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Biodiversité, biochimie et pharmacologie des peptides de venins de fourmis

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Sommaire

Avant-propos

Les venins sont des armes chimiques très sophistiquées, composées de plusieurs centaines de molécules bioactives qui ont été présélectionnées et pré-optimisées par la nature. Parmi ces composés chimiques, les peptides sont les toxines majoritaires qui possèdent une formidable diversité structurale et pharmacologique. De plus ces toxines peptidiques sont souvent structurées par des ponts disulfure leur conférant une remarquable stabilité chimique ainsi qu'une grande résistance aux dégradations enzymatiques. Ces peptides bioactifs sont promis à un grand avenir et pourraient trouver de nombreuses applications dans le domaine des biotechnologies et notamment pour l'industrie pharmaceutique. En effet les peptides présentent plusieurs avantages par rapport aux petites molécules qui constituent les médicaments traditionnels. Le principal atout des peptides est qu'ils offrent souvent une plus grande efficacité et surtout une plus haute sélectivité que les petites molécules. De surcroit, leur nature protéique leur apporte un temps de demi-vie très court bien que cela puisse également être une contrainte. De plus les produits de dégradation des peptides sont peu toxiques car ce sont des acides aminés. Tout cela offre l'espoir d'en dériver des agents thérapeutiques efficaces possédant des activités très ciblées et avec moins d'effets secondaires [King, 2011; Lewis and Garcia, 2003; Uhlig et al., 2014]. D'ailleurs plusieurs peptides issus de venins ont déjà permis le développement de médicaments et de nombreux autres sont actuellement en essais cliniques et précliniques. Le Prialt®, dérivé d'une conotoxine et le Captopril® issu d'un venin de serpent sont deux exemples remarquables de médicaments provenant de venin [Smith and Vane, 2003; Staats et al., 2004].

La composition biochimique des venins est strictement spécifique et donc l'ensemble des venins représentent une source presque inépuisable de peptides bioactifs. La grande biodiversité des organismes venimeux va de pair avec une grande diversité de peptides aux pharmacologies variées. L'ensemble de ces venins constitue une importante librairie de peptides bioactifs exploitable pour la recherche de molécules d'intérêt. Ainsi l'exploration de l'ensemble de la diversité taxonomique des organismes venimeux est la clé pour la découverte de nouvelles molécules. Dans cette optique, l'étude des venins de certains groupes d'animaux inexploités à ce jour, pourrait permettre la découverte de nombreuses molécules originales aux propriétés pharmacologiques uniques. Parmi les venins d'animaux, les venins de fourmis restent parmi les moins étudiés en raison de plusieurs désavantages. Le problème majeur dans l'étude des venins de fourmis est la petite taille des animaux, posant un défi majeur pour l'obtention d'une quantité de venin exploitable. Pour la reproductibilité des études biochimiques et pharmacologiques, il est primordial de pouvoir identifier de façon indiscutable les espèces étudiées mais l'identification des espèces de fourmis reste l'apanage de quelques rares spécialistes. Enfin, au contraire des venins d'araignées ou de cônes, l'absence d'une base de données spécifique aux toxines peptidiques provenant des venins de fourmis est aussi un frein à l'exploration des ces peptides. Malgré cela, les fourmis (Formicidae) constituent probablement la famille la plus facilement exploitable parmi les hyménoptères car elles offrent plusieurs avantages. Ce sont des insectes sociaux qui vivent en colonie allant de quelques individus à plusieurs millions. En plus de cela, la taxonomie des fourmis est probablement l'une des mieux connues de tous les hyménoptères et plusieurs bases de données taxonomiques existent [AntWeb, 2014b]. L'immense biodiversité des fourmis laisse envisager une grande diversité biochimique des toxines. En parallèle les avancées technologiques en termes de biochimie analytique et de

tests d'activité pharmacologique offrent désormais la perspective de travailler avec des quantités de venin infimes. Pour toutes ces raisons il devient pertinent de considérer que les venins de fourmis constituent un nouveau champ d'exploration des toxines peptidiques, ce qui est l'objet de ce travail.

Introduction générale

1. Sources de produits naturels pour la recherche pharmaceutique

La nature est un réservoir inépuisable de molécules et d'inspiration pour les chimistes, les biochimistes et l'industrie pharmaceutique. Les molécules naturelles de faible poids moléculaire (non-peptidiques), au même titre que les produits de synthèse, sont une source importante de molécules thérapeutiques en santé humaine. On estime à 60% la part de médicaments issus directement ou indirectement d'un produit naturel [Cragg and Newman, 2013; Newman and Cragg, 2012]. De nombreux squelettes naturels composent une grande partie des petites molécules de la pharmacopée mondiale et leur structure chimique originale n'aurait pas pu être imaginée par l'homme. Malgré ces considérations, la recherche en chimie des produits naturels ne rencontre plus aussi souvent le succès et le nombre de nouvelles molécules thérapeutiques mises sur le marché issu d'un produit naturel diminue chaque année. Ceci s'explique par le coût élevé et la difficulté de caractériser un nouveau produit naturel biologiquement actif dans de courts délais comparativement au criblage à haut-débit des chimiothèque composés de milliers de molécules. De nouvelles technologies sont en cours de développement pour améliorer l'échantillonnage d'organismes d'intérêts. D'autre part on constate que les principales sources naturelles des produits thérapeutiques sont les plantes et les microorganismes [Rates, 2001; Zhu et al., 2011]. Les plantes sont en effet à la base de la pharmacopée mondiale depuis l'Antiquité car elles présentent l'avantage d'être une ressource renouvelable, accessible et exploitable. De plus, les avancées scientifiques réalisées en microbiologie depuis le début du XXème siècle ont permis le développement de milieux adaptés à la culture des microorganismes producteurs de

molécules d'intérêt à l'échelle industrielle. Paradoxalement les insectes sont les organismes les plus diversifiés sur Terre (900.000 espèces), représentant environ 60 % de la biodiversité mondiale, néanmoins ils demeurent l'une des sources de produits naturels les moins exploitée [Dossey, 2010]. Peu d'études chimiques à visée thérapeutique s'intéressent aux métabolites des insectes et un seul médicament mis sur le marché a été identifié à partir d'un insecte. Il s'agit d'un peptide, l'alloféron®, isolé à partir de l'hémolymphe d'une mouche [Sidorova et al., 2005]. La principale difficulté pour isoler les substances naturelles provenant d'insectes est la récolte en grande quantité de ces organismes. Leur petite taille implique une faible quantité de métabolites, de plus, l'abondance des insectes varie considérablement dans l'espace et dans le temps. En général il est difficile de récolter rapidement un grand nombre d'individu d'une espèce spécifique hors élevage. Enfin, la taxonomie des insectes demeure complexe et l'identification des espèces est souvent délicate [Lupoli, 2010]

D'autre part il est surprenant de constater le nombre limité de peptides disponible pour un usage thérapeutique et ils représentent seulement 5% des médicaments mis sur le marché [Bartholow, 2012]. Or l'importance du rôle des peptides dans les mécanismes biochimiques constitutifs des organismes vivants est largement étudiée dans d'autres disciplines. Par exemple en physiologie végétale de nombreux peptides aux diverses fonctions biologiques ont été caractérisés [Fukuda and Higashiyama, 2011]. Le criblage des peptides naturels dans l'évaluation de leurs activités biologiques valorisable en santé humaine est par conséquent pertinent. Néanmoins la recherche en chimie thérapeutique continue de cribler majoritairement les petites molécules naturelles sur un grand nombre de cibles thérapeutiques alors que le nombre de cibles testées dans le criblage peptidiques est moins important. Les perspectives d'applications pharmaceutiques des peptides sont

pourtant nombreuses et les résultats concernant leurs activités analgésiques sont parmi les plus prometteurs [Craik et al., 2013; Diochot et al., 2012; Yang et al., 2013]. L'objectif est de développer des thérapeutiques de substitution aux alcaloïdes couramment utilisés actuellement et dont l'usage est associé à une accoutumance du patient au traitement médicamenteux. Le peptide ziconotide (Prialt®), actuellement utilisé pour sa remarquable activité analgésique ne semble pas créer d'accoutumance chez le patient mais malheureusement le mode d'administration par injection dans la moelle épinière limite son utilisation. La vectorisation est l'un des freins majeur aux développements des peptides à usage médical. En effet leur stabilité est faible dans les milieux biologiques complexes et la lyse provoquée par les enzymes, comme par exemple les enzymes digestives présentes dans l'estomac, contraint fortement le mode d'administration par ingestion. Pour palier à ces limitations, des stratégies innovantes s'intéressent à la vectorisation peptidique à l'aide par exemple, de leur l'encapsulation dans des nano-objets capables d'une part de les transporter vers leur cible thérapeutique et d'autre part d'éviter leurs dégradations [Gupta et al., 2013; Pinto Reis et al.]. Les résultats prometteurs dans ce domaine de recherche en pleine expansion justifient pleinement l'intérêt de rechercher des peptides bioactifs d'origine naturelle.

Afin d'augmenter les chances de découvrir un candidat médicament d'origine naturelle il est nécessaire de diversifier les approches méthodologiques, de développer des outils analytiques mais également d'élargir le spectre des sources naturelles et des structures chimiques recherchées.

Parmi les substances naturelles d'origine animale, les venins représentent un réservoir très prometteur pour la découverte de molécules aux propriétés biologiques intéressantes.

Ils constituent d'ailleurs l'une des sources principales de peptides thérapeutiques. Ces cocktails complexes de centaines voir de milliers de molécules ont été perfectionnées par la nature durant des millions d'années d'évolution. Les toxines des venins intéressent de façon croissante l'industrie pharmaceutique mais également l'industrie agronomique. En effet les toxines peptidiques sont d'excellents candidats bioinsecticides peu rémanents pouvant cibler spécifiquement certaines espèces d'insectes ravageurs et ne présentant pas de toxicité pour les vertébrés. Les venins (*e.g.* serpents, scolopendres, araignées, scorpions, cônes) ont d'ores et déjà montré leur potentiel pour la découverte de molécules d'intérêts avec de nombreuses applications dans les domaines pharmaceutique et agronomique. Les venins possèdent des toxines aux diverses activités biologiques :

- Antibiotiques [Kuhn-Nentwig, 2003; Moreau, 2012]
- Antifongiques [Gomes et al., 2005; Moerman et al., 2002]
- Anticoagulantes [Kini, 2006]
- Antidouleurs [Diochot et al., 2012; Staats et al., 2004; Yang et al., 2013]
- Anticancers [DeBin et al., 1993]
- Antipaludiques [Conde et al., 2000]
- Bioinsecticides [Herzig and Hodgson, 2008; Windley et al., 2012]
- Outils pharmacologiques [Dutertre and Lewis, 2010]

Les venins sont utilisés par de nombreux organismes mais les insectes et en particulier l'ordre des hyménoptères, constituent en nombre d'espèces la majorité de la biodiversité venimeuse. Une biodiversité qui demeure cependant largement inexploitée.

2. Les animaux venimeux

Au cours de l'évolution, certains organismes appartenant à des groupes taxonomiques très diversifiés ont élaboré tout un arsenal de toxines dans le but de se défendre ou de capturer des proies [Casewell et al., 2013]. Ces substances toxiques produites par des glandes spécialisées dans le but de perturber les systèmes physiologiques des proies et des adversaires sont appelés « venins ». Durant les dernières décennies, les venins de certains organismes ont été très étudiés, typiquement en raison de leur importance médicale. C'est le cas des venins de serpents, de scorpions, d'araignées ou encore des cônes. Si ces animaux ont la part belle dans les études toxicologiques, c'est surtout parce que ce sont les animaux venimeux qui représentent le plus grand danger pour l'homme et provoquent la majorité des décès par envenimation.

Les serpents sont évidement les organismes venimeux les plus dangereux et ceux qui occasionnent le plus d'accidents mortels. On estime à environ 125.000 le nombre de décès chaque année dans le monde ayant pour origine une morsure de serpent [Kasturiratne et al., 2008]. On dénombre quelques 3.500 espèces d'ophidiens dont l'appareil venimeux est composé d'une glande salivaire reliée généralement à un crochet. Quatre groupes de serpents peuvent être distingués selon l'anatomie de leurs crochets. Les aglyphes ne possèdent pas de crochets. Bien que ces serpents soient incapables d'inoculer leur venin, de récentes études ont tout de même démontré la présence de toxines bioactives dans leur salive, notamment chez les Colubridae [Fry et al., 2003]. Les opisthoglyphes possèdent des crochets sillonnés situés en arrière de la mâchoire, ce qui rend l'envenimation chez l'homme difficile. Les ophidiens protéroglyphes ont des crochets canaliculés qui sont fixes alors que les crochets des solénoglyphes sont mobiles. Ces deux derniers groupes de serpents sont par

conséquent les plus dangereux pour l'homme [Goyffon and Chippaux, 1990]. Les venins de serpents sont essentiellement composés d'enzymes, de protéines de haut poids moléculaire et de peptides. Parmi ces peptides, les toxines à « trois-doigts » constituent une famille de polypeptides caractéristiques des venins de serpents. Cette plateformes moléculaires porte des activités pharmacologiques très variées puisque les toxines peuvent être neurotoxiques, cytotoxiques ou encore anticoagulantes [Kini and Doley, 2010].

Les scorpions sont parmi les animaux terrestres les plus anciens; ils sont dotés d'un aiguillon venimeux au niveau du telson qui leur permet d'injecter des cocktails de peptides hautement neurotoxiques. Il existe environ 1.500 espèces de scorpions dans le monde réparties dans 13 familles et toutes les espèces sont venimeuses [Hallan, 2005]. Cependant toutes les espèces dangereuses pour l'homme se concentrent dans la famille des Buthidae [Goyffon and Chippaux, 1990].

Dans la classe des arachnides, le groupe des araignées est l'un des groupes d'organismes venimeux majeurs en termes d'abondance et de diversité. Il existe plus de 45.000 espèces d'araignées actuellement décrites réparties dans 110 familles et presque toutes sont venimeuses à l'exception des espèces appartenant à la famille des Uloboridae [Platnick, 2013]. L'appareil venimeux des araignées est constitué d'une paire de glandes à venin débouchant dans les chélicères. En dépit du grand nombre d'araignées, seul un petit nombre d'espèces possède un venin réellement dangereux. Les venins d'araignées contiennent un grand nombre de peptides neurotoxiques de motif ICK « Inhibitor Cystine Knot » dont les modes d'actions sur les canaux ioniques sont variables [Escoubas, 2006].

Les mollusques gastéropodes du genre *Conus* sont de redoutables prédateurs marins. Ils sont pourvus d'un proboscis transformé en appareil venimeux utilisant des dents

radulaires modifiées en forme de harpons, pour injecter un cocktail de peptides neurotoxiques capables d'immobiliser presque instantanément de petits poissons, des mollusques ou encore des vers marins. Les venins de cônes sont très étudiés pour leurs toxines que l'on appelle les conotoxines. Ces conotoxines sont de petits peptides réticulés par plusieurs ponts disulfure, qui possèdent une grande diversité de mécanismes d'action, notamment sur les canaux ioniques [Lewis et al., 2012].

En marge de ces animaux emblématiques, il existe une multitude d'autres organismes venimeux beaucoup moins étudiés du fait de leur relative innocuité pour l'homme. Ces organismes sont majoritairement des invertébrés et constituent une part très importante de la biodiversité des animaux venimeux [von Reumont et al., 2014a].

Ainsi il ne faut pas oublier le groupe des cnidaires qui rassemble environ 10.000 espèces de coraux, méduses et anémones de mer. L'épiderme de leurs tentacules est recouvert de cellules venimeuses spécialisés appelés les cnidocystes, lesquelles produisent et injectent un venin paralysant utilisé pour capturer des proies, mais également pour faire fuir les prédateurs. Les venins de cnidaires restent relativement mal connus [Rodríguez et al., 2012].

Chez les myriapodes, les scolopendres et les scutigères sont de redoutables prédateurs. Ils sont capables d'injecter un puissant venin par des crochets venimeux appelés forcipules [Ménez et al., 1990]. Bien que leurs venins soient très neurotoxiques ils restent néanmoins largement méconnus [Yang et al., 2012].

Certains poissons produisent également du venin. Le poisson pierre *Synanceia verrucosa* par exemple possède des épines dorsales reliées à une glande à venin. Ce venin a

un rôle uniquement défensif et provoque une vive douleur lorsqu'il est injecté [Gwee et al., 1994].

Étonnamment, il existe quelques rares mammifères venimeux comme l'ornithorhynque (*Ornithorhynchus anatinus*) [Whittington et al., 2008]. Les mâles ornithorhynques possèdent sur les pattes postérieures un aiguillon venimeux leur servant à se défendre. Aux Antilles ce sont de petits mammifères ressemblant aux musaraignes, les solénodons, qui produisent des toxines dans leurs glandes salivaires [Dufton, 1992].

La majorité des animaux venimeux appartiennent à la classe des insectes. On distingue trois principaux ordres d'insectes qui sont capables de produire du venin : les hémiptères, les lépidoptères et les hyménoptères. Parmi les hémiptères certaines familles comme les reduviidae utilisent un rostre pour injecter une salive neurotoxique afin de paralyser leurs proies [Sahayaraj and Muthukumar, 2011]. Chez les lépidoptères ce sont les chenilles et parfois les papillons de certaines espèces d'hétérocères (papillons de nuit) qui sont pourvus de poils urticants assimilés à de petites fléchettes et qui sont associés à des glandes venimeuses. Si beaucoup de ces espèces de papillons de nuit ne sont pas très dangereuses et ne provoquent seulement que de simple démangeaisons, d'autres espèces comme la chenille de *Lonomia obliqua* (Saturniidae) possèdent un venin puissant qui perturbe la coagulation sanguine et provoque de nombreuses hémorragies internes chez les personnes entrant en contact avec ses poils [Carrijo-Carvalho and Chudzinski-Tavassi, 2007].

Avec 120.000 espèces actuellement décrites, l'ordre des hyménoptères est de loin le groupe venimeux le plus vaste [van Emden, 2013]. Il comprend les guêpes, les abeilles, les bourdons, les mutiles et les fourmis. Chez les hyménoptères le venin est injecté par un aiguillon situé à l'extrémité de l'abdomen. Le système venimeux des hyménoptères est issu

du système de reproduction et l'aiguillon résulte d'une modification de l'ovipositeur. Ainsi, chez les hyménoptères, seules les femelles possèdent un appareil venimeux. Les venins d'hyménoptères possèdent tout un cortège d'enzymes qui s'avèrent être dans certains cas de puissants allergènes [Schmidt et al., 1986]. Par exemple, l'inoculation du venin d'hyménoptères peut déclencher un choc anaphylactique (réaction allergique extrême) pouvant être mortel pour l'homme. Les guêpes possèdent également un grand nombre de petits peptides linéaires tels que des mastoparanes qui augmentent la perméabilité membranaire ou des kinines aux propriétés neurotoxiques et algésiques [Piek, 1990]. Le venin de l'abeille méllifère (*Apis melifera*) est pour sa part principalement composé de deux peptides; la mellitine et l'apamine dont les activités sont respectivement cytotoxique et neurotoxique [Gauldie et al., 1976]. Au regard de la formidable biodiversité des hyménoptères, leurs venins demeurent cependant quasiment inexplorés.

L'univers des animaux venimeux est donc remarquablement vaste et diversifié mais seule une infime partie de cette biodiversité a été explorée (Figure 1) principalement en raison de la faible quantité de venins disponible dans la majorité des organismes venimeux. En fait, 90% de la biodiversité est représentée par des animaux venimeux dont taille est inférieure à 1 cm. La majorité des toxines animales décrites et caractérisées aujourd'hui proviennent des venins de serpents, de mygales, de scorpions et de cônes. Cela laisse donc de nombreux groupes venimeux inexplorés notamment chez les arthropodes.

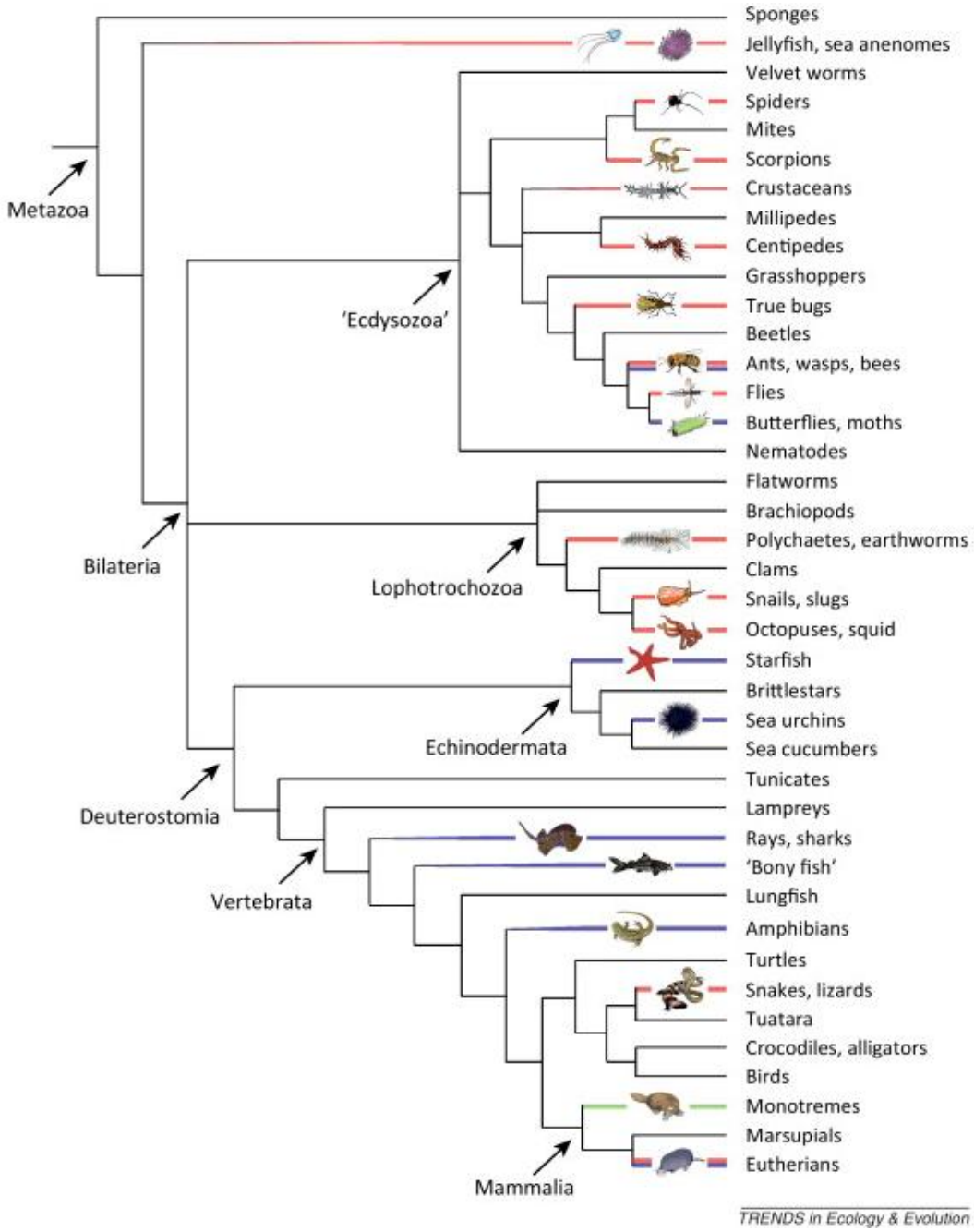

Figure 1 - Arbre schématique des organismes venimeux. Les branches colorées de l'arbre indiquent les organismes venimeux majeurs. Les branches rouges indiquent un rôle offensif du venin, les branches bleues indiquent un rôle défensif et les branches vertes un rôle dans la compétition intraspécifique. Schéma d'après [Casewell et al., 2013].

3. Les fourmis

Les fourmis se distinguent des animaux venimeux classiquement étudiés principalement par le fait que ce sont des insectes sociaux. Cette socialité leur a permis d'acquérir une incroyable diversité écologique et comportementale. Les fourmis sont des hyménoptères aculéates phylogénétiquement proches des Apidae [Johnson et al., 2013]. Toutes les espèces de fourmis sont sociales et on peut donc les considérer parmi les animaux venimeux terrestres les plus abondants. En effet, malgré leur poids de quelques milligrammes par individu, les fourmis représentent entre 15 et 20 % de la biomasse animale en forêt tropicale et leur masse totale dépasse celle de l'ensemble de l'humanité. Elles sont de nature ubiquiste et on les retrouve dans quasiment l'ensemble des biotopes terrestres de la planète [Hölldobler and Wilson, 1990; Wilson, 1990].

La diversité des fourmis est importante ; il existe environ 13.000 espèces de fourmis décrites mais beaucoup d'espèces restent à découvrir, particulièrement dans les forêts tropicales [AntWeb, 2014b]. On estime que le nombre total d'espèces de fourmis est supérieur à 25.000 [Ward, 2007]. L'appareil venimeux des fourmis se compose de deux glandes tubulaires et allongées qui sécrètent les différents composés du venin. La partie basale de ces glandes est convolutée avec la poche à venin de forme ovoïde qui sert de réservoir (Figure 2). Cette poche à venin est reliée à l'aiguillon via un canal excréteur [Ortiz and Mathias, 2006].

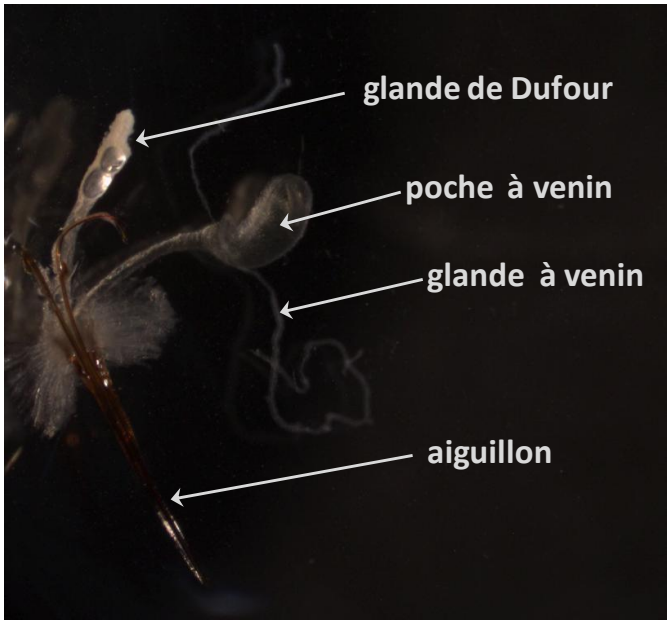

Figure 2- Structure de l'appareil venimeux de la fourmi *Paraponera clavata*.

Les fourmis sont toutes venimeuses, c'est-à-dire qu'elles possèdent une glande à venin. Néanmoins il faut distinguer les espèces pourvues d'un aiguillon bien développé et fonctionnel qui injectent leur venin et les fourmis qui ne possèdent plus d'aiguillon fonctionnel et dont le venin, non injectable, est pulvérisé ou appliqué sur la cuticule des proies. Ces venins non injectables sont supposés contenir peu de toxines peptidiques car ces dernières doivent être injectées pour être actives. Ainsi dans les sous-familles Formicinae, Dolichoderinae, Aneuretinae et la tribu des Dorylini chez les Dorylinae, l'aiguillon a disparu (Figure 3). On estime donc le nombre de fourmis qui possèdent un aiguillon fonctionnel à environ 9.100 espèces. Cette diversité est en fait supérieure au nombre total combiné d'espèces de scorpions (1.454 espèces), de serpents (3.496 espèces) et cônes marins (800 espèces) [Hallan, 2005; Olivera, 2006; Uetz et al., 2013]. Cependant cette diversité n'est pas répartie uniformément dans les sous-familles de fourmis à aiguillon. Par exemple, les Myrmicinae comprennent environ 6.500 espèces décrites et réparties sur l'ensemble de la

planète. La deuxième sous-famille de fourmis à aiguillon en termes de diversité est la sous-famille des Ponerinae (1.200 espèces) mais ces espèces ont une distribution principalement équatoriale. En outre les sous-familles néotropicales; Paraponerinae et Martialinae comprennent chacune une seule espèce de fourmis.

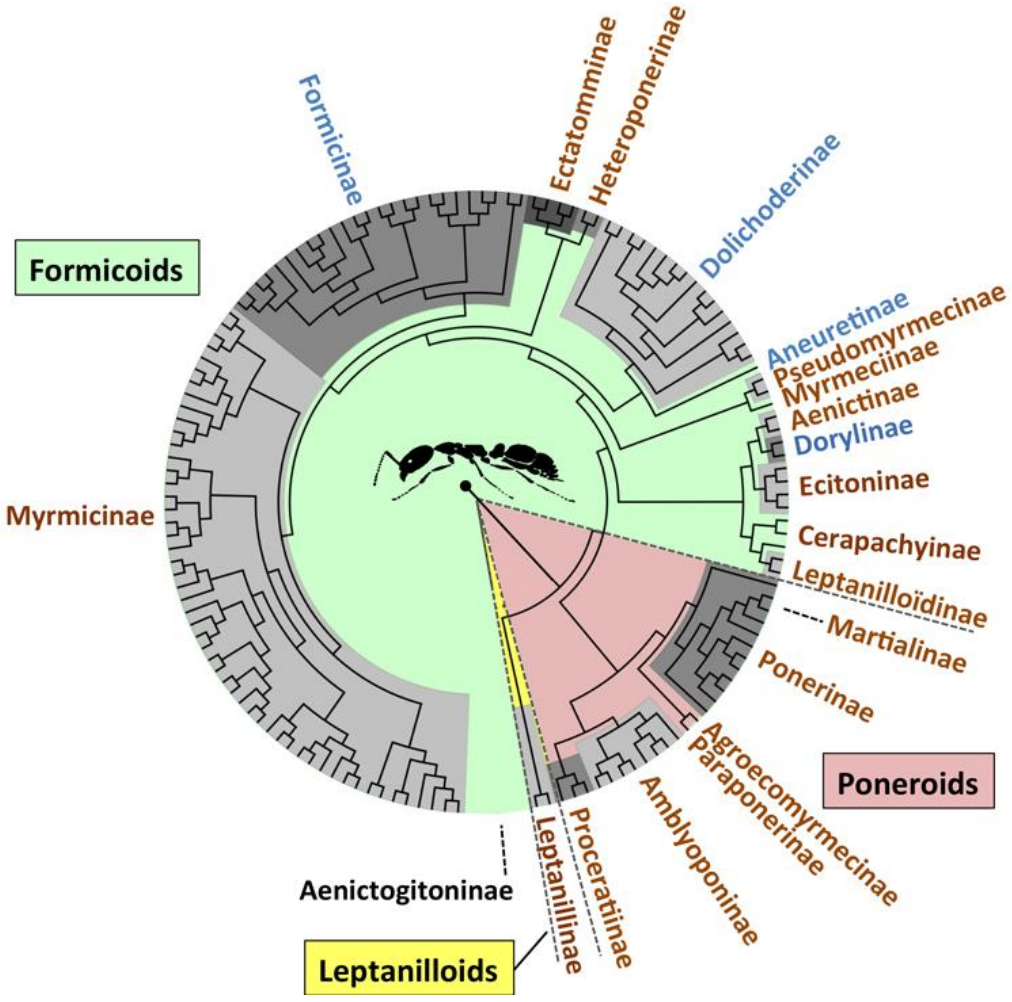

Figure 3- Relation phylogénétique des sous-familles de fourmis. Au cours de l'évolution, quatre sous-familles ont perdu leur capacité à piquer (texte en bleu). Les autres sous-familles représentent les fourmis à aiguillons (texte en marron). Les clades des fourmis sont représentés en vert (formicoides), rose (poneroides) et en jaune (leptanilloides).

Encadré 1. Changements taxonomiques récents chez les formicidae

Au cours de cette thèse, en 2014 deux publications majeures ont bouleversé la classification des fourmis à aiguillon.

Fractionnement du genre *Pachycondyla*

A la suite d'analyses phylogénétiques le genre *Pachycondyla* a été scindé en 19 genres distincts ; *Bothroponera, Brachyponera, Ectomomyrmex, Euponera, Hagensia, Megaponera, Mesoponera, Neoponera, Ophthalmopone, Pachycondyla, Paltothyreus, Pseudoneoponera, Pseudoponera, Austroponera, Buniapone, Fisheropone, Mayaponera, Parvaponera et Rasopone* [Schmidt and Shattuck, 2014]. Les articles soumis et publiés dans cette thèse utilisent la dénomination *Pachycondyla*. Cependant les articles en préparation ainsi que le reste du manuscrit utilisent les nouveaux noms de genre. Les espèces de Guyane française concernées sont listées dans la table ci-dessous :

Anciens noms	Nouveaux noms
Pachycondyla goeldii	*Neoponera goeldii*
Pachycondyla commutata	*Neoponera commutata*
Pachycondyla marginata	*Neoponera marginata*
Pachycondyla inversa	*Neoponera inversa*
Pachycondyla villosa	*Neoponera villosa*
Pachycondyla mesonotalis	*Neoponera mesonotalis*
Pachycondyla apicalis	*Neoponera apicalis*
Pachycondyla verenae	*Neoponera verenae*
Pachycondyla unidentata	*Neoponera unidentata*
Pachycondyla stigma	*Pseudoponera stigma*
Pachycondyla crassinoda	*Pachycondyla crassinoda*
Pachycondyla procidua	*Pachycondyla procidua*
Pachycondyla harpax	*Pachycondyla harpax*
Pachycondyla constricta	*Mayaponera constricta*
Pachycondyla arhuaca	*Rasopone arhuaca*

Fusion des sous-familles de fourmis légionnaires

Les sous-familles Ecitoninae, Cerapachyinae, Leptanilloïdinae, Dorylinae, Aenictinae et Aenictogitoninae ont récemment été regroupées en une seule sous-famille dénommée Dorylinae [Brady et al., 2014]. Cette nouvelle nomenclature a été prise en considération dans ce manuscrit mais pas dans les articles, notamment l'article 5.

A cette grande diversité taxonomique il faut également ajouter l'incroyable diversité écologique des fourmis qui a probablement amplement contribué à façonner les venins grâce à des phénomènes d'évolution adaptative et de course à l'armement. Bien que le régime alimentaire de la plupart des fourmis soit omnivore, beaucoup d'espèces sont de redoutables prédatrices et peuvent être considérées parmi les prédateurs majeurs d'invertébrés dans la plupart des écosystèmes terrestres. Beaucoup d'espèces de fourmis sont des prédateurs généralistes capables de capturer et de consommer un très large panel d'invertébrés ainsi que de petits vertébrés. D'autres espèces, principalement chez les myrmicines et les ponerines, sont des prédateurs spécialisés se nourrissant seulement aux dépens d'un groupe restreint d'invertébrés comme les vers de terre, les isopodes, les myriapodes, les collemboles, les termites ou bien encore les autres espèces de fourmis [Cerdá and Dejean, 2011]. L'évolution pourrait avoir sélectionné des toxines adaptées à la capture des ces proies spécifiques.

Les méthodes de chasse varient également en fonction des espèces. Beaucoup d'espèces de fourmis ont développé des techniques de chasse collective, ce qui augmente les chances de capture. Ce comportement collaboratif est parfois poussé à l'extrême comme c'est le cas chez les fourmis légionnaires qui forment des raids impressionnants comptant plusieurs dizaines de milliers d'individus pour chasser en groupe. Cependant, bien d'autres espèces de fourmis prédatrices ont des chasseuses solitaires notamment, chez les ponerines. Afin de capturer seules leurs proies, parfois de taille supérieure à elles, ces fourmis ont développé des armes performantes comme des mandibules hypertrophiées et des venins très puissants et neurotoxiques afin de pouvoir maitriser très rapidement les proies. Le choix

du site de nidification des fourmis peut également avoir influencé la composition des venins. En forêt tropicale les fourmis occupent toutes les strates. Ainsi on retrouve des fourmis aux mœurs souterraines, beaucoup d'espèces vivent dans la litière et au niveau du sol, tandis que d'autres sont arboricoles et occupent les différents étages de la végétation jusqu'à la canopée. La chasse en milieu arboricole nécessite certaines adaptations notamment parce que les possibilités de fuite des proies sont supérieures. L'hypothèse que les fourmis prédatrices aux mœurs arboricoles possèdent un venin plus puissant que celles du sol a été confirmée par une étude portant sur la toxicité des venins de plusieurs espèces arboricoles et terricoles dans l'ancien genre *Pachycondyla* [Orivel and Dejean, 2001]. Bien que la fonction offensive soit l'une des fonctions principales du venin permettant la capture des proies, le venin des fourmis est également une arme de défense [Schmidt, 2014]. Les fourmis du genre *Pogonomyrmex* sont exclusivement granivores et utilisent leur venin pour se défendre contre de nombreux rongeurs désireux de faire main basse sur leurs stocks de graines. Ainsi, *Pogonomyrmex badius* a développé un venin qui est essentiellement toxique pour les vertébrés et qui reste faiblement toxique pour les invertébrés [Schmidt and Blum, 1978a, c].

En plus des *Pogonomyrmex*, de nombreuses fourmis sont aussi réputées pour avoir des piqûres extrêmement douloureuses. Par exemple la piqûre de la fourmi flamande (*Paraponera clavata*) est considérée comme la plus douloureuse parmi tous les hyménoptères et l'effet perdure de longues heures [Starr, 1985]. Cette caractéristique est d'ailleurs exploitée par la tribu amérindienne des Wayana vivant dans la forêt Amazonienne. Les adultes utilisent la piqûre de cette fourmi lors d'un rite d'initiation appelé Maraké, durant lequel les jeunes hommes de la tribu doivent supporter la douleur induite par les piqûres de parfois plusieurs centaines de fourmis [Balée, 2000].

Le cas des fourmis de la sous-famille des Pseudomyrmecinae qui vivent dans les myrmécophytes (*i.e.*, plantes logeant des colonies de fourmis dans des structures creuses) est une autre illustration du rôle défensif des venins. Dans les savanes africaines, certaines espèces de fourmis du genre *Tetraponera* (Pseudomyrmecinae) vivent associées à des arbres myrmécophytes du genre *Vachelia* (anciennement *Acacia*). Ces fourmis sont capables, grâce à leur piqûre douloureuse de faire fuir de gros herbivores tel que des éléphants qui essayent de manger leur plantes hôtes [Palmer and Brody, 2007]. L'induction de la douleur par les venins semble être le principal mécanisme défensif. Cependant, chez les fourmis le rôle défensif des venins peut prendre une tournure inhabituelle dans certains cas. La fourmi *Pseudoneoponera tridentata* (anciennement *Pachycondyla tridentata*) est tout à fait capable d'injecter son venin normalement afin de paralyser ses proies. Mais lorsque qu'une ouvrière de cette espèce est menacée par de petits arthropodes, elle produit un venin moussant (Figure 3). La mousse ainsi excrétée se révèle être un mécanisme de défense à la fois original et très efficace contre les petites fourmis prédatrices [Maschwitz et al., 1981].

Figure 3- Certaines espèces de ponerinae produisent un venin moussant afin de se défendre contre les petits arthropodes. Ici *Pseudoneoponera tridentata* (photo A. Wild).

4. Les venins de fourmis

Les fourmis se sont remarquablement adaptées à de nombreuses niches écologiques et en conséquence elles utilisent leur venin pour des rôles multiples tels que la prédation, la protection contre les prédateurs, contre les pathogènes, pour la communication chimique et même comme herbicide. Cette importante diversité fonctionnelle des venins de fourmis a permis la sélection de nombreuses toxines très variées (Figure 4). La glande à venin des fourmis est donc capable de produire une incroyable diversité de molécules telles que de l'acide formique, des amines biogènes, des alcaloïdes, des acides aminés libres, des hydrocarbures, des aldéhydes, des enzymes, des protéines et des peptides [Morgan et al., 2003; Schmidt, 1982].

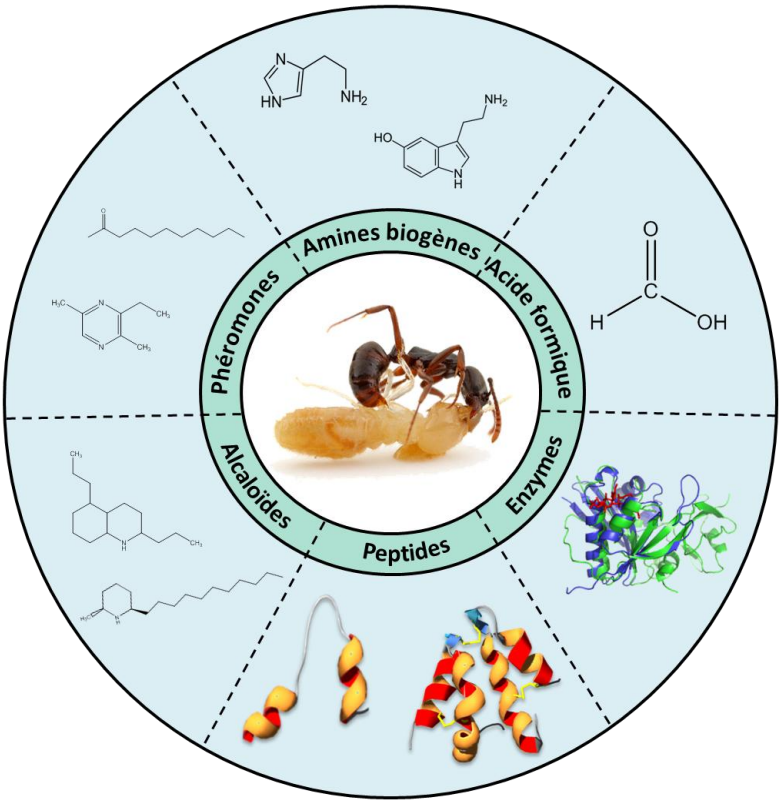

Figure 4- Diversité des molécules chimiques produites par les glandes à venin des fourmis.

L'acide formique, ou acide méthanoïque, est le principal composé chimique des venins de la sous-famille des Formicinae. Les fourmis appartenant à cette sous-famille sont dépourvues d'aiguillon et ne peuvent donc pas injecter leur venin. Ces fourmis ont développé un acidopore à la place de l'aiguillon, ce qui leur permet de projeter des jets d'acide formique. Les venins composés d'acide formique sont utilisés pour se défendre contre les prédateurs, pour capturer des proies et pour lutter contre les microorganismes. Cependant certaines espèces ont également adopté des utilisations originales de l'acide formique. Par exemples les fourmis du genre *Myrmelachista* sont à l'origine de ce que l'on appelle les jardins du diable. Ces fourmis habitent diverses plantes à fourmis dans les forêts tropicales sud américaines. Ces plantes fournissent aux fourmis à la fois un site de nidification et une ressource alimentaire. En retour les fourmis protègent la plante hôte en tuant toutes les autres plantes aux alentours du myrmécophyte. Pour cela elles injectent dans les plantes étrangères leur venin composé d'acide formique qui agit comme un herbicide [Frederickson and Gordon, 2007; Morawetz et al., 1992]. Un autre exemple d'utilisation originale de l'acide formique est celui de *Nylanderia fulva*. Cette fourmi applique son venin sur sa cuticule afin de se protéger des attaques des fourmis *Solenopsis invicta*. L'acide formique du venin de *Nylanderia fulva* permet de détoxifier le venin de *Solenopsis invicta* en dénaturant ses alcaloïdes [LeBrun et al., 2014].

Les amines biogènes sont une classe de molécules organiques comprenant un groupement amine. Elles sont souvent issues du catabolisme d'acides aminés. Dans les venins de fourmis on peut citer comme amine biogène l'histamine qui est impliquée dans la libération de signaux pro-inflammatoires [Matuszek et al., 1992].

Dans la plupart des animaux venimeux, les venins sont composés de protéines et de peptides qui représentent pour plus de 90% du poids sec du venin [Harvey, 2006; King and Hardy, 2013; Lewis et al., 2012; Palma, 2006; Possani and Rodríguez de la Vega, 2006]. De plus ces composés protéiques portent la majorité des propriétés toxiques du venin. Il a cependant été déterminé chez la fourmi *Solenopsis invicta* que la majeure partie du venin est de nature alcaloïdique et que les toxines protéiques ne représentent qu'une faible proportion du venin [Baer et al., 1979; Blum, 1992; Brand et al., 1972]. La solenopsine (Figure 5) est l'un des alcaloïdes majeurs et possède de nombreuses activités biologiques telles que des activités cytotoxiques, hémolytiques, nécrotiques, insecticides, antibactériennes et antifongiques [Blum et al., 1958; Javors et al., 1993]. De nombreux autres composés alcaloïdiques ont été caractérisés dans les venins des myrmicines des genres *Solenopsis, Monomorium*, *Megalomyrmex* et même chez une pseudomyrmecine du genre *Tetraponera* [Jones et al., 1996b; Jones et al., 1991; Jones et al., 2003; Merlin et al., 1988]. Des composés alcaloïdiques ont récemment été découverts dans le venin de la fourmi *Oecophylla smaragdina* (Formicinae) [Das et al., 2014].

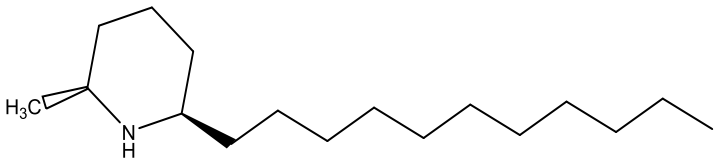

Figure 5- Structure chimique de la solenopsine.

Il est également intéressant de rappeler l'originalité du genre *Crematogaster* dans lequel certaines espèces possèdent un aiguillon en forme de spatule qui lui sert à déposer son venin. Ce venin, composé de petite molécules comme des acétates et des aldéhydes agit

comme un insecticide de contact et pénètre passivement dans l'organisme des ennemis et des proies [Daloze et al., 1998; Heredia et al., 2005; Marlier et al., 2004].

En ce qui concerne les composés protéiques, les venins de fourmis contiennent une batterie d'enzymes telles que des phospholipases, des phosphatases, des hyaluronidases, des lipases ou encore des estérases. L'activité enzymatique entraîne la lyse des parois cellulaires et agit comme facteur de diffusion tissulaire des autres composés toxiques.

La présence de nombreux peptides bioactifs a été révélée dans les venins de fourmis appartenant à différentes sous-familles : Paraponerinae, Ponerinae, Myrmicinae, Ectatomminae, Myrmeciinae, et Pseudomyrmecinae. Ces toxines peptidiques possèdent des activités insecticides, neurotoxiques, cytotoxiques et antibactériennes [Inagaki et al., 2004; Johnson et al., 2010; Orivel et al., 2001; Pan and Hink, 2000; Piek et al., 1991a; Pluzhnikov et al., 1994; Rifflet et al., 2012].

5. Les peptides des venins de fourmis

Parmi les molécules actives présentes dans les venins, les peptides sont les toxines majoritaires dans la plupart des espèces venimeuses. Les peptides sont définis comme de petites protéines ayant une chaîne d'acides aminés comprenant moins de 100 résidus. Il a été démontré que les venins de mygales, de scorpions, de cônes et de serpents sont incroyablement complexes. Certains venins peuvent contenir plusieurs centaines de peptides différents pour une seule espèce. Par exemple le venin de la mygale australienne *Atrax robustus* contient plus de 1.000 toxines peptidiques différentes [Escoubas et al., 2006]. Chez les fourmis, c'est plus de 300 peptides qui ont été trouvés dans le venin de *Dinoponera quadriceps* [Cologna et al., 2013].

A ce jour, seuls 72 peptides provenant du venin de 11 espèces de fourmis ont été caractérisés et séquencés. C'est très peu en comparaison des peptides des venins de serpents, de scorpions, d'araignées ou de cônes. Par exemple, 931 toxines peptidiques ont été séquencées à partir de 86 espèces d'araignées et sont disponibles dans la base de données ArachnoServer 2.0 [Herzig et al., 2011]. La principale raison qui explique le nombre limité d'études sur les peptides des venins de fourmis est leur petite taille et par conséquent la difficulté d'obtenir une quantité suffisante de venin. Cependant le développement des nouvelles technologies de biochimie analytique, telles que la nanochromatographie ou la spectrométrie de masse à haute résolution ouvrent la voie de l'exploration des venins de fourmis. On peut classifier les 72 peptides déjà caractérisés dans les venins de fourmis en trois groupes sur la base de leur structure : les peptides linéaires, les peptides dimériques et les peptides ICK « Inhibitor Cystine Knot ».

