**., Van Kerckvoorde M., Dekoninck W. (2013). First record of the myrmecophilous silverfish *Atelura formicaria* Heyden, 1855 in Belgium (Zygentoma: Nicoletiidae). *Bulletin de la Société royale belge d'Entomologie/Bulletin van de Koninklijke Belgische Vereniging voor Entomologie, 149*, 127-128.

A4 publications: science popularization

- **Parmentier Thomas**, Dekoninck, W., Wenseleers, T. (2014). Verstekelingen met een geurtje in de bosmierennesten van De Haan. *Bosreservatennieuws, INBO nieuwsbrief, 13*, 19-21.

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