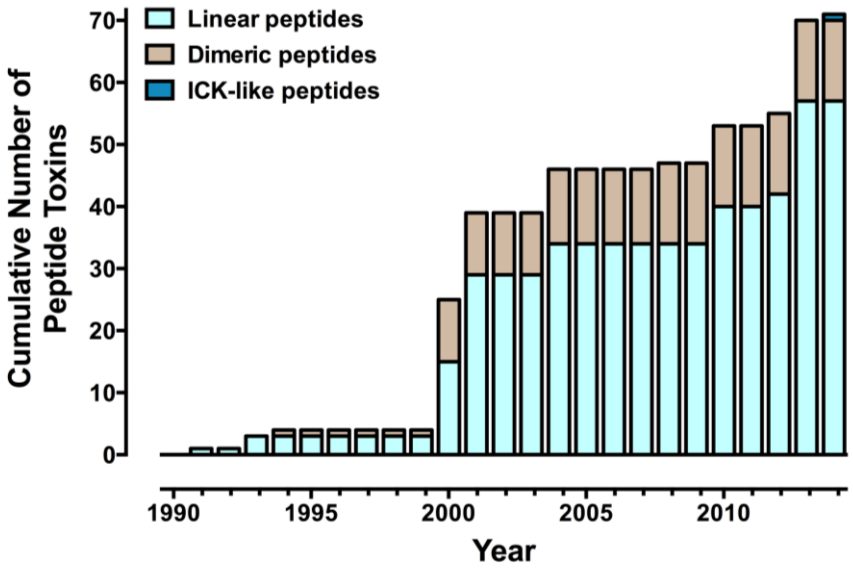

Figure 6 – Nombre total cumulé des séquences des toxines peptidiques publiées provenant des venins de fourmis. Cette figure montre les trois classes structurales : les peptides linéaires en bleu clair, les peptides dimériques en gris et les peptides ICK en bleu foncé.

5.1. Les peptides linéaires

Les peptides linéaires sont définis comme étant généralement de petite taille (moins de 35 acides aminés), souvent polycationiques et surtout dépourvus de ponts disulfures. Ces peptides sont souvent de nature amphiphile et adoptent typiquement des conformations en hélice α qui leur confèrent la capacité d'interagir avec la double couche phospholipidique des membranes cellulaires. De ce fait, les peptides linéaires possèdent souvent des activités cytotoxiques, antimicrobiennes et parfois insecticides. Les peptides linéaires sont présents dans les venins de scorpions et d'hyménoptères ainsi que dans les venins d'araignées aranéomorphes [Argiolas and Pisano, 1984, 1985; Baptista-Saidemberg et al., 2011; de Souza et al., 2004; Favreau et al., 2006; King and Hardy, 2013]. La plupart des études précédentes

sur les venins de fourmis ont révélé une grande majorité de petits peptides dépourvus de ponts disulfure et qui ont une masse inférieure à 4 kDa. Ces peptides sont généralement amphiphiles et adoptent des structures en hélice α [Cologna et al., 2013; Johnson et al., 2013; Orivel et al., 2001; Rifflet et al., 2012].

La poneratoxine, première toxine peptidique isolée et séquencée dans un venin de fourmi en 1991 est un peptide neurotoxique de 25 acides aminés provenant du venin de la fourmi néotropicale *Paraponera clavata* (Paraponerinae) [Piek, 1991; Piek et al., 1991a]. L'analyse structurale par RMN de la poneratoxine a révélé une forme en V du peptide avec deux hélices α connectées par un feuillet β [Szolajska et al., 2004]. Il a été démontré que cette toxine interagit avec les canaux sodium chez les vertébrés et les invertébrés [Duval et al., 1992; Hendrich et al., 2002].

Les ponericines sont un groupe de 27 peptides caractérisés dans les venins des fourmis ponerines *Neoponera goeldii*, *Neoponera apicalis* et *Neoponera inversa*. Les ponericines possèdent des structures amphiphiles en hélice α et montrent des activités insecticides, hémolytiques et antibactériennes [Orivel, 2000; Orivel et al., 2001].

Les fourmis géantes du genre *Dinoponera* sont des prédatrices qui chassent en solitaire et qui utilisent leur venin pour paralyser des invertébrés et pour provoquer une vive douleur chez les vertébrés. Les six peptides les plus abondants du venin de *Dinoponera australis* ont été caractérisés [Johnson et al., 2010]. Par la suite 15 autres peptides ont été isolés et séquencés dans le venin de *Dinoponera quadriceps* [Cologna et al., 2013]. L'ensemble de ces peptides forme un groupe de toxines appelées les dinoponeratoxines. Plusieurs de ces peptides possèdent des activités antibactériennes et antifongiques.

Dans la sous famille des Myrmicinae, une étude récente a permis de séquencer deux peptides antimicrobiens à partir du venin de *Tetramorium bicarinatum*, les bicarinalines 1 et 2 [Rifflet, 2012; Rifflet et al., 2012].

La pilosuline 1 est un peptide allergène isolé du venin de la fourmi australienne *Myrmecia pilosula*. Il s'agit d'un peptide de grande taille par rapport aux autres peptides linéaires des venins de fourmis. Il a une masse de 6048 Da et possède une forte activité cytotoxique et antimicrobienne. Le venin de cette fourmi a été particulièrement bien étudié en Australie car elle occasionne des problèmes allergiques pouvant entraîner des décès. La pilosuline 1 a été identifiée comme l'un des allergènes majeurs de ce venin [Davies et al., 2004; Donovan et al., 1993; Donovan et al., 1995; Wu et al., 1998].

5.2. Les peptides dimériques

Les peptides dimériques sont des complexes formés par deux chaînes polypeptidiques reliées entre elles par un ou plusieurs ponts disulfures. Dans les venins de serpents plusieurs peptides dimériques ont été décrits. Les désintégrines forment une famille de polypeptides isolés à partir du venin de la vipère *Echis carinatus*. Ces peptides sont capables d'inhiber les protéines transmembranaires intégrines [Calvete et al., 2003; Marcinkiewicz et al., 1999]. Le venin du cobra *Naja kaouthia* possède également des peptides dimériques qui ont des activités neurotoxiques [Osipov et al., 2008]. A l'exception de ces venins de serpents, la structure dimérique des peptides est assez rare, bien que quelques peptides dimériques aient été décrits dans les venins de scorpions [Zamudio et al., 1997], d'araignées [Santos et al., 1992] et de cônes [Loughnan et al., 2006]. Chez les venins de fourmis, la structure dimérique des peptides semble être assez commune dans les sous-familles des

Ectatomminae [Pluzhnikov et al., 2000; Pluzhnikov et al., 1994], Myrmeciinae [Inagaki et al., 2004; Inagaki et al., 2008a] et Pseudomyrmecinae [Pan and Hink, 2000].

L'un des peptides les plus neurotoxiques isolés dans les venins de fourmis est l'ectatomine Et-1 qui est un peptide dimérique caractérisé dans le venin d'*Ectatomma tuberculatum* (Ectatomminae) [Pluzhnikov et al., 1994]. Un autre peptide homologue a été identifié dans ce venin, il s'agit de l'ectatomine Et-2. Ces peptides hétérodimériques sont composés de deux chaînes polypeptidiques fortement homologues et reliées par un pont disulfure inter-caténaire [Arseniev et al., 1994]. Chacune des chaînes possède également un pont disulfure intra-caténaire. La structure en trois dimensions de l'ectatomine Et-1 a été déterminée par RMN et consiste en un paquet de quatre hélices α [Nolde et al., 1995]. Ce peptide neurotoxique forme des pores membranaires capables de moduler les courants calcium [Pluzhnikov et al., 1999; Pluzhnikov et al., 1994]. Deux autres ectatomines (Eq-1 et Eq-2) également dimériques, ont été isolées à partir du venin de la fourmi *Ectatomma brunneum*. Ces ectatomines possèdent également un pont disulfure inter-caténaire, mais ils sont dépourvus de pont disulfure intra-caténaire [Pluzhnikov et al., 2000].

Les myrmexines sont un groupe de six polypeptides identifiés dans le venin de *Pseudomyrmex triplarinus* (Pseudomyrmecinae). Ces peptides sont des complexes hétéro-dimériques stabilisés par deux ponts disulfure inter-caténaires [Pan and Hink, 2000].

Un autre groupe de peptides dimériques a été isolé dans le venin de *Myrmecia pilosula*, il s'agit des pilosulines 3, 4 et 5. La pilosuline 3 est un hétérodimère et possède deux ponts disulfure alors que les pilosulines 4 et 5 sont des homodimères. Bien que les pilosulines soient les principaux allergènes du venin de *Myrmecia pilosula,* leur rôle biologique reste encore inconnu [Inagaki et al., 2004; Inagaki et al., 2008a].

5.3. Les peptides ICK

La structure ICK d'un peptide est définie comme deux feuillets β anti-parallèles qui sont stabilisés par un « nœud de cystines ». Cette structure possède au moins trois ponts disulfure, deux ponts forment une boucle traversée par le troisième pont, formant alors le nœud de cystines (Figure 6). La structure ICK apporte aux peptides une grande stabilité et une résistance aux dégradations enzymatiques [Craik et al., 2001]. Cette structure est retrouvée dans les venins d'un grand nombre d'organismes venimeux différents [Zhu et al., 2003], tels que les araignées [Escoubas and Rash, 2004], les scorpions [Smith et al., 2011], les cônes [Gilly et al., 2011] et les anémones de mer [Rodríguez et al., 2014]. Cependant, de tous ces organismes, les araignées sont les animaux venimeux qui produisent le plus grand nombre de peptides ICK ainsi que la plus grande diversité pharmacologique.

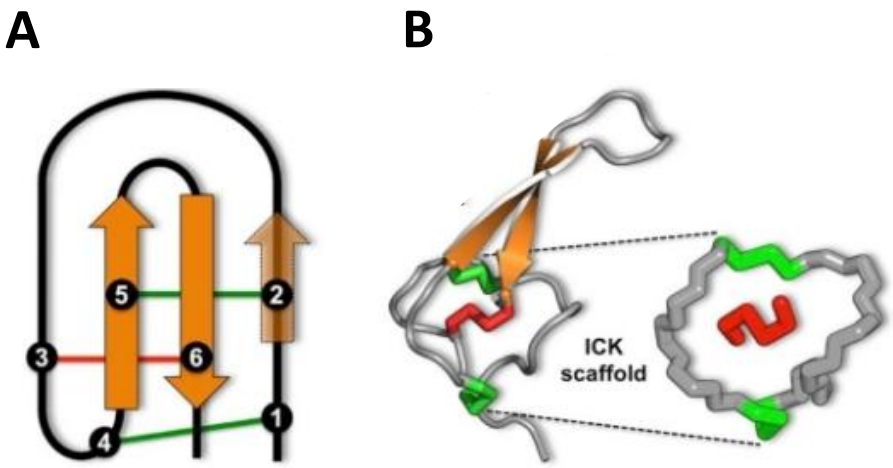

Figure 6- Le motif ICK (Inhibitor Cystine Knot). (A) Schéma d'un peptide ICK avec les six cystéines numérotées de 1 à 6. (B) Structure 3D du peptide ICK, ω-hexatoxin-Hv1a isolé à partir du venin de l'araignée *Hadronyche versuta*. Ce motif est composé de deux feuillets β antiparallèles (en orange) stabilisés par un nœud de cystines (Cystine Knot). Le nœud de cystines se compose d'un anneau formé par deux ponts disulfure (en vert) par lequel un troisième pont disulfure (en rouge) passe au travers.

Dans les venins de fourmis seuls deux types de peptides ICK ont été décrits; il s'agit d'une dinoponeratoxine et des peptides SKTXs. L'analyse récente du transcriptome des glandes à venin de la fourmi *Dinoponera quadriceps* (Ponerinae) a confirmé la présence et permis d'établir la première séquence d'un peptide ICK dans un venin de fourmis [Torres et al., 2014]. La dinoponeratoxine ICK est un composé mineur du venin de *Dinoponera quadriceps* et son rôle est encore inconnu.

Le venin de la fourmi *Strumigenys kumadori* contient aussi des peptides ICK qui ont été nommés SKTXs. Ces peptides possèdent une forte homologie avec certains peptides ICK trouvés dans les venins de mygales connus pour leurs activités neurotoxiques. Les peptides SKTXs sont capables de moduler les canaux ioniques des drosophiles ; cependant les résultats de cette étude ne sont toujours pas publiés et les séquences restent inconnues [Inagaki et al., 2008b].

En conclusion, on peut résumer les connaissances actuelles sur les peptides des venins de fourmis en trois points :

- Les peptides caractérisés dans les venins de fourmis sont majoritairement petits et linéaires avec des activités cytotoxiques et antibactériennes.
- Quelques peptides à ponts disulfure ont été séquencés, il s'agit presque uniquement de peptides dimériques à l'exception d'un peptide ICK.
- Leur pharmacologie reste essentiellement inconnue.

Les venins de fourmis constituent donc un vaste champ d'exploration pour la découverte de nouvelles toxines peptidiques aux propriétés originales. En effet la grande diversité taxonomique et l'énorme diversité écologique des fourmis permettent de former l'hypothèse que la diversité peptidique des venins de fourmis est très importante.

La synthèse bibliographique réalisée lors de cette introduction a été publiée dans l'article 1 (Annexe 1).

- **Article 1: « Diversity of peptide toxins from stinging ant venoms »**

6. Objectifs généraux

L'étude menée dans le cadre de cette thèse s'inscrit dans un projet collaboratif entre l'UMR EcoFoG et l'entreprise de biotechnologie VenomeTech. Ce travail a été financé par le projet FEDER BI-Appli (115/SGAR-DE/2011/052274) et le projet BIOPEPMED qui ont pour objectif général la découverte et le développement de molécules d'intérêt thérapeutique basés sur les toxines peptidiques des venins de fourmis.

Les fourmis se distinguent des autres animaux venimeux classiquement étudiés, par certaines caractéristiques telles que la socialité, une grande diversité spécifique, une omniprésence dans tous les milieux terrestres, ainsi qu'une étonnante diversité écologique et comportementale. Bien que la composition et les propriétés de leurs venins restent presque inexplorées, leurs caractéristiques écologiques et taxonomiques suggèrent une grande diversité biochimique et pharmacologique de leurs toxines. Une investigation détaillée de la composition des venins de fourmis en relation avec le rôle de ces venins dans l'écologie des fourmis permettra de déterminer leur potentiel pour la découverte de nouvelles molécules aux propriétés biologiques intéressantes. Cette thèse constitue l'une des plus importantes études de protéomique et pharmacologique entreprises sur les venins de fourmis à ce jour. Ce manuscrit se divise en quatre chapitres :

- **Chapitre 1 : Biodiversité**
- **Chapitre 2 : Ecologie**
- **Chapitre 3 : Biochimie**
- **Chapitre 4 : Pharmacologie**

L'un des facteurs majeur qui limite les études sur les substances naturelles provenant d'insectes est la nécessité de collecter les espèces en grand nombre. Pour cela, l'identification taxonomique est essentielle bien que souvent complexe. L'objectif des travaux décrits dans le chapitre 1 était de développer et de tester l'applicabilité d'une méthode générale de chimiotaxonomie basée sur les profils peptidiques des venins de fourmis. L'article 2 présente l'application de cette méthode d'analyse par spectrométrie de masse à des venins bruts de fourmis appartenant aux genres *Pachycondyla* et *Odontomachus* qui a servi de base pour la suite des études réalisées lors de cette thèse. Le travail présenté dans ce chapitre vise à démontrer que les peptides des venins de fourmis sont de bons marqueurs chimiotaxonomiques afin d'identifier les espèces de fourmis et permettent donc d'apprécier la diversité spécifique des peptidomes.

- **Article 2: « Elucidation of the unexplored biodiversity of ant venom peptidomes via MALDI-TOF mass spectrometry and its application for chemotaxonomy »**

Il y a une grande variabilité de la composition des venins entre des espèces phylogénétiquement proches, mais également au niveau intraspécifique. Dans le chapitre 2 nous nous sommes intéressés aux facteurs écologiques impliqués dans la diversification des toxines des fourmis. Pour cela nous avons étudié la composition et la toxicité des venins de fourmis suivant différents traits liés à l'écologie des espèces. Dans une première partie, la composition du venin de la fourmi *Odontomachus haematodus* a été étudiée en relation avec le polyéthisme (distribution des tâches dans la colonie). Dans la seconde partie du chapitre, la toxicité des venins d'espèces de fourmis qui ont des régimes alimentaires spécialisés ou généralistes a été caractérisée afin de déterminer si l'écologie trophique des

fourmis a joué un rôle dans la diversification des toxines. Pour finir, la troisième partie de ce chapitre est consacrée aux différences de composition des venins de trois espèces de fourmis du genre *Pseudomyrmex* avec des utilisations principalement offensives du venin pour deux espèces et une utilisation uniquement défensive du venin pour la troisième espèce. Ce chapitre rassemble les deux articles suivants:

- **Article 3 : « Intraspecific variation of the venom peptidome of the ant *Odontomachus haematodus* »**
- **Article 4 : « Venom toxicity and composition in three *Pseudomyrmex* ant species having different nesting modes »**

Les peptides structurés par des ponts disulfure sont des toxines caractéristiques des venins des autres organismes venimeux. Chez les fourmis très peu de peptides à ponts disulfure sont connus. Une étude biochimique de grande ampleur portant sur la diversité structurale des toxines peptidiques des venins de fourmis a été menée et est décrite dans le chapitre 3. L'objectif de ce travail a été d'explorer la diversité des peptides dans des venins appartenant aux neuf principales sous-familles de fourmis à aiguillon afin de découvrir puis de caractériser de nouvelles toxines appartenant à des familles structurales originales en recherchant tout particulièrement les peptides structurés par des ponts disulfure. Les résultats de ce chapitre sont présentés dans l'articles 5:

- **Article 5 : « The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling »**

La recherche de nouvelles molécules possédant des activités intéressantes peut se faire selon deux approches : un screening à l'aveugle sur une cible pharmacologique d'intérêt ; ou bien un guidage bio-rationnel qui permet de sélectionner certaines cibles pharmacologiques basées sur les caractéristiques écologiques de l'organisme. Les résultats du chapitre 2 laissent à penser que les venins de fourmis possèdent des neurotoxines et également des toxines défensives impliquées dans l'algésie des mammifères. Le chapitre 4 est donc consacré à l'évaluation de l'activité pharmacologique des peptides originaux (structurés par des ponts disulfure) que nous avons sélectionnés et caractérisés dans le chapitre 3. L'objectif de ce chapitre est de déterminer l'activité biologique et la pharmacologie de ces nouveaux peptides en particulier sur les canaux ioniques et les récepteurs TRP, cibles récurrentes des neurotoxines et des nocitoxines. La découverte de nouvelles molécules dotées de propriétés agonistes ou antagonistes pour ces récepteurs (canaux ioniques, récepteurs TRP) pourrait conduire au développement futur de molécules thérapeutiques en lien avec certaines pathologies humaines telles que la douleur. Les résultats de ces études sont résumés dans le chapitre 4 et ont donné lieu à un article en préparation :

- **Article 6 : « Formicitoxins : a novel family of disulfide-rich, neurotoxic peptides from ant venom»**

Chapitre 1. Biodiversité

La diversité des fourmis est importante, le nombre d'espèces de fourmis actuellement décrites dans le monde est d'environ 13.000 et beaucoup d'autres espèces restent à découvrir, particulièrement dans les forêts tropicales [Ward, 2007]. Ce grand nombre d'espèces, ainsi que leur proximité morphologique rend l'identification des espèces de fourmis complexe, de sorte que cette tâche reste réservée aux myrmécologues avertis. Les espèces cryptiques ajoutent un problème supplémentaire [Bickford et al., 2007] qui requiert l'utilisation de méthodes complémentaires de l'approche classique basée sur la morphologie. L'avènement de la taxonomie intégrative, qui se base sur la combinaison de plusieurs approches (*e.g.* morphologie, barcode ADN, chimiotaxonomie), permet désormais de pallier les problèmes liés aux espèces cryptiques [Schlick-Steiner et al., 2010]. Il a été nécessaire de développer diverses méthodes de taxonomie permettant de délimiter les espèces. L'utilisation des techniques de biologie moléculaire telles que le barcoding ADN a apporté des outils simples et efficaces pour identifier les espèces. La technique du barcoding moléculaire consiste à séquencer une petite partie de l'ADN (généralement le gène mitochondrial de la Cytochrome Oxydase I) qui servira de code-barres spécifique [Smith et al., 2005]. Dans le contexte actuel où l'estimation de la biodiversité est devenue une course contre la montre, le développement de nouvelles techniques de taxonomie permet également d'accélérer le processus de recensement des espèces. Ces dernières années une multitude d'autres techniques a vu le jour. En ce qui concerne les fourmis, le caractère spécifique des profils d'hydrocarbures cuticulaires [Schlick-Steiner et al., 2006] ou bien encore la comparaison des séquences de stridulation chez certaine espèces de ponérines [Ferreira et al., 2010] ont été utilisés dans le cadre d'études taxonomiques.

1. Taxonomie par spectrométrie de masse

La spectrométrie de masse est une technique d'analyse permettant de détecter et d'identifier des molécules par mesure de leurs masses. Plusieurs études de chimiotaxonomie ont notamment utilisé la spectrométrie de masse MALDI-TOF pour identifier des espèces de bactéries pathogènes [Hsieh et al., 2008] et des pollens [Krause et al., 2012]. Cette méthode de classification reposant sur l'analyse des profils peptidiques des venins par spectrométrie de masse a également été utilisée avec succès pour identifier divers organismes venimeux. Les venins sont des mélanges complexes qui caractérisent les espèces qui les produisent car ils sont toujours liés par leur composition peptidique à une expression directe du génome. Par exemple l'identification morphologique des mygales est une tâche ardue et une nouvelle méthode d'identification a été proposée. La carte des masses moléculaires des peptides du venin est caractéristique de l'espèce, et cela même pour des espèces proches [Escoubas et al., 1997; Escoubas et al., 1999; Escoubas et al., 2002; Escoubas et al., 1998b]. Cette technique repose sur le principe que les animaux venimeux d'une même espèce vont exprimer essentiellement des peptides avec les mêmes séquences d'acides aminés et que la composition peptidique va différer entre espèces.

Par la suite, cette méthode d'identification a été appliquée aux serpents venimeux en montrant l'intérêt du couplage HPLC/MALDI-TOF ou ESI-TOF pour la taxonomie [Stöcklin et al., 2000]. Cependant peu d'études de chimiotaxonomie ont été menées sur les serpents en raison de problèmes de taxonomie moins importants que pour les invertébrés.

➢ **Les peptides des venins de fourmis, à l'instar des venins de serpents ou de mygales, sont-ils de bons marqueurs taxonomiques afin d'identifier les différentes espèces ?**

2. Application aux venins de fourmis

L'article 2 propose une méthode d'analyse par spectrométrie de masse MALDI-TOF des venins bruts de fourmis, laquelle pourrait être utilisée comme un outil de chimiotaxonomie. Chez les fourmis, la chimiotaxonomie des venins à déjà été testée sur les venins alcaloïdiques, particulièrement sur les genres *Solenopsis* et *Monomorium*. Cependant les venins alcaloïdiques ne sont pas majoritaires chez les fourmis à aiguillon. La majorité des venins de ces fourmis contiennent un grand nombre de peptides qui pourraient servir de marqueurs chimiotaxonomiques. À ce jour, seul un nombre limité d'études portant sur les peptides des venins de fourmis et utilisant la spectrométrie de masse MALDI-TOF ont été réalisées. De plus, très peu de matrices MALDI ont été testées. Nous avons donc effectué une étude comparative en testant plusieurs matrices, ainsi que des additifs, afin d'optimiser la détection des peptides. Cela nous a permis de mettre au point une méthode qui servira de base pour l'exploration future des peptidomes des venins de fourmis. La seconde application de cette méthode est son utilisation pour l'identification des espèces de fourmis à aiguillon. Nous avons montré une variation presque totale des profils peptidiques entre les différentes espèces de fourmis alors qu'une très forte similarité intraspécifique des profils de peptides a été observée. Néanmoins pour trois espèces de fourmis, *Pseudoponera stigma*, *Neoponera apicalis* et *Odontomachus haematodus*, une variation intraspécifique plus importante des profils peptidiques a été notée entre les échantillons. Une analyse génétique

menée en parallèle nous a permis de séquencer le gène mitochondrial de la cytochrome oxydase I en complément de l'analyse biochimique pour les échantillons de ces fourmis.

La congruence des variations génétiques du COI avec les différences de profils peptidiques observées dans les venins *Neoponera apicalis* et *Pseudoponera stigma* nous permet de conclure que ces espèces sont des complexes d'espèces cryptiques. Une analyse plus approfondie par LC/MS de deux échantillons de venin de *Pseudoponera stigma* appartenant aux deux morphes montre que très peu de peptides sont communs (Figure 7).

Les conclusions pour les échantillons d'*Odontomachus haematodus* sont moins évidentes car il n'y a pas de congruence entre l'analyse génétique et l'analyse biochimique. Les variations observées dans le venin d'*Odontomachus haematodus* doivent probablement être attribuées à d'autres facteurs tels que l'ontogénie ou la géographie (Voir chapitre 2.1).

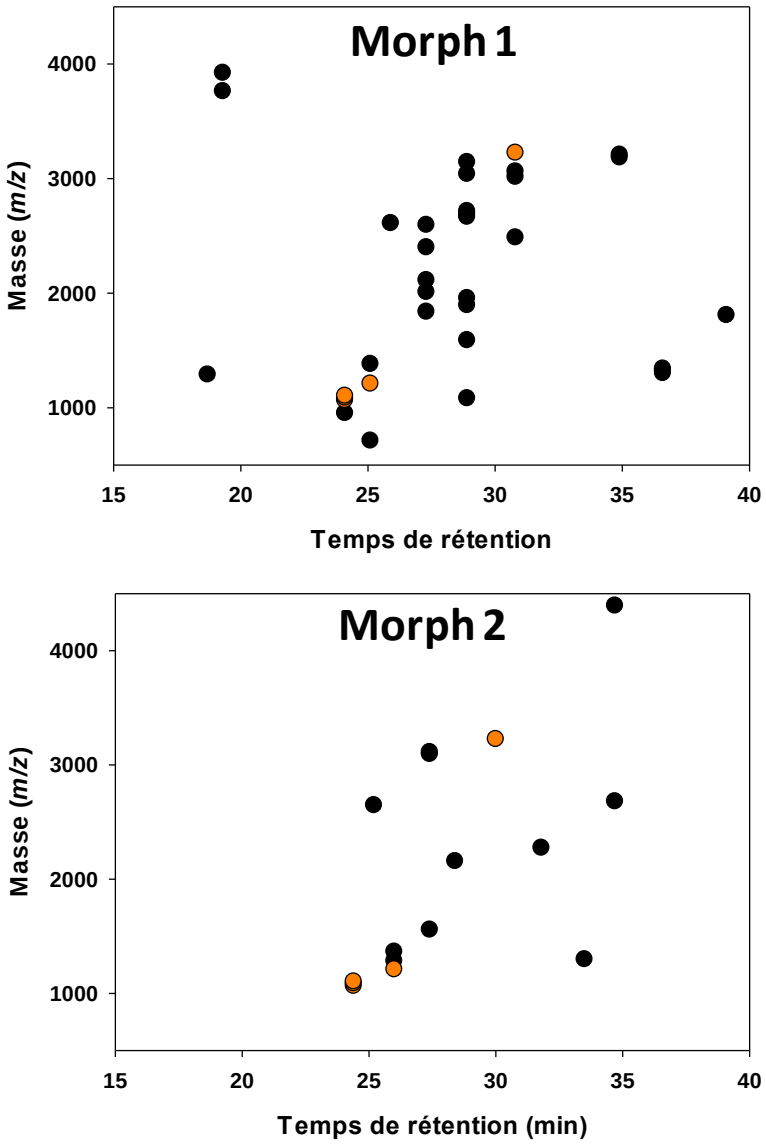

Figure 7- Cartographie des peptides par LC/MS des venins des deux morphes de *Pseudoponera stigma*. Chaque point représente un peptide en fonction de son temps de rétention (min) et de sa masse (*m/z*). Les points en orange correspondent aux peptides communs entre les deux morphes.

Article 2

Elucidation of the unexplored biodiversity of ant venom peptidomes via MALDI-TOF mass spectrometry and its application for chemotaxonomy

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Elucidation of the unexplored biodiversity of ant venom peptidomes via MALDI-TOF mass spectrometry and its application for chemotaxonomy

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Keywords: Peptidome, Ponerinae, Ant Venom, Peptide, Chemotaxonomy, MALDI-TOF MS

Abstract

The rise of integrative taxonomy, a multi-criteria approach used in characterizing species, fosters the development of new tools facilitating species delimitation. Mass spectrometric (MS) analysis of venom peptides from venomous animals has previously been demonstrated to be a valid method for identifying species. Here we aimed to develop a rapid chemotaxonomic tool for identifying ants based on venom peptide mass fingerprinting. The study focused on the biodiversity of ponerine ants (Hymenoptera: Formicidae: Ponerinae) in French Guiana. Initial experiments optimized the use of automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine variations in the mass profiles of ant venoms using several MALDI matrices and additives. Data were then analyzed *via* a hierarchical cluster analysis to classify the venoms of 17 ant species. In addition, phylogenetic relationships were assessed and were highly correlated with methods using DNA sequencing of the mitochondrial gene cytochrome *c* oxidase subunit 1. By combining a molecular genetics approach with this chemotaxonomic approach, we were able to improve the accuracy of the taxonomic findings to reveal cryptic ant species within species complexes. This chemotaxonomic tool can therefore contribute to more rapid species identification and more accurate taxonomies.

Significance

This is the first extensive study concerning the peptide analysis of the venom of both *Pachycondyla* and *Odontomachus* ants. We studied the venoms of 17 ant species from French Guiana that permitted us to fine-tune the venom analysis of ponerine ants *via* MALDI-TOF mass spectrometry. We explored the peptidomes of crude ant venom and demonstrated that venom peptides can be used in the identification of ant species. In addition, the application of this novel chemotaxonomic method combined with a parallel genetic approach using COI sequencing permitted us to reveal the presence of cryptic ants within both the *Pachycondyla apicalis* and *Pachycondyla stigma* species complexes. This adds a new dimension to the search for means of exploiting the enormous biodiversity of venomous ants as a source for novel therapeutic drugs or biopesticides.

1. Introduction

Animal venoms are currently under intense investigation for their potential as unique sources of novel therapeutic drugs. The molecular diversity of venoms, particularly peptide toxins, is linked to a formidable array of targets and pharmacological properties. Several drugs have already been developed based on the high selectivity and potency of venom peptides and the in-depth exploration of new venoms will undoubtedly lead to more discoveries [King, 2011].

In this context, ant venoms represent an unexplored world of potentially valuable peptides. Moreover, with ~13,000 extant valid taxa [Antweb, 2013], among which ~9,000 are stinging species, ants are a hyper-diverse group accounting for the greatest number of

species and ecological richness among social insects [Hölldobler and Wilson, 1990]. As a complex group of animals where problems of systematics and identification exist, correct identification of ant specimens is necessary for reproducibility in the characterization of venoms. Ants employ venoms for a variety of purposes including the paralysis of prey species, self-defense from predators and for communication within colonies [Schmidt, 1982]. Ant venoms are chemically diverse, with peptides, alkaloids or formic acid being the main molecular classes encountered. They are also often complex cocktails of species-specific chemicals that have been used as markers for taxonomic purposes. For example, many alkaloidal components in Myrmicinae ant venoms belonging to the genera *Solenopsis* [DeFauw et al., 2010] and *Monomorium* [Jones et al., 2009; Jones et al., 2003] have been identified using gas chromatography–mass spectrometry (GC-MS) and used as taxonomic markers to characterize different taxa belonging to closely related species [Jones et al., 2009; Jones et al., 2003]. Nevertheless, the majority of ant venoms are mainly composed of proteins and peptides [Davies et al., 2004; Inagaki et al., 2008a; Inagaki et al., 2008b; Johnson et al., 2010; Orivel et al., 2001; Pan and Hink, 2000; Piek et al., 1991a; Pluzhnikov et al., 1994], requiring a different methodological approach to their investigation.

The advent of modern biological mass spectrometry (MS), using soft ionization techniques such as matrix-assisted laser desorption/ionization (MALDI-MS) or electrospray ionization (ESI-MS) now permits the true complexity of venom peptidomes to be revealed and can provide a rapid snapshot of crude venom composition. The MS analysis of venom peptides and proteins has previously been employed to identify various species of snakes [Souza et al., 2008; Stöcklin, 1997; Stöcklin et al., 2000], spiders [Escoubas et al., 1997; Escoubas et al., 1999; Escoubas et al., 2002; Escoubas et al., 1998b; Palagi et al., 2013] and scorpions [Dyason et al., 2002; Nascimento et al., 2006], but not cone snails [Jones et al.,

1996a; Krishnamurthy et al., 1996] as too much individual variation was observed [Davis et al., 2009]. Moreover, the peptide mass fingerprinting of venoms represents a simple and rapid tool for identifying venomous species without biological destruction when the venom is collected through electrical stimulation [Escoubas et al., 1997].

Chemotaxonomy originated at the end of the 19th century when secondary plant metabolites were shown to play a role in plant morphology and evolution [Abbott, 1886, 1887] and was first developed for plant systematics [Bisby et al., 1980]. With the rise of integrative taxonomy, a multi-criteria approach used in characterizing species [Schlick-Steiner et al., 2010], the development of new tools facilitating species identification and delimitation are needed. Chemotaxonomy can greatly contribute to this approach [DeFauw et al., 2010; Howse and Bradshaw, 1980; Jones et al., 2009; Jones et al., 1988; Vander Meer, 1986]. This taxonomic challenge holds true for ants for which morphological identification needs to be fine-tuned to better discriminate species [Balakrishnan and Paterson, 2005; Bickford et al., 2007]. To date, such integrative approaches included DNA barcoding [Hebert et al., 2003; Smith et al., 2005], stridulatory patterns [Ferreira et al., 2010], cuticular hydrocarbon composition [Schlick-Steiner et al., 2006] and morphometric analysis [Fisher and Smith, 2008], but not venoms. Thus, we aimed to investigate the potential of peptidic ant venoms as chemotaxonomic markers using MALDI-TOF MS. To this end, we focused on the optimization of a rapid automated MALDI-TOF MS analysis technique using the Neotropical ponerine ants of the genera *Pachycondyla* and *Odontomachus* as models. Ponerinae are a large and diverse ant sub-family, widespread in tropical habitats, comprising of ~1,100 extant species. These predatory ants are either specialized hunters or generalists and generally use venom in prey capture. However, their taxonomy, especially that of the heterogeneous genus *Pachycondyla,* is still unclear [Schmidt, 2009]. Previous studies of

ponerine venoms revealed that they are rich in peptides [Johnson et al., 2010; Orivel et al., 2001], which makes them suitable models to test the use of MALDI-TOF mass spectrometry in determining ant biodiversity. The combined use of mass spectrometry venom profiling and a parallel molecular genetics approach, using DNA barcoding to validate the chemotaxonomic approach, has also revealed the existence of cryptic ant species.

2. Materials and methods

2.1. Ants

Live specimens of worker ants from different species of *Pachycondyla* and *Odontomachus* were collected from several regions of French Guiana (Fig. 1). A worker ant specimen from each colony was also stored in 96% ethanol for later morphological identification according to the current species classification [Delabie JHC et al., 2008]. Ants were stored at –20°C prior to the dissection of the venom glands. Between three and 30 venom glands from worker ants from each colony were dissected and pooled in 10% acetonitrile (ACN) / water (v/v). To determine intraspecific variations, single venom glands were collected from individual ants belonging to the same colony. Samples were centrifuged for 5 min at 14,400 rpm (12,000 g_{av}), the supernatant collected and lyophilized prior to storage at –20°C. Following the dissection of the venom glands, all remaining ant tissues were preserved in 96% v/v % ethanol for subsequent mDNA barcoding analysis.

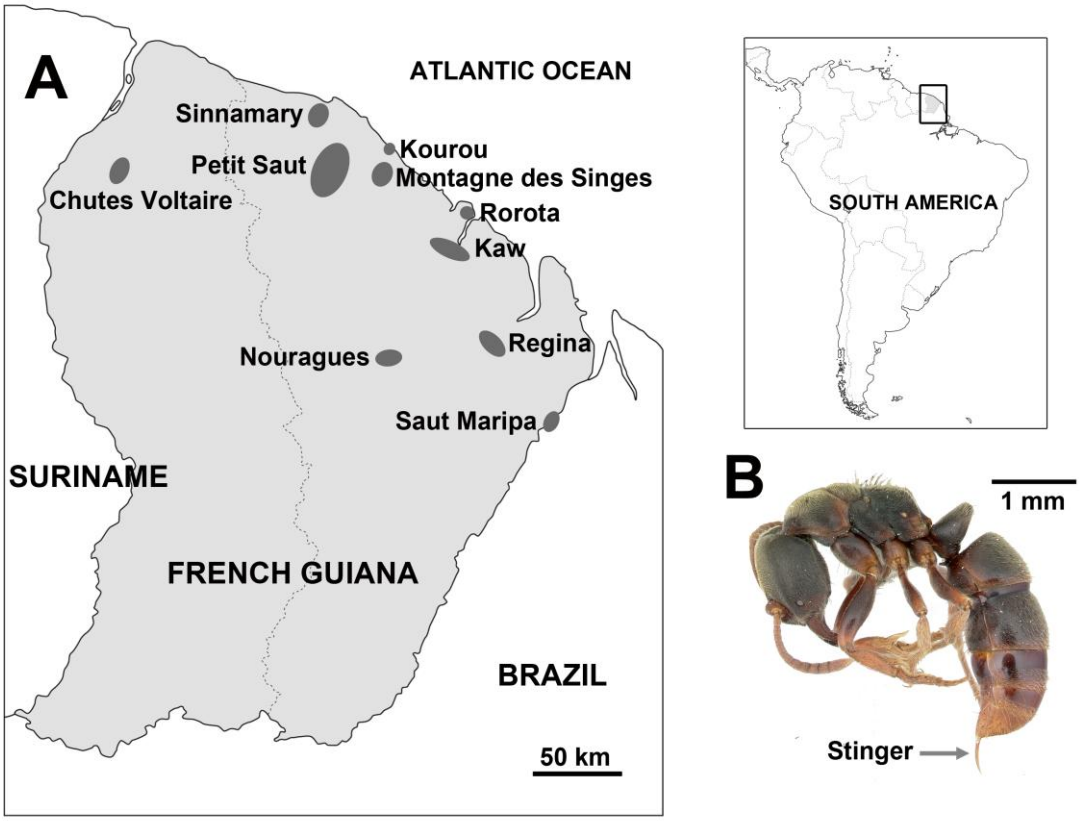

Fig. 1 –Ant species collection sites in French Guiana. (A) Map showing the locations of the 17 ponerine ant species collected for the present study. (B) Profile view of *Pachycondyla stigma* showing the stinger. Photo by April Nobile. Available from: http://www.antweb.org//specimenImages.do?name=casent0178180. Accessed 23 July 2013.

2.2 Mass spectrometry (MS)

MS analyses were performed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems; Forster City, CA, USA). In the initial experiments, several MALDI matrices were evaluated for optimum crystallization and ionization. All matrices tested were dissolved in water/ACN/trifluoroacetic acid (TFA) diluent at a concentration of 10 mg/ml, except α-cyano-4-hydroxycinnamic acid (CHCA) that was used at 5 mg/ml. The MALDI matrices and diluent ratios were: CHCA 50/50/0.1; 2,4,6-trihydroxyacetophenone monohydrate (THAP) 50/50/0.1; caffeic acid (CA) 60/40/0.1, sinapinic acid (SA) 80/20/0.1; ferulic acid (FA) 80/20/0.1; 2,5-dihydroxybenzoic acid (DHB) 80/20/0.1, and 5-methoxysalicylic acid (sDHB) 80/20/0.1. In order to minimize the influence of salt, ion adducts and improve the quality of

the spectra, 10 mM ammonium phosphate [Delvolve and Woods, 2009] or 100 mM serine [Nishikaze and Takayama, 2007] were tested as additives to the various MALDI matrices. Before MS analysis, the ant venom peptides were desalted using ZipTip® C18 (Millipore) pipette tips. Then, 0.5 µl of the desalted sample was deposited on the MALDI target plate, to which 0.5 µl of the matrix was added. Each spectrum was calibrated externally using a mixture of peptides of known molecular masses in the same *m*/*z* range (Peptide calibration Mix 4; LaserBio Labs; Sophia-Antipolis, France). External calibration was performed by depositing 0.5 µl of the calibration mixture adjacent to each sample along with 0.5 µl of the matrix. The spectra for all venoms were acquired with the following parameter settings: acceleration voltage, 20 kV; 50 shots per spectrum. Spectra were acquired in linear or reflector mode and calibrated automatically using the sequence module of the Voyager® control software (Applied Biosystems; Forster City, CA, USA). Five spectra of 50 laser shots each were accumulated based on adequate signal intensity in the desired *m*/*z* range and acceptance parameters. The initial mass spectra were collected over the range 500–10,000 *m*/*z* in positive ion mode. Signals below 500 *m*/*z* were masked by matrix-related ion clusters [Escoubas et al., 2006].

2.3 Spectra analysis

Data Explorer® v4.11 software (AB SCIEX) was used to analyze the spectra. The spectra were subjected to baseline correction with a correlation factor of 0.7 and Gaussian smoothing to reduce noise with a 5-point filter width. All mass attributions were verified manually and a mass list created for each MALDI-TOF MS run. Potential sodium and potassium adducts were manually removed from all mass lists. The percentage of similarity between venoms from different species was determined by matching masses that differed by no more than ± 1.0

Da. Following the methodology recently used in the identification of pollen [Krause et al., 2012] and human pathogenic bacteria [Hsieh et al., 2008], we performed a hierarchical cluster analysis (HCA) of the presence or absence of every distinct mass collected from all ant venom samples. For the HCA, Euclidean distance measures and Ward's algorithm were used and analyses performed with Orange 2.7.1 software (Bioinformatics Laboratory, Faculty of Computer & Information Science, University of Ljubljana, Slovenia) [Demšar, 2004]. The HCA analysis was performed on mass data and did not take into account the abundance of the masses, as peak intensities varied due to the heterogeneity of the MALDI-TOF ionization.

2.4 Molecular genetic analysis

'DNA barcoding' is a technique used in species identification based on the sequencing of a fragment (~650 bp) of the mitochondrial DNA (mDNA) gene of cytochrome *c* oxidase 1 (CO1). This technique is an efficient method for identifying species and has recently been used successfully in solving taxonomic issues, as well as greatly contributing to assessing biodiversity [Hebert et al., 2003]. We used this approach for all ant samples in the present study to confirm the validity of the MS-based chemotaxonomic method developed for ant identification. DNA was extracted from the tissue of representative worker ants using a NucleoSpin® kit 96 Tissue kit (Macherey-Nalgel; Dueren, Germany) in 96-well plates as described in the user manual. Mitochondrial DNA (mDNA) variation was assessed by amplifying a ~650 bp fragment of the CO1 gene [Derocles et al., 2012; Hebert et al., 2003] using a cocktail of primers (Table 1) as described in Folmer et al. [Folmer et al., 1994] and modified as described in Meusnier et al. [Meusnier et al., 2008]. We attached universal M13-FP 5'-TGTAAAACGACGGCCAGT-3' forward and reverse 5' CAGGAAACAGCTATGACC-3' tails to our forward and reverse primers, respectively, to facilitate the high throughput sequencing

process. These tails did not decrease the PCR success. The primers were synthesized by Eurogentec. Each PCR reaction was carried out in a volume of 35 µl in a Bio-Rad thermal cycler (Hercules, CA, USA) using the following thermal cycler profile: 2 min at 94°C followed by five cycles (30 s at 94°C / 40 s and 45°C / 1 min at 72°C) and a further 35 cycles (30 s at 94°C / 40 s and 51°C / 1 min at 72°C) finishing with 10 min at 72°C. The amplified PCR products were sequenced by GATC Biotech (Konstanz, Germany) using an Applied Biosystems ABI 3730XL sequencer (Forster City, CA, USA). DNA sequences were aligned and phylogenetic trees inferred using Geneious software (Biomatters Ltd, Auckland, New Zealand) implementing the Neighbor-Joining (NJ) algorithm for tree reconstruction. Genetic distances were calculated using a Tamura-Nei (TN93) model [Tamura and Nei, 1993]. Jukes-Cantor (JC69) and Hasegawa-Kishino-Yano (HKY85) models were also applied, but produced similar results. The robustness of the trees was tested using 1,000 bootstrap replications with a 50% consensus support threshold. We followed the procedure developed by Mallet [Mallet, 1995] in considering the sympatric existence of separate genotypic clades as an indication of full species status and we employed a 3% sequence divergence threshold typically used in DNA barcoding studies to acknowledge distinct genetic clades as different valid species [Song et al., 2008].

Table 1- List of primers used in this study.

Name	Sequence 5'→ 3'
1-LCO1490puc_t1	F : TGTAAAACGACGGCCAGTTTTCAACWAATCATAAAGATATTGG
2-LCO1490Hem1_t1	F: TGTAAAACGACGGCCAGTTTTCAACTAAYCATAARGATATYGG
3-HCO2198puc_t1	R : CAGGAAACAGCTATGACTAAACTTCWGGRTGWCCAAARAATCA
4-HCO2198Hem2_t1	R : CAGGAAACAGCTATGACTAAACYTCAGGATGACCAAAAAAYCA
5-HCO2198Hem1_t1	R : CAGGAAACAGCTATGACTAAACYTCDGGATGBCCAAARAATCA

3. Results

3.1 Optimization of the MALDI matrix and additives for ponerine ant venoms

Sample preparation can greatly influence the quality of the MALDI ionization of peptides particularly when analyzing a complex mixture of peptides in venoms. CHCA and DHB are two of the most commonly used MALDI matrices for peptide mass fingerprint analysis using MALDI-TOF MS. Yet, the two studies previously conducted on ant venoms both used DHB matrix for MALDI-TOF MS analysis [Orivel et al., 2001; Pan and Hink, 2000]. Therefore, based on four venoms from three *Pachycondyla* species (i.e., *P. laevigata*, *P. goeldii* and two samples taken from *P. verenae*), we tested seven matrices to determine which would best permit us to most easily detect peptides. The use of CHCA, SA and FA permits the detection of a greater number of peptides than other matrices, with 52 ± 37, 58 ± 44 and 46 ± 12 peptides (mean ± S.D.), respectively (Fig. 2). We also noted that the FA matrix produced mass profiles with markedly higher intensities (Fig. 2). DHB and sDHB detected mainly small peptides with masses below 1,500 Da. The remaining matrices CA and THAP detected only an average of 20 ± 6 and 29 ± 7 peptides (mean ± S.D.), respectively. As we wished to develop a technique that would enable the acquisition of MALDI-TOF MS spectra in automated mode, the homogeneity of matrix crystallization leading to faster data acquisition, and more homogeneous spectra, was an important factor to consider. Homogeneous matrix crystallization leads to more even peptide distribution, therefore avoiding the need to hunt for a "hot spot" where signal intensity is higher. In turn, this minimizes the acquisition time as informative spectra can rapidly be acquired from any position on the sample spot. Therefore, SA was not used further due to the heterogeneity of crystallization [Lemaire et

al., 2006], so that we selected CHCA and FA for the remainder of this study and carried out all further data acquisitions in automated mode.

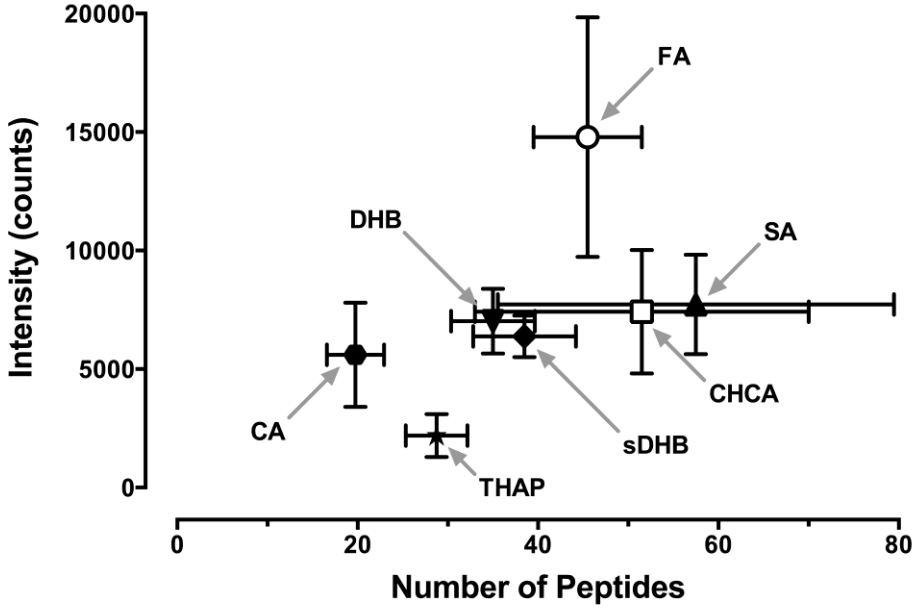

Fig. 2 – Performance of seven different matrices in the detection of peptides from *Pachycondyla* ant venoms. Symbols represent the average number of peptides detected (abscissa) and average signal intensity (ordinate) from the venoms of four *Pachycondyla* spp. Data represent the mean ± SEM (*n* = 4).

It is also known that ions generated by the MALDI ionization of peptides are often contaminated by trace amounts of metals resulting in additional adduct peaks in mass spectra. This leads to spectra that are more complex while lowering signal intensity for each peptide. This arises because a heterogeneous population of ions is formed for each peptide according to the number of adducts. Previous studies have demonstrated that the use of matrix additives such as ammonium phosphate or serine can greatly reduce or eliminate alkali metal adducts [Nishikaze and Takayama, 2007; Smirnov et al., 2004] resulting in substantially increased MALDI MS sensitivity and an improvement in mass accuracy and signal-to-noise ratio. The addition of ammonium phosphate to the CHCA matrix improved the quality of the MS signals by reducing sodium and potassium adducts (Fig. 3A,B), while

the addition of serine to the FA matrix improved the intensity of low mass ions (Fig. 3F). We therefore chose a combination of CHCA/ammonium phosphate or FA/serine for the remainder of this study. However, only the results obtained using the CHCA/ammonium phosphate matrix are shown as similar results were obtained with FA/serine.

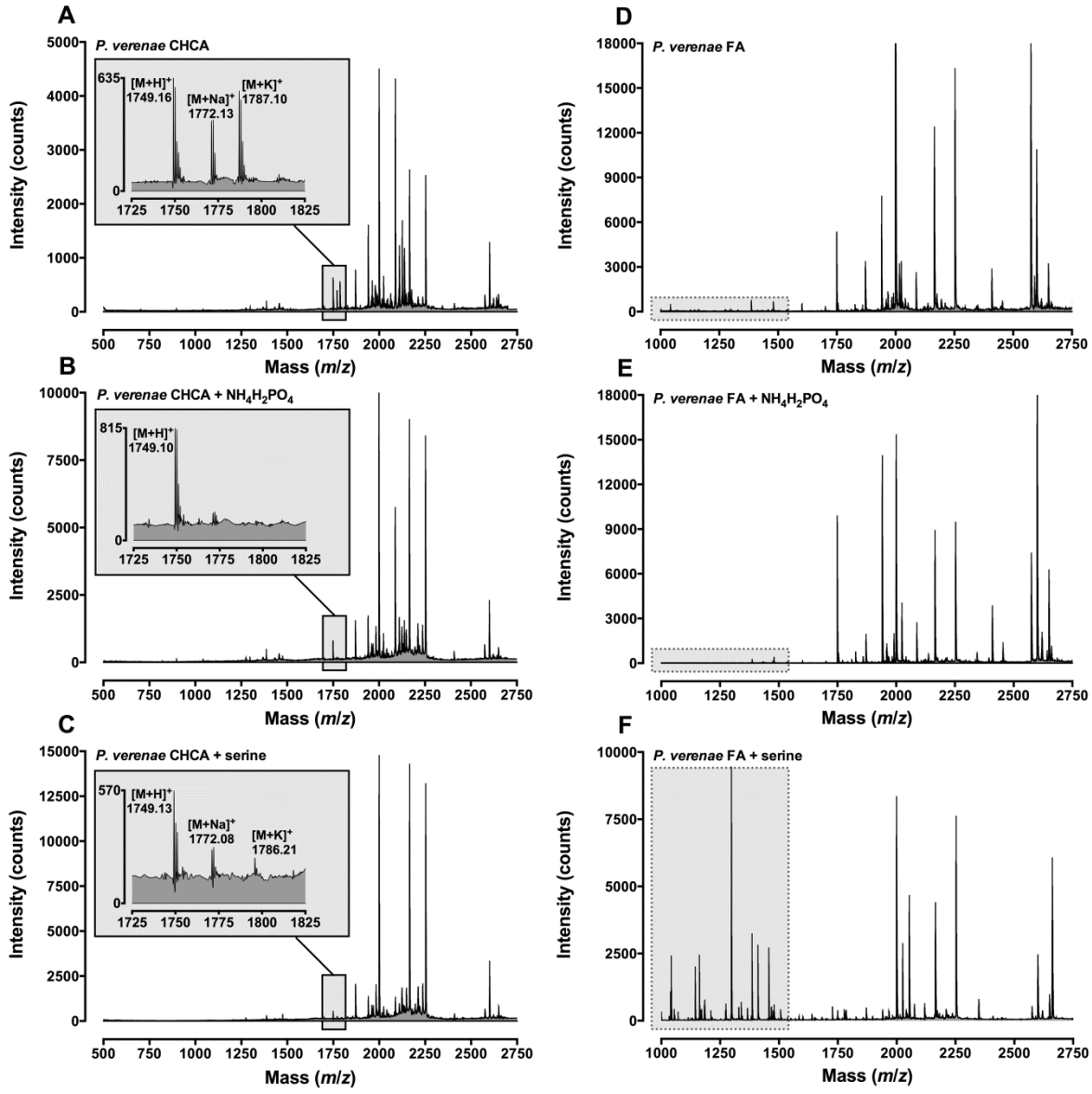

Fig. 3 – MALDI-TOF mass spectra of *P. verenae* venom showing the effect of ammonium phosphate and serine additives on FA and CHCA MALDI matrix performance. (A) CHCA, (B) CHCA with 10 mM ammonium phosphate, (C) CHCA with 100 mM serine, (D) FA, (E) FA with 10 mM ammonium phosphate and (F) FA with 100 mM serine. (A-C) Note the reduction in alkali metal adducts with ammonium phosphate. (D-F) Note the increase in the intensity of low mass ions with serine.

3.2 Intra-species variation in ponerine ant venom mass profiles

The individual venom profiles of 10 ants, labeled 'A' to 'J' and belonging to a single colony of *P. goeldii,* were compared with a pooled venom sample of eight ant glands from individuals from the same colony. Although some peptides detected within the pooled sample were not in certain individual samples, these variations were minor as the individual profiles presented between 67.4% (ant 'E') and 88% (ant 'J') similarity with the pooled sample (Fig. 4A). As previously reported for spider venoms [Escoubas et al., 2002; Escoubas et al., 1998b], quantitative variations in peak intensity (abundance) between individual spectra were more obvious (Fig. 5). Indeed, venom samples from individual *P. goeldii* ants C, D, E, G and J showed only two or three major peaks with other peptides producing only ions of minor intensity. In contrast, venom from *P. goeldii* ants A, B, F, H and I showed 20 major ions while the pooled sample had an intermediate profile. It thus appears that the MALDI-TOF analysis of pooled venom samples creates a homogenizing effect that is more representative of the overall mass profile of a given colony.

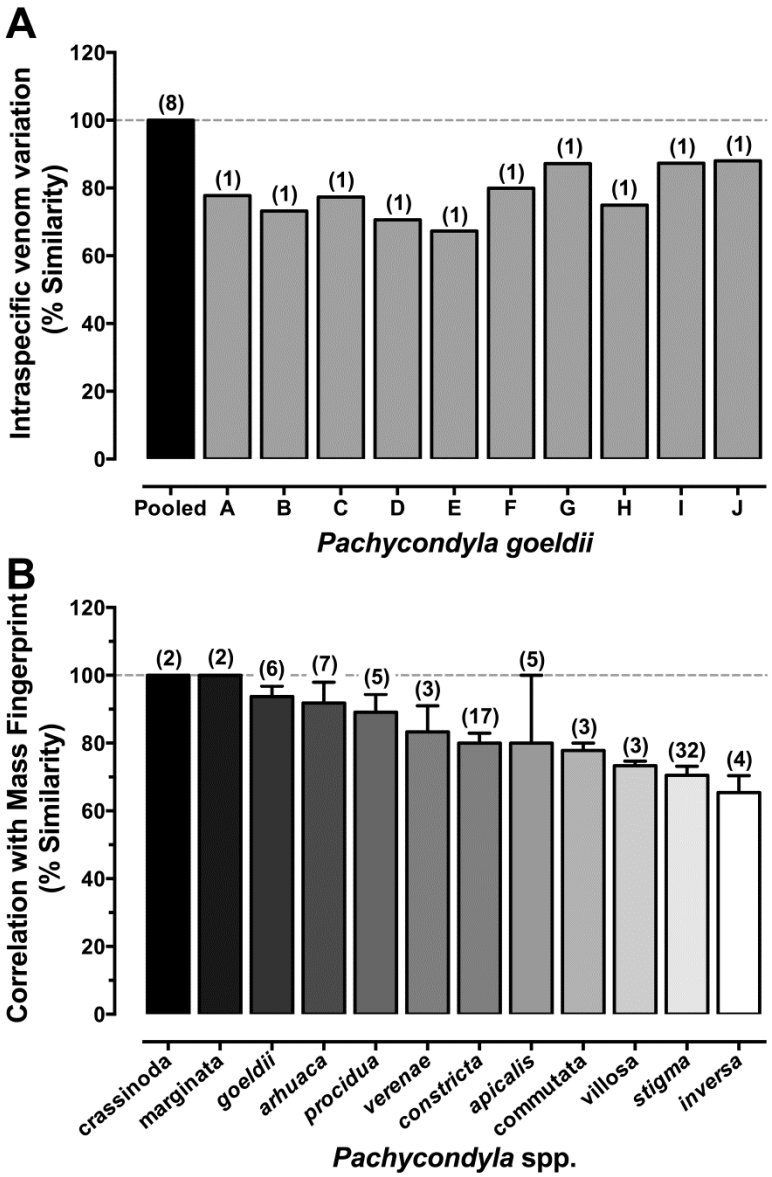

Fig. 4 – Intra- and interspecific variation in *Pachycondyla* spp. venoms. (A) Intraspecific variation for 10 individual venom samples (gray columns labeled 'A-J') *versus* a pool of venom from eight ants ('Pooled', black column) from the same colony of *P. goeldii*. (B) Correlation of *Pachycondyla* ant venom mass profiles with their respective mass fingerprint (see Tables 2 and 3). For *P. apicalis* and *P. stigma*, mass fingerprints for Morph 1 (see Table 3) were utilized in the calculation. Data represent the mean ± SEM. Values above each column represent the number of replicates (*n*).

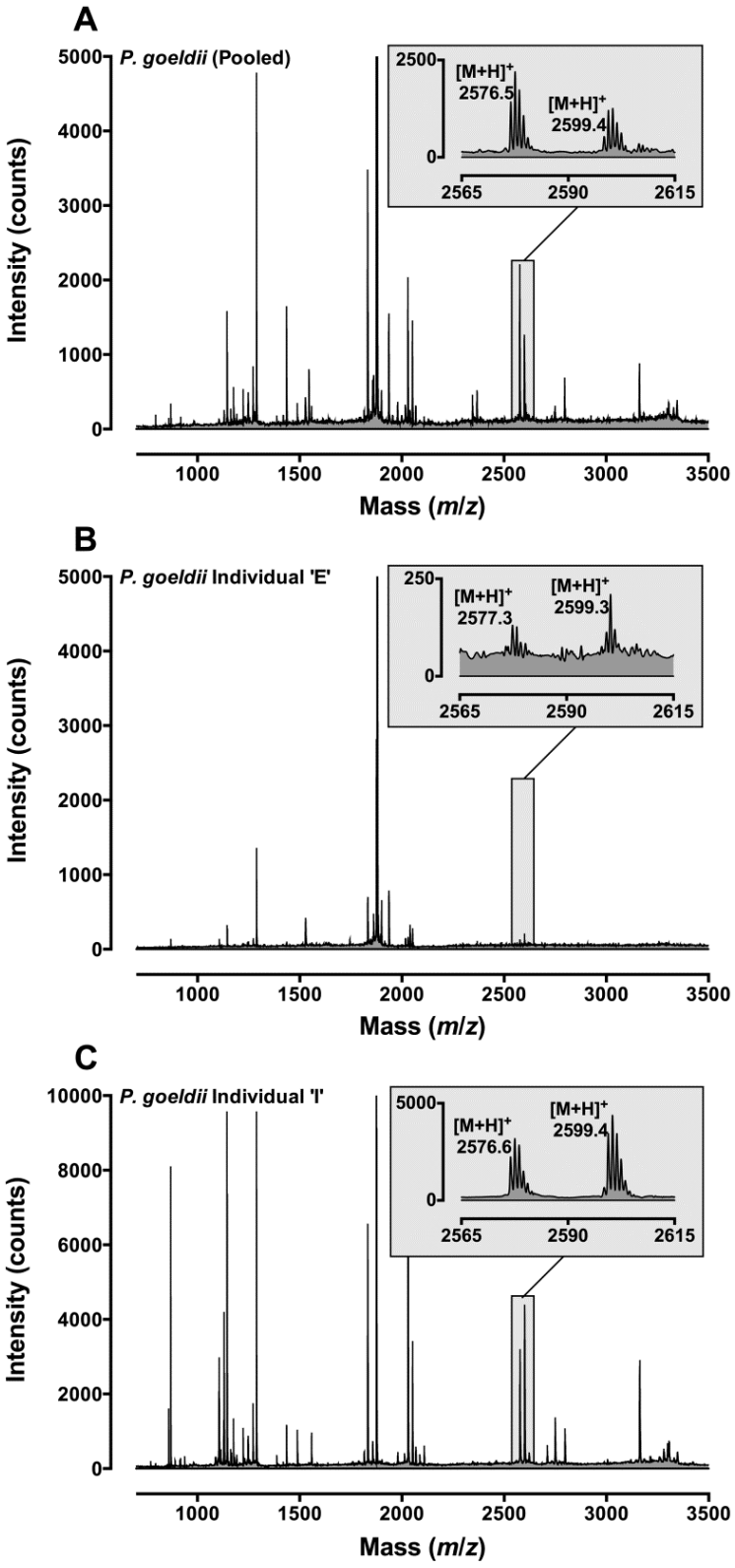

Fig. 5 – MALDI-TOF mass spectra highlighting variation in *P. goeldii* venom peptide ion intensity in ants from the same colony. Panels show the mass spectra of (A) pooled venom from eight ants, (B) individual ant venom 'E' and (C) individual ant venom 'I'. Insets highlight the variation in the two representative masses.

3.3 Peptide mass distribution in ponerine ant venoms

Using CHCA and FA MALDI matrices, we analyzed 147 crude venom samples from 17 *Pachycondyla* and *Odontomachus* species (Fig. 6). In both genera, the mass range of detected peptides remains quite narrow. Between 73% and 90% of all peptides detected were found in the mass range of 1000–3000 Da for *Pachycondyla* using FA and *Odontomachus* using CHCA, respectively. These results differ from those noted for other venomous animals including spiders, snakes and scorpions where masses >5 kDa are commonly observed [Escoubas et al., 2006; Kini and Doley, 2010; Palagi et al., 2013; Rodríguez de la Vega et al., 2010]. Indeed, there were no peptides with a mass beyond 5000 Da in *Odontomachus* spp. venoms, and very few in *Pachycondyla* spp. venoms; one using CHCA (5742.3 Da; 0.24%) and four using FA (5354.0, 5484.9, 5742.1, 6773.2 and 6896.1 Da; 0.97%; Fig. 6). Consequently, for the remainder of the study, we limited our analysis to a mass range of 500–5000 Da.

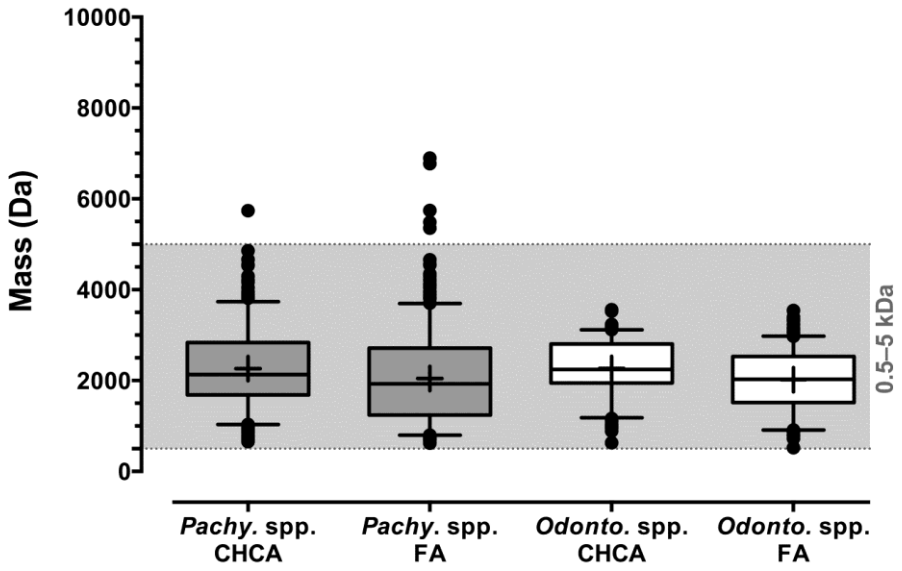

Fig. 6 – Box-and-whisker plot of the peptide mass distribution for two genera of ponerine ant venoms. Data show all peptide masses from 12 *Pachycondyla* (*Pachy*.) spp. venoms and five *Odontomachus* (*Odonto*.) spp. venoms determined using both CHCA and FA matrices. The bottom and top of each grey box represents the first and third quartiles, respectively, while the bar and plus sign inside each box represents the median and mean masses, respectively. The ends of the whiskers represent the 5-95 percentile range while the filled circles represent masses outside the 5-95 percentile range. Note: >99% of all masses are between 0.5–5 kDa.

3.4 Ponerine ant venom mass fingerprinting

We then developed a manual identification method based on the peptide mass fingerprinting of crude venoms belonging to 12 species from the genus *Pachycondyla* (i.e., *P. apicalis, P. arhuaca, P. commutata, P. constricta, P. crassinoda, P. goeldii, P. inversa, P. marginata, P. procidua, P. stigma, P. verenae,* and *P. villosa*). The most frequent and abundant peaks detected in the MALDI-TOF mass spectra of venom samples from each species were used to construct a unique mass fingerprint for each species (Table 2). The lowest average similarity was found for the venom samples of *P. stigma* and *P. inversa* with a 70.5 ± 2.7% (mean ± SE, n = 32) and 65.4 ± 5.0% (n = 7) similarity, respectively (Fig. 4).

Venom samples from the species complex *P. apicalis* displayed an 80.0 ± 20.0% (*n* = 5) similarity. However, the high standard error is due to a mismatch between *P. apicalis* 3 venom and the other four *P. apicalis* venom mass fingerprints (see Table 3). Indeed, when comparing only these latter four samples, the similarity reached 100 ± 0%. As a consequence, the overall high similarity of each venom with its corresponding unique peptide mass fingerprint indicates that the MALDI-TOF MS approach can readily identify an ant species based on its venom mass profile, and even provide insights into the existence of cryptic species.

Table 2. Specific mass fingerprints of the most common masses in the venoms of eight *Pachycondyla* species showing unique peptide masses. Mass (± 0.1 Da) was measured using MALDI-TOF MS with CHCA + ammonium phosphate matrix. Gray shaded masses are common to multiple venoms.

Pachycondyla spp. Peptide Mass Fingerprint (Da)									
arhuaca (*n* = 7)	*commutata* (*n* = 3)	*constricta* (*n* = 17)	*crassinoda* (*n* = 2)	*goeldii* (*n* = 6)	*inversa* (*n* = 4)	*marginata* (*n* = 2)	*procidua* (*n* = 5)	*verenae* (*n* = 3)	*villosa* (*n* = 3)
1466.8	1850.1	1939.1	963.7	869.5	1533.8	2055.2	778.4	1474.6	1533.8
1556.9	1864.2	2096.1	1117.7	1104.7	2043.3	2175.8	1198.8	1998.3	2043.3
1606.8	2130.2	2247.2	1133.7	1106.7	2590.8	2192.1	1328.7	2023.4	2599.5
2146.1	2463.2	2645.6	1975.3	1128.4	2604.7	2742.6	1485.7	2052.9	3476.5
2706.5	2479.3	2694.4	2001.0	1130.7	2724.9	3305.5	1864.9	2069.3	4299.0
3694.0	2726.4	2714.4	2017.3	1144.7	2727.7	3860.1	1888.6	2186.4	
4212.3	3260.5	2722.5	2059.1	1246.7	2747.9	3875.6	1905.0	2210.2	
	3350.5	2752.5	2075.3	1272.4	2925.8		2543.1	2236.1	
	3367.7	2834.4	2834.6	1288.7	3194.9		2559.4	2252.4	
		3064.6	2850.9	1435.7	3205.9		2920.8	2599.8	
		4049.1	3347.0	1557.8	3333.0		3000.4	2986.7	
				1832.0	3339.0			3703.4	
				1874.0	4662.2			3820.5	
				2029.3					
				3161.7					

We further validated the potential of the technique by confirming the identification of several specimens of ants that were not previously identified based on morphological analysis. The comparison of the venom profile of *P. constricta* and *P. arhuaca*, two species

that are difficult to distinguish morphologically, revealed the presence of homologous peptides in *P. constricta* that were absent from *P. arhuaca* venom (Fig. 7). Therefore, the MALDI-MS approach was able to clearly identify the correct species and solve the problematic morphology-based identification. The venom profiles of *P. arhuaca* and *P. constricta* are sufficiently dissimilar so that any possible ambiguity in morphological characters is completely resolved by the biochemical approach.

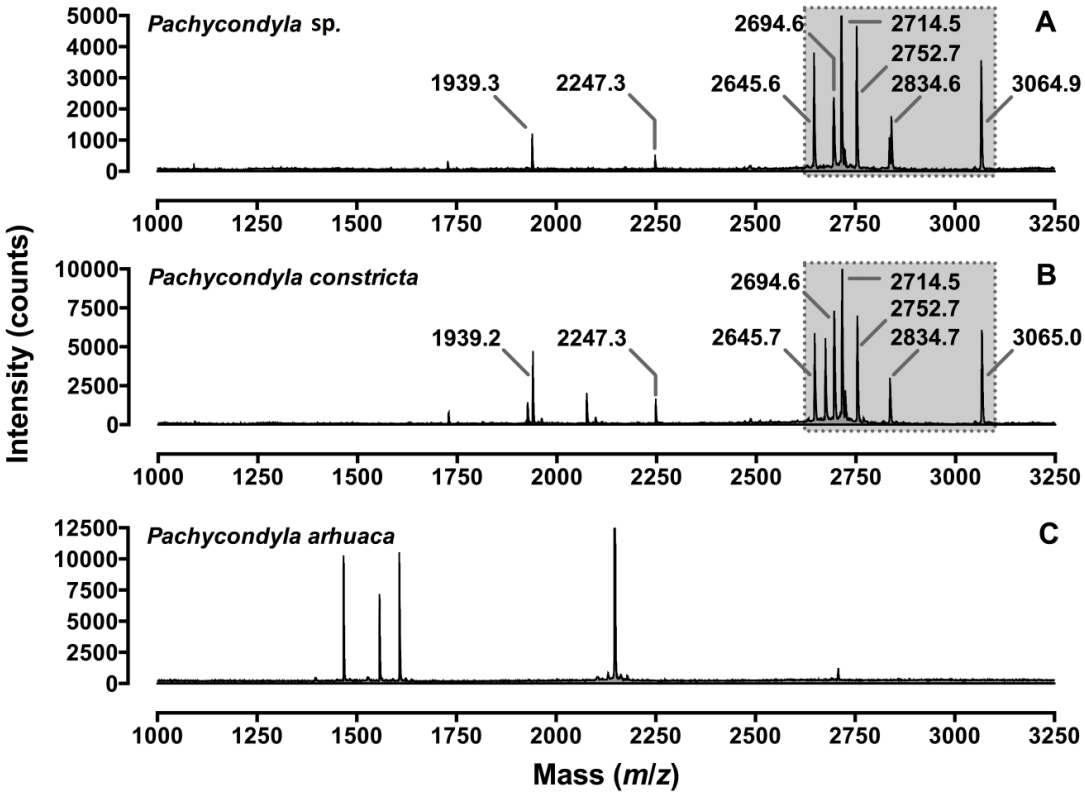

Fig. 7. – Use of MALDI-TOF MS to identify ants based on the mass fingerprinting of venom. The *Pachycondyla* species in panel A was an unknown species that was not morphologically identified. (A-C) Comparison of its venom (A) with the venom mass profile of (B) *P. constricta* and (C) *P. arhuaca* venoms revealed that it contained a number of homologous peptides consistent with that of *P. constricta* that were absent from *P. arhuaca* venom, particularly those masses highlighted in the shaded area.

Table 3. Specific mass fingerprints of venoms from two *Pachycondyla* species complexes showing unique peptide masses. Mass (± 0.1 Da) was measured using the CHCA + ammonium phosphate matrix. †Morph 1 comprises *P. stigma* 1–25 while Morph 2 comprises *P. stigma* 26–32. ‡Morph 1 comprises *P. apicalis* 1, 2, 4 and 5 while Morph 2 comprises *P. apicalis* 3. Gray shaded masses for *P. stigma* are common to the two morphs.

Peptide Mass Fingerprint (Da)			
‡*P. apicalis* Morph 1	*P. apicalis* Morph 2	†*P. stigma* Morph 1	*P. stigma* Morph 2
	1307.6	1065.6	1065.6
1572.9		1081.7	
	1615.8	1087.6	1087.6
	1630.9	1210.6	1210.6
	1646.8	1274.4	1274.4
	1671.8	1364.6	1364.6
	1687.9	1382.7	1382.7
	1701.9	3001.2	
	1816.9	3017.6	
2043.2		3047.4	
2065.2		3063.5	
2450.6			3096.3
2696.7			3112.4
2747.4		3225.5	3225.5
2894.3			
2910.5			
	3166.4		
3930.0			

3.5 Automated ponerine ant venom identification by hierarchical cluster analysis

We extended our study to additional ponerine species using an automated venom classification method and a HCA based on the presence/absence of peptides in each venom sample. The study involved 147 venom samples from the genera *Odontomachus* and *Pachycondyla*. Morphological identifications allowed 17 distinct species to be differentiated. These consisted of five species of *Odontomachus* (i.e., *O. biumbonatus*, *O. haematodus*, *O.*

hastatus, *O. mayi*, and *O. scalptus*) and 12 species of *Pachycondyla* (i.e., *P. apicalis, P. arhuaca, P. commutata, P. constricta, P. crassinoda, P. goeldii, P. inversa, P. marginata, P. procidua, P. stigma, P. verenae,* and *P. villosa*) (Fig. 8).

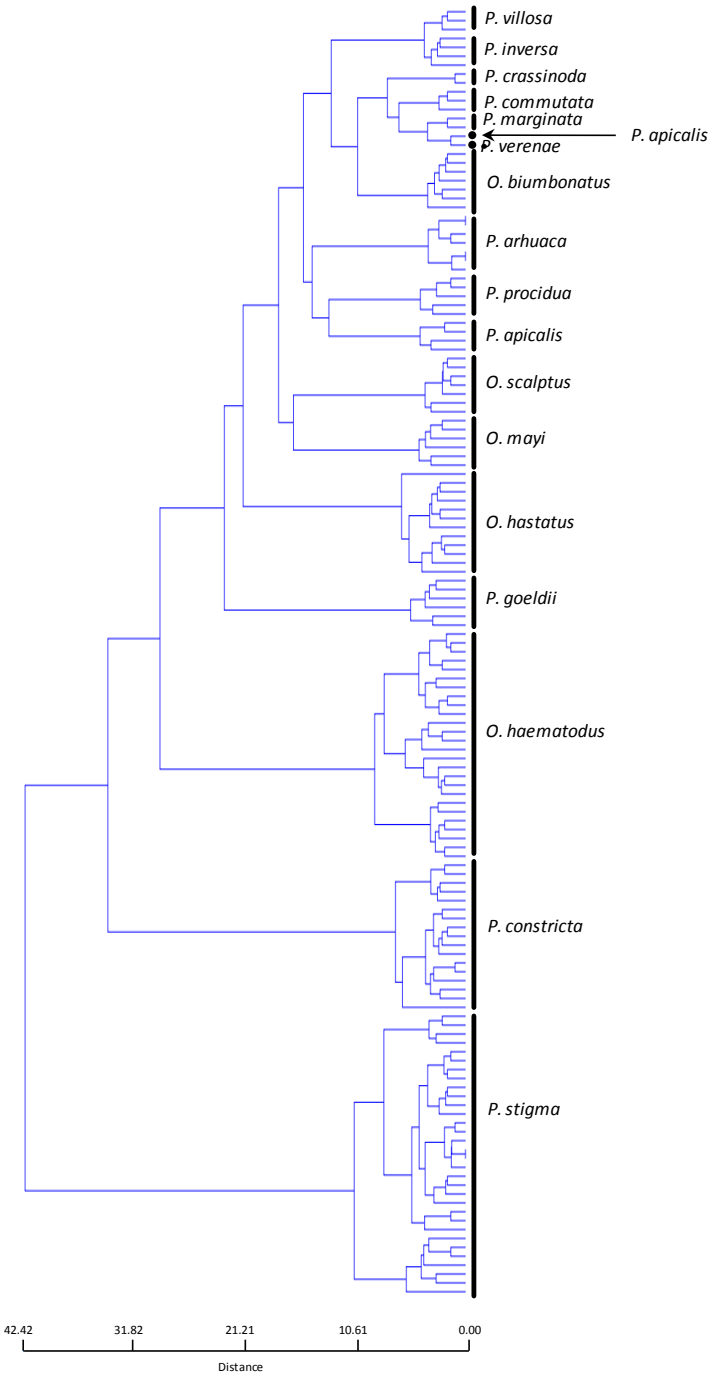

Fig. 8. – Hierarchical cluster analysis of the venom peptide masses belonging to the ponerine ant genera *Odontomachus* (*O.* spp) and *Pachycondyla* (*P.* spp). The black bars in front of each dendrogram indicate the morphological identification of each sample.

Venom samples from the same species displayed high homology and the samples were clustered based on morphological identification. Even the closely related-species *P. villosa* and *P. inversa*, despite sharing some common masses, were separated in the HCA analysis. We noted just a single outlier in Fig. 8, with one venom sample, *P. apicalis* 3 (already noted in section 3.4), which was not grouped within the cluster constituted by the other *P. apicalis* venom samples. It likely represents a distinct *P. apicalis* morph.

Some clusters displayed greater distances between venoms belonging to the same morphological species and could be divided into sub-clusters, highlighting potential cryptic species. This was the case for clusters corresponding to *P. stigma* (Fig. 9B) and *O. haematodus* (Fig. 10B). This is consistent with the hypothesis that *P. stigma* is a species complex widespread in Neotropical regions comprising cryptic species [Ferreira, 2010; Wetterer, 2012] and would indicate that *O. haematodus* may also represent a species complex. This shows that some species cannot be identified through morphological methods alone and that the venom-based approach reveals hidden biodiversity.

3.6 Correlation with genetic analysis

Our biochemical approach was validated since the HCA clusters were perfectly correlated with the clades determined through genetic analysis using the mDNA gene cytochrome *c* oxidase subunit 1 (CO1). Moreover, we found distinct genotypic lineages in *P. apicalis* and *P. stigma* identified through morphological approaches (Fig. 9A and 12A), while in *O. haematodus* the two clades were only marginally distinct (Fig. 10A).

Further examination of mass spectra showed that the *P. apicalis* 3 venom was markedly different from those of other *P. apicalis* (Fig. 11). Consistent with that observation, the NJ phylogenetic tree analysis showed that the genetic distances between *P. apicalis* 3 and other

P. apicalis samples accounted for 12.73 ± 0.1% (*n* = 5; Fig. 12), consistent with the mass spectra differences illustrated in Fig. 11 and Table 2. These results are strongly suggestive that *P. apicalis* should be split into two morphs; Morph 1 containing *P. apicalis* 1, 2, 4 and 5 and Morph 2 containing *P. apicalis* 3.

Similar findings were observed for the *P. stigma* species complex. The two major sub-clusters displayed in the *P. stigma* HCA clusters from the mass spectra matched with the NJ phylogenetic tree from the mDNA CO1 analysis (Fig. 9) in which the genetic distance between clades is about 14.72 ± 1.04% (*n* = 32), allowing them to be separated into two distinct morphs. Although displaying high similarity, a comparison of the mass spectra from the venom of *P. stigma* Morph 1 (*P. stigma* 1–25) with *P. stigma* Morph 2 (*P. stigma* 26–32) showed mass differences enabling us to assign characteristic masses to each morph (Table 2).

The NJ phylogenetic tree inferred from the mDNA CO1 analysis of the *O. haematodus* complex displayed two distinct clades genetically distant by just 3.66 ± 0.32% (*n* = 24; Fig. 10A). The second clade comprised samples *O. haematodus* 9, 10, and 11, all collected from the same location (Chutes Voltaire). However, the separation of clades is poor and not reflected in the venom peptide mass variations in the HCA dendrogram for *O. haematodus* (Fig. 10B). This likely reflects intraspecific variation rather than morph separation.

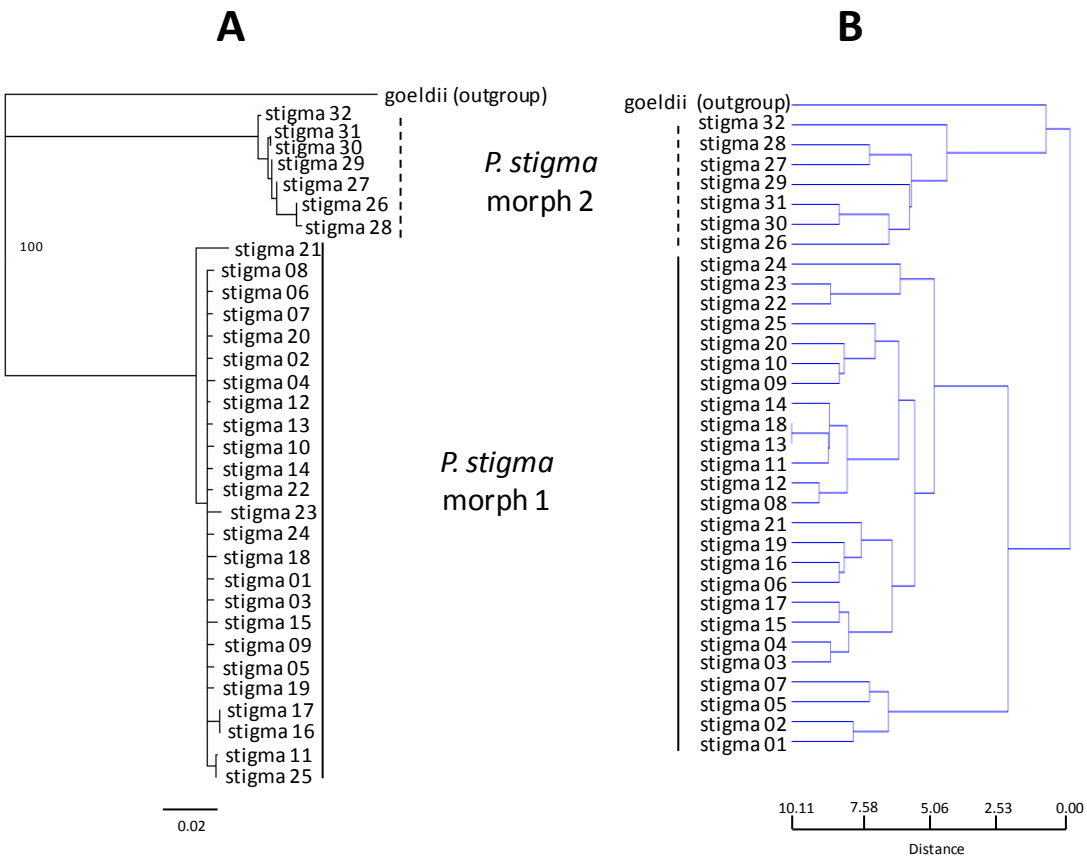

Fig. 9 – Neighbor-joining phylogenetic tree of the mDNA CO1 gene (A) *versus* the HCA dendrogram of venoms (B) for the *P. stigma* samples. The tree is rooted using the *P. goeldii* sequence as an outgroup.

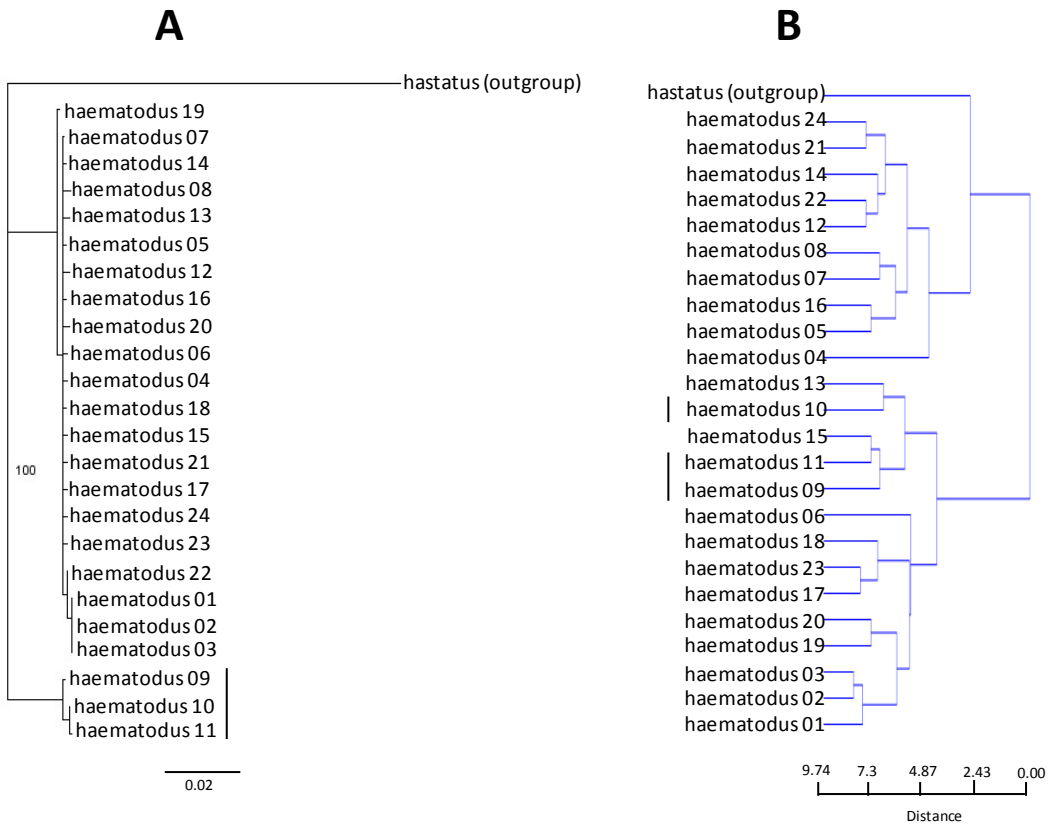

Fig. 10 – Neighbour-joining phylogenetic tree of the mDNA CO1 (A) *versus* the HCA dendrogram of venoms (B) for the *O. haematodus* samples.

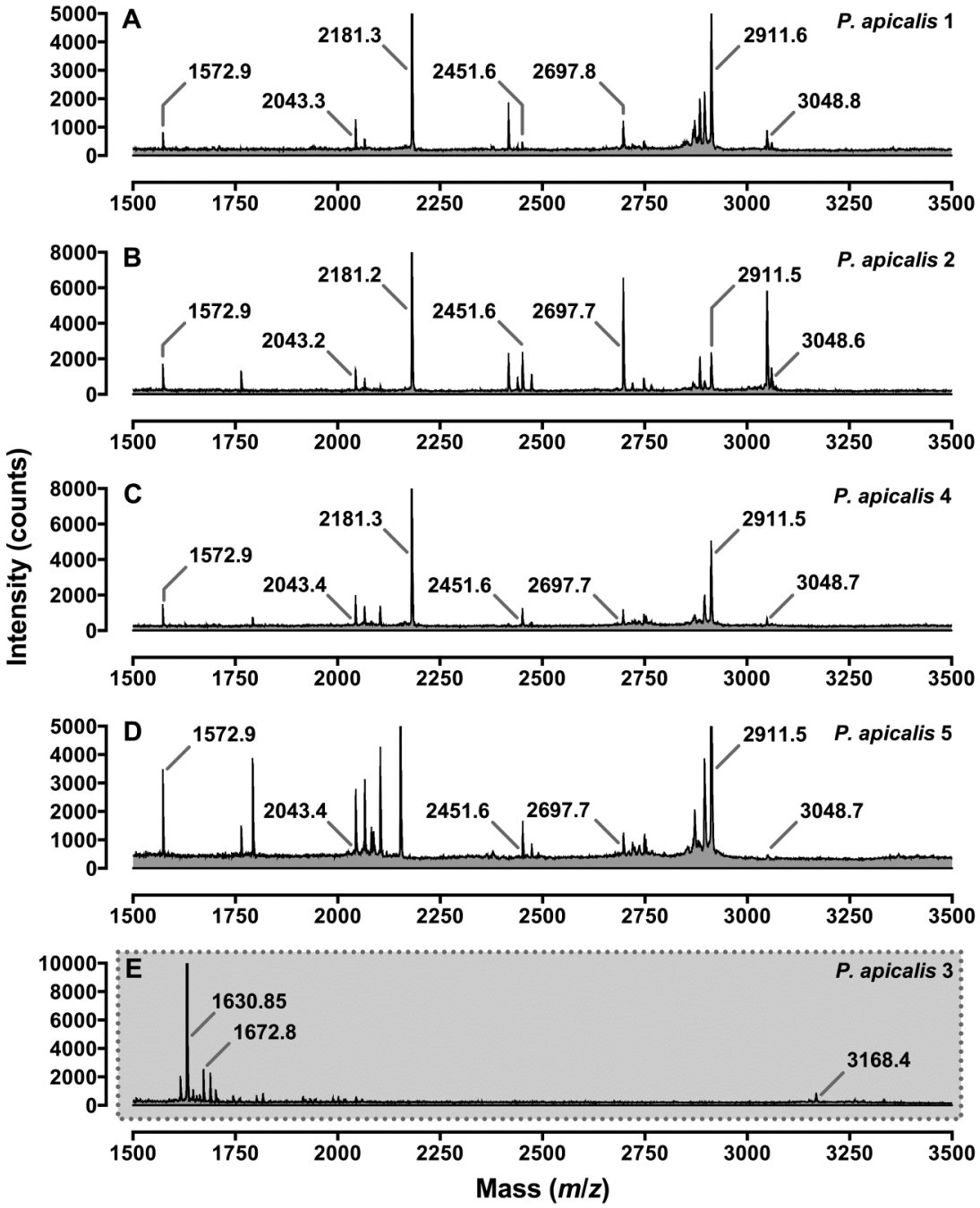

Fig. 11 – Alignment of MALDI-TOF mass spectra from all *P. apicalis* venoms. Note that the mass profile of *P. apicalis* 3 venom (shaded spectra E) is totally different to remaining *P. apicalis* venoms 1, 2, 4 and 5 (A-D).

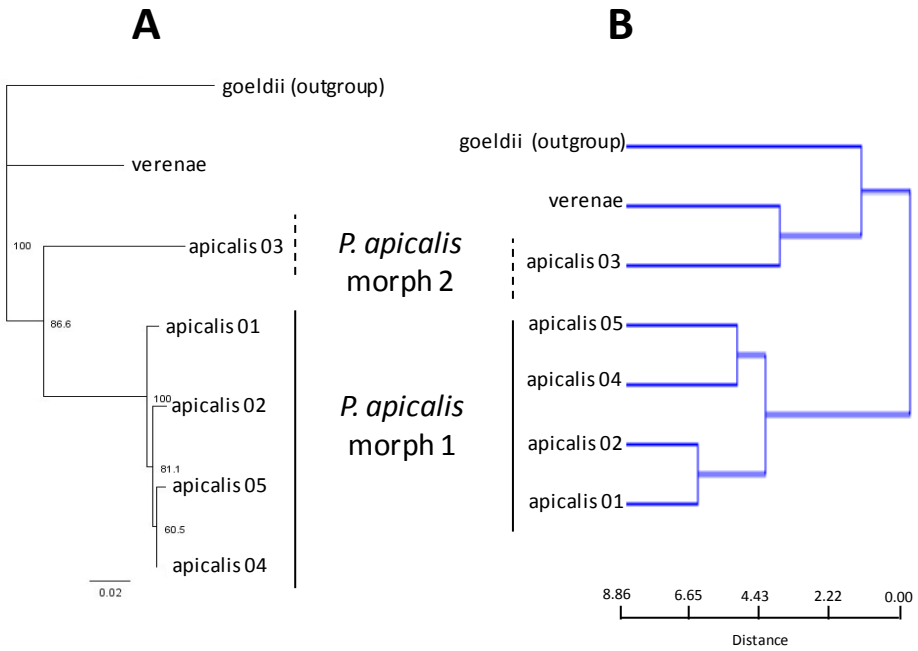

Fig. 12– Neighbor-joining phylogenetic trees comparing the mitochondrial DNA CO1 sequence (A) *versus* the HCA dendrogram of venom peptides (B) of *P. apicalis* species complex samples. Numbers in (A) represent bootstrap values. The tree is rooted using the *P. goeldii* sequence as an outgroup.

4. Discussion

4.1 Ant venom MS analysis

Initial experiments optimized the matrices for the detection of ant venom peptides by MALDI-TOF MS analysis leading us to select two matrix-additive combinations: CHCA-ammonium phosphate and FA-serine. Therefore, in order to obtain optimum MS signals, ant venom peptides appear to require additional sample preparation techniques that differ from standard methods. While CHCA is one of the most commonly employed matrices in MALDI-TOF MS analysis, the addition of ammonium phosphate to suppress alkali metal adducts is uncommon. Furthermore, FA is rarely used in MALDI-TOF MS analysis and its association with serine has not previously been reported. This latter matrix-additive combination is

unique and permitted the acquisition of high intensity spectra even for minute samples such as individual ant venom samples. Both combinations therefore made the acquisition of high quality spectra possible in the automated mode. Using these matrix-additive combinations, it was determined that >99% of the peptides in ponerine ant venoms are in a mass range below 5000 Da, which is the mass range reported to be ideal for both of these matrices [Gusev et al., 1995; Sze et al., 1998].

4.2 The chemotaxonomic approach

Most of the ~9,000 extant stinging ant species are thought to contain large numbers of peptides in their venoms. Given that venom peptides are species-specific gene products, they provide a useful tool for determining the genetic relationship between specimens. In the present study, we optimized the chemotaxonomic method to identify ants based on their venom peptide profiles. Some minor qualitative and quantitative venom variations were revealed between *P. goeldii* individuals from the same colony, possibly due to factors such as age and caste. Indeed the role of the worker caste may change over time [Fresneau, 1994] and the composition of their venom may vary between ants of the same colony. Nevertheless, by pooling venom from at least three workers from each colony, we were able to correctly identify ants based on their venom sample profile. We have shown that MALDI-TOF mass profiles of venoms displayed sufficient qualitative differences between species and enough intraspecific similarity to permit taxonomic discrimination even in closely related species. This chemotaxonomic tool therefore adds a new dimension to biochemical- and genetics-based ant taxonomic methods. The automated MALDI-TOF MS and HCA analysis of venom peptides provides a taxonomic tool that permits the rapid analysis of a large number of samples. While this method has previously been used for the identification of other

venomous species [Escoubas et al., 1997; Escoubas et al., 1999; Escoubas et al., 2002; Escoubas et al., 1998b; Nascimento et al., 2006; Souza et al., 2008; Stöcklin, 1997; Stöcklin et al., 2000], it has never before been tested for any venomous social hymenopteran. Ants belong to the order Hymenoptera, which also includes social wasps and bees, that forms the most speciose venomous taxonomic group with ~120,000 venomous species [van Emden, 2013]. Therefore, MALDI-TOF MS venom profiling can be envisioned as a general chemotaxonomic method for hymenopteran classification and could be extended to thousands of other hymenopteran species because their venoms are mostly composed of proteins and peptides [Argiolas and Pisano, 1985; Baptista-Saidemberg et al., 2011; de Souza et al., 2004; Vincent et al., 2010; Yamamoto et al., 2007].

4.3 Cryptic species

Cryptic species are a major problem in ant taxonomy [Bickford et al., 2007]. The taxonomic challenge posed by cryptic species underlines the importance of using multiple criteria in delimiting species as the validation of a new species requires additional evidence beyond genetic or morphological data only. The use of venom peptide mass profiles therefore provides a powerful systematics tool for solving the complex and challenging task of delimiting ant species. The biochemical approach developed in this study, in parallel with a genetic analysis, permitted us to reveal cryptic ponerine species and to discover undetected ant diversity. This was the case for both *P. apicalis* and *P. stigma* as this study provides evidence of two valid taxa belonging to the *P. apicalis* species complex and also two valid taxa within the *P. stigma* species complex present in French Guiana. Venom peptide mass profiles therefore provide a new and rapid tool for separating morphs among a species complex.

However, in some cases, this tool does not provide enough evidence to separate taxa within species complexes. This was the case for *O. haematodus* where the divergence between clades within the phylogenetic tree was marginally higher than the 3% threshold commonly used for delimiting species in genetic DNA barcoding studies [Song et al., 2008]. This genetic variation is too weak to conclude that a separation exists, particularly concerning differences in the composition of venoms. The observed differences in the mass spectrometry data may thus reflect local environmental factors that subtly influence the expression and levels of peptides in intra-specific analyses of species complexes [Pekár et al., 2012].

In conclusion, the large number of studies assessing the current level of biodiversity on Earth attests to the increasing concern over the rapid loss of that biodiversity. This effort requires additional tools to improve the accuracy of taxonomic identification to complete an inventory of all ant species. As ants constitute a hyperdiverse taxonomic group, they are often difficult to identify through standard morphological means. In the present study, the determination of mitochondrial DNA variation in CO1 supported the observed venom peptide mass differences, validating our contention that venom peptides are a useful chemotaxonomic tool in delimiting ant species. We have developed a powerful tool for use in ant taxonomy that can reveal true ant biodiversity even among closely related species such as cryptic ant species. This is also important if we are to exploit the enormous biodiversity of ant and other hymenopteran venoms as sources for novel drugs or biopesticides. Such a chemotaxonomic approach also opens perspectives on the use of venoms to investigate the phylogenetic relationships between species. Using peptide and protein sequencing a thorough understanding of the origin and evolution of ant venom

toxins is possible, as previously employed for snake venoms [Fry and Wüster, 2004; Juárez et al., 2008].

Acknowledgements

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Chapitre 2. Écologie

Les venins sont des cocktails de toxines et leur composition varie fortement entre les différentes espèces, même si elles sont phylogénétiquement très proches. En plus de cette diversité interspécifique, il a été observé des variations intraspécifiques de la composition des venins chez de nombreux organismes venimeux tels que les serpents, les cônes, les scorpions ou les mygales. Ces variations intraspécifiques sont généralement liées au sexe [Escoubas et al., 1997; Herzig and Hodgson, 2009; Herzig et al., 2002; Herzig et al., 2008], à la géographie [Duda et al., 2009; Núñez et al., 2009; Shashidharamurthy et al., 2002], ou encore à l'ontogénie [Escoubas et al., 2002; Guércio et al., 2006].

La duplication des gènes suivie de la néo-fonctionalisation sont les principaux processus évolutifs généralement évoqués pour expliquer la diversification rapide des venins [Kordiš and Gubenšek, 2000; Wong and Belov, 2012]. Ainsi les nouveaux gènes dupliqués vont accumuler les mutations génétiques non délétères permettant la genèse de nouvelles toxines et de nouvelles fonctions lesquelles seront soumises ensuite à la sélection naturelle et à la dérive génétique. La sélection naturelle va donc au fur et à mesure de l'évolution éliminer les toxines les moins efficaces et conserver les mieux adaptées à l'écologie et aux besoins des espèces venimeuses [Casewell et al., 2013; Fry et al., 2008].

Les fourmis sont des organismes dominants de la planète et elles ont colonisé presque tous les milieux terrestres. Elles montrent ainsi une formidable diversité écologique [Lach et al., 2010; Wilson, 1990]. Il y a peu de doute que cette diversité écologique, via la sélection positive, a joué un rôle important dans la diversification et la sélection des toxines.

- **Chez les fourmis quels sont les facteurs écologiques qui ont joué un rôle dans la diversification des venins au niveau intra et interspécifique ?**

Dans cette partie du projet, nous avons étudié les différences de composition et de toxicité des venins de fourmis en fonction de plusieurs aspects écologiques, leur polyéthisme, et le rôle offensif ou défensif du venin. Dans un premier temps, nous nous sommes intéressés aux variations intraspécifiques par l'étude de la composition peptidique du venin de la fourmi *Odontomachus haematodus* en relation avec le polyéthisme (la division des tâches dans la colonie). La deuxième partie de ce travail a porté sur les différences de composition et toxicité des venins en relation avec le régime alimentaire des fourmis, certaines espèces étant des prédatrices généralistes et d'autres spécialistes. La dernière partie a consisté en l'étude des différences de composition des venins en fonction du rôle offensif ou défensif en prenant comme modèle d'étude trois espèces du genre *Pseudomyrmex*.

1. Venin et polyéthisme

Le succès écologique éclatant des fourmis est en grande partie attribuable à leur mode de vie eusocial [Hölldobler and Wilson, 1990]. Les colonies de fourmis sont des superorganismes, c'est-à-dire des unités composées de plusieurs individus dans lesquelles la division du travail est hautement spécialisée (polyéthisme) [Wilson and Sober, 1989]. Cette vie en société des hyménoptères sociaux est l'une des différences majeures avec les autres animaux venimeux généralement étudiés (serpents, araignées, scorpions et cônes) et elle pourrait avoir influencé la composition des venins. En effet le rôle des fourmis change entre les individus d'une même colonie, et en fonction de ce rôle, l'utilisation du venin peut changer. Chez les fourmis la division du travail peut être associée avec la morphologie et être par conséquent corrélée avec la taille des ouvrières, connue sous le nom de

polymorphisme de caste [Oster and Wilson, 1978]. Quand il n'y a pas de caste morphologique, la division du travail peut être associée avec une caste temporelle où un groupe d'individus est spécialisé dans des tâches basées sur l'âge, c'est le polyéthisme temporel [Fresneau, 1994; Fresneau et al., 1982].

- **Est-ce que la composition des venins varie dans une colonie de fourmis en fonction du polyéthisme ?**

La synthèse des toxines d'un venin représente un fort coût métabolique pour l'organisme. On émet alors l'hypothèse que les fourmis ouvrières qui ont des tâches assignées à l'intérieur du nid (ouvrières nourrices) et donc qui n'utilisent pas leur venin pour chasser, tout comme les reines, vont avoir un venin moins complexe, ou tout au moins différent de celui des ouvrières chasseuses.

Afin de tester cette hypothèse nous avons étudié la composition peptidique du venin de l'espèce *Odontomachus haematodus* en fonction du polyéthisme (article 3). Dans cette étude nous avons séparé les ouvrières de plusieurs colonies en fonction de leur rôle de nourricières ou de fourrageuses. Deux autres groupes composés des reines fécondées et des reines non fécondées sont venus compléter l'étude. Les résultats n'ont pas montré de différences dans la composition des venins en relation avec le polyéthisme pour cette espèce, suggérant que le polyéthisme n'a pas d'effet sur la composition des venins. Des études précédentes ont néanmoins démontré que les poches à venins des nourrices sont moins remplies que celle des fourrageuses chez les espèces *Harpegnathos saltator* [Haight, 2012] et *Neoponera commutata* [Schmidt and Overal, 2009]. Le coût métabolique du venin

semble donc être diminué chez les nourrices en produisant moins de venin mais avec la même composition que celui des fourrageuses.

Chez *Odontomachus haematodus*, des variations de composition des venins ont cependant été observées entre les différentes colonies et particulièrement entre les colonies provenant de zones géographiques différentes. De telles observations avaient précédemment été faites entre des colonies géographiquement distantes de la fourmi *Dinoponera quadriceps* [Cologna et al., 2013]. Ces auteurs expliquent ces variations par une adaptation aux proies locales. Les variations intraspécifiques de composition des venins pourraient induire des variations d'efficacités contre les différentes proies. Ainsi ces variations intercoloniales des venins apparaissent comme un pré-requis nécessaire à la sélection naturelle et la diversification des toxines.

Article 3

Intraspecific variation of the venom peptidome of the ant *Odontomachus haematodus* (Formicidae: Ponerinae)

Axel Touchard, Alain Dejean, Pierre Escoubas, Jérôme Orivel

Peptides (Article en préparation)

For submission to *Peptides*

Intraspecific variations in the venom peptidome of the ant *Odontomachus haematodus* (Formicidae: Ponerinae)

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Abstract

Ant venoms are complex cocktails of toxins employed to subdue prey and to protect the colony from predators and microbial pathogens. Previous studies have revealed the presence of numerous bioactive peptides in most stinging ant venoms, although the extant of ant venom peptide diversity remains largely unexplored. In this study, we investigated the venom peptidome of *Odontomachus haematodus* using LC-MS analysis and then verified whether the division of labor in the colonies and their geographical location are correlated with venom composition changes. Our results reveal that *O. haematodus* venom is comprised of 105 small linear peptides. Venom composition does not differ between the different castes (i.e., nurses, foragers and queens), but an intraspecific variation in peptides contents was observed, particularly when the colonies are separated by large distances. Geographical variation appears to increase the venom peptide repertoire of this ant species, showing intraspecific venom plasticity.

1. Introduction

There are ca. 9100 stinging species of ants belonging to 16 different subfamilies. Moreover, ants constitute 15-20% of the animal biomass in tropical forests [Hölldobler and Wilson, 1990] which makes them one of the most abundant venomous animal taxa on Earth due to their ubiquity in terrestrial environments. Ants use complex venoms containing a variety of toxins to paralyze prey, ward off predators and protect their colony from microbial pathogens [Orivel et al., 2001; Schmidt, 1982]. Ant venoms therefore represent a promising source of unique bioactive molecules with original scaffolds and potentially novel pharmacological activities. Although ant venoms remain little studied, previous work by our group and others [Aili et al., 2014; Touchard et al., 2014a; Touchard et al., 2015] have demonstrated that venoms of stinging ants are essentially composed of small peptides, similarly to spiders, scorpions, and cone snails. Indeed venoms from ponerine ants have been shown to be rich in small linear peptides [Aili et al., 2014; Touchard et al., 2014a] and peptidic toxins from several subfamilies such as the Paraponerinae [Piek et al., 1991a], Ponerinae [Cologna et al., 2013; Johnson et al., 2010; Orivel et al., 2001], Ectatomminae [Arseniev et al., 1994; Pluzhnikov et al., 2000], Myrmicinae [Rifflet et al., 2012], Myrmeciinae [Inagaki et al., 2004; Inagaki et al., 2008a] and Pseudomyrmecinae [Pan and Hink, 2000] have been characterized.

One of the major issues in biochemical and pharmacological studies is the reproducibility of experiments, which requires accurate species identification. At the species level, peptide fingerprints of venoms are reliable chemotaxonomic markers for ant species determination and possibly the discrimination of unelucidated species complexes [Touchard et al., 2014a]. However, intraspecific variations can also occur as shown recently in

Dinoponera quadriceps for which only 48 peptides were shared between colonies, out of more than 300 peptides found in whole venom [Cologna et al., 2013]. In other venomous species, such as snakes, scorpions, tarantulas and cone snails, intraspecific variations in the venom composition has been observed for some species and been linked to geographical distribution [Núñez et al., 2009; Shashidharamurthy et al., 2002], age [Escoubas et al., 2002]or sex [Escoubas et al., 1997; Herzig and Hodgson, 2009; Herzig et al., 2002; Herzig et al., 2008]. However, it remains unclear whether such variation is a common denominator of all venomous animals or remains restricted to some species. There is abundant debate among specialists about this key point, since sampling conditions are often limiting and species-wide broad surveys of venom composition are in general not possible.

Since in hymenopteran colonies only the females are venomous, sex can be excluded as a factor of variation. Therefore, intracolonial variations in venom composition could possibly be related to geographical distribution, diet, age or division of labor (polyethism). In ants, reproduction is carried out by the queen(s), while all other tasks are performed by the workers. The division of labor amongst workers is based on physical castes, or most often, on age [Fresneau, 1994]. Usually, the youngest workers are involved in intranidal activities, whereas older workers are assigned to tasks outside the nest such as defense and foraging [da Silva-Melo and Giannotti, 2012; Sendova-Franks and Franks, 1999; Wilson, 1963].

As the venom is mostly used by workers performing extranidal activities, one can hypothesize that polyethism could affect venom composition. To test this hypothesis, we investigated intracolonial variations in venom composition in the Neotropical ponerine species *Odontomachus haematodus*. The monomorphic workers of this species possess a peptide-rich venom [Touchard et al., 2014a] used in colony defense and prey capture. To assess possible venom variations, we characterized the whole venom peptidome by

combining HPLC chromatographic separation with offline MALDI-TOF mass spectrometry analysis and explored the putative differences in venom composition between castes (i.e., nurses, foragers and queens) and colonies from different geographical locations by comparing venom peptide fingerprints.

2. Materials and methods

2.1. Ant collection and taxonomy

Odontomachus haematodus colonies were collected from three different areas in French Guiana: six colonies were collected on the *Campus Agronomique*, Kourou; three in Sinnamary; and one in Angoulême (Fig. 1). Voucher specimens were deposited in the *Laboratorio de Mirmecologia*, Cocoa research Centre, Ilhéus, Bahia, Brazil. In the laboratory, the ant colonies were conserved in artificial nests made of plastic boxes (11 cm X 11 cm x 6 cm) filled with 2 cm of plaster to create chambers, and covered by a plate of red glass. These boxes were connected to a foraging arena consisting of a second, similar plastic box without plaster. Colonies were kept a 25°C and provided with dead mealworms and honey twice a week.

2.2. Behavioral observations and ants grouping

To investigate the division of labor between workers, individual ants were marked with dots of paint on their thoraxes and gasters. Worker tasks were determined by scan sampling their behavior (three scans per day at 9 am, 2 pm and 5 pm; 5 days per week over 3 weeks).Each time, the identity of the workers present either in the foraging area or inside the nest was recorded. Then the percentage of presence in the foraging area of each

individual over the three weeks period was calculated to define behavioral groups. Workers that had either never been seen in the foraging area or been there between 0% to 25% of the time were considered nurses ([group 0%] and [group 25%], respectively). Those observed between 25% to 50% of the time in the foraging area were considered intermediates [group 50%], and those observed between 50% to 75% or between 75% to 100% of the time in the foraging area were considered foragers ([group 75%] and [group 100%], respectively). Moreover, winged females present in the colonies were named “virgin queens” in order to differentiate them from “reproductive queens” devoid of wings (see Fig. 1).

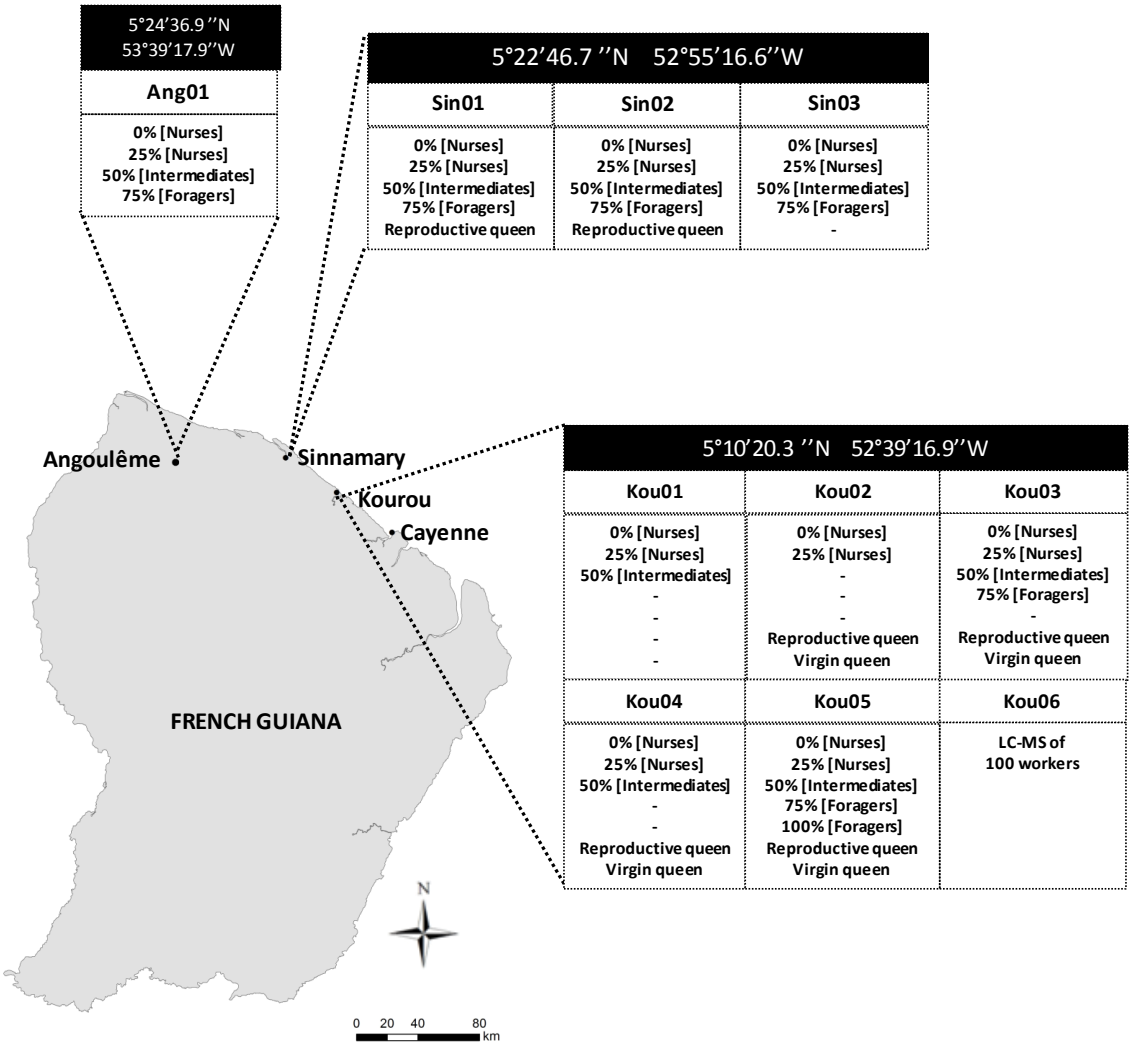

Fig. 1- Sites where the 10 *Odontomachus haematodus* colonies were collected in French Guiana. Table panels show information about each colony, including GPS coordinates, colony code and the different behavioral groups. One hundred dissected workers from colony Kou06 were used for LC-MS investigation.

2.3. Venom analysis

Ants were killed by freezing at -20°C prior to dissecting their venom glands. The venom glands were dissected and stored in 10% acetonitrile (ACN)/water (v/v). Then, samples were centrifugated for 5 min at 14,400 rpm and the supernatant was collected and lyophilized prior to storage at -20°C for subsequent biochemical analysis To study intra-colonial variations and the influence of the role of individual ants in the colony, five venom glands

from each behavioral group (cf. Fig. 1) were dissected and pooled for each colony. Furthermore, 100 venom glands from the workers of one colony (Kou06; 3.55mg of dry crude venom in total) were dissected to carry out an in-depth exploration of the whole venom by LC-MS.

2.4. Chromatographic separation

In order to fully explore the peptidome of *O. haematodus*, a venom sample pooled from 100 workers was fractionated using C18 reversed-phase high performance liquid chromatography (RP-HPLC). The separation of venom peptides was performed using a Waters Xterra-C18 5µm, 2.1 x 100 mm column on an Agilent HP 1100 HPLC system. Fractionation was achieved using a gradient of solvent A (water / 0.1% trifluoroacetic acid TFA) and solvent B (ACN / 0.1% TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0-60% for 60 min, 60-90% for 10 min and 90-0% for 15 min, at a flow rate of 0.3 mL/min. The absorbance of the column effluent was monitored at 215 nm on a diode-array detector. Signal was monitored in real time, and fractions collected manually for each eluting peak. Individual fractions were then dried and reconstituted in 50µL of water/0.1% TFA for subsequent off-line MALDI-TOF MS analysis and disulfide bonds reduction.

2.5. Chemical reduction of disulfide bonds

To map the distribution of disulfide bonds in venom peptides, 5µL of each fraction were incubated in 10µL of reducing buffer (100 mM Tris, pH 8, 6M guanidine) with 10 mM dithiothreitol (DTT) for 1h at 56°C in darkness. The reaction was stopped by the addition of 5µL of water / TFA 0.1%. Prior to mass spectrometry analysis, reduced fractions were desalted using Ziptip® C18 (Millipore) pipette tips. Chemical reduction results in a mass

increase of 2 Da for each disulfide bond. Comparison of mass shifts between native and reduced venom fractions allowed determination of the presence and number of disulfide bonds for each each peptide.

2.6. Mass spectrometry analysis

Mass spectrometry analyses were performed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems; CA, USA) using α-cyano-4-hydroxycinnamic acid (CHCA) dissolved at 5 mg/mL in a solution of water/ACN/TFA (50/50/0.1 v/v/v) as matrix. Prior to MS analysis, crude venoms were desalted using Ziptip® C18 (Millipore) pipette tips. Then, 1µL of each reconstituted HPLC fraction or desalted crude venom was deposited on the MALDI target plate followed by 1 µL of the matrix. Each spectrum was calibrated externally using a mixture of peptides of known molecular masses in the same *m/z* range (Peptide calibration Mix 4, LaserBio Labs, Sophia-Antipolis, France). External calibration was performed by depositing adjacent to each sample, 0.5 µL of the calibration mixture co-crystallised with 0.5 µL of CHCA matrix. All spectra were acquired in reflector mode to maximise accuracy of mass determination. Spectra were collected over the *m/z* 500–10,000 range in positive ion mode (200 shots per spectrum) and were automatically calibrated using the sequence module of the Voyager® control software (Applied Biosystems, USA).

2.7. Data analysis

The spectra were subjected to baseline correction (0.7 correlation factor) and Gaussian smoothing (5-point filter width) using Data Explorer® 4.11 software. Potential sodium and potassium adducts were manually removed from all mass lists. Masses matching within ± 1.0 Da were defined as identical peptides in this study. Identical masses in adjoining HPLC

fractions, which were interpreted as reflecting an incomplete separation, were also removed. Two-dimensional scatter plots, termed “2D venom landscapes”, were constructed using SigmaPlot 12.0 software. All peptide masses detected in the HPLC fraction spectra were plotted in dot graphs with masses on the y-axis and RP-HPLC elution time on the x-axis. A Principal Component Analysis (PCA) of the relative abundance of peptides from the venom MS spectra was performed using PAST 3.02 software.

3. Results

3.1 Venom peptidome analysis

LC-MS analysis of *O. haematodus* venom revealed the presence of 105 peptides (Table 1). All peptides are small, falling within a narrow mass range of *m/z* 777.49 to 2978.5 ($M+H^+$) (Fig. 2A and B). We estimated that the number of residues varied between 7 and 27 based on a theoretical estimate of MW_{av} of 111.1254 Da for an average amino acid (Averagine). The value is derived from the statistical occurrence of amino acids in proteins [Senko et al., 1995], and calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}S_{0.0417}$. All the peptides eluted between 15 % and 45%ACN (retention time 20-50 min), with the most abundant peptides in the venom eluting between 35% and 45% of ACN (retention time 40-50 min) (Fig. 2C).

Mass analysis of the chemically reduced HPLC fractions did not show any mass shift between native and reduced peptides, demonstrating that *O. haematodus* venom is exclusively composed of linear peptides (i.e. devoid of disulfide bonds).

Table 1. Mass list of peptides (*m/z*) from *O. haematodus* venom collected at Kourou (Kou06).							
777.49	800.84	809.31	822.3	877.45	888.6	890.5	932.5
937.4	945.4	950.49	973.73	987.76	1003.5	1008.4	1010.5
1016.43	1044.73	1047.5	1058.75	1064.7	1078.65	1107.79	1283.67
1127.6	1130.56	1134.57	1186.93	1194.65	1248.6	1274.79	1383.67
1316.85	1320.81	1358.8	1360.6	1370.7	1375.7	1376.78	1380.62
1421.7	1429.9	1447.8	1473.87	1497.87	1500.92	1518.84	1522.8
1694.07	1714.04	1725.12	1729.92	1731.93	1756.06	1774.1	1792.07
1803.06	1818.96	1854.07	1857.9	1863.2	1872.07	1906.24	1917.09
1933.01	1963.01	1968.14	1978.03	1979.02	2010.27	2020.02	2044.97
2048.02	2063.09	2078.04	2079.15	2086.73	2088.3	2096.12	2111.17
2117.24	2137.08	2139.08	2157.2	2245.25	2254.22	2272.47	2430.3
2448.2	2461.28	2473.3	2515.39	2590.34	2637.36	2655.33	2766.48
2784.62	2785.8	2789.47	2790.4	2802.38	2805.4	2944.5	2960.52
2978.5							

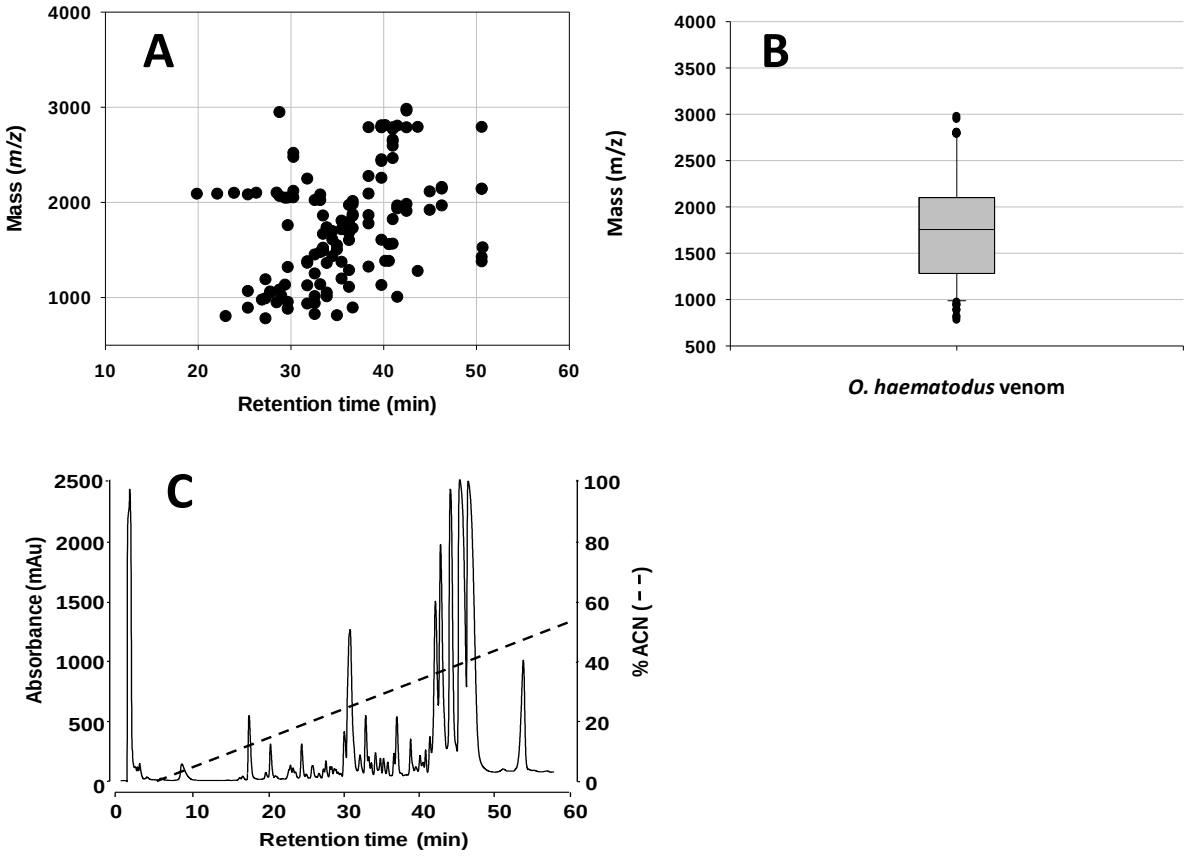

Fig. 2- Investigation of the whole *Odontomachus haematodus* venom peptidome by LC-MS. (A) Two-dimensional landscape of the venom. Black spots indicate peptides. (B) Box-and-whisker plot of the peptide mass distribution presented in the 2D venom landscape. The bottom and top end of the box represent the first and third quartiles, respectively, while the line inside each box represents the median mass. The ends of the whiskers represent the 5-95 percentile range while the black dots represent masses outside the 5-95 percentile range. (C) C18 RP-HPLC chromatogram of the venom. The black dotted line shows the slope of the ACN gradient.

3.2 Venom peptidome variations

We collected 43 venom samples from the nine *O. haematodus* colonies monitored: 18 venoms from nurses; eight from intermediates, seven from foragers, six from fertilized queens and four from virgin queens. MALDI-TOF MS peptide mass fingerprinting of these 43 crude venoms resulted in the selection of the 20 characteristic peptides masses (i.e. showing the most abundant signals) which constituted the matrix used for the principal component statistical analysis (PCA) (*m/z* 1842.6, 1861.1, 1916.91, 1962.89, 2019.91, 2044.69, 2062.27, 2086.03, 2095.89, 2117.03, 2219.16, 2245.32, 2387.28, 2473.22, 2515.32, 2590.18, 2679.79, 2784.34, 2789.25, 2802.39).

PCA based on the relative abundance of the selected peptides revealed that the first two principal components accounted for 68.1% of the variance (Fig. 3).The venoms from Angoulême, which contained two specific peptides (*m/z* 1861.1 and 2062.27), were separated from those from the two other localities, and differed only by the relative proportions of the mass 2019.91 *m/z*(Fig. 3).

PCA showed that the venom composition was not related to castes (Fig. 3). Indeed, venoms from different behavioral groups showed similar patterns, indicating that polyethism and reproductive status did not affect the peptidic composition of the venoms (Fig. 5). Yet, some qualitative intracolonial differences were noted in the nurse caste (Fig. 4).

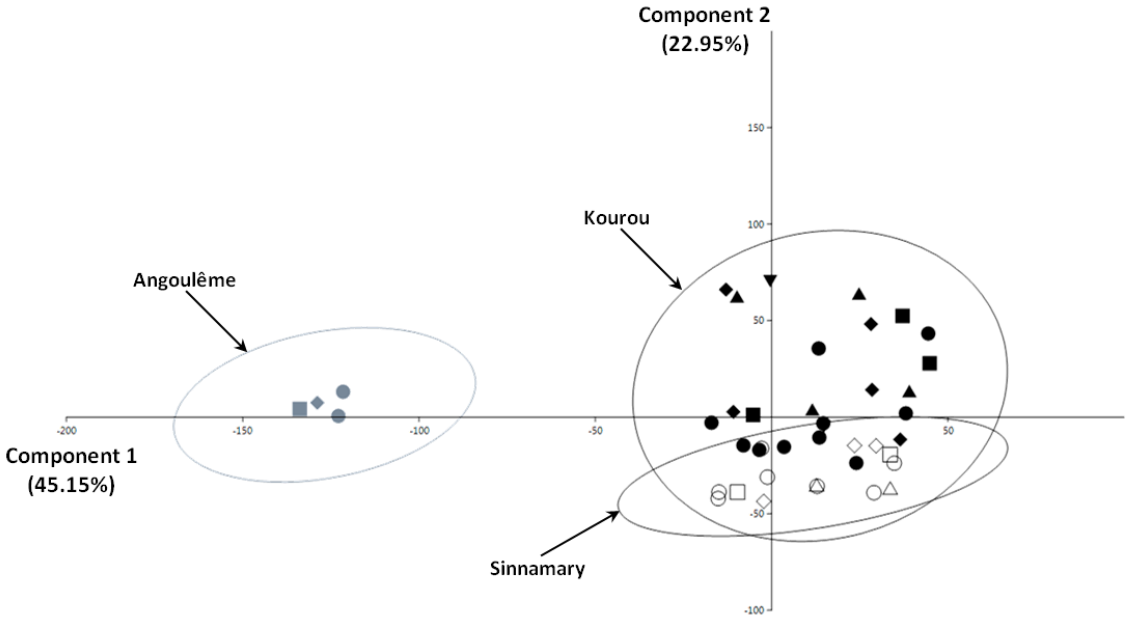

Fig. 3- Ordination diagram based on the principal components of the relative abundance of peptides from 43*Odontomachus haematodus* venoms (PAST 3.02 software). Black-filled symbols represent ant venoms from Kourou, empty symbols represent ant venoms from Sinnamary and grey symbols are venoms from Angoulême. The 95% confidence ellipses are displayed. The different behavioral groups and casts are shown by the following symbol shapes: dot [Nurses]; diamond [intermediates]; square [foragers]; triangle [reproductive queens] and inversed triangle [virgin queens].

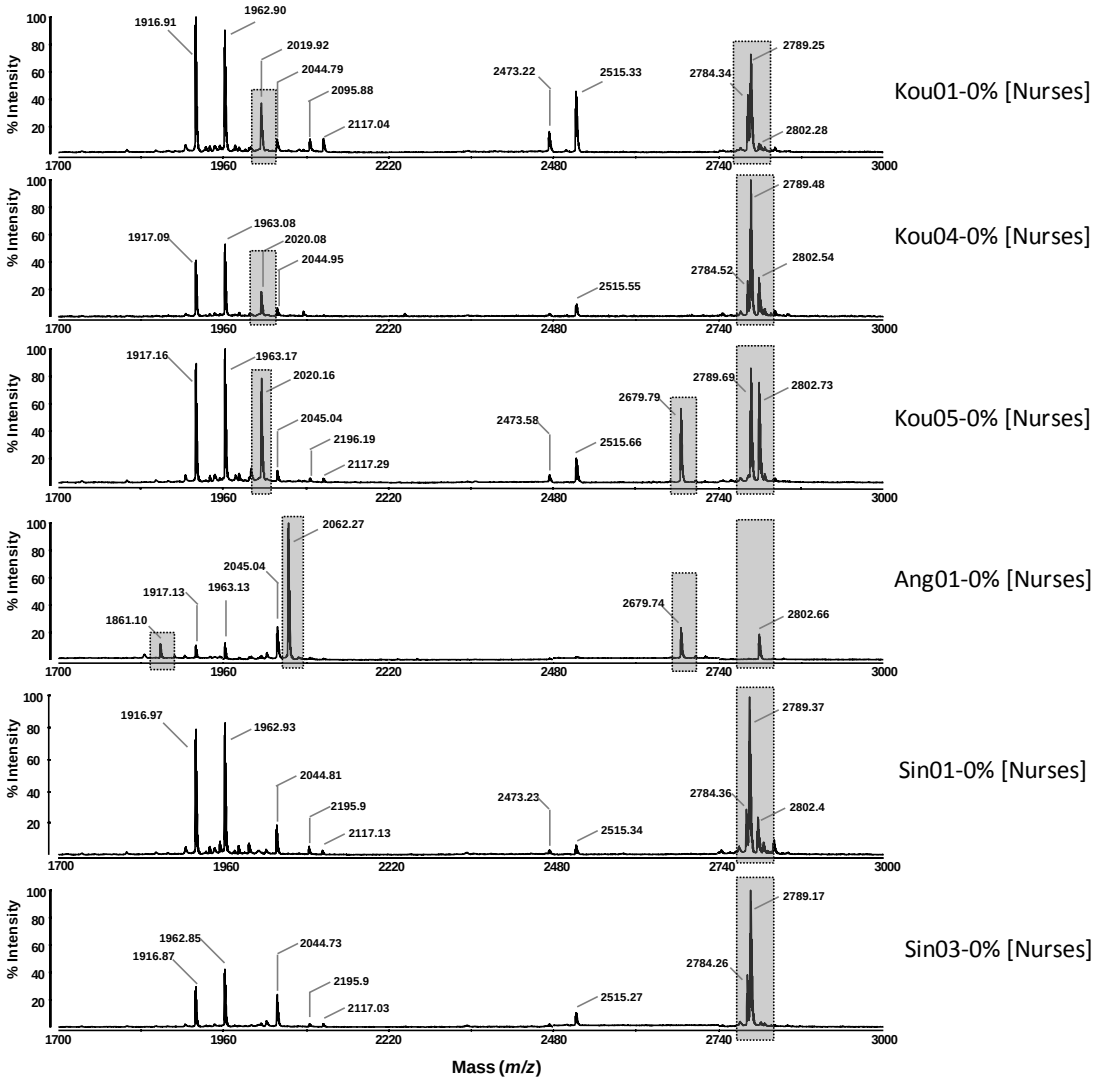

Fig. 4- Inter-colonial mass spectra variation among the nurses [0% group]. Shaded areas highlight major mass variations among the different *Odontomachus haematodus* colonies.

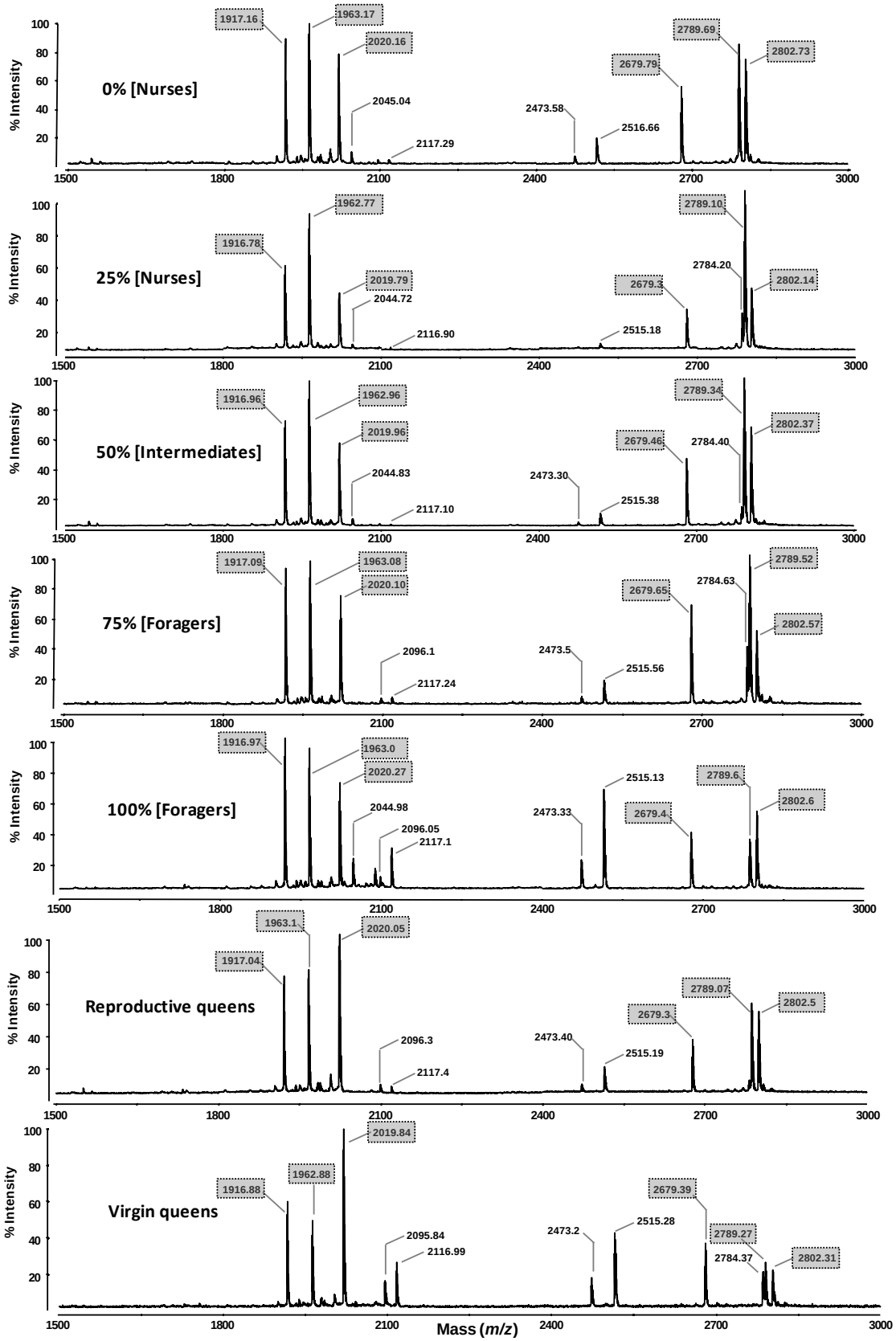

Fig. 5- Intra-colonial mass spectra variations among the different behavioral groups of colony Kou05. Few qualitative variations can be observed and many dominant peptides were present in all groups, particularly the shaded masses.

4. Discussion

Ant venoms are complex cocktails of peptides which have evolved to act on multiple biological targets. By combining MALDI-TOF MS with chromatographic separation, we have shown that *O. haematodus* venom peptidome is composed of more than one hundred small and linear peptides in the 700-3000 *m/z* mass range. This feature is consistent with a previous study on five Neotropical *Odontomachus* species venoms [Touchard et al., 2014a] as well as wasp and cone snail venoms which usually contain less than 35 residues [Baptista-Saidemberg et al., 2011; Cologna et al., 2013; de Souza et al., 2004; Gomes et al., 2014; Johnson et al., 2010; Lewis et al., 2012; Orivel et al., 2001]. In contrast, spider, scorpion or snake venoms possess peptides containing typically between 40 and 100 amino acids [Olivera et al., 1990].

Because these toxins are associated with the metabolic cost of venom production, we hypothesized that ants dedicated to tasks inside the nest, typically nurses and queens, would possess less complex venoms than foragers, the latter using their venom to subdue prey and deter enemies. Yet, our results show that the venom composition does not differ between nurses, intermediates, foragers and even queens in *O. haematodus*. As such, the toxicity of *Neoponera commutata* (Ponerinae) worker venoms was not related to age or task specialization, but the workers from different behavioral castes contain different amounts of venom in their reservoir[Schmidt and Overal, 2009]. Also, *Harpegnathos saltator* (Ponerinae) callow workers have empty venom sacks, and workers dedicated to tasks inside the nest have lower amounts of venom than older ones [Haight, 2012]. Therefore, it is likely that in most Ponerinae, nurses limit the metabolic cost of venom by producing lower amounts, but with the same peptidic composition as the foragers. These results contrast with the case of

fire ants whose alkaloid venom composition changes with the size and age of the workers [Deslippe and Guo, 2000; Haight and Tschinkel, 2003]. Also, *Neoponera commutata* (Ponerinae) and *Pogonomyrmex* spp. (Myrmicinae) queens produce less venom than do workers and their venom is significantly less lethal and paralytic than that of the workers, suggesting differences in venom composition [Schmidt and Overal, 2009; Schmidt and Schmidt, 1985].

The differences in venom composition seem rather associated with geographic variations. Indeed, *O. haematodus* venom peptide fingerprints clearly differed between colonies, particularly if they come from locations separated by large distances. Such inter-colonial variations have previously been reported for *Dinoponera quadriceps* (Ponerinae) collected from different areas in Brazil [Cologna et al., 2013].Among animal venoms, intraspecific variations related to geography are a common phenomenon and have been reported in snakes [Shashidharamurthy et al., 2002], cone snails [Duda et al., 2009], scorpions [Omran and McVean, 2000], spiders [Escoubas et al., 1998a] and both social and [Dias et al., 2014] parasitoid wasps [Poirié et al., 2014]. In snakes, intraspecific variations have been shown to exhibit differential venom effectiveness towards different prey [Casewell et al., 2013].This may be the result of allelic variations in the genes coding the peptides as shown in *Conus ebraeus* venom [Duda et al., 2009], increasing the venom peptidic diversity in this species. This intraspecific diversity was essential for natural selection and ant venom diversification.

Acknowledgments

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2. Spécialisation trophique

L'évolution rapide des toxines des venins est souvent expliquée comme le résultat d'une « course aux armements » entre les prédateurs venimeux et leurs proies [Casewell et al., 2013; Jansa and Voss, 2011]. La variation des proies est donc présumée être le principal facteur affectant la diversification et la sélection des toxines des animaux venimeux. Plusieurs études ont montré que chez les serpents et les cônes, la composition et la toxicité de leur venin sont parfaitement adaptées à leur écologie trophique [Barlow et al., 2009; Daltry et al., 1996; Duda et al., 2009; Remigio and Duda Jr, 2008].

Les fourmis sont parmi les principaux prédateurs d'arthropodes dans tous les écosystèmes terrestres et elles utilisent largement leur venin pour paralyser et capturer des proies appartenant à divers taxa [Brady et al., 2006; Maschwitz et al., 1979]. La grande majorité des fourmis prédatrices ont des régimes alimentaires généralistes et consomment un large panel d'invertébrés. Il existe cependant certaines espèces de fourmis aux mœurs alimentaires spécialisées qui chassent strictement un certain type de proies tel que des termites, des collemboles, des fourmis, ou encore des myriapodes [Cerdá and Dejean, 2011].

- **Les fourmis qui ont des régimes alimentaires très spécialisées ont-elles des venins spécifiquement adaptés à leurs proies naturelles ?**

Pour répondre à cette question nous avons étudié la composition et l'effet des venins de quatre espèces de fourmis (Figure 8) aux régimes alimentaires distincts (Tableau 1).

Tableau 1. Liste des espèces étudiées et de leur régime alimentaire

Espèces	Régime	Proie
Neoponera commutata	Spécialisée	*Syntermes* sp. [Mill, 1984]
Neoponera goeldii	Généraliste	Divers arthropodes
Odontomachus haematodus	Généraliste	Divers arthropodes
Eciton burchelli	Spécialisée	Principalement autres hyménoptères

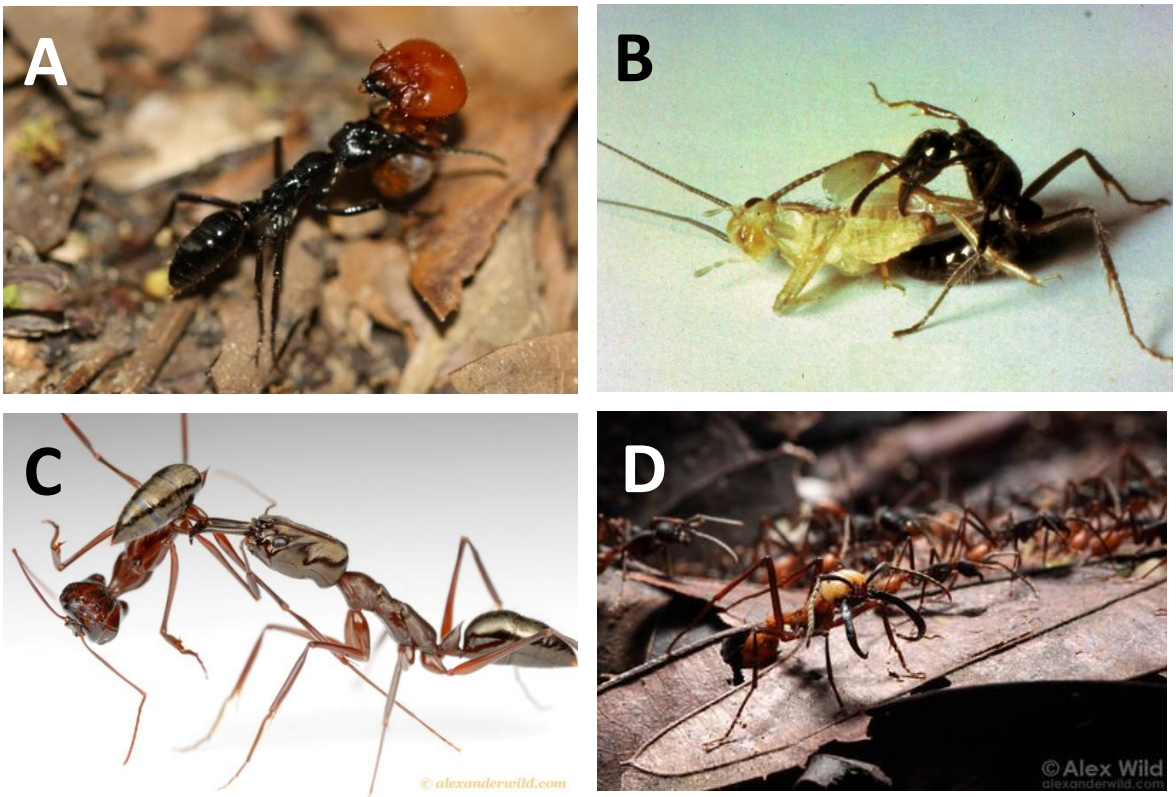

Figure 8- Photographies des quatre espèces de fourmis étudiées pour la spécialisation trophique. A) *Neoponera commutata* avec un termite *Syntermes* sp. capturé (photo V. Cabrol). B) *Neoponera goeldii* qui utilise son aiguillon pour paralyser un grillon (photo J. Orivel). C) *Odontomachus haematodus* (photo A. Wild). D) *Eciton burchelli* (photo A. Wild).

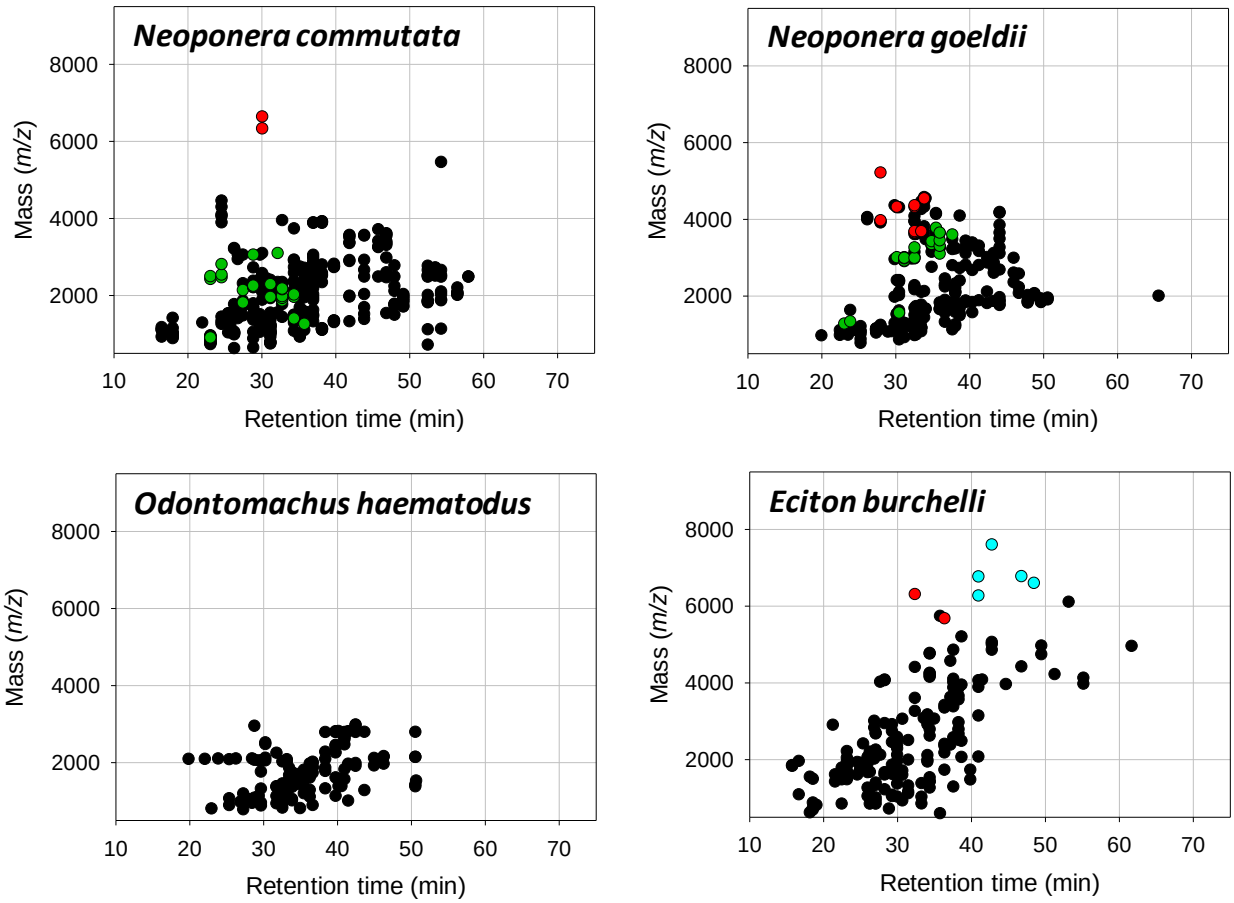

Figure 9- Cartographie des peptides des venins des quatre espèces de fourmis par LC/MS. Chaque point représente un peptide en fonction de sa masse moléculaire et de son temps de rétention. Les points noirs correspondent aux peptides linéaires (sans pont disulfure), les points verts correspondent aux peptides avec un pont disulfure, les points bleus correspondent aux peptides avec deux ponts disulfure et les points rouges sont les peptides réticulés par trois ponts disulfure.

L'analyse de la composition peptidique de ces quatre venins a été effectuée par LC/MS couplée en parallèle à une expérience de réduction chimique afin de révéler les peptides à ponts disulfure. On observe d'importantes variations dans les peptidomes et cela même entre les deux espèces phylogénétiquement très proches du genre *Neoponera*. Les peptides dans les venins varient en nombre (356 pour *N. commutata* ; 288 pour *N. goeldii* ; 105 pour *O. haematodus* et 174 pour *E. burchelli*), en taille, en hydrophobicité et en nombre de ponts disulfure (Figure 9).

La toxicité de ces venins a ensuite été testée sur différentes proies ; *Syntermes* sp. (termite), *Nasutitermes* sp. (termite), *Acheta domesticus* (grillon domestique), *Camponotus atriceps* (fourmi) et *Tenebrio molitor* (larve de coléoptère).

Tableau 2. Dose effective 50% (DE_{50}), dose létale 50% (DL_{50}) et capacité paralysante (CP) des quatre venins sur les différentes proies. Le poids sec moyen des venins par glande à venin est indiqué entre parenthèses pour chaque espèce.

Proie	*Neoponera commutata* (362 µg / fourmi)				
	DE_{50} (0.5 h) µg/g	DE_{50} (2 h) µg/g	DL_{50} (24 h) µg/g	CP (0.5 h) mg/piqûre	CP (0.5 h) proie/piqûre
***Syntermes* sp.**	93.11	71.3	-	3890	84
***Nasutitermes* sp.**	203.7	88.92	-	1770	78
Camponotus atriceps	282.16	204.18	244.51	1280	945
Acheta domesticus	82.83	79.8	144.54	4370	204
Tenebrio molitor	212.52	242.72	328.7	1700	81

Proie	*Neoponera goeldii* (49.6 µg / fourmi)				
	DE_{50} (0.5 h) µg/g	DE_{50} (2 h) µg/g	DL_{50} (24 h) µg/g	CP (0.5 h) mg/piqûre	CP (0.5 h) proie/piqûre
***Syntermes* sp.**	287.27	128.38	-	172	3
***Nasutitermes* sp.**	406.26		-	122	65
Camponotus atriceps	575.3	434.91	462.38	86	6
Acheta domesticus	-	-	-	-	-
Tenebrio molitor	321.95	330.29	794.32	154	7

Proie	*Odontomachus haematodus* (29.7 µg / fourmi)				
	DE_{50} (0.5 h) µg/g	DE_{50} (2 h) µg/g	DL_{50} (24 h) µg/g	CP (0.5 h) mg/piqûre	CP (0.5 h) proie/piqûre
***Syntermes* sp.**	> 400	> 400	-	-	-
***Nasutitermes* sp.**	595.66	592.92	-	50	27
Camponotus atriceps	656.14	614.75	409.26	45	3
Acheta domesticus	-	-	-	-	-
Tenebrio molitor	378.18	398.16	267.3	78	4

Proie	*Eciton burchelli* (47.1 µg / fourmi)				
	DE_{50} (0.5 h) µg/g	DE_{50} (2 h) µg/g	DL_{50} (24 h) µg/g	CP (0.5 h) mg/piqûre	CP (0.5 h) proie/piqûre
***Syntermes* sp.**	> 800	> 800	-	-	-
***Nasutitermes* sp.**	394.91	378.66	-	120	63
Camponotus atriceps	> 1315	> 1315	-	-	-
Acheta domesticus	> 935	> 935	-	-	-
Tenebrio molitor	> 1000	> 1000	-	-	-

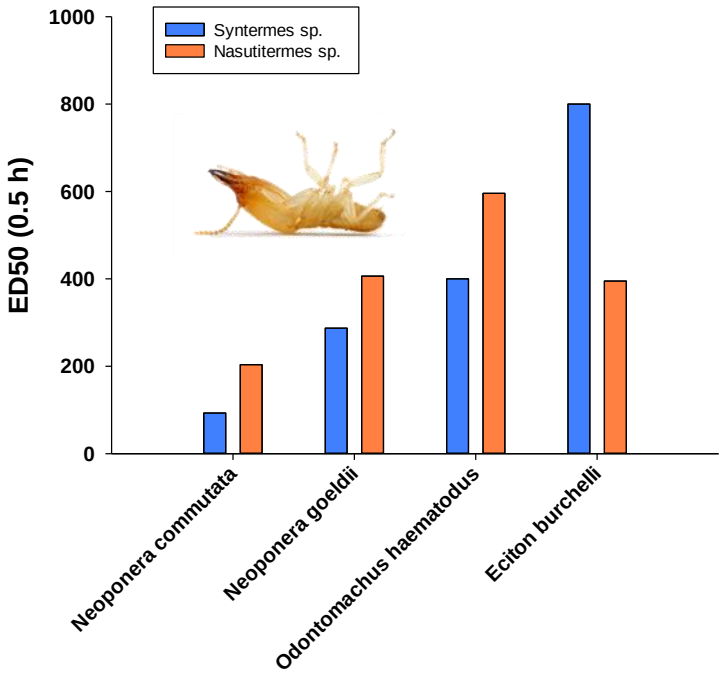

Figure 10- Doses effectives à 50% (0.5h) des différents venins sur les deux espèces de termites.

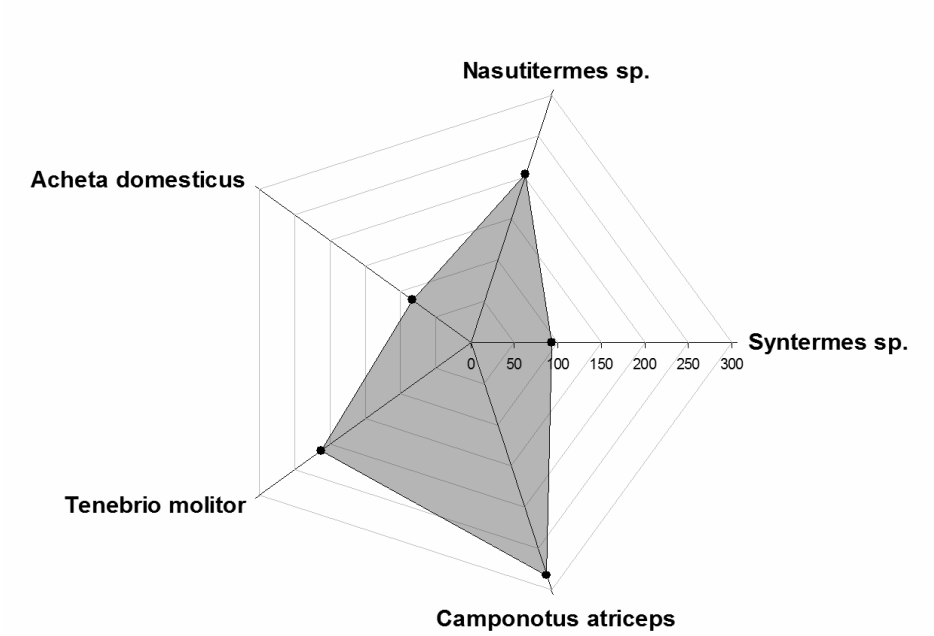

Figure 11- Dose effectives du venin de Neoponera commutata sur les différentes proies.

Le venin de la fourmi légionnaire *E. burchelli* semble très faiblement neurotoxique sur presque toutes les proies testées (Tableau 2). *Eciton burchelli* appartient à la sous-famille des Dorylinae (anciennement Ecitoninae). Les espèces de cette sous-famille se caractérisent par un mode de vie nomade et une méthode de chasse en groupe. Ainsi cette méthode de chasse en masse où les proies ont peu de chances de s'échapper pourrait avoir défavorisé la sélection de neurotoxines. Les fourmis légionnaires africaine du genre *Dorylus* adoptant la même méthode de chasse n'ont même plus de système venimeux fonctionnel. On pourrait donc émettre l'hypothèse que le venin des fourmis légionnaires néotropicales est utilisé pour des fonctions autres que la capture des proies telles que la prédigestion des proies ou encore pour une action antiseptique (antimicrobienne et antifongique) ou défensive contre les vertébrés prédateurs de fourmis (fourmiliers) car la piqûre est douloureuse (pour l'Homme).

En comparaison des trois autres espèces de fourmis, *N. commutata* (termitophage) possède le venin le mieux adapté pour capturer des termites car son venin induit un effet neurotoxique rapide. Il s'agit des valeurs de DL_{50} les plus faibles vis-à-vis des termites (*Syntermes* sp. et *Nasutitermes* sp.), si l'on compare aux valeurs obtenues pour les trois autres espèces de fourmis (Fig. 10). L'évaluation de la toxicité du venin de *N. commutata* sur les différentes proies indique que ce venin est très efficace pour paralyser *Acheta domesticus* (Dose effective 50% ; DE_{50} 82.83 µg/g à 30 min) et *Syntermes* sp. (DE_{50} 93.11 µg/g à 30 min) mais moins efficace contre *Nasutitermes* sp. (DE_{50} 203.7 µg/g à 30 min), *Tenebrio molitor* (DE_{50} 212.52 µg/g à 30 min) et *Camponotus atriceps* (DE_{50} 282.16 µg/g à 30 min) (Fig. 11). Le régime termitophage ultra spécialisé et dirigé uniquement contre les termites du genre *Syntermes* pourrait avoir favorisé la sélection de toxines adaptées pour la paralysie de ce genre de termites. Cependant, la toxicité n'est pas spécifique à cette espèce

et semble être forte sur toutes les proies testées. De plus il s'agit du venin qui possède la plus forte diversité de toxines observée avec 356 peptides. Une analyse LC-MS utilisant un spectromètre de masse plus résolutif (Spectromètre de masse MALDI-TOF/TOF 5800, AB SCIEX) a permis de révéler plus de 1000 peptides dans le venin de *N. commutata* (Aili. S communication personnelle). Cela laisse supposer que la fonction offensive du venin n'est pas l'unique facteur écologique affectant la diversification des venins de fourmis. Bien que très spécialisées, les ouvrières de *N. commutata* utilisent leur également venin pour se défendre contre des prédateurs vertébrés et invertébrés ainsi que d'autres fourmis compétitrices. Le rôle défensif du venin a donc probablement également joué un rôle très important dans la sélection des toxines.

3. Spécialisation défensive

Le rôle de la fonction défensive dans l'évolution des toxines des venins a été moins étudié que celui de la fonction offensive [Casewell et al., 2013]. En effet l'aspect défensif est généralement un rôle secondaire du venin dans la majorité des taxons venimeux étudiés. Les quelques études réalisées sur les venins défensifs ont montré une plus faible variation et une moins grande complexité que celle des venins offensifs [Fry et al., 2009]. Cela laisse supposer que l'aspect défensif joue un rôle secondaire dans la diversification des venins chez la majorité des taxons venimeux étudiés (serpents, cône venimeux, scorpions, araignées).

Si les fourmis sont de redoutables prédatrices, elles sont également la proie de nombreux invertébrés et vertébrés. De plus il y a une très forte compétition intraspécifique chez les fourmis. Elles se livrent à de véritables guerres pour dominer les espèces de fourmis avoisinantes et s'accaparer les ressources alimentaires à proximité de leur nid. La coévolution a donc conduit à l'élaboration d'armes défensives efficaces afin de se protéger.

Les hyménoptères aculéates ont ainsi développé des venins puissants afin de se prémunir de la prédation des vertébrés. Les venins d'hyménoptères sont très toxiques et provoquent une vive douleur pour mettre en fuite les prédateurs vertébrés (tout au moins les mammifères) qui sont entre plusieurs centaines de fois jusqu'à plusieurs millions de fois plus imposant qu'eux [Schmidt, 2014]. Les fourmis sont confrontées à une multitude de prédateurs potentiels, intéressés par l'importante ressource nutritive que représente un nid d'insectes sociaux. La pression de sélection exercée par les prédateurs et les compétiteurs semble donc plus importante chez les hyménoptères que chez les autres taxons venimeux communément étudiés (serpents, scorpions, araignées et cônes) et donc la fonction défensive a indubitablement joué un rôle très important dans l'évolution des venins d'hyménoptères afin de mettre au point des armes biochimiques défensives efficaces.

Chez les fourmis, toutes les espèces sont sociales et la protection de leur colonie, tout particulièrement des sexués et du couvain, est l'une des préoccupations majeures des ouvrières. Afin d'assurer la sécurité de leur nid elles disposent d'une piqûre très toxique envers les invertébrés et souvent douloureuse pour les vertébrés (tout au moins les mammifères). Cette arme biochimique est souvent associée avec un comportement très agressif à l'encontre des intrus. Certaines espèces de fourmis du genre *Pogonomyrmex* (Myrmicinae) dont le venin est uniquement défensif ont même développé un venin particulièrement toxique pour les vertébrés et faiblement toxique contre les invertébrés [Schmidt and Blum, 1978a, c].

- **Le rôle défensif des venins de fourmis a-t-il favorisé la diversification et la sélection de certaines toxines ?**

Pour savoir si la composante défensive du venin a joué un rôle majeur dans l'innovation des toxines chez les fourmis, nous nous sommes intéressés aux venins des fourmis du genre *Pseudomyrmex* (Pseudomyrmecinae). Dans la sous-famille des Pseudomyrmecinae, alors que quelques rares espèces nidifient au sol, la majorité des espèces vivent dans les arbres et certaines espèces sont même associées avec des plantes myrmécophytes. Ces plantes myrmécophytes sont des espèces de plantes associées avec certaines espèces de fourmis dans une relation mutualiste au cours de laquelle la plante fournit un abri aux fourmis grâce à certaines structures creuses nommées les domaties (poches foliaires, tiges, pétioles ou épines creuses) et souvent de la nourriture (nectaires extra-floraux ou corps nourriciers). En retour les fourmis protègent la plante hôte contre les

animaux phytophages, les plantes compétitrices et les microorganismes pathogènes grâce à un venin puissant et à un comportement très agressif [Heil and McKey, 2003].

Nous avons choisi d'étudier les peptidomes des venins de trois espèces du genre *Pseudomyrmex* : *P. termitarius* est une espèce terricole qui utilise son venin essentiellement pour capturer de petits arthropodes, *P. gracilis* est une espèce arboricole dont le venin à une fonction principalement offensive et *P. penetrator* est une espèce strictement associée avec un myrmécophyte *Tachigali paniculata* [Ward, 1999]. Le venin de cette dernière espèce possède un rôle uniquement défensif car cette espèce refuse tout apport alimentaire extérieur à la plante hôte [Dejean et al., 2014].

Alors que le venin de *P. termitarius* contient un total de 87 peptides linéaires, les venins de *P. gracilis* et *P. penetrator* contiennent respectivement 23 et 26 peptides, dont certains sont structurés par des ponts disulfure. Le venin de *P. penetrator* n'est pas moins complexe que les autres venins bien au contraire. Ce venin contient notamment des peptides dimériques réticulés par deux ponts disulfure (PP-aa, PP-bb et PP-ab). Il existe donc des différences structurales importantes entre les peptides des trois venins. Le peptidome du venin uniquement défensif possède une plus grande diversité de structure que les deux autres venins. Ensuite une étude de la cytotoxicité du venin brut et des fractions HPLC du venin de *P. penetrator* a été effectuée sur des cellules d'insectes (*Aedes albopictus*). La fraction 6 est la fraction la plus active avec une DL_{50} de 3.2 µg/mL à 24 H (Figure 12). Une analyse en spectrométrie de masse des fractions a montré que le peptide hétéro-dimérique PP-ab était le composé principal de la fraction 6. Nous avons nommé ce peptide, la penetratine et les chaines peptidiques qui le composent (PP-a et PP-b) ont été séquencées et sont reportées dans le tableau 3. Dans une étude précédente, un groupe de six polypeptides hétéro-dimériques avait déjà été découvert dans le venin de *Pseudomyrmex triplarinus* [Pan

and Hink, 2000]. Cette espèce de fourmi, qui est également associée avec un myrmécophyte, possède un venin uniquement à vocation défensive. Les peptides dimériques ne semblent donc pas présents chez toutes les espèces de *Pseudomyrmex* et la sélection de tels peptides pourrait avoir été associée avec le rôle défensif extrême des espèces de Pseudomyrmecinae vivant dans les myrmécophytes. Dans de futures études il serait intéressant de tester l'effet algésique de ces peptides sur les mammifères ou leur toxicité sur les insectes phytophages.

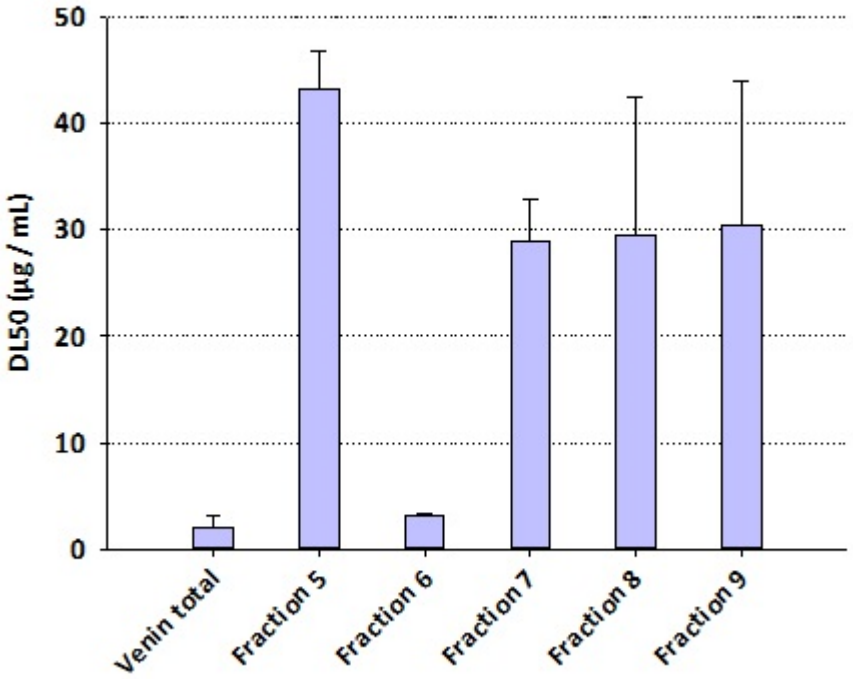

Figure 12. Cytotoxicité à 24 h du venin total et des fractions HPLC actives du venin de *Pseudomyrmex penetrator* sur les cellules d'insectes (*Aedes albopictus*). Les fractions HPLC 1, 2, 3 et 4 n'ont pas montré de cytotoxicité significative aux concentrations testées. La fraction 6 a montré une forte cytotoxicité avec une DL_{50} de 3.2 µg/mL.

Tableau 3. Séquences des chaines peptidiques de la penetratine (PP-ab). Les deux chaines sont fortement homologues (en gris) et sont reliées par deux ponts disulfure inter-caténaires (Cys^1—Cys^4/ Cys^2—Cys^3).

Peptide	Espèce	Séquence	Masse (Da)
PP-a	*P. penetrator*	KIPNILKGGLKSI**C**^{1}KHRKYLDKA**C**^{2}PAS-NH_2	2977.68
PP-b	*P. penetrator*	IDPLTILKILKGGLKSI**C**^{3}KHRKYLDKA**C**^{4}ASIGQ	3621.06

Article 4

Venom toxicity and composition in three *Pseudomyrmex* ant species having different nesting modes

Axel Touchard, Nicolas Labrière, Olivier Roux, Frederic Petitclerc, Jérôme Orivel, Pierre Escoubas, Jennifer M.S. Koh, Graham M. Nicholson, Alain Dejean

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Venom toxicity and composition in three *Pseudomyrmex* ant species having different nesting modes

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Running head: venom activity and composition in an ant genus

[1] contributed equally.

ABSTRACT

We aimed to determine whether the nesting habits of ants have influenced their venom toxicity and composition. We focused on the genus *Pseudomyrmex* (Pseudomyrmecinae) comprising terrestrial and arboreal species, and, among the latter, plant-ants that are obligate inhabitants of myrmecophytes (i.e., plants sheltering ants in hollow structures). Contrary to our hypothesis, the venom of the ground-dwelling species, *P. termitarius*, was as efficacious in paralyzing prey as the venoms of the arboreal and the plant-ant species, *P. penetrator* and *P. gracilis*. The lethal potency of *P. termitarius* venom was equipotent with that of *P. gracilis* whereas the venom of *P. penetrator* was less potent. The MALDI-TOF MS analysis of each HPLC fraction of the venoms showed that *P. termitarius* venom is composed of 87 linear peptides, while both *P. gracilis* and *P. penetrator* venoms (23 and 26 peptides, respectively) possess peptides with disulfide bonds. Furthermore, *P. penetrator* venom contains three hetero- and homodimeric peptides consisting of two short peptidic chains linked together by two interchain disulfide bonds. The large number of peptides in *P. termitarius* venom is likely related to the large diversity of potential prey plus the antibacterial peptides required for nesting in the ground. Whereas predation involves only the prey and predator, *P. penetrator* venom has evolved in an environment where trees, defoliating insects, browsing mammals and ants live in equilibrium, likely explaining the diversity of the peptide structures.

Key-words: ants, ant venoms, arboreal and ground-nesting ants, evolution, peptides, *Pseudomyrmex*

1. Introduction

Venoms are fluid animal secretions consisting of a complex mixture of compounds produced in specialized glands and secreted through specialized delivery systems (e.g., nematocysts, spines, fangs or stingers) or that are sprayed to disable prey, predators and competitors. Venoms are mostly composed of proteins, peptides, amino acids, salts, alkaloids and neurotransmitters (Morgan, 2008; Escoubas and King, 2009; Casewell et al., 2013; Cologna et al., 2013; King and Hardy, 2013; Smith et al., 2013). While 'predatory venoms' are complex mixtures that are often highly variable in their composition and physiological effects, 'defensive venoms', such as those used by fish and bees, are 'streamlined and highly conserved' (Fry et al., 2009; Casewell et al., 2013).

Ants are venomous animals with diverse terrestrial or arboreal nesting habits. Compared to ground-dwelling species, arboreal ants have developed innovative hunting behaviors to efficiently capture insect prey that are able to escape by flying away, jumping or dropping and whose presence is unpredictable in tree foliage. So, the rapid paralysis of prey after the injection or spraying of venom is primordial for arboreal species (Cerda and Dejean, 2011) and hence may influence venom composition. Within the group of arboreal species, specialized 'plant-ants' exist in an obligate mutualism with myrmecophytes (i.e., plants sheltering plant-ants in hollow structures). In return for a nesting place and frequently food rewards, the ant colony helps protect its host myrmecophyte from enemies such as herbivores. Depending on the species, plant-ants often feed solely upon resources provided by the host plant or have evolved elaborate means of prey capture such as the construction of traps (Rico-Gray and Oliveira, 2007; Cerda and Dejean, 2011).

In this general context, we focused the present study on the monophyletic genus *Pseudomyrmex* (Pseudomyrmecinae) that comprises terrestrial and arboreal species, and, among the latter, plant-ants (Ward and Downie, 2005). Within this genus, nesting habits influence ant feeding preferences. Thus, ground-nesting species feed mostly on prey, arboreal species mostly on sugary substances, while plant-ant species live in a kind of food autarchy feeding strictly on rewards gathered on, or directly produced by, their host myrmecophyte. Nevertheless, arboreal species are not more efficient at capturing prey than are ground-nesting species, something likely due to the influence of worker size in this group (Dejean et al*.,* 2014).

In this study, we aimed to determine whether the venoms of foraging workers from three *Pseudomyrmex* species having different nesting habits (i.e., ground, arboreal, or restricted to a myrmecophyte) (*i*) act differently on standardized prey and (*ii*) have different compositions. The venom of the only two *Pseudomyrmex* species studied so far, *P. pallidus* and *P. triplarinus*, are proteinaceous (Blum and Callahan, 1963; Hink et al., 1994; Pan and Hink, 2000) and hence an in-depth peptide mass analysis was performed. The three species were carefully chosen to represent each of the three nesting habits. The colonies of the terrestrial species, *P. termitarius*, build underground nests up to 80 cm in depth (Jaffé, Lopez and Aragort*,* 1986); the workers mostly forage diurnally on the ground, hunting or scavenging for a wide range of arthropods and occasionally exploiting extrafloral nectar on low vegetation (Mill, 1981; Orivel and Dejean, 2001a). The colonies of the arboreal species, *P. gracilis*, typically nest in dead, hollow twigs or branches (Ward, 1993); the workers are good hunters and frequently exploit extrafloral nectar (Dejean et al*.,* 2014). The plant-ant, *P. penetrator*, is typically associated with myrmecophytes of the genus *Tachigali*

(Cesalpiniaceae) (Ward, 1999); its colonies are lodged in hollow, compound leaf rachis, however, the very aggressive workers do not hunt (Dejean et al., 2014).

2. Materials and Methods

2.1. *Study sites and ant gathering*

The present study was conducted in Kourou (5°09'30''N; 52°38'34''W), French Guiana, between January 2010 and September 2013. Live specimens of *Pseudomyrmex* workers were collected around Petit Saut (5°03’39”N; 53°02’36”W) and on La Montagne des Singes (05°04'20"N; 52°41'43"W). Voucher specimens of the ants were deposited in the *Laboratório de Mirmecologia* collection, Cocoa Research Centre, Ilhéus, Bahia, Brazil, and the Department of Entomology, University of California, Davis, CA 95616, U.S.A.

We chose sandy areas to rapidly unearth *P. termitarius* nests using small shovels. The colonies were immediately placed into plastic boxes whose walls were coated with Fluon® to prevent the workers from climbing out. We cut up dry *Irlbachia alata* stems (Gentianaceae), which frequently shelter *P. gracilis* colonies (Dejean et al., 2011), and placed those containing a colony in separate plastic bags. We used pruning scissors to cut up *Tachigali* aff. *paniculata* compound leaves containing parts of *P. penetrator* colonies and also placed them in plastic bags. The boxes and plastic bags containing the colonies were immediately transported to the laboratory where the workers were separated and killed by freezing (12–15 colonies each time).

2.2. Acute Pseudomyrmex *venom toxicity*

To prevent the degradation of the toxins, the venom reservoirs of >100 ants from each species were dissected and pooled in 10% acetonitrile (ACN) / distilled water (v/v) (see protocol in Touchard et al., 2014). They were then centrifuged for 5 min at 14,400 rpm (12,000 g_{av}) to pellet empty reservoirs and membranes. The supernatant was evaporated in a centrifugal concentrator and the dried extract was dissolved in insect saline of the following composition (in mM): NaCl 270, KCl 3.2; $CaCl_2$ 1.6; $MgCl_2$ 10; 3-[N-morpholino] propane-sulfonic acid 10, then freeze-dried and stored at –20°C. To determine the dry venom weight per worker, the venom from 100 workers per species was pooled, dried and weighed using a microscale (Mettler® AE 260).

To determine the acute insect toxicity of the *Pseudomyrmex* venoms, a series of sequential dilutions with insect saline were injected into *Nasutitermes costalis* termite workers using an Eppendorf CellTram oil® micro-injector (mean weight of the individuals: 7.67 ± 0.05 mg; n = 50). Note that the *Pseudomyrmex* species were previously tested in different insect prey, including flies, cricket, katydids and different termite species (Dejean et al., 2014; pers. comm.). Each termite was injected with 0.5 µL of diluted venom in the dorsal part of its thorax. Each *Pseudomyrmex* venom dilution was tested on 10 termites and each dose-response curve was repeated three times (n = 3). The number of termites that were still mobile, knocked down (i.e., on their back, with their legs and antennae continuing to move or vibrate), or dead were recorded over a 24-h period. For each series of experiments, three lots of 10 control termites were injected with 0.5 µL of the insect saline to act as a negative control. There were no signs of paralysis in any of these control groups.

2.3. Pseudomyrmex *venom biochemistry*

Twenty venom reservoirs from each *Pseudomyrmex* species were dissected, pooled and centrifuged as described in section 2.2, and the supernatant collected and lyophilized prior to storage at –20°C before biochemical analysis.

The venom peptides were separated by reversed-phase HPLC using a Waters Xterra-C18 5 µm, 2.1 x 100 mm column on an Agilent HP 1100 HPLC system. The venoms were fractionated using a gradient of solvent A (0.1% v/v TFA) and solvent B (ACN/0.1% v/v TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0–60% for 60 min, 60–90% for 10 min and 90–0% for 15 min, all at a flow rate of 0.3 mL/min. The absorbance of the column effluent was monitored at 215 nm on a diode-array detector. We monitored the signal in real time, collected the fractions manually for each eluting peak, and then freeze-dried and reconstituted them in 50 µL of 50% ACN/0.1% TFA for subsequent off-line MALDI-TOF MS analysis and disulfide bond reduction.

Mass spectrometry analyses were performed on a Voyager® DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems; CA, USA) using the matrix α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in a solution of water/ACN/TFA (50/50/0.1) at 5 mg/mL. Subsequently, 1 µL of each reconstituted HPLC fraction was deposited on the MALDI target plate followed by 0.5 µL of the matrix. Each spectrum was calibrated externally using a mixture of peptides of known molecular masses in the same *m*/*z* range (Peptide calibration Mix 2, LaserBio Labs, Sophia-Antipolis, France). External calibration was performed by depositing 0.5 µL of the calibration mixture co-crystallized with 0.5 µL of the CHCA matrix, adjacent to each sample. All spectra were acquired in reflector mode to maximize peak resolution. They were

collected over the range 500–10,000 *m/z* in positive ion mode (200 shots per spectrum) and were automatically calibrated using the sequence module of the Voyager® control software (Applied Biosystems).

To map the peptide disulfide bond distribution, 5 µL of each fraction was incubated in 10 µL of the reducing buffer (100 mM ammonium bicarbonate, pH 8, 6 M guanidine) with 10 mM dithiothreitol (DTT) for 1 h at 56°C. The reduction results in a mass increase of 2 Da for each disulfide bond as revealed by MALDI-TOF MS analysis. When the presence of dimeric peptides was suspected we subsequently carried out alkylation of the fraction by the addition of 50 mM iodoacetic acid (IAA) for 30 min in the dark. The reaction was terminated by the addition of 5 µL of 0.1% v/v TFA. Prior to mass spectrometry analysis, the reduced/alkylated fractions were desalted using Ziptip® C18 (Millipore) pipette tips. Alkylation with IAA results in a mass increase of 116 Da for each disulfide bond (+58 Da for each alkylated cysteine residue). A comparison of mass shifts between native and reduced/alkylated venom fractions allowed the number of disulfide bonds in each peptide to be determined. Dimeric peptides were identified by the disappearance of the parent peptide mass after reduction/alkylation and the appearance of two lower masses in reduced/alkylated spectra.

The spectra were subjected to a baseline correction (0.7 correlation factor) and Gaussian smoothing (5-point filter width) in Data Explorer® 4.11 software (AB SCIEX). Potential sodium and potassium adducts were manually removed from all mass lists. Mass matches within ±1.0 Da were defined as identical peptides in this study. Identical masses in adjoining HPLC fractions, which reflected an incomplete separation, were also removed.

2.4. *Data analysis*

The doses required to knockdown (KD_{50}) or kill (LD_{50}) half of the termites were calculated at each time point. These values were obtained by fitting the dose-response data using the following form of the logistic equation: $y = 1/(1+[x]/Dose_{50})^{nH}$ where x is the toxin dose, n_H is the Hill coefficient (slope parameter), and $Dose_{50}$ is the median inhibitory dose causing lethality (LD_{50}) or knockdown (KD_{50}). Non-linear curve-fitting of data was performed using GraphPad Prism version 6.00c software for Macintosh (GraphPad Software, San Diego). The statistical comparisons of the dry venom weights per venom reservoir were carried out using an ANOVA and a Newman-Keuls *post-hoc* test (GraphPad Prism). Area-proportional Euler plots depicting overlapping peptide masses from multiple venoms were constructed using eulerAPE software (www. eulerdiagrams.org/eulerAPE/).

The effectiveness of venoms in paralysing or killing termites were compared at 1, 5 and 60 min, and 2, 4, 8 and 24 h. Binary data (knockdown or not and dead or not) were analyzed using the Generalized Linear Model (GLM) procedure with quasibinomial errors to correct for data overdispersion and logit link. Species and the log of venom concentrations and their interactions were used as fixed effects. When the species effect was significant, a Tukey's *post-hoc* test was used to compare species ("glht" function of the "multcomp" package). For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests. Term removals that significantly reduced explanatory power ($P < 0.05$) were retained in the minimal adequate model (Crawley, 2007). Two-dimensional scatter plots, also termed “2D venom landscapes”, were constructed for the three venoms using SigmaPlot 12.0 software (Systat, CA, USA).

3. Results

3.1. *Paralyzing and lethal properties of* Pseudomyrmex *venoms*

The knockdown induced by all *Pseudomyrmex* venoms was always irreversible with no termites recovering over a 24 h period at any dose (Fig. 1A, B). The degree of knockdown did not differ between the three compared species at 1 and 5 min following the injection (GLM: species effect at t = 1 min: F = 1.76; df = 2; P = 0.17 and at t = 5 min: F = 2.39; df = 2; P = 0.09). The KD_{50} value decreased with time for the two predatory species, *P. termitarius* and *P. gracilis*, but this was less evident for the plant-ant, *P. penetrator* (Fig. 1B). Necrosis, which appeared as a blackish area surrounding the zone of the injection, was noted for low venom doses even before death of termites.

In terms of lethal effects, all venoms caused limited effects at t = 1 min with less than 3% lethality at 2 mg/g or less. However, at t = 5 min the venom of *P. termitarius* is more potent than that of the two other species (GLM: species effect: F = 9.05; df = 2; P < 0.001. Tukey's *post-hoc* test: *P. termitarius vs. P. gracilis*: z = 3.66, P < 0.001; *P. termitarius vs. P. penetrator*: z = 3.15, P = 0.004; and *P. penetrator vs. P. gracilis*: z = 1.16, P = 0.47; see also Fig. 2). At t = 60 min and later, the venom of *P. penetrator* is less potent than that of the two other species (Fig. 2A; GLM at 60 min: species*dose effect: F = 5.18; df = 2; P < 0.005. Tukey's *post-hoc* test: *P. termitarius vs. P. gracilis*: z = 1.03, P = 0.54; *P. termitarius vs. P. penetrator*: z = 3.81, P < 0.001; and *P. penetrator vs. P. gracilis*: z = -3.15, P = 0.003).

The dry venom weight per venom reservoir was 23.39 ± 0.58 µg, 7.44 ± 0.29 µg and 5.56 ± 0.25 µg (n = 100) for *P. penetrator*, *P. termitarius* and *P. gracilis*, respectively, all of the differences being significant (ANOVA: F = 591; df = 2; P < 0.0001; Newman-Keuls multiple comparison test: P < 0.01 for each comparison). One minute after injection, 4.1% of the contents of the venom reservoir were necessary for a *P. gracilis* worker to knock down 50%

of the termites (KD_{50}), whereas 2.6% was sufficient for *P. termitarius* and only 0.7% for *P. penetrator* (Fig. 1C). The relative quantity of venom resulting in the death of 50% of the termites (LD_{50}) after 1 h represented 11.8% of the contents of the venom reservoir for a *P. gracilis* worker, and 7.3% and 8.3% for *P. termitarius* and *P. penetrator*, respectively (Fig. 2C).

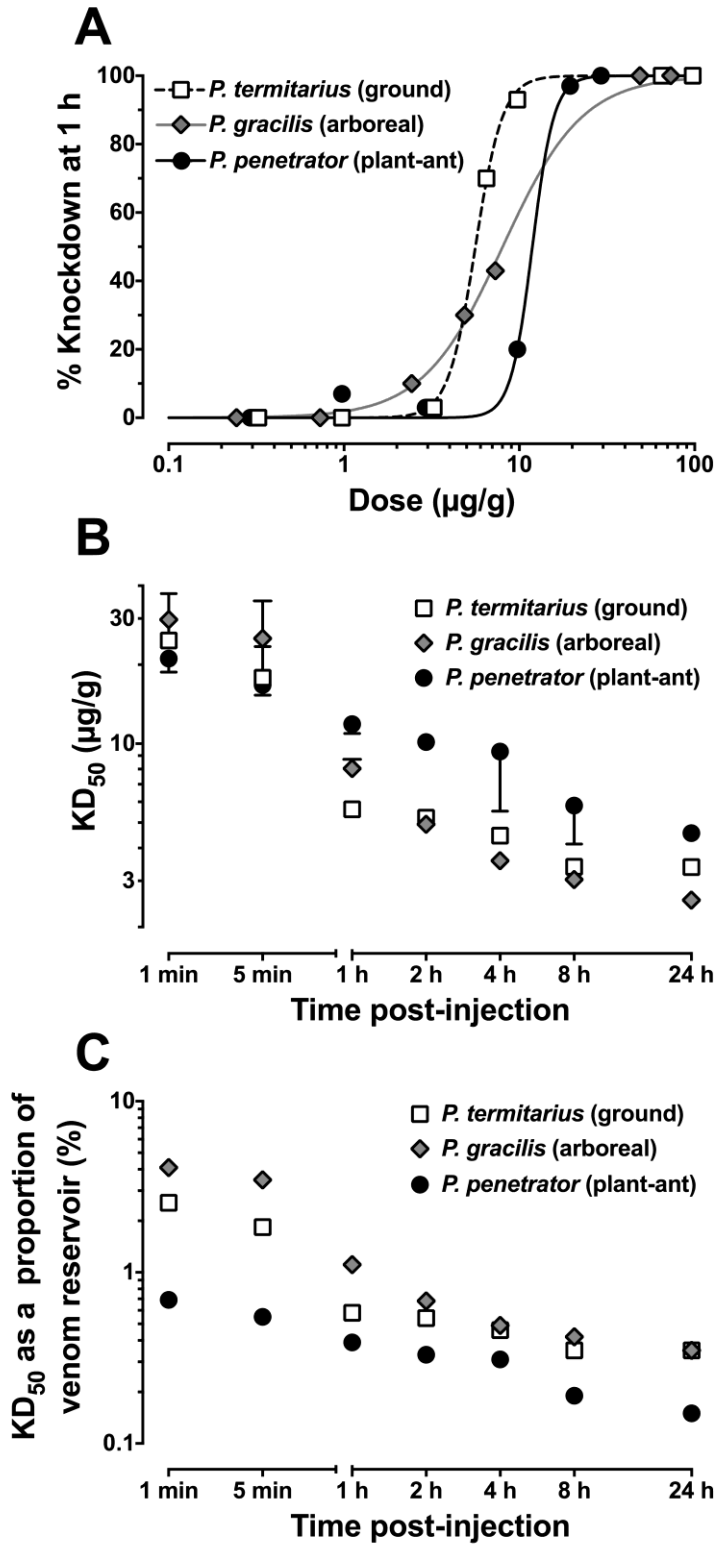

Fig. 1. Knockdown effect of *Pseudomyrmex* venoms on termites. (A) Dose-response curve for the excitatory paralytic effect of the three venoms at 1 min. (B) Comparison of the median knockdown dose (KD_{50}) as a function of time post-injection. (C) Relative proportion of the venom reservoir required to achieve the KD_{50} as a function of time post-injection.

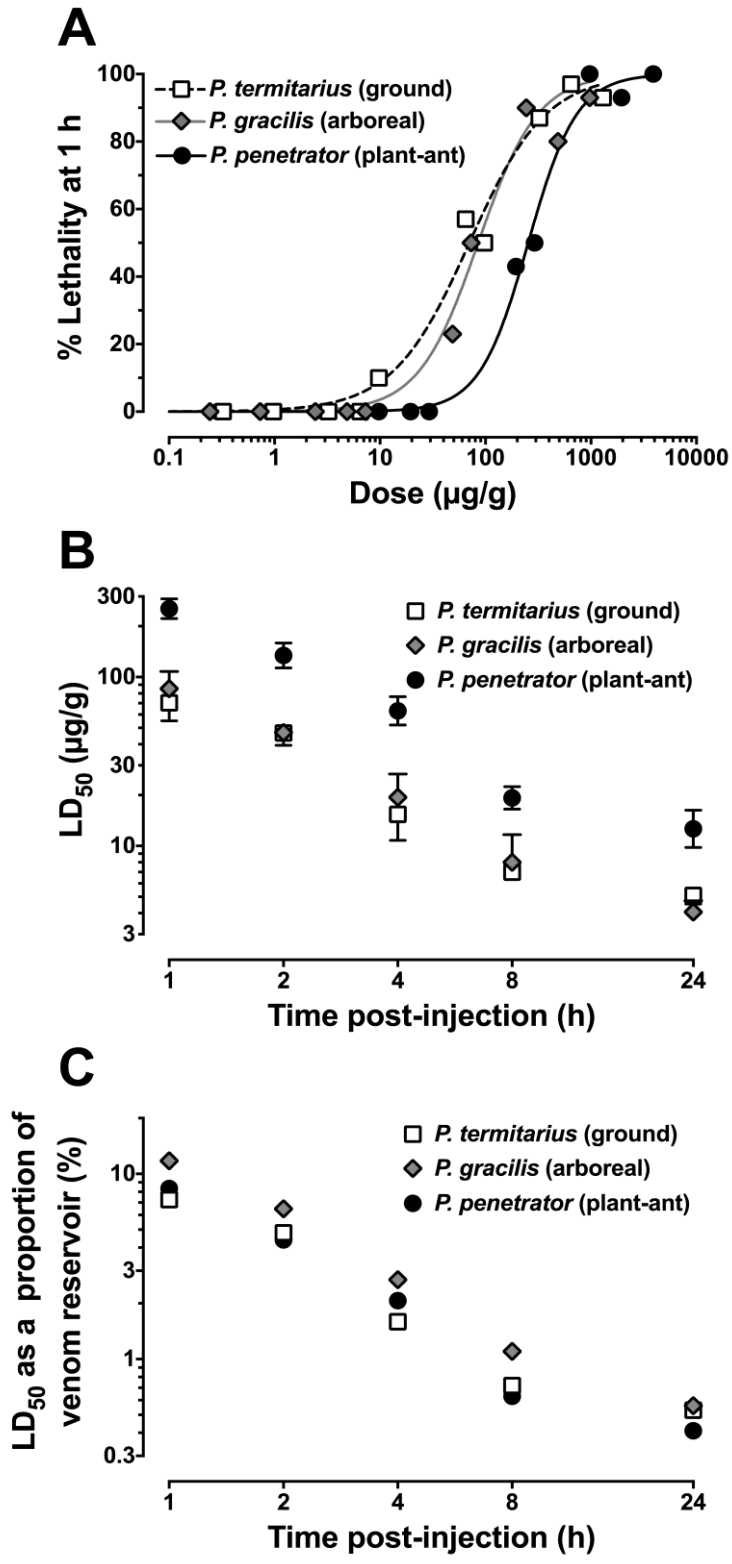

Fig. 2. Lethal effect of *Pseudomyrmex* venoms on termites. (A) Dose-response curve for the lethal effect of the three venoms at 1 h. (B) Comparison of the median lethal dose (LD_{50}) as a function of time post-injection. (C) Relative proportion of the venom reservoir required to achieve the LD_{50} as a function of time post-injection.

3.2. *LC-MALDI-TOF MS analysis*

The HPLC chromatograms of the venoms of the three *Pseudomyrmex* species showed a similar pattern of peptide elution where the majority of the components eluted between 40 and 60 min, corresponding to 35–55% ACN (Fig. 3). The number of HPLC peaks (fractions) collected from the venoms of *P. termitarius*, *P. gracilis* and *P. penetrator* was 20, 18 and 21, respectively.

The MALDI-TOF MS analysis of each HPLC fraction often showed the presence of multiple peptides co-eluting in the same LC fraction, particularly for *P. termitarius*, despite sharp single LC peaks (Fig. 3). We recorded 87 peptides in the *P. termitarius* venom, but only 23 and 26 in the *P. gracilis* and *P. penetrator* venoms, respectively (Table 1).

Although the masses of most peptides from the venoms of the three *Pseudomyrmex* species are relatively similar, only a few can be considered identical. If we consider that a mass tolerance of less than 1.0 Da indicates identical peptides on the condition that they do not differ by the presence of a disulfide bond (Table 1), the same peptide is represented, for example, by the masses of 1796.92, 3167.80 and 3213.76 Da in the *P. gracilis* venom and the peptides of 1797.84, 3167.89 and 3213.63 Da in the *P. termitarius* venom (Table 1). All other peptides found in the three venoms were therefore exclusive, so that only 2.2% of the peptides were common between the *P. termitarius* and the *P. gracilis* venoms, while there were no common peptides between the *P. gracilis* and the *P. penetrator* venoms or between the *P. termitarius* and the *P. penetrator* venoms.

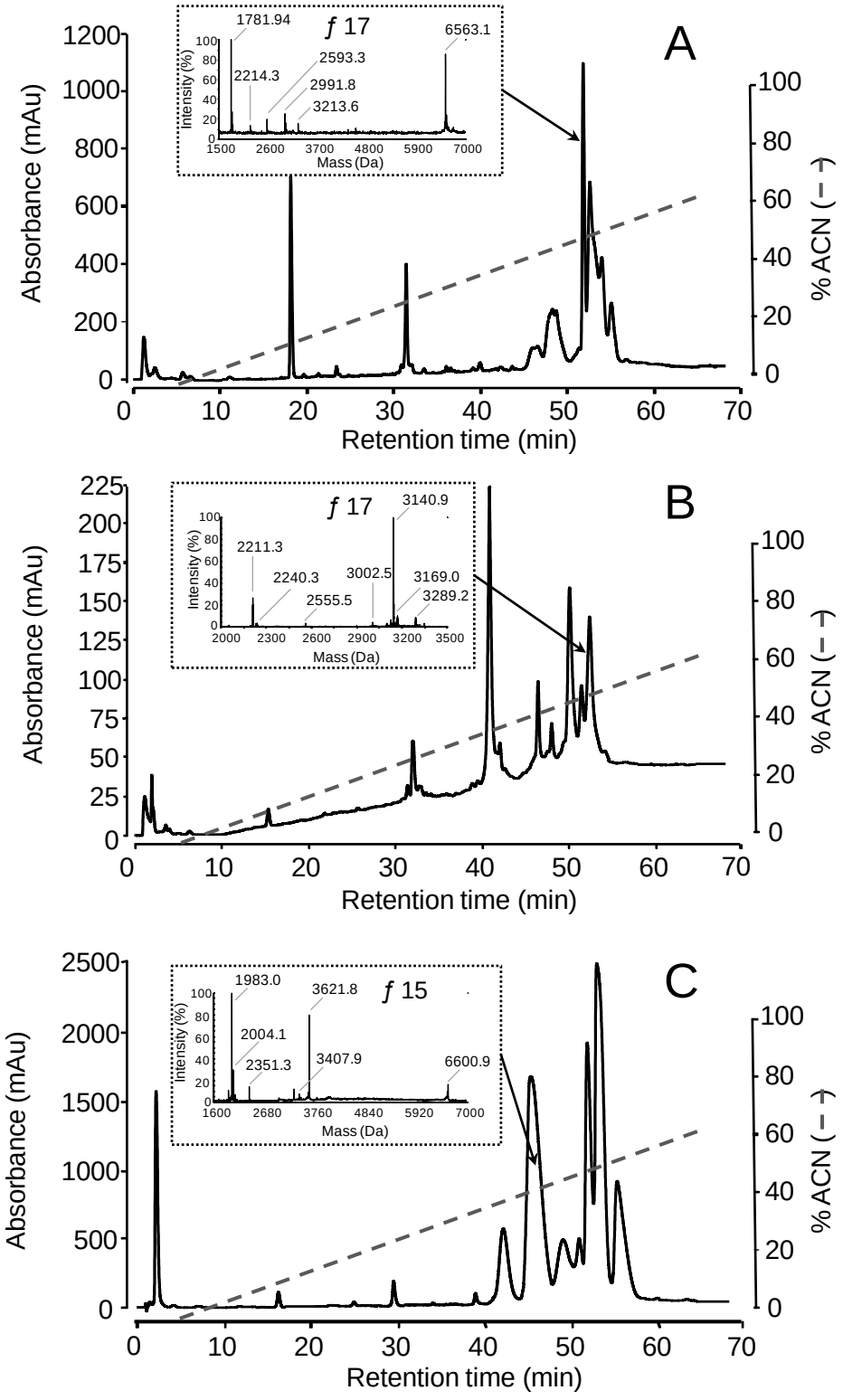

Fig. 3. RP-HPLC chromatograms of pseudomyrmecine ant venoms. Venoms were separated by analytical C18 RP-HPLC separation using an acetonitrile/0.1% TFA gradient of 1%/min (grey dotted line) at 0.3 mL/min. Panels show the chromatographic elution profile of venoms from (A) *P. termitarius*, (B) *P. gracilis*, and (C) *P. penetrator*. Chromatographic peaks were monitored at 215 nm. LC-MALDI-TOF mass spectra of *Pseudomyrmex* venoms. MALDI-TOF MS spectra for fractions (A) ƒ17, (B) ƒ17 and (C) ƒ15 are shown in boxes.

3.3. *Molecular mass distribution*

The peptide mapping of the venoms by LC-MALDI-TOF MS revealed a majority of small peptides (91% in the 1000–5000 Da mass range) (Fig. 4). However, *Pseudomyrmex* venoms also contain peptides in the 5239–7244 Da mass range. While *P. gracilis* venom contains only one large peptide (5362.72 Da), the venoms of the two other *Pseudomyrmex* species contain five large peptides (Table 1). It must be noted, however, that the MALDI matrix used in this study (CHCA) is better suited to the ionization of peptides in the 500–5000 Da range (Sze et al., 1998) and thus may not reveal the full extent of the peptides of a higher molecular weight. More isoforms or other peptides in the mass range above 5 kDa may therefore be present in *Pseudomyrmex* venoms.

Table 1. Peptide mass fingerprint (Da) from *Pseudomyrmex* spp. venoms following LC-MALDI-TOF MS. Shaded masses are common between venoms. Masses in a single box are monomeric peptides with a single disulfide bond. Masses surrounded by a double box are dimeric peptides with two interchain disulfide bonds. * Note that the masses of 1557.74 and 1557.90 Da found in the *P. gracilis* and *P. penetrator* venoms, respectively, differ due to the presence of a disulfide bond in the latter case.

Mass (Da)					Peptide
P. termitarius			*P. gracilis*	*P. penetrator*	name for *P. penetrator*
1381.76	2716.23	3167.89	1162.47	1011.52	
1521.72	2772.54	3173.22	1288.58	1127.70	
1781.94	2783.50	3179.75	1529.77	1250.84	
1797.84	2786.31	3182.61	1557.74*	1557.90*	
1798.22	2788.58	3200.63	1796.92	1911.05	
1813.90	2804.36	3205.80	1809.07	1982.11	
1821.03	2864.89	3213.63	1825.04	2004.10	
1837.03	2918.41	3339.80	2211.30	2351.30	
1852.98	2934.42	3397.84	2240.30	2684.34	
1892.38	2946.26	3427.72	2485.60	2706.46	
1897.01	2946.40	3497.87	2555.54	2722.41	
1913.01	2962.21	3514.12	3002.22	2952.50	
1949.38	2991.79	3589.75	3140.97	2980.60	
1965.38	3005.00	3603.74	3167.80	3407.94	
1997.13	3019.28	3733.09	3195.03	3440.16	
2133.42	3021.80	3845.77	3213.76	3621.86	PP-b
2184.00	3027.58	3853.80	3214.88	3639.12	
2189.04	3033.30	3867.20	3218.42	3915.95	
2205.08	3044.57	4378.00	3250.90	3917.31	
2214.30	3063.82	4510.00	3289.25	4044.44	
2220.46	3081.50	4554.30	3477.64	4316.70	
2447.24	3092.60	4673.00	3652.74	5239.07	
2498.33	3099.46	4714.00	5362.72	5255.58	
2593.32	3106.66	4731.20		5956.20	PP-aa
2610.41	3116.28	5470.00		6600.94	PP-ab
2616.48	3142.77	6328.00		7244.30	PP-bb
2624.55	3153.83	6339.00			
2632.45	3162.53	6563.10			
2642.43	3164.65	6569.00			

3.4. *Structural diversity*

The comparisons of the spectra before and after reduction/alkylation did not support the presence of dimeric peptides in the *P. termitarius* and *P. gracilis* venoms, but resulted in the detection of three dimeric peptides in the *P. penetrator* venom. These dimeric peptides consist of two short peptidic chains linked together by two interchain disulfide bonds (Fig. 5) that we named PP-a and PP-b. Mass analysis demonstrated that the *P. penetrator* venom contains both the homodimeric peptides PP-aa and PP-bb (5956.20 and 7244.30 Da, respectively), the heterodimeric peptide, PP-ab (6600.94 Da) (Fig. 5), as well as the peptide PP-b in its monomeric form (3621.86 Da), which is structured by the presence of one intrachain disulfide bond. Other monomeric peptides with one disulfide bond were found in the *P. penetrator* venom (1557.90, 2952.50, 2980.60 and 3407.94 Da) as well as in the *P. gracilis* venom (3218.42, 3477.64 and 3652.74 Da), but not in the *P. termitarius* venom (Table 1).

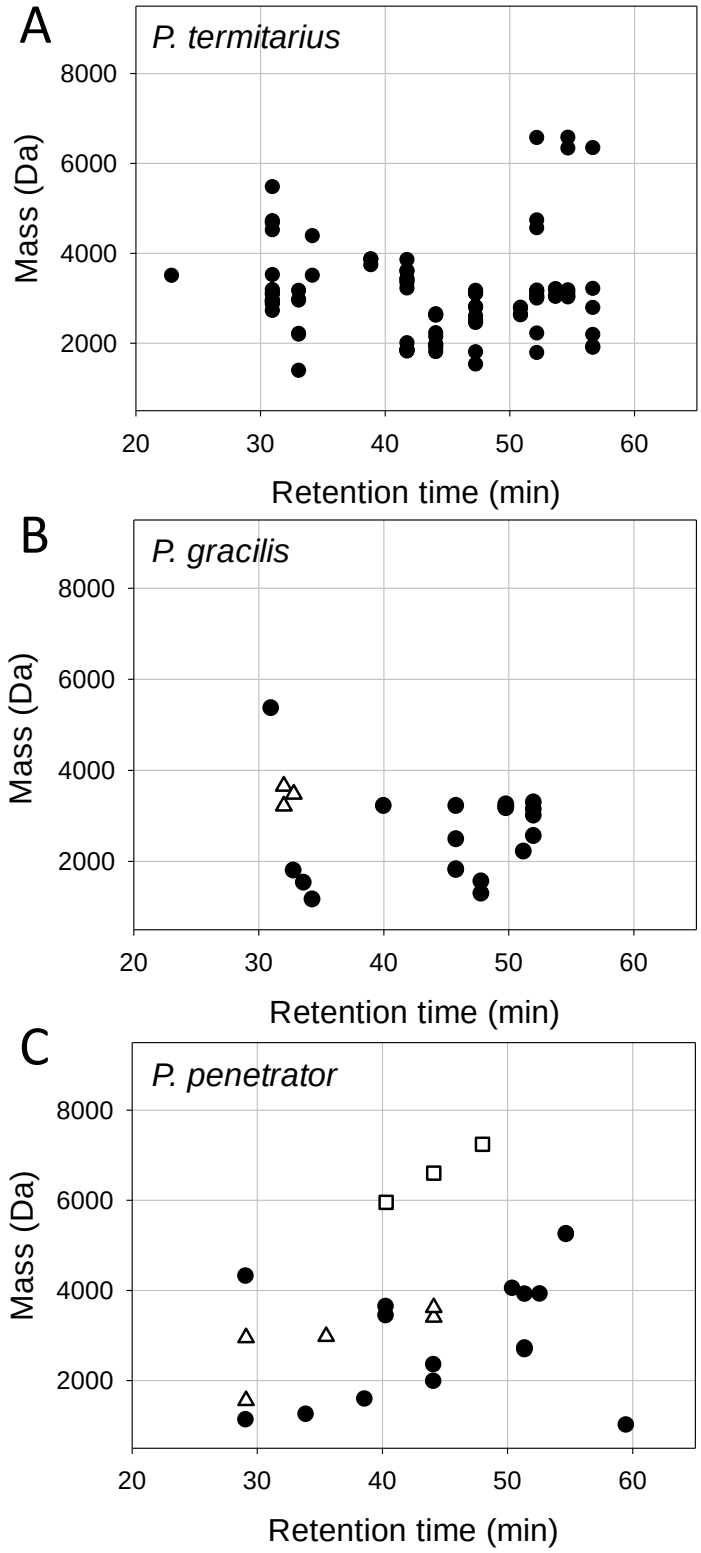

Fig. 4. Two-dimensional venom landscapes of pseudomyrmecine ant venoms. LC-MALDI-TOF MS of (A) *P. termitarius*, (B) *P. gracilis* and (C) *P. penetrator* venoms depicting components in the venoms according to their mass and retention times. Filled circles represent peptides where no disulfide bond was detected, while empty triangles represent peptides with one disulfide bond. The empty squares in the 2D venom landscape of *P. penetrator* indicate the presence of dimeric peptides with two disulfide bonds.

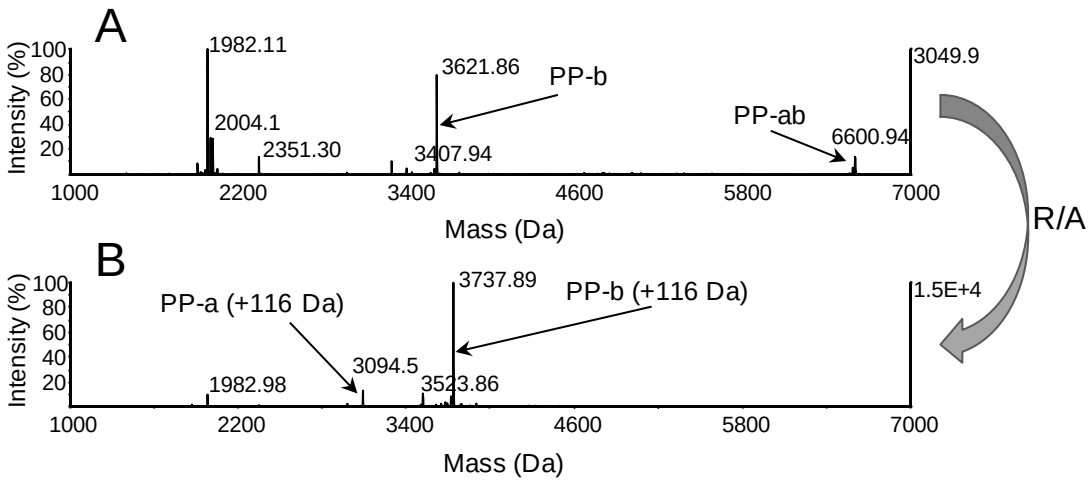

Fig. 5. Determination of disulfide bonding in *P. penetrator* venom peptides. Panels show the MALDI-TOF mass spectra from ƒ15 of *P. penetrator* venom before (A) and after (B) the reduction/alkylation (R/A) process. A single disulfide bond was identified in the 3407.94 Da peptide (A) following the +116 Da gain in mass between the native and reduced/alkylated peptide (B; 3523.86 Da) consistent with the S-carboxymethylation of two cysteines. Comparison of panels A and B also revealed the presence of the heterodimeric peptide PP-ab in the *P. penetrator* venom. The heterodimer PP-ab (6600.94 Da), consisting of the monomers PP-a and PP-b and native monomeric PP-b at 3621.86 Da, were present before R/A. After R/A, peptide PP-ab disappeared, the mass of the monomer PP-b shifted by +116 Da to 3737.86 Da, (reflecting the alkylation of two cysteines) and the mass of the alkylated monomeric PP-a (+116 Da) at 3094.5 Da also appeared.

4. Discussion

Although numerous studies examining predatory ant behavior have demonstrated the paralytic properties of ant venoms (see Cerda and Dejean, 2011), the present study is only one of a few that has combined the analysis of ant venom toxicity and composition (Orivel and Dejean, 2001b; Lai et al 2012; dos Santos Pinto et al., 2012; Torres et al., 2014).

Consistent with the recent results on *Pseudomyrmex* predatory behavior (Dejean et al., 2014), the present study has shown that the venom of the ground-dwelling species, *P. termitarius*, is equi-effective at causing rapid (within 5 min) knockdown of prey as venom from the arboreal species, *P. gracilis* and *P. penetrator* (Fig. 1B). Indeed the irreversible excitatory paralysis of the prey occurred within the first minute regardless of the venom

tested (Fig. 1B). After 5 min the KD_{50} values decreased over time for the two predatory species, *P. termitarius* and *P. gracilis*, but were less evident for the plant-ant, *P. penetrator.* Nevertheless, this slight difference was negated by the fact that the venom yield is significantly greater for *P. penetrator* and hence it may inject larger quantities of venom that would compensate for the slightly lower activity. In support of these findings, Orivel and Dejean (2001b) found that there were no differences in the paralytic doses between ground-dwelling and arboreal species in ponerine ants from the genus *Pachycondyla*. In terms of the lethality of the three *Pseudomyrmex* venoms, there were only limited deaths in termites within the first 5 min following injection, and only at doses greater than 300 µg/g. Nevertheless, the lethal activity of the ground-dwelling species *P. termitarius* was significantly greater at 5 min than the remaining two venoms. After this period the two predatory ants *P. termitarius* and *P. gracilis* both had significantly lower LD_{50} values than the plant-ant *P. penetrator*. Overall, however, the LD_{50} values as a proportion of the venom reservoir showed remarkably similar values between all three venoms, suggesting that the lethal actions are not markedly different. In contrast, Orivel and Dejean (2001b) found that the lethality of ponerine ant venoms was significantly lower for arboreal species. However for predatory ants, rapid immobilization of a prey species is much more important than a slower lethal action if they are to prevent prey escaping. Thus, the main offensive effect of their venoms is paralysis, rather than death, of their target (Schmidt, 1986; Piek et al., 1991; Orivel and Dejean, 2001b). The same is true for the plant-ant, *P. penetrator*, whose venom is more effective at causing the knockdown of termites, but not in killing them. Indeed, this ant species, which is not a predator, can therefore eliminate insects, including defoliators, landing in its territory.

Previous studies on *Pseudomyrmex* venom composition have shown that they are proteinaceous (Blum and Callahan, 1963; Hink et al., 1994; Pan and Hink, 2000), although a polysaccharide was noted in the venom of an unidentified *Pseudomyrmex* species (Schultz et al., 1979). The venoms of all three *Pseudomyrmex* species studied here appear to contain both neurotoxic and cytotoxic compounds given the rapid paralytic and necrotic effect observed following injection. Despite the limited knowledge of ant venom composition, many other ground-dwelling and arboreal ants have been shown to contain peptide toxins with this type of neurotoxic and cytotoxic activity. For example, ectatomin, from *Ectatomma tuberculatum* (a ground-nesting, arboreal-foraging Ectatomminae), is a powerful neurotoxic calcium channel blocker that can also cause pores to form in the cytoplasmic membrane producing a cytotoxic action (Pluzhnikov et al., 1994, 1999). Poneratoxin, isolated from the venom of the ground-nesting, arboreal-foraging predatory species *Paraponera clavata* (Paraponerinae), is also known to be a potent insect-specific neurotoxin which affects voltage-gated sodium channels and blocks synaptic transmissions in the central nervous system, paralyzing the prey (Piek et al., 1991; Duval et al., 1992). Lastly, a wide range of different ponericins, from the arboreal species *Pachycondyla goeldii*, have already been shown to possess cytolytic, insecticidal and antimicrobial properties (Orivel et al., 2001, Orivel and Dejean, 2001b).

The three venoms examined in the present study reveal different types of ant peptide structures: linear peptides, single-chain peptides with intrachain disulfide bonds, and dimeric peptides. The venom of the ground-dwelling species, *P. termitarius*, is composed only of linear peptides, but in comparatively large numbers (87 *versus* 23 and 26 peptides for *P. gracilis* and *P. penetrator*, respectively). This higher number of peptides could be due to the necessity of targeting a wider variety of molecular targets in more prey types (e.g., crawling

and flying insects) or to the presence of antifungal or antibacterial peptides, given that this species is much more exposed to such microbial pathogens from the soil than are arboreal species.

Both the *P. gracilis* and *P. penetrator* venoms contain peptides with disulfide bonds. These disulfide bonds contribute to the stability of small peptides and provide strong resistance to proteases (King and Hardy, 2013). Such peptides often act on ion channels and receptor targets and are common in the venoms of a wide range of evolutionarily unrelated predators including sea anemones, cone snails, centipedes, scorpions, spiders and snakes (Yang et al., 2012; Smith et al., 2013; King and Hardy, 2013). Nevertheless, all of the peptides with disulfide bonds previously described and characterized in ant venoms were dimeric complexes formed from intrachain disulfide-linked monomers or, more commonly, linear monomers. Here we report for the first time the presence, in ant venoms, of (*i*) monomeric peptides structured by one intrachain disulfide bond (e.g., PP-b) and (*ii*) dimeric peptides comprised of hetero- and homomeric combinations of the same peptides (PP-a and PP-b) structured by two interchain disulfide bonds. Except for snake venoms (Osipov et al., 2008 and papers cited therein), peptides with a dimeric scaffold are quite rare in other venomous animals, although they have occasionally been noted in the venoms of some scorpions (Zamudio et al., 1997), spiders (Santos et al., 1992) and marine cone snails (Loughnan et al., 2006). For ant venoms, heterodimeric peptides formed from intrachain disulfide-linked monomers such as ectatomin from *E. tuberculatum* venom have been described (Pluzhinikov et al., 1994). Heterodimeric and homodimeric pilosulin peptides formed from linear monomers with one or two interchain disulfide bonds have also been reported in the venom of *Myrmecia pilosula* (Wiese et al., 2007; Inagaki et al., 2008), while heterodimeric peptides reticulated by two interchain disulfide bonds are present in a range of myrmexins in the

venom of *P. triplarinus* (Pan and Hink, 2000), another plant-ant species strictly associated with the myrmecophyte *Triplaris americana* (Polygonaceae), but belonging to another group of *Pseudomyrmex* (Ward and Downie, 2005). However, in none of these venoms are there examples of peptides where both homodimeric (e.g., PP-aa and PP-bb) and heterodimeric (e.g., PP-ab) combinations of the same two peptides exist. Indeed the present study has shown that at least one of these peptides (PP-b) also exists as a disulfide-bonded monomer, adding further structural complexity.

Ants have thus evolved a biochemically economical way of adding structural and pharmacological diversity to their venoms by using post-translational modifications of single-chain peptides to form homo- or heteromeric dimers. As both monomers and dimers are found in the venom, the biosynthesis of two single peptide chains can potentially result in the presence of five mature peptides in the venom, most likely endowed with different pharmacological properties. This appears to be different to the majority of other arthropod venoms, such as those from spiders and scorpions, that appear to contain mostly peptide monomers with 1–7 intrachain disulfide bonds and often a defined structural motif e.g., inhibitory cystine-knot or disulfide-directed β-hairpin (Nicholson, 2013).

Using venom potency assays and MS analysis, we have shown that there is likely a strong difference in the venom composition of *Pseudomyrmex* species based on their nesting mode. In the search for environmentally safe insecticides and peptides with antimicrobial activities, the diversified venoms of ground-dwelling species may be a good source of useful bioactive molecules. Yet, the structural diversity in the venom peptides of the plant-ant *P. penetrator* presents strong similarities with that of mymexins I–VI from *P. triplarinus*, another plant-ant. Because the association with a myrmecophyte requires that it protects

the host plant from phytophagous insects as well as browsing mammals, the venoms of plant-ants may be a promising source of peptides that modulate both insect and vertebrate molecular targets. This is exemplified by the presence of peptides with both interchain disulfide bonds (heterodimeric or homodimeric forms) and intrachain disulfide bonds (monomeric form) in the plant-ant venom – a novel class of ant peptides.

Ethical statement

The experiments comply with the current laws of the country in which they were conducted. The authors declare that they have no conflict of interest.

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Chapitre 3. Biochimie

Les pressions de sélection pour la capture des proies et la défense contre les prédateurs ont ainsi permis aux fourmis d'élaborer un arsenal complexe de toxines qui possèdent probablement une variabilité pharmacologique importante. Alors que la structure primaire des peptides varie considérablement dans les venins, les structures tertiaires restent relativement bien conservées. On retrouve ainsi des motifs structuraux partagés par un large groupe de polypeptides dont les séquences et les pharmacologies diffèrent [Casewell et al., 2013; Mouhat et al., 2004].

En moyenne seuls 3 à 4 % des acides aminés sont cruciaux pour le maintien d'une structure protéique fonctionnelle. Cependant pour les toxines animales, une plus large proportion d'acides aminés est nécessaire, particulièrement en raison des cystéines formant les ponts disulfure qui ont un rôle dans la détermination et la stabilisation des structures tridimensionnelles [Kordiš and Gubenšek, 2000]. Les ponts disulfure assurent aux peptides une grande stabilité chimique et apportent une plus grande résistance aux dégradations enzymatiques et permettent aux peptides d'adopter certaine conformation tridimensionnelles [Zhou et al., 1993]. On retrouve de nombreux peptides riches en ponts disulfure dans les venins des différents organismes venimeux. Certains motifs structuraux comme les peptides ICK, sont présents dans les venins de nombreux organismes (araignées, serpents, scorpions, cnidaires) et d'autres motifs peuvent être associés spécifiquement avec un certain type d'organisme venimeux telles que les conotoxines (*e.g*. ω-conotoxine) avec les cônes [Olivera and Teichert, 2007] ou encore les toxines à trois-doigts caractéristiques des venins de serpents (Elapidae) [Kini and Doley, 2010].

Dans les venins de fourmis peu de structures ont été décrites et la majorité des peptides séquencés à ce jour sont linéaires. Pourtant l'importante diversité écologique et taxonomique des fourmis ainsi que la grande diversité fonctionnelle de leur venin laissent espérer une plus grande diversité structurale dans les peptidomes.

- **Quelle est la diversité structurale des toxines dans les peptidomes des venins de fourmis ?**

1. Diversité structurale des peptides

La diversité structurale des peptides dans les venins de fourmis reste très méconnue. Seules trois grandes classes de peptides ont été découvertes : les peptides linéaires, les peptides dimériques et les peptides ICK. Dans ce chapitre nous avons réalisé une étude extensive de la diversité des peptides dans les venins de fourmis en recherchant tout particulièrement la présence de ponts disulfure.

L'analyse des venins bruts de 82 espèces appartenant aux 9 principales sous-familles de fourmis à aiguillon à été effectuée par spectrométrie de masse MALDI-TOF. Dans un deuxième temps, une étude plus détaillée des peptidomes par LC/MS couplée avec la réduction chimique des peptides a également été effectuée sur plusieurs espèces sélectionnées. Les résultats indiquent que les peptidomes de fourmis sont majoritairement composés de petits peptides dont la taille est inférieure à 4 kDa, soit une estimation de moins de 35 acides aminés. On retrouve cependant des peptides de taille supérieure (> 4 kDa) principalement dans les sous-familles appartenant au clade des formicoides et ceci est particulièrement vrai dans la sous-famille des Dorylinae néotropicales qui produisent un

groupe de peptides dont la masse est comprise entre 6000 et 8000 Da. Les réductions chimiques des peptides ont permis de démontrer que la majorité des peptides dans les venins étudiés sont linéaires (dépourvus de ponts disulfure). Les peptides structurés par des ponts disulfure sont des composés minoritaires des peptidomes de fourmis à l'exception de certains venins qui semblent essentiellement composés de peptides à ponts disulfure (*Prionopelta* cf. *amabilis* et le genre *Anochetus*). Bien que minoritaires dans les venins, on a retrouvé des peptides à ponts disulfure dans les venins de presque toutes les sous-familles de fourmis étudiées, à l'exception de la sous-famille Paraponerinae. Ces peptides à ponts disulfure sont soit monomériques soit dimériques et ils sont structurés par 1, 2 ou 3 ponts disulfure. Ces résultats ont mis au jour la grande diversité moléculaire des toxines des venins de fourmis et ils ont permis la découverte de nouvelles familles structurales de toxines qui possèdent probablement de nouvelles activités biologiques et pharmacologiques.

Article 5

The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling

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The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling

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ABSTRACT

RATIONALE: Compared with other animal venoms, ant venoms remain little explored. Ants have evolved complex venoms to rapidly immobilize arthropod prey and to protect their colonies from predators and pathogens. Many ants have retained peptide-rich venoms that are similar to other arthropod groups.

METHODS: With the goal of conducting a broad and comprehensive survey of ant venom peptide diversity, we investigated the peptide composition of venoms from 82 stinging ant species from nine subfamilies using MALDI-TOF mass spectrometry. We also conducted an in-depth investigation of eight venoms using reversed-phase (RP)-HPLC separation coupled with offline MALDI-TOF mass spectrometry.

RESULTS: Our results reveal that the peptide composition of ant venom peptidomes from both poneroid and formicoid ant clades are comprised of hundreds of small peptides (<4 kDa), while large peptides (>4 kDa) are also present in the venom of formicoids. Chemical reduction revealed the presence of disulfide-linked peptides in most ant subfamilies, including peptides structured by one, two or three disulfide bonds as well as dimeric peptides reticulated by three disulfide bonds.

CONCLUSIONS: The biochemical complexity of ant venoms, associated with an enormous ecological and taxonomic diversity, suggests that stinging ant venoms constitute a promising source of bioactive molecules that could be exploited in the search for novel drug and biopesticide leads.

Introduction

Animal venoms are sophisticated biochemical weapons that are currently under intense investigation for their potential development into novel sources or lead compounds for therapeutic agents and insect-selective biopesticides. The molecular diversity of venoms, particularly peptide toxins, is linked to a formidable array of molecular targets and pharmacological properties. Several drugs and a biopesticide have already been developed based on the high selectivity and potency of venom peptides and an in-depth exploration of animal venoms will undoubtedly lead to further discoveries [1-3].

Venoms exhibit an extraordinary biochemical complexity ranging from small molecules to large proteins that have been fine-tuned by nature for greater efficacy and target selectivity. In most animal venoms, peptides are the predominant class of toxins and have been investigated intensively in snakes [Kini and Doley, 2010], scorpions [Ma et al., 2012], cone snails [Biass et al., 2009; Davis et al., 2009] and spiders [Escoubas et al., 2006; King and Hardy, 2013; Palagi et al., 2013]. However, as the venomous animal biodiversity encompasses ca. 173,000 species, the vast majority of animal venoms remain unexplored in spite of their potential. Several large venomous animal groups have been largely understudied including centipedes [Yang et al., 2012], ticks [Nicholson et al., 2006], sea anemones [Rodríguez et al., 2012], wasps [Baptista-Saidemberg et al., 2011] and ants. This is largely due to the small size and difficulty of collecting venoms or glands from these invertebrates. However, cutting-edge technologies such as transcriptomic and proteomic approaches now offer the possibility of exploring these venoms in detail, with samples of a limited size. Many species are also cryptic, difficult to access or can only be identified by specialized taxonomists. In addition, they may not be seen as a potential health threat to

humans and therefore have attracted less attention than snakes, scorpions, spiders and cone snails that have been responsible for many human fatalities.

Ants (Hymenoptera: Formicidae) are a good example of such a neglected group. Ants are extremely diverse and ubiquitous in terrestrial environments [Hölldobler and Wilson, 1990] and can be considered one of the most abundant groups of venomous animals on Earth. Ants are known to use venoms for both offensive and defensive purposes and are also among the leading predators of other invertebrates in most ecosystems [Brady et al., 2006]. Some ant subfamilies lack stingers and spray secretions containing formic acid from their venom glands (Formicinae) or deposit small chemicals from their pygidial glands (Dolichoderinae) onto their targets. However, *ca*. 70% of all ant species (*ca*. 9,000 species) are capable of stinging and inject their venoms *via* an abdominal apparatus including a stinger connected to a venom gland [AntWeb, 2014b]. Despite this, it is fascinating to note that although the total number of stinging ant species is actually higher than the combined number of snake, scorpion and cone snail species, very little is known about their venom composition. Apart from those individuals with an allergy to ant stings, this is largely due to their innocuity and their small size. So far, most work on ant venoms has concentrated on small organic molecules such as hydrocarbons and alkaloids [Brand et al., 1972; Jones et al., 1991; Morgan et al., 2003]. However, ant venoms also contain a variety of peptides and proteins [Schmidt, 1978; Touchard et al., 2014a] and therefore are potentially as complex as venoms from other groups of arthropods. This variability in the composition of ant venoms may be further enhanced by differing selection pressures due to their enormous ecological diversity [Pluzhnikov et al., 2000; Schmidt, 1978].

Recent studies have revealed that stinging ant venoms are rich in peptides, similar to other venomous animals [Touchard et al., 2014a]. However, very few ant venom peptides

have been characterised and only 72 peptide sequences have been reported to date. These are mainly linear peptides lacking disulfide bonds that have antimicrobial, cytolytic and insecticidal activities. Examples include poneratoxin [Piek et al., 1991a; Szolajska et al., 2004], ponericins [Orivel et al., 2001], bicarinalins [Rifflet et al., 2012] and dinoponeratoxins [Cologna et al., 2013; Johnson et al., 2010]. The venoms of other arthropods as well as mollusks and snakes are known to contain disulfide-rich peptides as their main constituents [Harvey, 2006; King and Hardy, 2013; Lewis and Garcia, 2003; Possani and Rodríguez de la Vega, 2006; Ueberheide et al., 2009]. Although broad coverage of ant peptides have not been broadly studied so far, some preliminary data indicate that ant venoms may also contain various types of peptides cross-linked by disulfide bridges. Disulfide bonds constrain the peptide backbone into rigid three-dimensional scaffolds which endow the peptides with a better chemical stability, increased resistance to *in vivo* proteolytic degradation and form tightly defined pharmacophores that can be finely modulated by single amino acid mutations [Zhou et al., 1993]. These characteristics make cysteine-rich venom peptides the major active components of animal venoms that are behind their pharmacological activities [King and Hardy, 2013]. In particular, the inhibitor cystine knot (ICK) structural motif is relatively common in small cysteine-rich peptide toxins from a variety of animal venoms and plants [Gilly et al., 2011; Pallaghy et al., 1994; Rodríguez et al., 2014; Zhu et al., 2003]. While large numbers of ICK peptide toxins have been reported in other arthropods such as cone snails, sea anemones, spiders and scorpions, only two such ant venom peptides with a disulfide connectivity consistent with ICK toxins are currently described : a *Dinoponera* ICK-like peptide and SKTXs [Inagaki et al., 2008b; Torres et al., 2014]. Indeed, all of the disulfide-rich peptides previously isolated from ant venoms were homo- and heterodimeric complexes such as the myrmexins, pilosulins, and ectatomins found in venoms from the

subfamilies Myrmeciinae [Inagaki et al., 2004; Inagaki et al., 2008a], Pseudomyrmecinae [Pan and Hink, 2000] and Ectatomminae [Pluzhnikov et al., 1994] (for a complete review see [Aili et al., 2014]).

In light of the enormous chemical, taxonomical and ecological diversity of ants, we therefore hypothesized that ant venoms likely represent a promising source of unique peptides with original scaffolds and novel pharmacologies. The study of ant venoms may therefore open up a new, and largely unexplored, field in toxinology that may hold great potential in the search for novel drug leads and genetically engineered biopesticides.

In earlier studies, we reported a novel method for the investigation of venom peptides and have successfully applied it to the chemotaxonomic study of selected ant species [Touchard et al., 2014a]. The present study embarked on a broader investigation of ant venoms, with the aim of conducting a wide survey of ant venom peptidomes among nine different stinging ant subfamilies. The study was designed to provide the comprehensive coverage of ant venom peptide composition, particularly focusing on the discovery of disulfide-linked peptides in these venoms. The MS profiling of these ant venoms has revealed the occurrence of hundreds of unknown small linear peptides as well as many novel peptides cross-linked by one, two or three disulfide bonds, suggesting the great depth of structural and probable pharmacological diversity in ant venoms.

Materials and methods

Ant Collection and Taxonomy

Venoms from 82 ant species were investigated in the present study, covering 31 genera from 9 of the 16 stinging ant subfamilies. Field collections of live worker ants were conducted in various areas of French Guiana, with additional samples taken from continental

France as well as Trinidad and Tobago. In order to include the largest possible biodiversity and phylogenetic range, additional venoms were purchased from a commercial supplier (Southwest Venoms, Tucson, AZ, USA). The samples included in the present study represent *ca*. 1% of the total stinging ant species currently described and *ca*. 13% of all stinging ant genera (Table 1 and Fig. 1A). Other subfamilies not included in the study are either rare or not present in the collection areas accessible to our group (South America, Australia and France), such as African ants. Some subfamilies such as the Amblyoponinae and Cerapachyinae are difficult to investigate because they are subterranean, which makes collection work more prone to serendipity. Also the subfamily Myrmicinae is very large and diversified with *ca*. 6,500 species [AntWeb, 2014b] and therefore it is difficult to obtain a representative coverage of this subfamily. Phylogenetic analyses were conducted according to the most recent phylogeny of ants which describes 21 subfamilies and divides ant subfamilies into three clades: leptanilloid, poneroid and formicoid [Brady et al., 2006; Moreau et al., 2006]. Ants belonging to the leptanilloid clade were not included in this study. The complete list of the ant species investigated in the present study is provided in Supplementary Tables 1 and 2.

Collected ants were stored at –20°C prior to the dissection of the venom glands and specimens from each of the 82 species were also stored in 96% ethanol for later morphological identification. Three to thirty venom glands from worker ants were dissected for each species, pooled and stored in 10% acetonitrile (ACN) / water (v/v) for whole venom analysis. For further exploration by liquid chromatography coupled to mass spectrometry (LC/MS), 8–50 venom glands per species were dissected and pooled. Samples were centrifuged for 5 min at 14,400 rpm; the supernatant was collected and the freeze-dried prior to storage at –20°C. Freeze-dried venoms of *Myrmecia* sp., *Diacamma* sp., *Dinoponera*

grandis, *Streblognathus aethiopicus*, *Tetraponera* sp. and *Pogonomyrmex maricopa* were purchased from Southwest Venoms.

Table 1. Genera and extant species of stinging ants

Clade	Subfamily	No. of extant genera	No. of genera studied	% of genera studied	No. of extant species	No. of species studied	% of species studied
poneroid	Amblyoponinae	13	1		121	1	
	Paraponerinae	1	1		1	1	
	Ponerinae	28	10		1157	39	
	Martialinae	1	0		1	0	
	Proceratinae	3	0		135	0	
	Agroecomyrmecinae	2	0		3	0	
formicoid	Cerapachyinae	7	1		267	2	
	Ecitoninae	5	4		151	8	
	Myrmeciinae	2	1		92	4	
	Pseudomyrmecinae	3	2		229	4	
	Ectatomminae	4	2		270	10	
	Myrmicinae	142	9		6443	13	
	Leptanilloidinae	3	0		15	0	
	Aenictinae	1	0		174	0	
	Heteroponerinae	3	0		24	0	
leptanilloid	Leptanillinae	6	0		59	0	
	Total	**224**	**31**	**13.84%**	**9142**	**82**	**0.90%**

Mass spectrometry analysis of crude venoms

MS analyses were performed on a Voyager DE-Pro MALDI-TOF (Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight) mass spectrometer (Applied Biosystems, Inc, Foster City, CA USA). Samples were prepared as previously reported using a ferulic acid (FA) matrix dissolved in 20% ACN / water (v/v) containing 0.1% v/v trifluoroacetic acid (TFA) at a concentration of 10 mg/mL with the addition of 100 mM serine [Touchard et al., 2014a]. The FA/serine combination has previously been shown to be the most efficient matrix for the analysis of crude ponerine ant venoms by MALDI-TOF MS [Touchard et al., 2014a]. Prior to MS analysis, crude venoms were desalted using ZipTip® C18 pipette tips (Millipore, Billerica,

MA USA). Then 0.5 µL of the desalted sample was deposited on the MALDI target plate followed by 0.5µL of matrix. Each mass spectrum was calibrated externally using a mixture of peptides of known mass values in the same *m/z* range (Peptide Calibration Mix 4, LaserBio Labs, Sophia-Antipolis, France). Then 0.5 µL of the calibration mixture was co-crystallized with 0.5µL of the matrix, spotted adjacent to each sample and measured separately. All calibration spectra were acquired in the automated mode, to maximize mass accuracy and reproducibility, and were calibrated automatically. Spectra of crude venoms were acquired in linear or reflector mode and calibrated automatically using the sequence module of the Voyager® Control Software (Applied Biosystems, Foster City, CA USA). Five spectra of 50 laser shots per spectrum were accumulated for each sample based on the acceptance parameters and adequate signal intensity in the *m/z* 500–10,000 range. Mass spectra were collected in positive ion mode with 20 kHz acceleration. Signals below *m/z* 500 were not recorded as they were comprised of mostly matrix-related ion clusters [Escoubas et al., 2006]. All ions observed were singly charged ([M+H+]), as for peptides in the mass range observed. No doubly charged ions could be observed under the ionisation conditions used.

RP-HPLC separation

In order to obtain a representative picture of ant venom diversity, we selected one venom each from eight different ant subfamilies to be further analysed by LC/MALDI MS. The C18 reversed-phase high-performance liquid chromatography (RP-HPLC) separation of venoms of the following species was therefore conducted: *Prionopelta* cf. *amabilis* (Amblyoponinae), *Pachycondyla goeldii* (Ponerinae), *Acanthostichus* sp. 1 (Cerapachyinae), *Eciton burchelli* (Ecitoninae), *Myrmecia pilosula* (Myrmeciinae), *Tetraponera* sp. (Pseudomyrmecinae), *Gnamptogenys sulcata* (Ectatomminae) and *Manica rubida* (Myrmicinae). These selected ant

venoms were fractionated using an Xterra-C18 5 µm, 2.1 x 100 mm column (Waters, Milford, MA USA) using a gradient of solvent A (0.1% v/v TFA) and solvent B (ACN/0.1% v/v TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0–60% over 60 min, 60–90% over 10 min and 90–0% over 15 min at a flow rate of 0.3 mL/min. The eluate was monitored by UV absorbance at 215 nm on a diode-array detector. All analyses were performed on an HP 1100 HPLC system (Agilent, Santa Clara, CA, USA). Peptide elution was monitored in real time and fractions were collected manually for each eluting peak. Each fraction was then dried and reconstituted in 50 µL of 0.1% v/v TFA for offline MALDI-TOF MS analysis and disulfide bond reduction.

Disulfide bond reduction and alkylation

The presence of disulfide-bonded peptides in ant venoms was determined through the chemical reduction of crude venoms and HPLC fractions. 5 µL of crude venom or selected HPLC fractions were incubated with 10 µL of 100 mM ammonium bicarbonate buffer (pH 8) containing 6 M guanidine and 10 mM dithiothreitol (DTT) for 30 min at 56°C. The reaction was stopped by the addition of 5 µL of 0.1% v/v TFA. Prior to MS analysis, reduced venoms or fractions were desalted using ZipTip® C18 pipette tips (Millipore, Billerica, MA USA). Chemical reduction results in a mass increase of 2 Da for each disulfide bond. Thus, by comparing the mass spectra of native and reduced samples, the number of disulfide bonds in ant venom peptides can be determined. However, due to the use of a single-stage MALDI-TOF MS instrument, the resolution of the instrument did not permit the detection of +2 Da mass differences in peptides with masses above 5kDa. Therefore, the venom fractions which contained peptide masses over 5 kDa were also alkylated, following DTT reduction, by incubating the mixture with 50 mM iodoacetic acid (IAA) for 15 min at room temperature in

the dark. The formation of the *S*-carboxymethyl derivative of cysteine results in a mass increase of 116 Da for each disulfide bond (58 Da for each cysteine residue) that allows the determination of the number of disulfide bonds in larger venom peptides.

Mass spectra analysis

All mass spectra were processed with Data Explorer® 4.11 software (AB SCIEX, Framingham, MA USA) and subjected to a baseline correction with a correlation factor of 0.7 and Gaussian smoothing to reduce noise with a 5-point filter width. Supplementary $[M+H^+]$ ions resulting from sodium or potassium adducts were manually removed from all mass lists. Values within *m/z* ± 1.0 of neighbouring HPLC fractions were considered as identical peptides, reflecting incomplete separation, and were also removed. Two-dimensional scatter plots, termed '2D venom landscapes', were constructed using SigmaPlot 12.0 software (Systat, CA, USA). All peptide masses detected in each HPLC fraction were plotted as a function of their *m*/*z* values (*x*-axis) and their HPLC retention time reflecting their hydrophobicity (*y*-axis). Averaged data represents the mean ± S.D. unless otherwise stated.

Results

Crude venom analysis

The crude venoms of the 82 ant species were initially analyzed by MALDI-TOF mass spectrometry and a total of 1396 distinct masses were detected in all venoms after the elimination of masses consistent with doubly charged ions, or potassium and sodium adducts. The mean number of peptides detected in crude venom profiles was 17 ± 9 (mean ±

S.D) and varied widely, ranging from 4 to 42 in *Chalepoxenus muellerianus* (Myrmicinae) and *Solenopsis saevissima* (Myrmicinae) venoms, respectively.

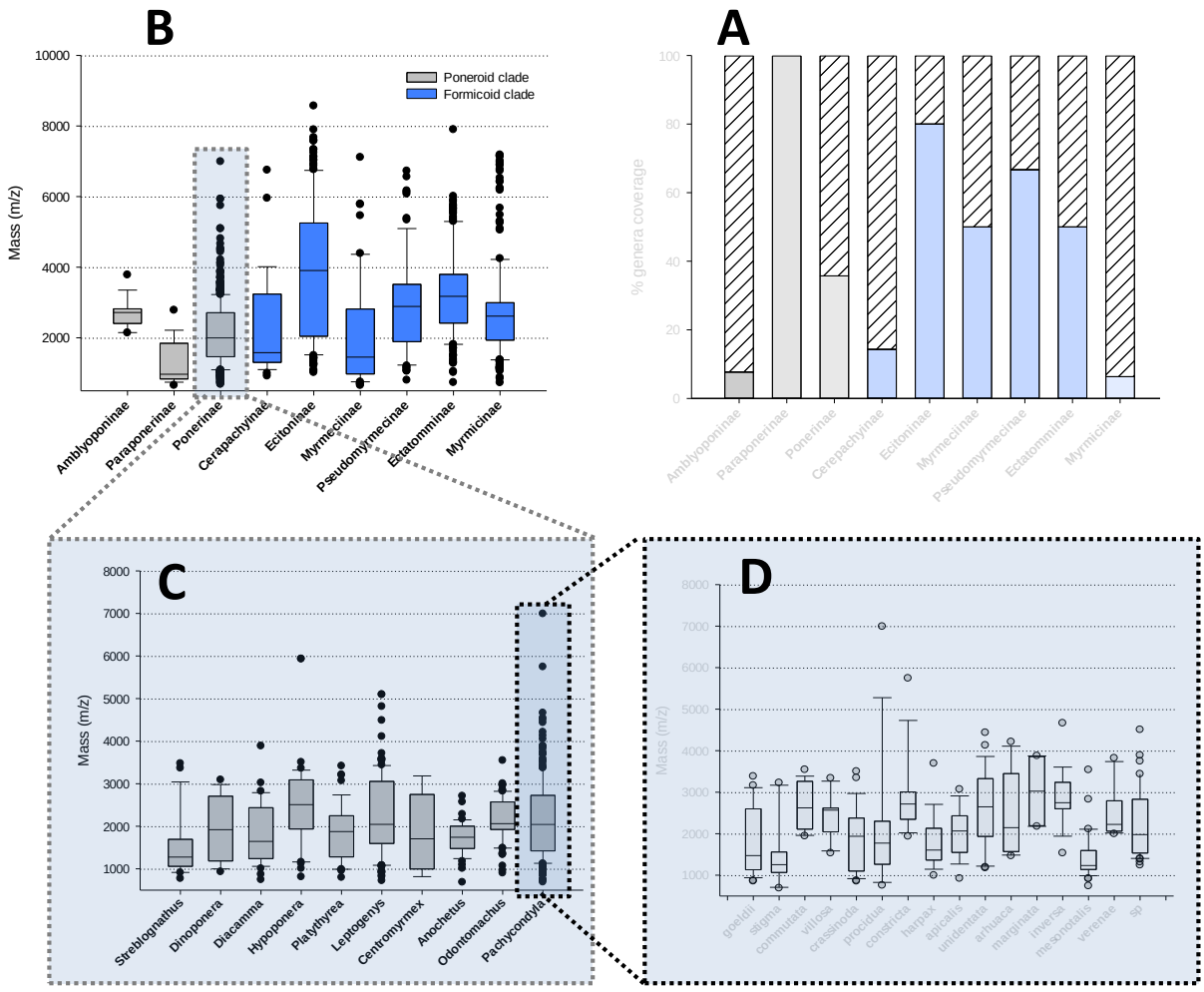

Fig. 1 – MALDI-TOF MS analysis of 82 crude venoms belonging to 9 stinging ant subfamilies from poneroid and formicoid clades. (A) Diversity coverage (%) of the nine subfamilies of stinging ants studied from poneroid (filled grey bars) and formicoid clades (filled blue bars). The white striped bars represent the percentage of genera not studied within each subfamily. (B) Box-and-whisker plot of the peptide mass distribution of all 82 ant venoms organised by subfamilies. The bottom and top end of each box represent the first and third quartiles, respectively, while the line inside each box represents the median mass. The ends of the whiskers represent the 5–95 percentile range while the black circles represent masses outside the 5–95 percentile range. (C) Box-and-whisker plot of the peptide mass distribution of ant venom peptides in the subfamily Ponerinae. (D) Box-and-whisker plot of the peptide mass distribution of ant venom peptides from 16 species of the genus *Pachycondyla*.

The analysis of crude venoms revealed a great heterogeneity in the peptide molecular weight between subfamilies with an overall range of *m/z* 652.2–8569.4 [M+H$^+$]. The huge variation in peptide composition between subfamilies is illustrated by the narrow *m/z* range of Amblyoponinae (*m/z* 2138.8–3782.6) as opposed to the broad *m/z* range observed in both Ponerinae and Ecitoninae subfamilies (*m/z* 685.7–6992.0 and *m/z* 1019.5–8569.4, respectively). The mass analysis of all crude venoms revealed the presence of mostly small ions with 87% of all *m/z* values below 4000 (Fig. 1B). However, larger ions with *m/z* values >4000 were also observed in specific venoms, notably in subfamilies belonging to the formicoid clade, where 22.9 ± 23.7% (*n* = 41 species investigated) of the ions have *m/z* values > 4000. The proportion of large peptides in ant venoms is even greater in Ecitoninae venoms with 56.3 ± 24.3% (*n* = 8 species investigated) of ions [M+H$^+$] in the *m/z* range 4000.8–8569.4. This finding is consistent with previous reports of large peptides described and sequenced from formicoid ant venoms such as ectatomins [Arseniev et al., 1994], myrmexins [Pan and Hink, 2000] and pilosulins [Inagaki et al., 2004; Inagaki et al., 2008a; Wu et al., 1998]. It should be noted that the larger peptide masses are associated with dimeric forms of peptides, often associating two linear chains linked by interchain disulfide bonds [Aili et al., 2014]. In the range of species studied, the proportion of large peptides (*m/z* > 4000) found in ant venoms from the poneroid clade was significantly lower than in the formicoid clade (Mann-Whitney U = 280, $n_1 = n_2 = 41$, $P < 0.001$). Only 3.0 ± 6.1% (*n* = 39 species investigated) of the peptides of ponerine ants were found to have *m/z* values > 4000 (*m/z* range 4049.8–6992.0). This result confirms our earlier observations of Ponerinae ant venom composition, particularly from the *Pachycondyla* and *Odontomachus* genera, where 99% of all masses detected were in the mass range 0.5–5.0 kDa [Touchard et al., 2014a]. However, despite the narrow range of masses, there are significant differences between genera and

between species belonging to the same genus as illustrated by results from ten different genera in the subfamily Ponerinae (Fig. 1C) and species from the large genus *Pachycondyla* (Fig. 1D).

Overall, the examination of crude ant venom peptidomes indicates that ant venoms may contain primarily small peptides in the mass range 0.5–4.0 kDa with larger peptides observed mostly in the formicoid clade. We can therefore estimate that the lengths of most ant venom peptides are below 35 residues. This estimate was determined from a MW_{av} of 111.1254 Da using the statistical occurrences of amino acids in the proteins of an average amino acid, averagine, calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}O_{1.4773}S_{0.0417}$ [Senko et al., 1995]. This finding is similar to those of conotoxins that are typically between 10 and 30 amino acids in length [Lewis et al., 2012] but is in contrast to spider, scorpion and snake venom peptide toxins that are normally between 40 and 100 amino acids [Olivera et al., 1990].

Disulfide bond mapping in crude venoms

To broadly map the presence of disulfide-bonded peptides in crude venoms, the venoms were reduced. The comparative MALDI-TOF MS profiling of native venoms revealed the presence of *ca*. 50 peptides linked by disulfide bonds. We detected disulfide-bonded peptides among five distinct ant subfamilies from both the formicoid and poneroid clades (*i.e*. Ponerinae, Amblyoponinae, Ectatomminae, Myrmeciinae and Myrmicinae). A comparison of the spectra before and after reduction permitted us to detect the presence of peptides with one, two or three disulfide bonds (Fig. 2A-C). These masses are listed in the supplementary Table 3.

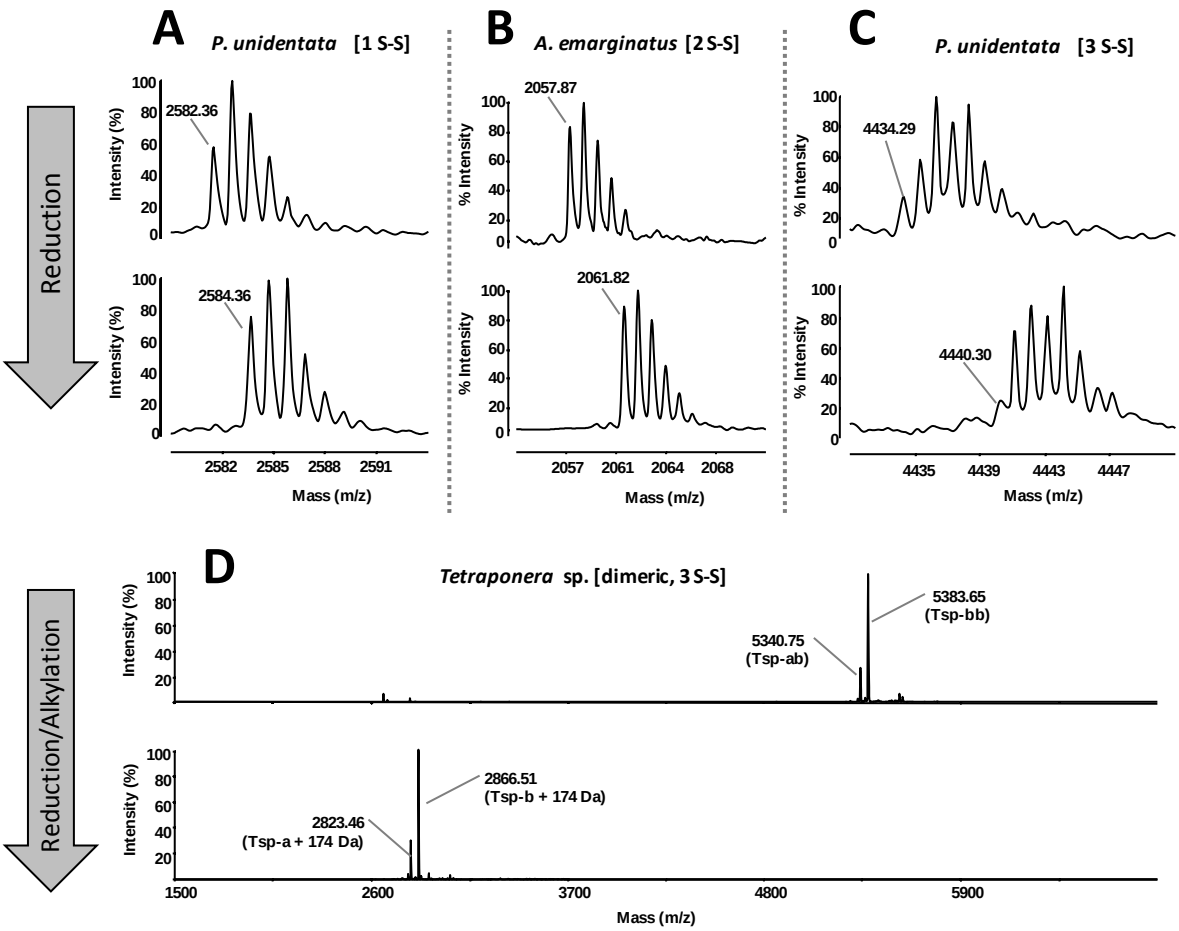

Fig. 2 – Identification of disulfide-bonded peptides in ant venoms. Typical MALDI-TOF MS spectra recorded in reflector mode showing [M+H$^+$] ions of peptides increases of 2 Da (A; *Pachycondyla unidentata*), 4 Da (B; *Anochetus emarginatus*) and 6 Da (C; *Pachycondyla. unidentata*) consistent with the presence of one, two and three disulfide bonds, respectively. Upper panels show native peptide isotopic clusters, while lower panels show the same peptide in reduced form obtained after reduction with DTT. (D) MALDI-TOF mass spectra from *f*16 of *Tetraponera* sp. venom before, and after, reduction and alkylation with IAA (R/A). Comparison of spectra before, and after, R/A revealed the presence of the heterodimeric peptide Tsp-ab (*m*/*z* 5340.75) and the homodimeric peptide Tsp-bb (*m*/*z* 5383.65). After R/A, [M+H$^+$] ions of peptides Tsp-ab and Tsp-bb disappeared and the [M+H$^+$] ion of the alkylated monomeric Tsp-a (*m*/*z* 2823.46) and Tsp-b (*m*/*z* 2866.51) appeared. This is consistent with *S*-carboxymethylation of three cysteines in each monomer.

Only 32 peptides with one disulfide bond were detected in 14 crude venoms (*i.e*. *Pachycondyla commutata*, *P. mesonotalis*, *P. unidentata*, *Odontomachus hastatus*, *O. scalptus*, *Anochetus horridus*, *A.* cf. *diegensis*, *Streblognathus aethiopicus*, *Prionopelta* cf.

amabilis, *Ectatomma brunneum*, *Myrmecia rufinodis*, *M.. similima*, *Pogonomyrmex maricopa*, and *Manica rubida*); 17 peptides with two disulfide bonds were detected only in venoms from the genus *Anochetus* (*i.e. A. horridus*, *A.* cf. *diegensis* and *A. emarginatus*); and one peptide with three disulfide bonds was detected in *P. unidentata* venom. From our sample survey, more disulfide-linked peptides were detected in venoms from the poneroid clade (42 disulfide-bonded peptides) than in the formicoid clade (8 disulfide-bonded peptides). Overall, 33 disulfide-bonded peptides were found in Ponerinae venoms plus nine in Amblyoponinae venoms (all poneroids), whereas only one disulfide-bonded peptide was found in Ectatomminae, two in Myrmeciinae and five in Myrmicinae venoms (from the formicoid clade). This may indicate a greater structural diversity in the Ponerinae, although a larger venom sample set encompassing more species is needed for any definitive conclusion to be drawn.

While disulfide-bonded peptides seem to be minor components in most ant venoms, some venoms appeared to deviate from this pattern and were composed mostly of disulfide-bonded peptides. Of the 37 peptides detected in the venoms of the three *Anochetus* species (Ponerinae) 54% were structured by one and two disulfide bonds and 90% of the 10 peptides detected in the venom of *Prionopelta* cf. *amabilis* (Amblyoponinae) were structured by one disulfide bond. This suggests that our study, despite sampling a broad range of ant subfamilies, cannot reveal the full extent of ant peptidome diversity. The vast number of stinging ant species (*ca*. 9,000 species) may reveal other peptide classes, structures and pharmacological properties. This is because specific genera like *Anochetus* and *Prionopelta* may have evolved different venoms based on atypical structural motifs.

LC/MALDI analysis and 2D landscapes

The mass fingerprinting of crude venoms revealed up to a maximum of only 42 peptides, as seen with *Solenopsis saevissima* venom. However, this low peptide count is consistent with results obtained from a series of tarantula venoms where the examination of crude venoms by MALDI-TOF MS revealed a maximum of 50 to 70 peptides [Escoubas et al., 1997; Escoubas et al., 1999; Escoubas et al., 2002; Escoubas et al., 1998b]. Further examination of the HPLC fractions later revealed the full complexity of these tarantula venoms [Escoubas et al., 2006]. Crude venom mass fingerprinting is therefore a suitable tool for preliminary investigation and for comparing venom profiles in a given taxonomic group. However, it cannot reveal the full extent of the venom peptidome, most likely due to ion suppression effects where large numbers of peptides compete for proton capture during the MALDI ionization process. Consequently, the most abundant peptides, or the ones with the highest proton affinity, are likely to be over-represented in the mass spectra, while peptides with low abundance or lesser proton affinity may not ionize, or ionize in amounts below the detection limit. This results in spectra displaying only a subset of the crude venom complexity. The key to revealing the full extent of a venom peptidome diversity is chromatographic separation of the venom prior to mass analysis, using hyphenated chromatography either online (LC coupled to electrospray ionization MS) or offline (LC coupled to sample deposition and MALDI-TOF MS). We therefore proceeded to fractionate a range of ant venoms into fractions containing fewer peptides to reveal the true venom complexity.

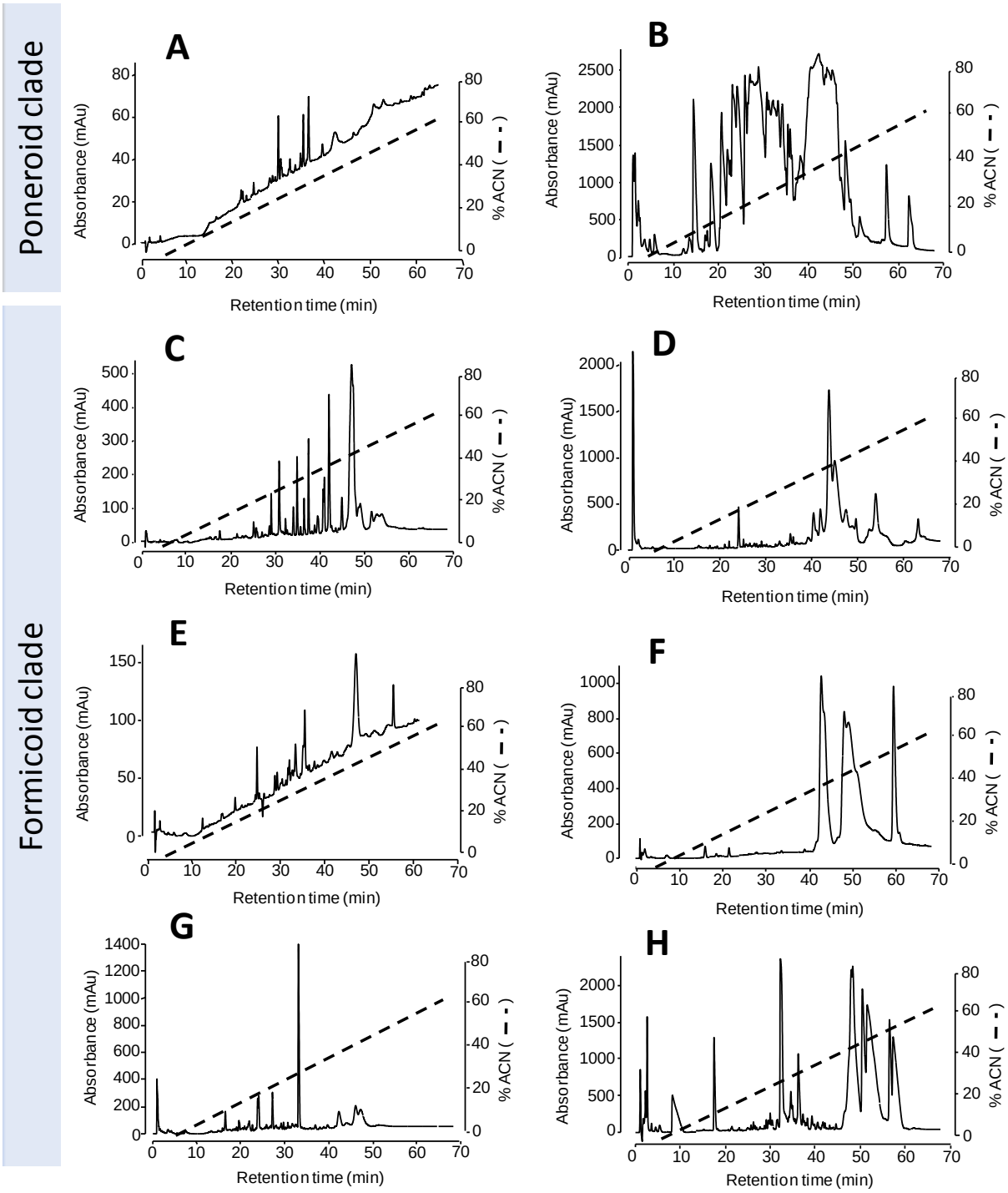

Fig. 3– Representative RP-HPLC chromatograms of formicoid and poneroid ant venoms. Venoms were separated by C18 RP-HPLC using an ACN gradient of 1%/min (black dotted line). Panels show the chromatographic profile of venoms from (A) *Prionopelta* cf. *amabilis* (Amblyoponinae),(B) *Pachycondyla goeldii* (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E) *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G) *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae).

The resulting chromatographic profiles showed few similarities between the various subfamilies (Fig. 3) and varied in their complexity. This is reflected by the number of fractions collected from the chromatograms. A total of 27 fractions were collected from the least complex venom (*Tetraponera* sp.) and 61 from the most complex venom (*Eciton burchelli*). The overall complexity and range of elution patterns observed in ant venoms is parallel to that observed in the RP-HPLC profiles of other venomous arthropods such as Australian funnel-web spiders [Palagi et al., 2013]. The eight ant venoms that were fractionated showed peptides eluting in a broad range of hydrophobicity. The most hydrophilic fraction eluted at 10.8% ACN (Fraction *f*1 from the venom of *Eciton burchelli* at 15.8 min) and the most hydrophobic fraction eluted at 60% ACN (*f*43 from the venom of *Pachycondyla goeldii* at 65 min). *Prionopelta* cf. *amabilis* venom eluted over a narrow hydrophobicity range (15.9–35.2% ACN) while both *Manica rubida* and *Pachycondyla goeldii* venoms eluted in a broad hydrophobicity range (13.1–57% ACN and 15–60% ACN, respectively). This highlights the broad physicochemical diversity among ant venom peptides, suggesting that peptides with different structural scaffolds or widely differing amino acid compositions are present.

The chromatographic fractions were then analysed by offline MALDI-TOF MS leading to the construction of bi-dimensional graphs termed '2D venom landscapes' in which the molecular mass of each peptide in a given venom was plotted against the HPLC retention time reflecting hydrophobicity. The 2D landscapes revealed the enormous peptide complexity in ant venoms (Fig. 4). The total number of masses detected in the venoms analysed varied from 31 to 288 for *Prionopelta* cf. *amabilis* and *Pachycondyla goeldii*, respectively (Fig. 5). Some fractions were highly complex and although they were collected as single peaks based on signal monitoring, mass analysis revealed the presence of up to 26

peptides in a single fraction (*f*18 at a retention time 32.6 min from *Pachycondyla goeldii*). This clearly demonstrates the power of the 2D analytical approach, as true venom complexity is not revealed by single-dimensional analysis.

Although many more peptides were detected, the mass distribution of peptides from the LC/MALDI-TOF MS analyses was quite similar to that previously observed in the analysis of the corresponding crude venoms. A total number of 1112 peptides were detected in the eight venoms by LC/MALDI-TOF MS with 94.4% of all peptides having a mass < 5 kDa and 87.5% < 4 kDa. However, most peptides in the venoms of formicoids fall into a broad *m/z* range such as in *Eciton burchelli* (*m/z* 592.2–7595.0), *Myrmecia pilosula* (*m/z* 679.5–6246.4), *Tetraponera* sp. (*m/z* 1012.2–5773.2) and *Manica rubida* (*m/z* 535.4–6570.0), with many peptides having masses >4 kDa, while the other formicoid and poneroid venoms contain peptides that were almost exclusively lower than 4 kDa (Fig.4). In contrast, some venoms were mostly composed of peptides in a remarkably narrow mass range. This included venoms such as *Prionopelta* cf. *amabilis* that contained only 31 peptides ranging from *m/z* 1411.1 to *m/z* 4265.2 (Fig. 5).

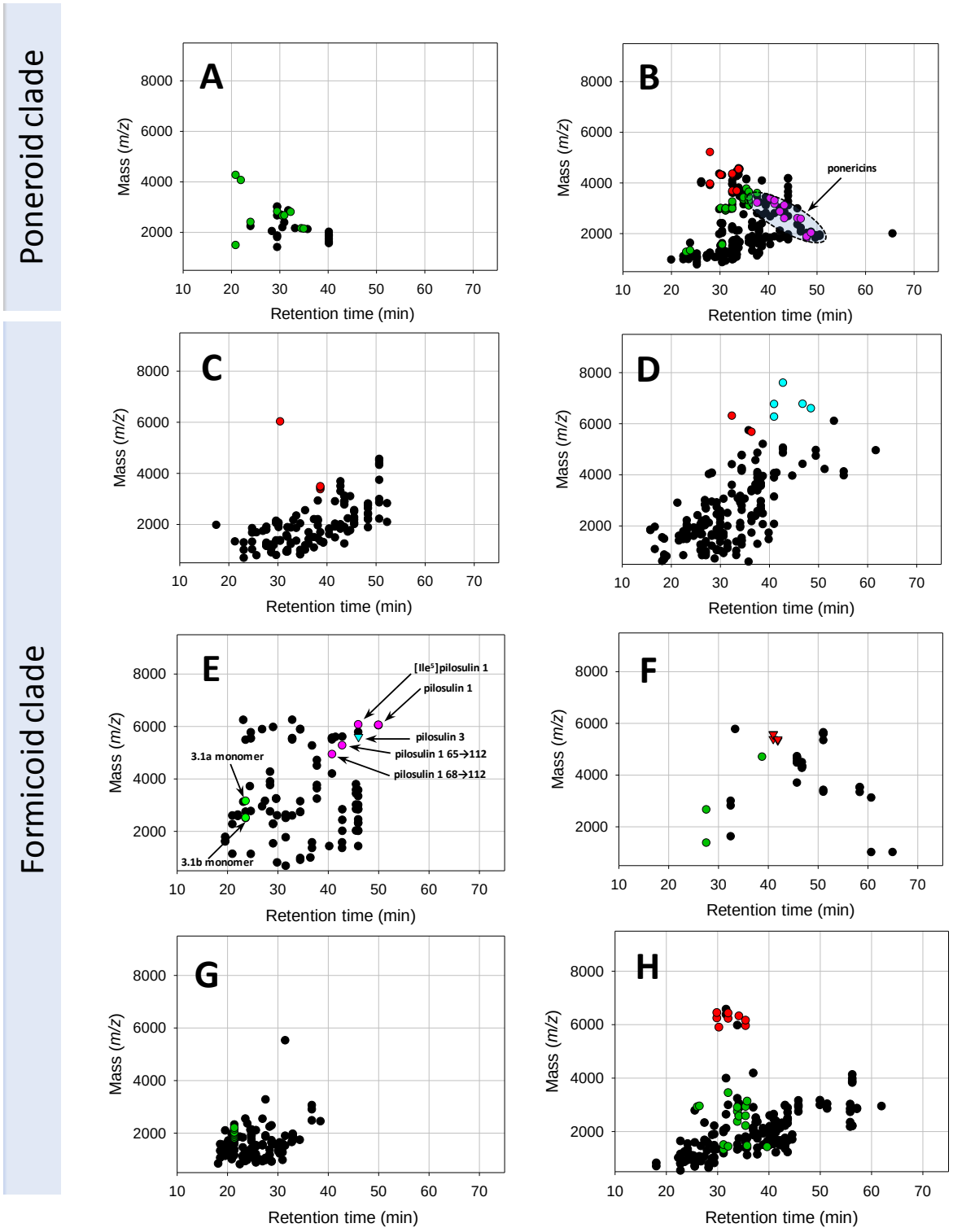

Fig. 4 – 2D Landscapes of ant venoms from formicoid and poneroid ants. (A-H) Representative species from eight different subfamilies showing the LC/MALDI-TOF MS venom profiles of (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla goeldii* (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E) *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G) *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae). Black circles indicate $[M+H^+]$ ions of peptides without disulfide bonds, green circles represent peptides with one disulfide bond, cyan circles $[M+H^+]$ ions of peptides with two disulfide bonds and red circles $[M+H^+]$ ions of peptides with three disulfide bonds. Red inversed

triangles in the 2D landscape of *Tetraponera* sp. (panel F) show the presence of $[M+H^+]$ ions of dimeric peptides with three disulfide bonds while the cyan inversed triangle in the 2D landscape of *M. pilosula* (panel E) represents heterodimeric pilosulin 3 reticulated by two disulfide bonds. Purple circles highlight previously characterised pilosulins (panel E) and ponericins (panel B) found in *M. pilosula* and *P. goeldii* venoms, respectively.

Disulfide bond mapping

For a more precise characterization of disulfide-bonded peptides in each venom, the chemical reduction of each HPLC fraction was carried out to map the presence and number of disulfide bonds. We detected the presence of monomeric peptides with one, two or three disulfide bonds within all the venoms tested (Fig. 4). Peptides reticulated by one disulfide bond were found in a wide range of venoms including *Prionopelta* cf. *amabilis*, *Pachycondyla goeldii*, *Tetraponera* sp., *Myrmecia pilosula*, *Gnamptogenys sulcata* and *Manica rubida*. The *m/z* values of these ions ranged from 1274.2 (*Pachycondyla goeldii*) to 4701.8 (*Tetraponera* sp.). Peptides with 2 disulfide bonds were identified in the venom of *Eciton burchelli* within the range *m/z* 6594–7595 (Fig. 4D). Peptides with two disulfide bonds were also identified in the crude venom analysis of *Anochetus emarginatus*, *A.* cf. *diegensis* and *A. horridus* venoms. However, the *m/z* values of these peptides were much lower compared to those of *Eciton burchelli* (*m/z* 1623.6–2709.1), as shown in the supplementary Table 3. This difference in *m/z* ranges may indicate that these peptides belong to distinct toxin families. A group of 15 linear ponericins were previously characterized from the venom of *Pachycondyla goeldii* [Orivel et al., 2001]. In the present study, 11 of these ponericins (*i.e.* G1, G2, G3, G4, G5, G6, L1, L2, W3 and W5) were also detected within the 2D venom landscape of *P. goeldii* (Fig. 4B).

The disulfide bond mapping of HPLC fractions revealed peptides with three disulfide bonds in the venoms of *Pachycondyla goeldii*, *Acanthostichus* sp. 1, *Eciton burchelli* and

Manica rubida within the broad range of *m/z* 3651.2–6440.8. Both homo- and heterodimeric peptides (Tsp-ab and Tsp-bb) were also found in the venom of *Tetraponera* sp. (Pseudomyrmecinae) with a mass shift, after reduction and alkylation, consistent with the presence of three disulfide bonds (Fig. 2D). It is presently unclear, however, if each Tsp monomer is linked to its other subunit by three inter-chain disulfide bonds or each Tsp monomer has only one inter-chain disulfide and an additional intra-chain disulfide bond. Myrmexins are dimeric peptides that have been previously reported in the venoms of *Pseudomyrmex triplarinus* and *P. penetrator* (Pseudomyrmecinae) [Pan and Hink, 2000; Touchard et al., 2014b]. However, myrmexin peptides only have two cysteine residues per monomer, not three. Although the linear pilosulin 1 (and its isoforms [Ile^{5}]pilosulin 1, pilosulin 1 65→112 and pilosulin 1 68→112) and the heterodimeric pilosulin 3 were found in the venom of *Myrmecia pilosula* (Fig. 4E), the homodimeric pilosulins 4 and 5 were not detected, even though these peptides have been previously reported in *M. pilosula* venom [Inagaki et al., 2004; Inagaki et al., 2008a]. Overall, the percentage of disulfide-linked peptides in venoms ranges from 3 to 20% for *M. pilosula* and *Tetraponera* sp., respectively (Fig. 5), highlighted by the dominance of linear peptides in ant venoms. However, the venom of *Prionopelta* cf. *amabilis* stood out with 32.3% of the 31 peptides having 1 disulfide bond (Fig. 5).

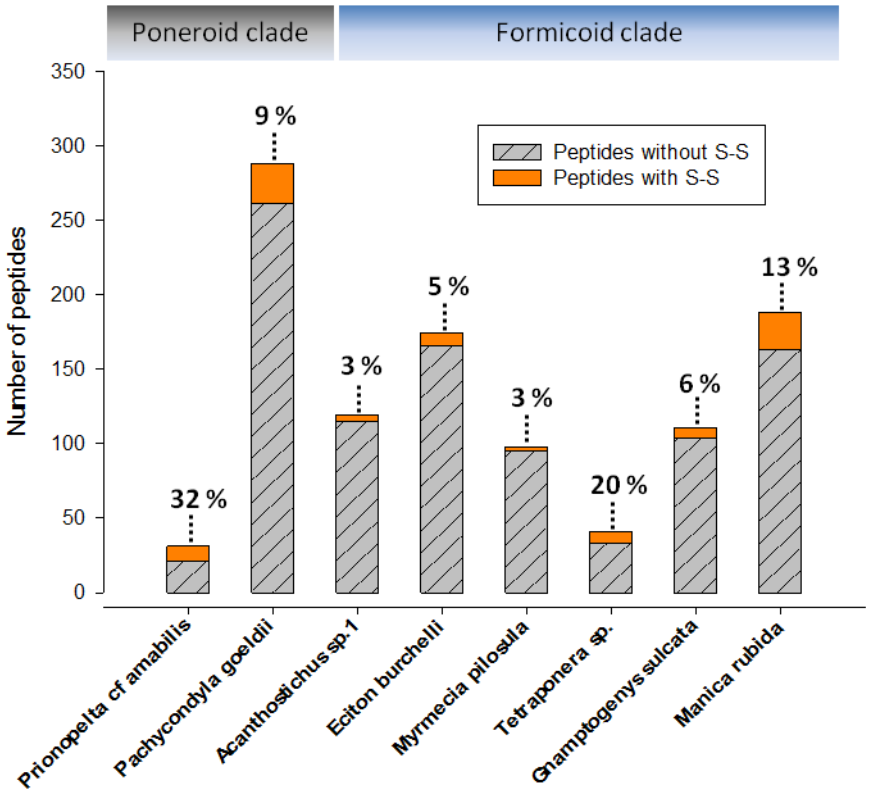

Fig. 5 – Total numbers of peptides in eight representative ant venoms revealed using LC/MALDI-TOF MS. Orange bars show the proportion of disulfide-bonded peptides detected by a mass shift after reduction of each RP-HPLC fraction with DTT. The percentages of disulfide-bonded peptides in whole venoms are indicated above each bar.

Discussion

Ant venoms evolved bioactive peptides in order to disrupt multiple biological targets and permit the capture of arthropod prey, deter predators, communicate with other members of the colony and also act as antimicrobials. It is therefore not surprising that the present study of the ant venom peptidome has revealed enormous variation in the complexity of ant venoms highlighting the potentially diverse pharmacologies and functions of ant venom peptides. It is now well established from other taxonomic groups [Davis et al., 2009;

Escoubas et al., 2006] that venoms are highly complex, containing up to 1000 peptides, and that many peptide classes as well as numerous isoforms in each class combine into a complex biochemical cocktail. Our investigation of ant venoms reveals that a wide range of ants produce venoms with a peptidic composition as complex as those of spiders, scorpions or cone snails as previously demonstrated in the venom of the ponerine ant *Dinoponera quadriceps* [Cologna et al., 2013]. The LC/MALDI-TOF mass fingerprinting of a wide range of poneroid and formicoid ant venoms detected total numbers of peptides ranging from 31 to 288 peptides. Indeed, the venoms of *Pachycondyla* (Ponerinae) ants seem to be particularly rich in peptide components and the total number of peptides appear to be as complex as the previously described venom from the related ponerine ant, *Dinoponera quadriceps* [Cologna et al., 2013]. Based on the present study, we would estimate that ant venoms contain on average *ca*. 130 unique peptides. If we assume a total of *ca*. 9,000 stinging ant species, we can calculate the total number to be more than 1 million peptides in all stinging ant venoms. However, this number is probably an under-estimate, as this does not take into account the intra-species variations observed in ant venoms [Cologna et al., 2013]. Moreover, cryptic ant species also contribute to a hidden peptide diversity as the venom peptide profile can vary among cryptic ant species [Touchard et al., 2014a]. Indeed, many ant species are still undiscovered particularly within tropical areas and the total number of ants could reach 25,000 species (currently *ca*. 13,000 species described) [Ward, 2010].

Hymenopteran venom peptides have not been extensively investigated to date. However, past studies on wasp venoms revealed small linear peptides [Baptista-Saidemberg et al., 2011; de Souza et al., 2004; Gomes et al., 2014; Mendes et al., 2004] and some rare single disulfide-linked peptides such as pallipine-I, -II and –III from the venom of the wasp *Agelaia p. pallipes* [Baptista-Saidemberg et al., 2011] and sylverin from the venom of

Protonectarina sylveirae [Dohtsu et al., 1993]. A wide survey of ant venom peptidomes has never been attempted before and this study therefore constitutes the most extensive overview conducted to date, providing a broad overview of the variability of ant venom peptide composition. Ant venom peptides appear to be similar in mass to those of wasps as the majority of their peptides was also less than 4 kDa and devoid of disulfide bonds. We have also shown that formicoid ants still possess some large peptides, which is consistent with earlier studies on ant venoms that showed peptides larger than 4 kDa among the formicoid subfamilies Ectatomminae, Pseudomyrmecinae and Myrmeciinae [Donovan et al., 1993; Inagaki et al., 2004; Inagaki et al., 2008a; Pan and Hink, 2000; Pluzhnikov et al., 1994]. Given their impressive ecological diversity, formicoid ants may therefore have enhanced their venom arsenal by developing of more complex and larger peptides that permit them to modulate novel pharmacological targets.

Disulfide-bonded peptides often act on ion channels and receptor targets and are common and dominant components in the venoms of a wide range of evolutionarily unrelated predators including sea anemones, cone snails, centipedes, scorpions, spiders and snakes [Ueberheide et al., 2009; Yang et al., 2012]. The present study has highlighted the distribution of monomeric peptides structured by one, two or three disulfide bonds in a wide range of ant venom peptidomes. Based on the present study, such peptides appear to be minor components within most ant venom peptidomes except for some venoms such as *Anochetus emarginatus*, *A.* cf. *diegensis*, *A. horridus* and *Prionopelta* cf. *amabilis* that are mostly composed of disulfide-linked peptides. Peptides structured by one disulfide bond have only been previously identified in the venoms of *Pseudomyrmex gracilis* and *P. penetrator* [Touchard et al., 2014b]. In the present study, peptides containing a single disulfide bond were extended to include venoms from five additional ant subfamilies

encompassing 14 ant species (Supplementary Table 3). In hymenopteran venoms, monomeric peptides reticulated by two disulfide bonds, such as apamin, were only described from the defensive venom of the honey bee *Apis mellifera* [Gauldie et al., 1976]. Importantly, the presence of 17 such peptides from three species of *Anochetus* is the first report of peptides with two disulfide bonds in ant venoms.

The *Dinoponera* ICK-like peptide [Torres et al., 2014] is the only monomeric peptide structured by more than one disulfide bond previously described from ant venoms. Peptides sharing high homology with an ICK peptide from tarantula venom have been found in the venom of the myrmecine ant, *Strumigenys kumadori* [Inagaki et al., 2008b]; however this data remains unpublished. Importantly, we found one peptide in the crude venom of *Pachycondyla unidentata* and several peptides from the venom of *P. goeldii* reticulated by three disulfide bonds that had masses within the most common peptide mass range of other ICK toxins (3500–4500 Da). Therefore, this strongly suggests that this toxin scaffold, which has evolved many times in unrelated venomous animals [Zhu et al., 2003], may also be present in many other ant venoms. However, the definitive identification of an ICK structural motif will require the purification of the peptides and determination of the disulfide linkage and tertiary structure. All the remaining disulfide-bonded peptides identified in ant venoms were homo- or heterodimeric complexes formed from monomers linked by one or two disulfide bonds from *Pseudomyrmex* spp. (Pseudomyrmecinae) and *Ectatomma* spp. (Ectatomminae) or *Myrmecia pilosula* (Myrmeciinae) [Pan and Hink, 2000; Pluzhinikov et al., 1994; Pluzhnikov et al., 2000; Touchard et al., 2014b; Wiese et al., 2006]. We also found dimeric peptides within the venom of the related pseudomyrmecinae ant, *Tetraponera* sp.

Except for the heterodimeric peptide pilosulin 3 (*m/z* 5603), no other dimeric peptides were identified in either *Myrmecia pilosula*, *Ectatomma tuberculatum* or *E. brunneum*

venoms, even though several different dimeric pilosulins (*m/z* 8192-8540) and ectatomins (*m/z* 7923-9419) peptides have been fully characterised from these venoms [Inagaki et al., 2004; Inagaki et al., 2008a]. The ability of our MALDI-TOF MS method using a FA/serine matrix to mainly detect ions in the *m/z* range *ca*. 500–8000 may explain why the dimeric ectatomins and pilosulins were not detected in our investigation. This shows that the peptide richness is likely to be even more extensive. The use of complementary analyses, employing other techniques such as ESI mass spectrometry or using different matrices, may reveal the presence of larger peptides.

Conclusion

The present work constitutes the most extensive study of ant venom peptidomes and demonstrates the diversity in mass and disulfide connectivity of peptides in ant subfamilies and species. Ant venoms remain little investigated and the impressive diversity of peptides from ant venoms highlighted in this work combined with the improvement of mass spectrometry technology should boost future studies on ant venom toxins. Furthermore, the small sizes of ant venom peptides make the sequencing and synthesis of these toxins relatively simple therefore facilitating the characterisation of their pharmacological targets. Ant venoms may therefore provide a novel source of bioactive peptides to develop drug and bioinsecticide lead compounds.

Acknowledgements

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Supplementary Table 1. List of poneroid ants and their origin

Subfamily	Genus	Species	Origin
Amblyoponinae	*Prionopelta*	cf. *amabilis*	French Guiana
Paraponerinae	*Paraponera*	*clavata*	French Guiana
Ponerinae	*Pachycondyla*	*goeldii*	French Guiana
	Pachycondyla	*stigma*	French Guiana
	Pachycondyla	*commutata*	French Guiana
	Pachycondyla	*villosa*	French Guiana
	Pachycondyla	*crassinoda*	French Guiana
	Pachycondyla	*procidua*	French Guiana
	Pachycondyla	*constricta*	French Guiana
	Pachycondyla	*harpax*	French Guiana
	Pachycondyla	*apicalis*	French Guiana
	Pachycondyla	*unidentata*	French Guiana
	Pachycondyla	*arhuca*	French Guiana
	Pachycondyla	*marginata*	French Guiana
	Pachycondyla	*inversa*	French Guiana
	Pachycondyla	*mesonotalis*	French Guiana
	Pachycondyla	*verenae*	French Guiana
	Pachycondyla	sp.	French Guiana
	Odontomachus	*hastatus*	French Guiana
	Odontomachus	*haematodus*	French Guiana
	Odontomachus	*scalptus*	French Guiana
	Odontomachus	*biumbonatus*	French Guiana
	Odontomachus	*mayi*	French Guiana
	Odontomachus	cf. *ruginodis*	French Guiana
	Anochetus	*emarginatus*	French Guiana
	Anochetus	cf. *diegensis*	French Guiana
	Anochetus	*horridus*	French Guiana
	Centromyrmex	sp.	French Guiana
	Leptogenys	*unistimulosa*	French Guiana
	Leptogenys	sp. 1	French Guiana
	Leptogenys	sp. 2	French Guiana
	Leptogenys	sp. 3	French Guiana
	Platythyrea	sp. 1	French Guiana
	Platythyrea	sp. 2	French Guiana
	Platythyrea	sp. 3	French Guiana
	Hypoponera	sp. 1	French Guiana
	Hypoponera	sp. 2	French Guiana
	Hypoponera	sp. 3	French Guiana
	Diacamma	sp.	Unknown
	Dinoponera	*grandis*	Brazil
	Streblognathus	*aethiopicus*	Unknown

Supplementary Table 2. List of formicoid ants and their origin

Subfamily	Genus	Species	Origin
Cerapachyinae	*Acanthostichus*	sp. 1	French Guiana
	Acanthostichus	sp. 2	French Guiana
Ecitoninae	*Eciton*	*burchelli*	French Guiana
	Eciton	*mexicanum*	French Guiana
	Neivamyrmex	sp. 1	French Guiana
	Neivamyrmex	sp. 2	French Guiana
	Neivamyrmex	sp. 3	French Guiana
	Neivamyrmex	sp. 4	French Guiana
	Labidus	*coecus*	French Guiana
	Nomamyrmex	*hartigi*	French Guiana
Ectatomminae	*Ectatomma*	*tuberculatum*	French Guiana
	Ectatomma	*brunneum*	French Guiana
	Ectatomma	*edentatum*	French Guiana
	Ectatomma	cf. *ruidum*	Trinidad and Tobago
	Gnamptogenys	*sulcata*	French Guiana
	Gnamptogenys	*striatula*	French Guiana
	Gnamptogenys	*triangularis*	French Guiana
	Gnamptogenys	*mordax*	French Guiana
	Gnamptogenys	sp. 1	French Guiana
	Gnamptogenys	sp. 2	French Guiana
Myrmeciinae	*Myrmecia*	*gulosa*	Australia
	Myrmecia	*rufinodis*	Australia
	Myrmecia	*simillima*	Australia
	Myrmecia	*pilosula*	Australia
Myrmicinae	*Manica*	*rubida*	France
	Pogonomyrmex	*maricopa*	North America
	Strumigenys	sp.	French Guiana
	Daceton	*armigerum*	French Guiana
	Solenopsis	*saevissima*	French Guiana
	Myrmica	sp.	France
	Myrmica	*lobulicornis*	France
	Myrmica	*ruginodis*	France
	Myrmica	*sabuleti*	France
	Myrmica	*sulcinodis*	France
	Tetramorium	sp.	France
	Chalepoxenus	*muellerianus*	France
	Strongylognathus	*testaceus*	France
Pseudomyrmecinae	*Pseudomyrmex*	*termitarius*	French Guiana
	Pseudomyrmex	*tenuis*	French Guiana
	Pseudomyrmex	*gracillis*	French Guiana
	Tetraponera	sp.	Unknown

Supplementary Table 3. List of monomeric disulfide bonded peptides detected in crude ant venoms. Data shows presence of one, two and three disulfide bonds detected by shifting of peptide masses (+ 2 Da for each disulfide bond) after reduction with DTT.

No of S-S	MW oxidised [M+H$^+$] *m/z*	MW reduced [M+H$^+$] *m/z*	Species	Subfamily	Clade
1	2243.34	2245.36	*Pachycondyla commutata*	**Ponerinae**	**poneroid**
	3059.45	3061.56	*Pachycondyla mesonotalis*		
	2582.36	2584.36	*Pachycondyla unidentata*		
	1777.74	1779.70	*Odontomachus hastatus*		
	2098.87	2100.92	*Odontomachus hastatus*		
	2282.86	2284.95	*Odontomachus hastatus*		
	3543.46	3545.62	*Odontomachus hastatus*		
	2000.74	2002.72	*Odontomachus scalptus*		
	2058.75	2060.72	*Odontomachus scalptus*		
	1459.65	1461.52	*Anochetus horridus*		
	1702.70	1704.67	*Anochetus horridus*		
	2283.06	2285.11	*Anochetus horridus*		
	1177.59	1179.43	*Anochetus* cf. *diegensis*		
	1493.76	1495.64	*Anochetus* cf. *diegensis*		
	1633.93	1635.91	*Streblognathus aethiopicus*		
	2138.83	2140.86	*Prionopelta* cf. *amabilis*	**Amblyoponinae**	
	2154.82	2156.85	*Prionopelta* cf. *amabilis*		
	2403.77	2405.80	*Prionopelta* cf. *amabilis*		
	2658.20	2660.97	*Prionopelta* cf. *amabilis*		
	2665.87	2667.92	*Prionopelta* cf. *amabilis*		
	2766.80	2768.91	*Prionopelta* cf. *amabilis*		
	2800.98	2802.97	*Prionopelta* cf. *amabilis*		
	2820.93	2823.02	*Prionopelta* cf. *amabilis*		
	2922.90	2925.00	*Prionopelta* cf. *amabilis*		
	1803.57	1805.52	*Ectatomma brunneum*	**Ectatomminae**	**formicoid**
	1391.56	1393.66	*Myrmecia rufinodis*	**Myrmeciinae**	
	1494.87	1496.76	*Myrmecia simillima*		
	1538.71	1540.62	*Pogonomyrmex maricopa*	**Myrmicinae**	
	2052.93	2054.95	*Pogonomyrmex maricopa*		
	2489.00	2491.12	*Pogonomyrmex maricopa*		
	1435.85	1437.89	*Manica rubida*		
	2745.30	2747.26	*Manica rubida*		
2	1687.70	1691.71	*Anochetus horridus*	**Ponerinae**	**poneroid**
	1832.80	1836.80	*Anochetus horridus*		
	2043.85	2047.89	*Anochetus horridus*		
	2709.10	2713.20	*Anochetus horridus*		
	1683.67	1687.58	*Anochetus* cf. *diegensis*		
	1702.79	1706.71	*Anochetus* cf. *diegensis*		
	1958.88	1962.80	*Anochetus* cf. *diegensis*		
	2134.88	2138.80	*Anochetus* cf. *diegensis*		
	1623.60	1627.59	*Anochetus emarginatus*		
	1716.65	1720.59	*Anochetus emarginatus*		
	1849.74	1853.74	*Anochetus emarginatus*		
	1866.76	1870.70	*Anochetus emarginatus*		
	1916.79	1920.71	*Anochetus emarginatus*		
	1975.71	1979.69	*Anochetus emarginatus*		
	2026.79	2030.77	*Anochetus emarginatus*		
	2057.87	2061.82	*Anochetus emarginatus*		
	2093.82	2097.80	*Anochetus emarginatus*		
3	4434.29	4440.30	*Pachycondyla unidentata*	**Ponerinae**	**poneroid**

2. Caractérisation de peptides originaux

Parmi les peptides structurés par des ponts disulfure révélés dans ce chapitre, les peptides monomériques réticulés par un et deux ponts disulfure constituent deux nouvelles classes structurales de toxines découvertes dans les venins de fourmis. Nous avons entrepris la purification de plusieurs de ces peptides (Figure 13) afin de les caractériser : un peptide avec un pont provenant du venin de *Neoponera commutata* (Ponerinae), un second peptide avec un pont disulfure du venin de *Manica rubida* (Myrmicinae), un troisième peptide avec un pont disulfure du venin d'*Odontomachus hastatus* (Ponerinae) et sept peptides possédant deux ponts disulfure isolés du venin d'*Anochetus emarginatus* (Ponerinae). Le séquençage de ces peptides a été effectué par dégradation d'Edman et les séquences sont présentées dans le Tableau 4. La synthèse et le repliement de ces peptides ont permis d'obtenir une quantité suffisante de matériel pour déterminer la structure tridimensionnelle par RMN des peptides Pc-1973 et Ae-1733, isolés respectivement du venin de *N. commutata* et d'*A. emarginatus* (Figure 14).

Tableau 4. Liste des peptides isolés et séquencés

Peptide	Espèce	Séquence	Masse (Da)
Pc-1973	*Neoponera commutata*	IDKKPHRL**C**TFKI**C**SW	1972.01
Mr-1435	*Manica rubida*	IIGP**C**PKKPIGIV**C**	1434.80
Oh-1777	*Odontomachus hastatus*	**C**HFGYKMVNGR**C**RPI-NH_2	1776.84
Ae-1733	*Anochetus emarginatus*	W**C**ASG**C**RKKRHGG**C**S**C**-NH_2	1732.73
Ae-1689	*Anochetus emarginatus*	DVG**C**SSG**C**HKVGGQ**C**R**C**-NH_2	1689.66
Ae-1790	*Anochetus emarginatus*	GTG**C**SSG**C**HRVGQQ**C**R**C**G-NH_2	1789.70
Ae-1852	*Anochetus emarginatus*	RSV**C**SNG**C**RPKPFGG**C**S**C**-NH_2	1851.77
Ae-1866	*Anochetus emarginatus*	RSL**C**SNG**C**RPKPFGG**C**S**C**-NH_2	1865.79
Ae-1975	*Anochetus emarginatus*	SFYA**C**TNG**C**WVKPGGG**C**Q**C**-NH_2	1975.76
Ae-1917	*Anochetus emarginatus*	RY**C**PSG**C**RKKPYGGG**C**S**C**-NH_2	1915.80

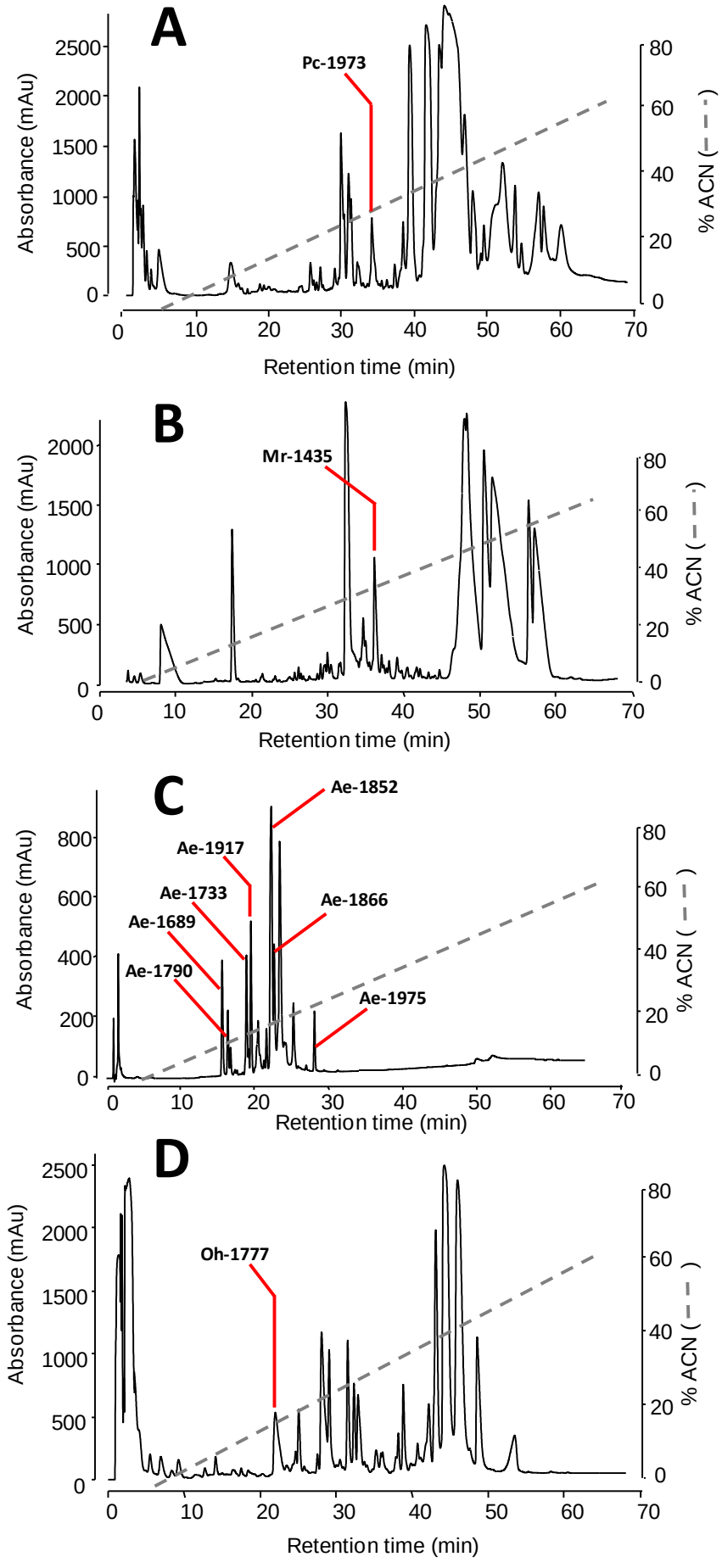

Figure 13- Séparation des venins par chromatographie liquide en phase inverse sur une colonne C18. (A) Venin de *Neoponera commutata*. (B) Venin de *Manica rubida*. (C) Venin d'*Anochetus emarginatus*. (D) Venin d'*Odontomachus hastatus*. Les pics contenant les peptides purifiés et séquencés sont indiqués sur les chromatogrammes.

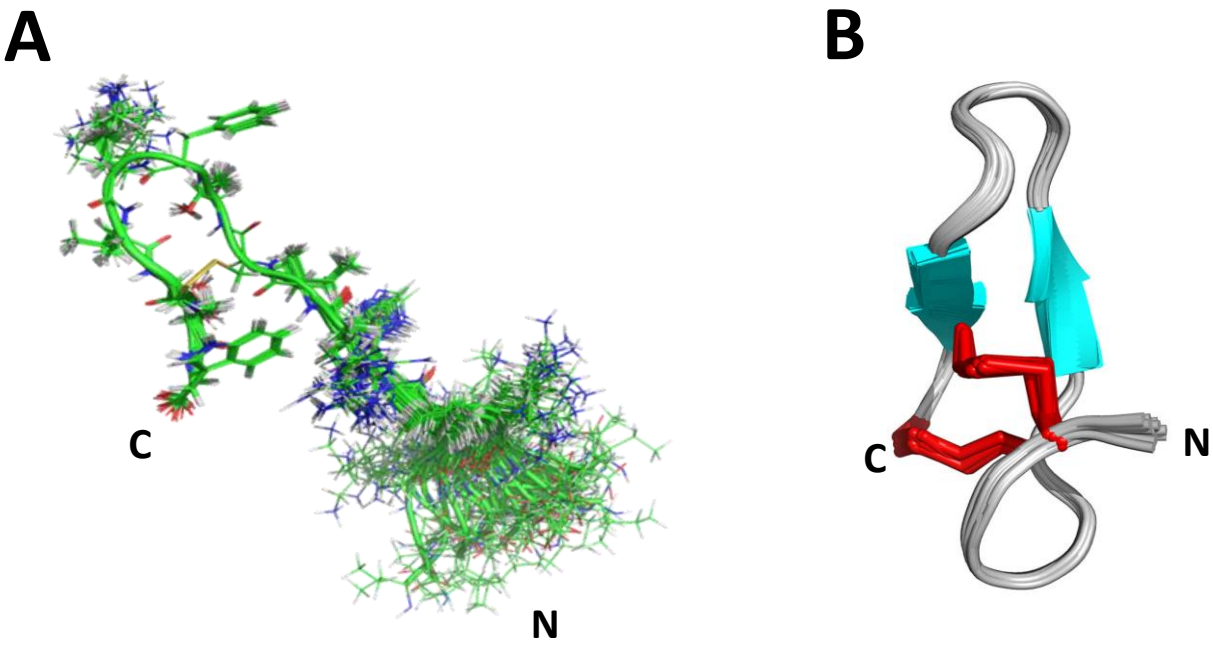

Figure 14- Structure RMN des toxines Pc-1973 (A) et Ae-1733 (B).

Chapitre 4. Pharmacologie

Les fourmis utilisent leur venin pour diverses fonctions biologiques [Schmidt, 1982]. Ainsi durant leurs 140 millions d'années d'évolution [Brady et al., 2006], les fourmis ont développé et sélectionné de multiples peptides neurotoxiques, insecticides, cytotoxiques, antimicrobiens, antifongiques et algésiques. On sait désormais que les venins de fourmis sont essentiellement composés de peptides linéaires (à l'exception des venins d'*Anochetus*). Ces derniers sont surtout connus pour avoir des activités antimicrobiennes, cytotoxiques et insecticides (*e.g*. ponericines, dinoponeratoxines, bicarinalines). Cependant la présence de nombreux peptides structurés par des ponts disulfure a été démontrée bien que leur rôle biologique et leur pharmacologie restent inconnus.

- **Quelle est la fonction biologique et la pharmacologie des peptides à ponts disulfure dans les venins de fourmis ?**

Les venins de mygales, de scorpions, de serpents, de cônes, d'anémones de mer ou encore de scolopendres possèdent de nombreux peptides structurés par des ponts disulfure, qui sont souvent des neurotoxines ciblant les canaux ioniques [King and Hardy, 2013; Lewis et al., 2012; Possani et al., 1999; Rodríguez et al., 2012; Tsetlin, 1999; Yang et al., 2012]. Certains peptides sont également capables d'activer les nocicepteurs des mammifères afin d'induire une sensation de douleur [Baron et al., 2013; Bohlen et al., 2011; Siemens and Hanack, 2014]. Les peptides structurés par des ponts disulfure découverts dans les venins de fourmis pourraient donc agir de la même façon sur les canaux ioniques et les nocicepteurs afin de paralyser les proies et repousser les prédateurs.

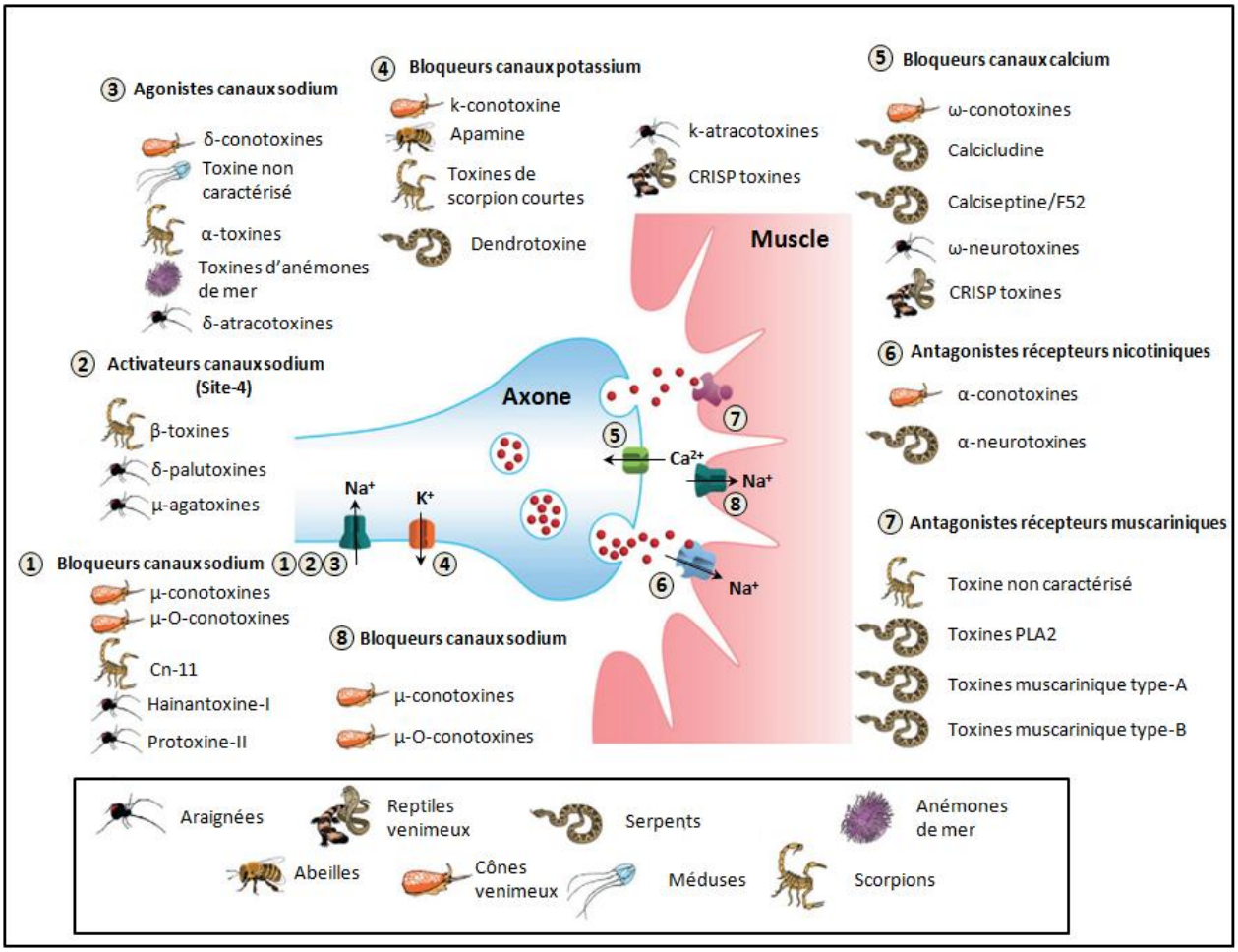

Figure 15- Diversité des cibles pharmacologiques des neurotoxines présentes dans les venins. Figure adaptée d'après [Casewell et al., 2013].

1. Neurotoxines

Les venins d'animaux sont une source extrêmement riche de peptides neurotoxiques. Ces neurotoxines sont capables de moduler une grande diversité de cibles moléculaires notamment les canaux ioniques avec différentes sélectivités, spécificités et efficacités (Figure 15). Ainsi de nombreuses toxines peptidiques modulant les canaux ioniques ont été découvertes dans les venins d'arthropodes [Beleboni et al., 2004; King and Hardy, 2013]. Dans les venins de fourmis, uniquement deux peptides neurotoxiques ont à ce jour été identifiés et caractérisés d'un point de vue pharmacologique. La poneratoxine isolée à partir du venin de *Paraponera clavata*, est capable de moduler les canaux sodium (Na_v) à la fois des invertébrés et des vertébrés [Duval et al., 1992; Piek et al., 1991a]. L'ectatomine est, quant à elle, une toxine peptidique dimérique isolée à partir du venin de la fourmi *Ectatomma tuberculatum*, qui module les courants calcium de type L (Ca_v) [Arseniev et al., 1994].

Afin d'évaluer l'activité neurotoxique de certains des peptides à ponts disulfure isolés de nos venins nous avons injecté les peptides synthétiques Pc-1973, Mr-1435 et Ae-1733 dans le thorax de mouches (*Lucilia cuprina*). Le peptide Pc-1973 a également été injecté dans la proie naturelle de *N. commutata* (*Syntermes* sp.) puisque cette espèce est un prédateur termitophage spécialisé. Seul le peptide Ae-1733 a montré un effet neurotoxique sur les mouches cependant cette paralysie est complètement réversible après 24 h (voir article 6).

Nous avons ensuite testé l'activité des venins bruts (*Manica rubida* et *Anochetus emarginatus*) ainsi que de trois peptides synthétiques (Pc-1973, Mr-1435 et Ae-1733) sur les canaux ioniques humains Ca_v, Na_v ainsi que sur le récepteur nicotinique nAChR α7 (le protocole est présenté dans l'article 6). Ces canaux ioniques étant une cible fréquente des

neurotoxines animales ainsi que des cibles potentielles pour la recherche de molécules thérapeutiques.

Le venin brut de *Manica rubida* est un puissant activateur de canaux ioniques et augmente dramatiquement les flux de Ca^{2+} des cellules nerveuses humaine SH-SY5Y (figure 16). Les canaux sodium semblent particulièrement activés. Il sera cependant nécessaire d'étendre la caractérisation pharmacologique de ce venin afin de déterminer quel type de canaux ioniques sont affectés. En effet les cellules SH-SY5Y possèdent de nombreux canaux ioniques et l'activation de certains de ces canaux peut indirectement provoquer l'activation des canaux sodium (Na_v). Le peptide synthétique Mr-1435, provenant de ce venin n'a aucune activité sur ces canaux ioniques. De même le peptide Pc-1973 n'a montré aucun effet sur les canaux ioniques Na_v, Ca_v et le récepteur nicotinique nAChR α7.

Le venin brut d'*Anochetus emarginatus* inhibent les canaux calcium Ca_v1 (Figure 16). Ce venin semble également inhiber le récepteur nicotinique nAChR α7, mais ce résultat pourrait être un effet indirect de l'inhibition des canaux calcium et nécessite. Une caractérisation pharmacologique étendu uniquement sur les récepteurs nicotiniques est donc nécessaire pour conclure sur l'inhibition des récepteurs nicotiniques. La caractérisation pharmacologique du peptide synthétique Ae-1733 a permis de montrer que ce peptide est un antagoniste des canaux calcium de type L (Ca_v1) et n'a pas d'effet sur les canaux calcium de type N (Ca_v2) (voir article 6).

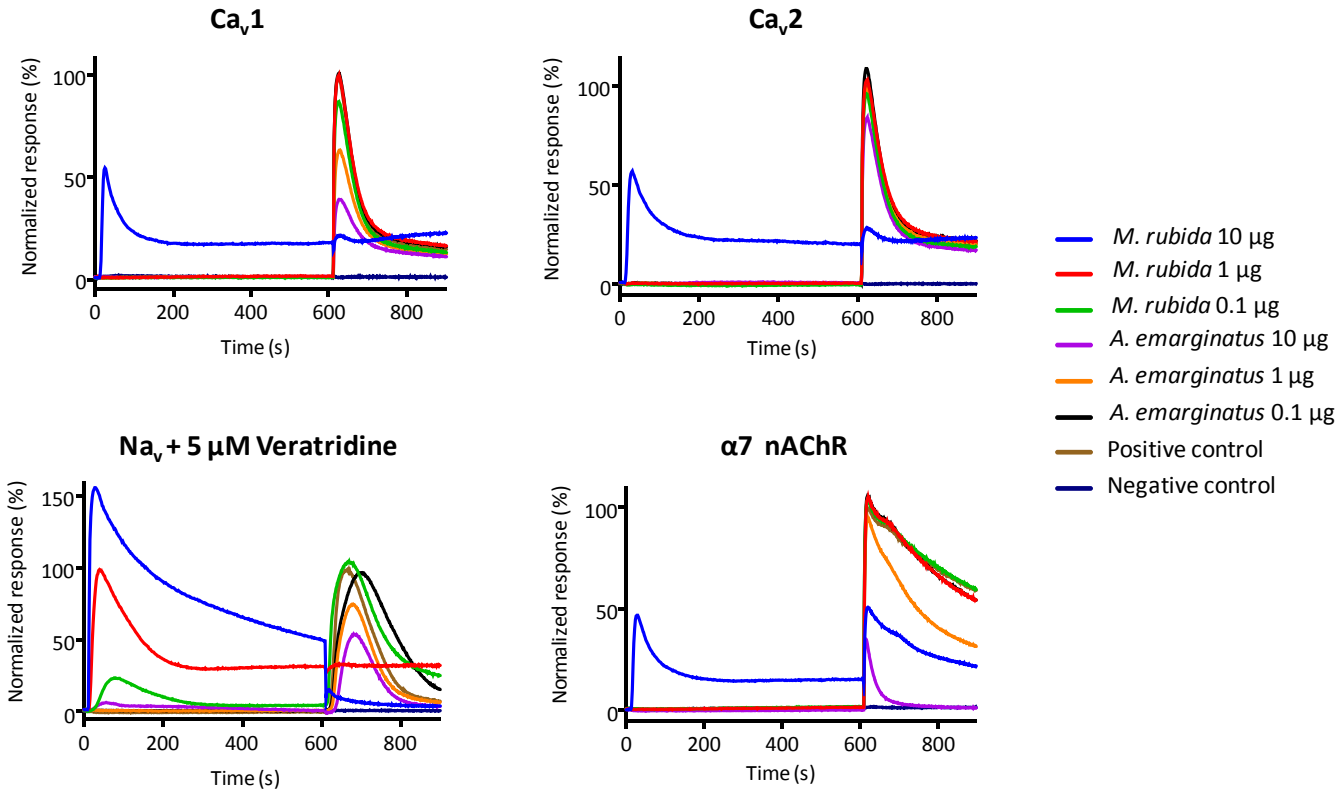

Figure 16- Evaluation des activités pharmacologiques des venins bruts de *Manica rubida* et d'*Anochetus emarginatus*. Les tests ont été effectués avec la lignée cellulaire humaine de neuroblastome SH-SY5Y comme décrit dans [Sousa et al., 2013; Vetter et al., 2012].

Article 6

Formicitoxins: a novel family of disulfide-rich, neurotoxic peptides from ant venom

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Formicitoxins: a novel family of disulfide-rich, neurotoxic peptides from ant venom

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Running title: Formicitoxins: a novel family of neurotoxins from ant venom

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Key words: peptides, disulfide bonds, neurotoxins, pharmacology, calcium channel, ants, *Anochetus*, venom

CAPTION

Background: *Anochetus* venoms, unlike other ant venoms, are mostly composed of disulfide-linked peptides (formicitoxins).

Results: The formicitoxin FOTX-Ae1 is a novel neurotoxin with a novel scaffold targeting L-type calcium channels.

Conclusion: *Anochetus* ants have evolved small, highly folded peptides targeting ion channels to capture their prey.

Significance: Ant venoms are the new frontier in the search for bioactive peptides.

ABSTRACT

Ants have evolved complex venoms to rapidly immobilize their prey and to defend themselves from predators and pathogens. Although most peptidic venoms from ants consist of small linear peptides, disulfide-linked peptides are also found as minor components. Unlike other ant venoms, *Anochetus* venoms are mostly composed of disulfide-linked peptides. In this study, the venom peptidome of *Anochetus emarginatus* was investigated using RP-HPLC and LC-MS. A total of seven disulfide-linked peptides named formicitoxins (FOTX) were isolated and their amino acid sequences characterized by both Edman degradation and *de novo* MS/MS sequencing. The toxin FOTX-Ae1 was selected for further analysis and chemically synthesized by Fmoc chemistry. FOTX-Ae1 exhibited a fully reversible paralytic activity on blowflies and also inhibited human L-type calcium channels (Ca_v1). Interestingly, C-terminal amidation was found to be crucial for its activity. The three-dimensional structure of FOTX-Ae1 was elucidated by NMR spectroscopy and revealed a compact structure in which a C-terminal β-hairpin is connected to the N-terminal region *via* two disulfide bonds which represents a novel toxin scaffold. This study demonstrates that ant venoms are a promising source of novel ligands for ion channels and a source of structural novelty for drug leads and biopesticides.

INTRODUCTION

Venomous animals employ complex biochemical cocktails of small molecules, peptides and proteins for the dual purpose of capturing prey and defending against predators. Venoms are typically comprised of a mixture of proteins, peptides, salts, biogenic amines and small molecules such as alkaloids, acylpolyamines or other structures. The proteinaceous components and particularly the peptide content are the dominant fractions in most animal venoms [King and Hardy, 2013]. In the main the animal venoms studied so far, such as scorpions, spiders, sea anemones, snakes and cone snails, are comprised of peptides that are disulfide-rich toxins acting on ion channels or other cellular receptors [Harvey et al., 1993; Yang et al., 2012].

The venom composition of other groups such as insects, centipedes, crustaceans or annelid worms has been the object of less attention, with some of the first investigations performed only very recently [Cherniack, 2011; von Reumont et al., 2014a; von Reumont et al., 2014b; von Reumont et al., 2014c; Yang et al., 2012]. Furthermore, despite the ubiquity, abundance and diversity of ants (Hymenoptera : Formicidae), with 13,029 species described [AntWeb, 2014b], their venoms remain largely unexplored. While a number of ant subfamilies rely on small-molecule chemical defenses, many other have retained the ability to sting, like wasps and bees, and those venoms appear to be essentially peptide-rich. To date, a total of 72 peptides have been sequenced from these ant subfamilies [Aili et al., 2014] and fall into three different structural classes: linear (*i.e.*, devoid of a disulfide bond); dimeric and ICK-like peptides. The recent investigation of 82 stinging ant species confirmed the prevalence of small linear peptides with less than 35 residues while highlighting the presence of disulfide-linked peptides in most subfamilies [Touchard et al., 2015].

Most interestingly, this study also showed that the venom of species from the genus *Anochetus* (Ponerinae) were almost solely composed of peptides with one or two disulfide bonds [Touchard et al., 2015]. *Anochetus* are predatory ant species that feed on small invertebrates using their trap-jaw mandibles and their venom to rapidly subdue their prey. To date, very little is known about the mode

of action of ant venom peptides, as there have been only a few pharmacological characterizations of isolated ant peptides. Poneratoxin, isolated from the venom of *Paraponera clavata* (Paraponerinae), modulates voltage-gated sodium (Na_v) channels used by both vertebrates and invertebrates [Duval et al., 1992]. Ectatomin, from the venom of *Ectatomma tuberculatum* (Ectatomminae), is both a pore-forming peptide and a modulator of L-type calcium channels [Pluzhinikov et al., 1994; Pluzhnikov et al., 1999]. Considering the unusual composition of *Anochetus* venoms, we investigated in detail the peptidome of the Neotropical species *A. emarginatus* by LC-MS prior to the isolation and characterization of a novel family of neurotoxic peptides, named formicitoxins.

EXPERIMENTAL PROCEDURES

Ant collection—Live *A. emarginatus* workers were collected from both the Nouragues biological station and Kaw in French Guiana, and voucher specimens were deposited in the *Laboratório de Mirmecologia* collection, Cocoa Research Centre, Ilhéus, Bahia, Brazil. Worker ants were stored at -20°C prior to the dissection. Fifty venom sacs were dissected and pooled in 10% v/v acetonitrile (ACN)/water. Samples were centrifuged for 5 min at 14,400 rpm; the supernatant was collected and freeze-dried prior to storage at -20°C for further whole venom analysis.

RP-HPLC separation and peptide purification—The *A. emarginatus* venom was separated through reversed-phase high-performance liquid chromatography (RP-HPLC) using an Xterra-C18 5µm, 2.1 x 100 mm column (Waters, USA). Fractionation was achieved using a gradient of solvent A (0.1% v/v trifluoroacetic acid (TFA)) and solvent B (acetonitrile (ACN)/0.1% v/v TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0-60% over 60 min, 60-90% over 10 min and 90-0% over 15 min at a flow rate of 0.3 mL/min. The eluate was monitored by UV absorbance at 215 nm on a diode-array detector. All analyses were performed on an Agilent HP 1100 system (Agilent, USA). Peptide elution was monitored in real time and fractions were collected manually for each eluting

peak. Each fraction was then dried and reconstituted in 50 µL of 0.1% v/v TFA/water for offline MALDI-TOF MS analysis and disulfide bond reduction. Further peptide purification was achieved by subjecting C18 fractions to a second reversed-phase chromatography on a Jupiter C4 5µm, 4.6 x 250 mm column (Phenomenex, USA).

Mass spectrometry analysis—Mass spectrometry (MS) analyses were performed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, CA, USA). Samples were prepared as previously reported using a ferulic acid (FA) matrix dissolved in 20% ACN/0.1% v/v TFA/water at a concentration of 10 mg/mL with 100 mM serine as an additive. The FA/serine combination has been shown to be the most efficient matrix for the analysis of crude ponerine ant venoms through MALDI-TOF MS [Touchard et al., 2014a]. Then 0.5 µL of each sample was deposited on the MALDI target plate followed by 0.5 µL of the matrix. Each mass spectrum was externally calibrated using a mixture of peptides of known molecular masses in the same *m/z* range (Peptide Calibration Mix 4, LaserBio Labs, Sophia-Antipolis, France). Lastly, 0.5 µL of the calibration mixture was co-crystallized with 0.5 µL of the matrix and placed adjacent to each sample and analyzed separately. All calibration spectra were acquired in automated mode to maximize mass accuracy and reproducibility. All spectra were acquired in reflector mode and sample spectra were calibrated automatically using the sequence module of the Voyager® Control Software (Applied Biosystems, CA, USA). Five spectra of 50 laser shots per spectrum were accumulated for each sample based on defined acceptance parameters and an adequate signal intensity in the 500–10,000 m/z range. Mass spectra were collected in the positive ion mode with 20 kHz acceleration. Signals below 500 *m/z* were not recorded as they were comprised of mostly matrix-related ion clusters.

Mass spectra analysis—All mass spectra were processed with Data Explorer® 4.11 software (AB SCIEX) and subjected to a baseline correction with a correlation factor of 0.7 and Gaussian smoothing to reduce noise with a 5-point filter width. Supplementary masses resulting from sodium (+22 Da)

and potassium (+38 Da) adducts were manually removed from all mass lists. Masses matching within ± 1.0 Da in neighboring HPLC fractions were considered identical peptides reflecting incomplete separation. Two-dimensional scatter plots, termed as "2D venom landscapes", were constructed using SigmaPlot 12.0 software (Systat, CA USA). All peptide masses detected in each HPLC fraction were plotted as a function of their *m*/*z* values (*x*-axis) and their HPLC retention time reflecting their hydrophobicity (*y*-axis).

The number of residues per peptides was estimated using the averagine value: this theoretical amino acid average molecular weight (111.1254 Da) is determined using the statistical occurrences of amino acids in proteins, calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}S_{0.0417}$.

Disulfide bond reduction and alkylation—The presence of disulfide-linked peptides in ant venoms was determined through the chemical reduction of crude venoms and HPLC fractions. 5 µL of crude venom or selected HPLC fractions were incubated with 10 µL of 100 mM ammonium bicarbonate buffer (pH 8) containing 6 M guanidine and 10 mM dithiothreitol (DTT) for 30 min at 56°C. Prior to MS analysis, reduced venoms or fractions were desalted using ZipTip® C18 pipette tips (Millipore, Billerica, MA USA). As chemical reduction results in a mass increase of 2 Da for each disulfide bond, the examination of mass shifts in the mass spectra of native and reduced samples permitted the number of disulfide bonds in the corresponding peptides to be determined. Targeted disulfide-linked peptides were purified. For further confirmation of the SS bond linkage, they were alkylated following DTT reduction by incubation in 50 mM iodoacetic acid (IAA) for 15 min at room temperature in the dark.

Peptide chemical sequencing—The purified peptides were subjected to Edman degradation on an Applied Biosystems gas-phase sequencer model 492. Phenylthiohydantoin amino acid derivatives generated at each sequence cycle were identified and quantified on-line with an Applied Biosystems Model 140C HPLC system using the data analysis system for protein sequencing from Applied

Biosystems Model 610A. The PTH-amino acid standard kit was used and reconstituted according to the manufacturer's instructions. The procedures and reagents were used as recommended by the manufacturer. Chromatography was used to identify and quantify the derivatized amino acid removed at each sequence cycle. The retention times and integration values of peaks were compared to the chromatographic profile obtained for a standard mixture of derivatized amino acids.

De novo mass spectrometry peptide sequencing—An aliquot (1/3) of each of the 6 purified peptides (*i.e.* FOTX-Ae2; Ae3; Ae4; Ae5; Ae6 and Ae7) after reduction and alkylation was subjected to *de novo* sequencing using AB Sciex 5600 TF system. The AB Sciex TripleTOF 5600 System is a hybrid quadrupole TOF MS equipped with a DuoSpray ionization source coupled to a Shimadzu 30 series HPLC system. The LC separation was achieved using a Agilent C_{18} 2.1 x 100 mm, 1.8 µm, 300 Å column at a fast gradient of 2-50% B (90% acetonitrile/0.1% formic acid (aq)) min^{-1} with a flow rate of 0.15 ml min^{-1} over 4 min. A cycle of one full scan of the mass range (MS) (300–1800 m/z) followed by multiple tandem mass spectra (MS/MS) (80-1400 m/z) was applied using a rolling collision energy relative to the *m/z* and charge state of the precursor ion up to a maximum of 80 eV. The full scan mass spectrometry had a duration of 14 min with a cycle time of 1.15 s (total of 729 cycles). The maximum number of candidate ions monitored per cycle was 20 and the ion tolerance was 0.1 Da. External calibration was applied before the acquisition. All data processing was conducted using Analyst1.6 software. The target ion spectra were manually interpreted.

Chemical synthesis—Protected Fmoc-amino acid derivatives were purchased from Novabiochem or Auspep (Melbourne, Australia). The following side chain protected amino acids were used: Cys(Trt), His(Trt), Hyp(tBu), Tyr(tBu), Lys(Boc), Trp(Boc), Arg(Pbf), Asn(Trt), Asp(OtBu), Glu(OtBu), Gln(Trt), Ser(tBu), Thr(tBu), Tyr(tBu). All other Fmoc amino acids were unprotected. Dimethylformamide (DMF), dichloromethane (DCM), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA) were supplied by Auspep P/L (Melbourne, Australia) as peptide synthesis grade. 2-(1H-

benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Triisopropyl silane (TIPS), HPLC grade acetonitrile, acetic anhydride and methanol were supplied by Sigma Aldrich (Sydney, Australia). The resin used was Fmoc-Rink amide resin (SV = 0.65 mmol/g) supplied by Auspep P/L. Ethane dithiol (EDT) was supplied by Merck.

Ant peptide FOTX-Ae1 is a peptide amide and was synthesized on a Protein Technology (Symphony) automated peptide synthesizer using Rink amide resin (0.1 mmol). The carboxyl version of FOTX-Ae1 was synthesized using Fmoc-Cys-Wang resin (SV =0.5mmol/g). The peptides were assembled using HBTU/DIEA in-situ activation protocols [Alewood et al., 1997] to couple the Fmoc-protected amino acid to the resin (5 equiv excess; coupling time: 5 minutes). Fmoc deprotection was performed with 30% Piperidine/DMF for 1min followed by a 2 min repeat. Washes were performed 10 times after each coupling as well as after each de-protection step. After chain assembly and final Fmoc deprotection the peptide resins were washed with methanol and dichloromethane and dried in a stream of nitrogen. Cleavage of the peptide resin was performed at room temperature in TFA/H_2O/TIPS/EDT (87.5/5/5/2.5) for 3 h. Cold diethyl ether (30 mL) was then added to the filtered cleavage mixture and the peptide precipitated. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% aqueous acetonitrile and lyophilized to yield a solid white product. The crude, reduced peptide was examined by reversed-phase HPLC for purity and the correct molecular weight confirmed by Electrospray mass spectrometry (ESMS).

Pure, reduced peptides FOTX-Ae1 (amidated and carboxyl version) (1 mg/ml) were oxidized by stirring at room temperature in 10% DMSO / 0.1M NH_4HCO_3 pH 8.0, for 16 h. The solutions were subsequently diluted to a DMSO concentration < 5 % prior to RP-HPLC purification. A single main oxidized product was purified to >95% purity and lyophilized.

Analytical HPLC runs were performed using a Shimadzu HPLC system LC10A with a dual wavelength UV detector set at 214 nm and 254 nm. A reversed-phase C-18 column (Zorbax 300-SB C-18; 4.6 x 50 mm) with a flow rate of 2 mL/min was used. Gradient elution was performed with the

following buffer systems: A, 0.05% TFA in water and B, 0.043% TFA in 90% ACN in water, from 0% B to 80% B in 20 min. The crude peptides and oxidized peptides were purified by semi-preparative HPLC on a Shimadzu HPLC system LC8A associated with a reversed-phase C-18 column (Vydac C-18, 25 cm x 10 mm) running at a flow rate of 5 mL/min with a 1%/min gradient of 5% B to 50% B. The purity of the final product was evaluated by analytical HPLC (Zorbax 300SB C-18: 4.6 x 100 mm) with a flow rate of 1 mL/min and a 1.67 %/min gradient of B (5–45%). The purity of the synthesized peptides were all greater than 95%.

Peptide concentration used during *in vitro* screening was calculated based on peak size detected at 214 nm by HPLC. Peak size was calibrated using a peptide standard with known peptide content established through amino acid analysis. Molecular extinction coefficients were calculated for the standard and the peptide of interest by applying increments established by Buck et al [Buck et al., 1989]. Using the Lambert Beer Law, the peptide concentration was calculated based on absorptions of standard and samples using calculated extinction coefficients.

Electrospray mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems API-150 spectrometer operating in the positive ion mode with an OR of 20, Rng of 220 and Turbospray of 350 degrees. Masses between 300 and 2200 amu were detected (Step 0.2 amu, Dwell 0.3 ms).

Insecticidal bioassay—The FOTX-Ae1 peptide dissolved in insect-saline (for details on composition, see [Eitan et al., 1990]) was injected into the ventro-lateral thoracic region of blowflies, *Lucilia cuprina* (mass 28.5–30.7 mg), using a 1.0 ml disposable syringe (B-D Ultra Fine, Terumo Medical Corporation, MD, USA) and a fixed 29 gauge needle, fitted to an Arnold hand micro-applicator (Burkard Manufacturing Co-Ltd., England). A maximum volume of 2 µl was injected per fly. Thereafter, flies were individually housed in 2 ml tubes and the paralytic activity was determined after 1 h and 24 h. A total of three tests was carried out and, for each test, seven doses of FOTX-Ae1 (n=10 flies per dose) and the appropriate control (insect saline; n= 20 flies each) were used. PD_{50} values were calculated as described previously [Bende et al., 2013; Herzig and Hodgson, 2008].

Measurement of intracellular Ca^{2+} responses—The Ca^{2+} responses were measured using a fluorescent imaging plate reader ($FLIPR^{TETRA}$) and Calcium 4 dye (Molecular Devices, Sunnyvale, CA, USA) and the neuroblastoma cell line SH-SY5Y as previously described for assaying voltage-gated ion channels Ca_v1, Ca_v2 and Na_v and the ligand-gated ion channel α7 nAChR [Sousa et al., 2013; Vetter et al., 2012]. SH-SY5Y cells were plated at 40,000 cells per well in a 384 well flat clear bottom black plate (Corning, NY, USA) and cultured at 37°C in a humidified 5% CO_2 incubator 48 h before assay. The medium was removed and the cells loaded with 20 µl per well of Calcium 4 dye reconstituted in an assay buffer containing (in mM) 140 NaCl, 11.5 glucose, 5.9 KCl, 1.4 $MgCl_2$, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 1.8 $CaCl_2$ and 10 HEPES pH 7.4 and incubated for 30 min at 37°C in a humidified 5% CO_2 incubator. For Ca_v1 assay, 1 µM CVID (Ca_v2 blocker) was added to the dye; for Ca_v2 assay, 10 µM nifedipine (Ca_v1 blocker) was added to the dye; and for α7 nAChR assay, 10 µM PNU-120596 (α7 agonist) was added to the dye.

Ca^{2+} fluorescence response was recorded at excitation 470-495 nm and emission 515-575 nm for 10 s to set the baseline, 600 s after the addition of *Anochetus emarginatus* crude venom at 10, 1 and 0.1 µg/well or FOTX-Ae1 peptide at various concentrations and for a further 300 s after the addition of the activators 90 mM $CaCl_2$ for Ca_vs, 30 µM choline for α7 nAChR or 30 µM veratridine for the Na_v1 assays. Maximum fluorescent responses were corrected against baseline, positive and negative controls, and used to plot the intracellular influx of calcium kinetics and curve fitting (nonlinear regression with log [inhibitor] *versus* normalized response and variable Hill slope) using GraphPad Prism Version 6 (GraphPad Software Inc, San Diego, CA, USA).

NMR structure determination—2D homonuclear NMR spectroscopy was used to determine the structure of FOTX-Ae1. Lyophilized, unlabelled FOTX-Ae1 toxin was resuspended at a final concentration of 2 mM in 300 µL of 20 mM sodium phosphate, pH6 containing 5% D_2O. An additional sample was prepared by lyophilizing the above sample and resuspending it in 100% D_2O. Spectra were acquired at 298 K on a Bruker AVANCE 600 MHz spectrometer equipped with a cryoprobe. Resonance assignments were obtained using a combination of 2D ^{15}N-HSQC, 2D ^{13}C-HSQC (in 100%

D_2O) and 2D TOCSY spectra. Inter-proton distance restraints were obtained from 2D NOESY spectrum acquired using a mixing time of 300 ms. Spectra were processed using Topspin (Version 3.2, Bruker) and analyzed using CcpNmr Analysis 2.4.1 [Vranken et al., 2005]. Dihedral-angle restraints were derived from TALOS+ chemical shift analysis [Shen et al., 2009] and the restraint range was set to twice the estimated standard deviation. The NOESY spectrum was manually peak picked, then the torsion angle dynamics package CYANA 3 [Güntert, 2004] was used to automatically assign the peak list, extract distance restraints, and calculate an ensemble of structures. During the process of automatic NOESY spectrum assignment, CYANA assigned 95.8% of all NOESY cross-peaks. The final structure was calculated using a total of 190 interproton distance restraints, 6 disulfide-bond distance restraints, and 27 dihedral-angle restraints; 200 structures were calculated and the top 20 were selected on the basis of final CYANA penalty function values and stereochemical quality as judged by MolProbity [Chen et al., 2010].

RESULTS

2D landscape of *A. emarginatus* venom—Previous investigation of *Anochetus* spp. crude venoms revealed the presence of numerous disulfide-rich peptides structured by one or two disulfide bonds. The analysis of *A. emarginatus* venom through liquid chromatography (RP-HPLC) coupled to offline mass spectrometry (MALDI-TOF), highlighted several peculiar features, making it very distinct from other ant venoms investigated so far.

First, the venom LC-MS analysis revealed only 35 masses (Table 1). Second, all of detected peptides were very small, falling into a narrow mass range of (*m/z)* 1462.89-2099.16 (Fig. 1). The determination of the approximate number of residues using the averagine molecular weight (111.1254 Da) resulted in an estimate of 13 to 19 residues per peptide. All these peptides were hydrophilic and eluted between 10 % and 25% ACN (retention time 15-30 min) (Fig. 2A). Third, it appeared that after the chemical reduction of each HPLC fraction, a mass shift of + 4 Da occurred for

almost all masses detected, indicating that *A. emarginatus* venom is mostly composed of peptides with two disulfide bonds. In this venom, only one linear peptide with a mass of 1623.58 *m/z* could be detected.

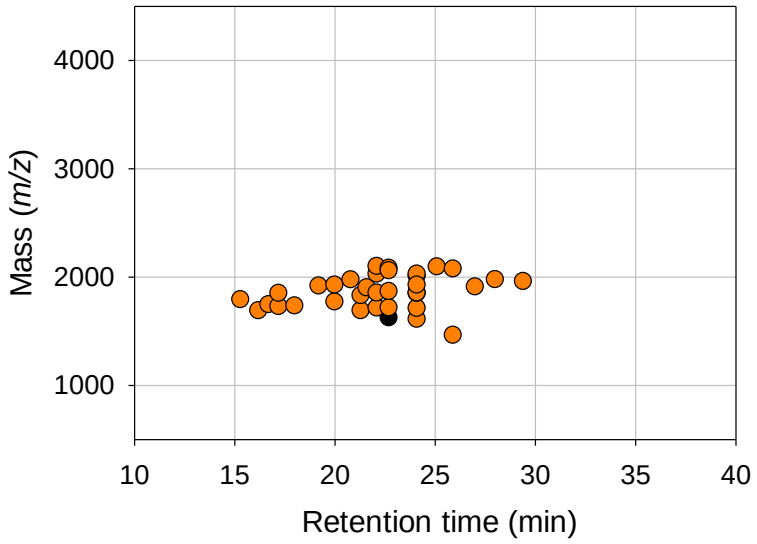

Figure 1. **Venom peptidome of *Anochetus emarginatus***. 2D Landscape showing the LC-MALDI-TOF MS venom profile of *A. emarginatus* which is mostly composed of disulfide-bonded peptides. Orange circles indicate peptides structured by two disulfide bonds while the black circle represents the peptide without a disulfide bond.

Table 1

Mass fingerprint of *Anochetus emarginatus* venom. Bold masses are the seven formicitoxins purified and sequenced. The shaded mass is the linear peptide. All remaining masses are structured by two disulfide bonds.

Fraction	RT (min)	*m/z* [M+H$^+$]
F 1	15.3	**1790.64**
F 2	16.2	**1689.58**
F 3	16.7	1745.85
F 4	17.2	1727.80
F 4	17.2	1850.90
F 5	18.0	**1733.64**
F 6	19.2	**1917.21**
F 7	20.0	1770.80
F 7	20.0	1926.90
F 8	20.8	1975.12
F 9	21.3	1689.80
F 9	21.3	1828.90
F 10	21.6	1901.05
F 11	22.1	1713.80
F 11	22.1	**1852.79**
F 11	22.1	2027.08
F 11	22.1	2099.16
F 12	22.7	1716.89
F 12	22.7	1623.58
F 12	22.7	**1866.81**
F 12	22.7	2081.11
F 12	22.7	2057.92
F 13	24.1	1609.90
F 13	24.1	1711.04
F 13	24.1	1850.15
F 13	24.1	1853.24
F 13	24.1	2009.26
F 13	24.1	2027.33
F 13	24.1	1926.10
F 14	25.1	2094.15
F 15	25.9	1462.89
F 15	25.9	2075.18
F 16	27.0	1909.07
F 17	28.0	**1975.74**
F 18	29.4	1959.01

Formicitoxin sequences—A total of seven disulfide-linked peptides (*e.g.*, Ae1a; Ae2a; Ae3a; Ae4a; Ae5a; Ae6a; Ae7a) were purified using a combination of C18 and C4 RP-HPLC. These formicitoxins (FOTX-Ae) are the first peptide toxins isolated and characterized from the *Anochetus*. The N-terminal Edman degradation of peptide FOTX-Ae1 yielded the 16-residue sequence WCASGCRKKRHGGCSC with the measured *m/z* value of 1733.64, consistent with C-terminal amidation (Fig. 2).

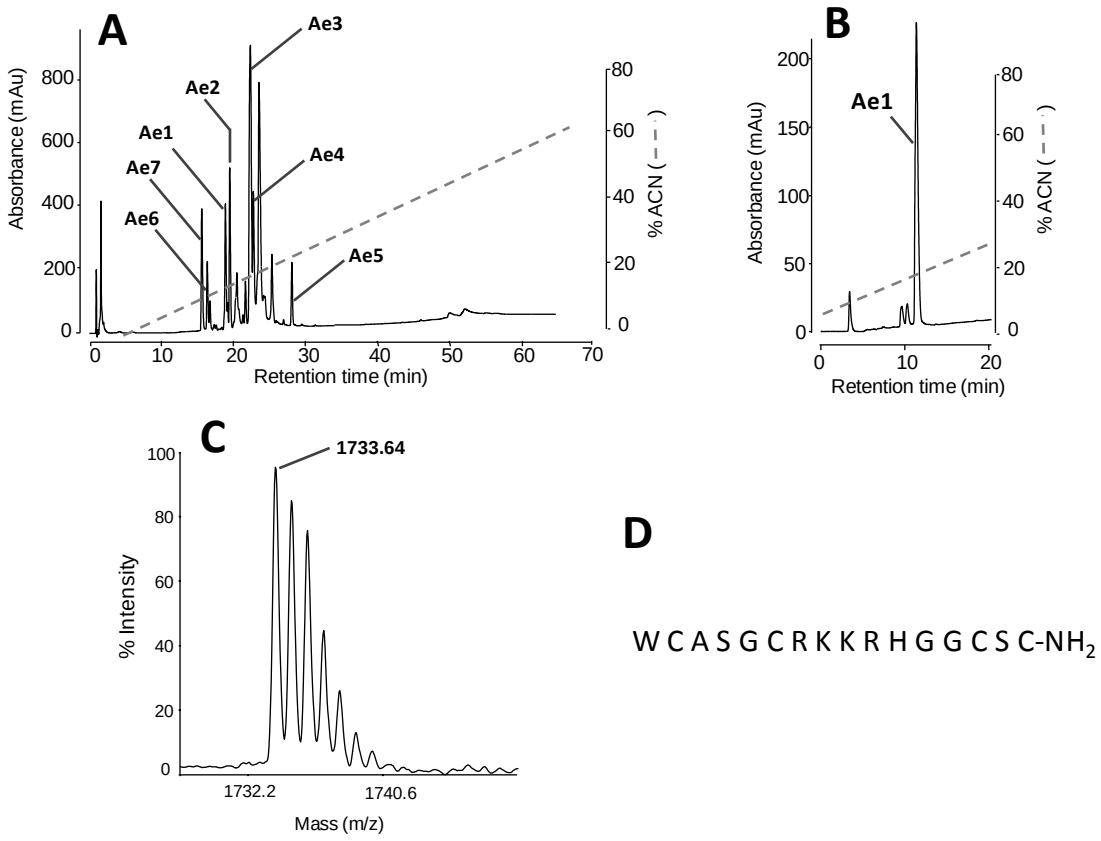

Figure 2. **Purification and amino acid sequence of FOTX-Ae1.** (A) Crude venom from *Anochetus emarginatus* was fractionated using C18 RP-HPLC and seven formicitoxin were selected for purification. (B) Purification of the peptide Ae1 by C4 RP-HPLC. (C) The mass of Ae1 was determined through MALDI-TOF MS. (D) The amino acid sequence of the novel toxin FOTX-Ae1 was obtained by Edman degradation.

The six remaining purified peptides were subjected to *de novo* mass spectrometry sequencing by high resolution AB Sciex TripleTOF 5600 mass spectrometry. The targeted peptides were individually selected for MS/MS analysis and fragmented by collision-induced dissociation. Taking advantage of the small size of the five peptides, sufficient fragments and good coverage have been obtained for each of the peptides that almost allowed their full sequence characterization. The sequences were further completed by Edman degradation (Fig. 3). As FOTX-Ae1 the six others formicitoxins were all C-terminally amidated and comprised 17 residues (FOTX-Ae7), 18 residues (FOTX-Ae2, FOTX-Ae3, FOTX-Ae4, FOTX-Ae6) and 19 residues (FOTX-Ae5) and shares between 41.2 % to 70.6 % sequence identity with the FOTX-Ae1. The FOTX (Ae1, Ae2, Ae3 and Ae4) have between 61.1 % to 94 % identity and are very basic (pI between 17.22 to 17.27). The two peptides Ae6 and Ae7 have a 76.5 % identity. The last FOTX-Ae5 is the most hydrophobic and the less basic peptide (pI 10.36) and shares no more than 55.6 % sequence identity with both FOTX-Ae3 and FOTX-Ae4.

Peptide	Sequence	identity	Mass [M]
FOTX-Ae1	WCASGCRKKRHGG-CSC ★	100 %	1732.73 Da
FOTX-Ae2	RYCPSGCRKKPYGGGCSC ★	70.6 %	1915.80 Da
FOTX-Ae3	RSVCSNGCRPKPFGG-CSC ★	62.5 %	1851.77 Da
FOTX-Ae4	RSLCSNGCRPKPFGG-CSC ★	62.5 %	1865.79 Da
FOTX-Ae5	SFYACTNGCWVKPGGG-CQC ★	50.0 %	1975.76 Da
FOTX-Ae6	GTGCSSGCHR--VGQQCRCG ★	41.2 %	1789.70 Da
FOTX-Ae7	DVGCSSGCHK--VGGQCRC ★	52.9 %	1689.66 Da

Figure 3. **Alignment of the fomicitoxins**. Gaps were introduced to optimize the alignment. Identical residues are boxed in yellow and the red stars indicate an amidated C-terminus. Percentage identity is relative to the first peptide FOTX-Ae1. Theoretical monoisotopic masses were calculated using GPMAW 10.0 software.

Insecticidal activities—The synthetic peptide FOTX-Ae1 exhibited a paralytic activity against the blowflies, *L. cuprina*, with a PD_{50} (after 1h) of 8.9 ± 3.1 nmol/g (Fig. 4). However the activity of the toxin was fully reversible, as all flies recovered within 24 h following the injection. So, no lethal effects of FOTX-Ae1 were observed within the dose range tested.

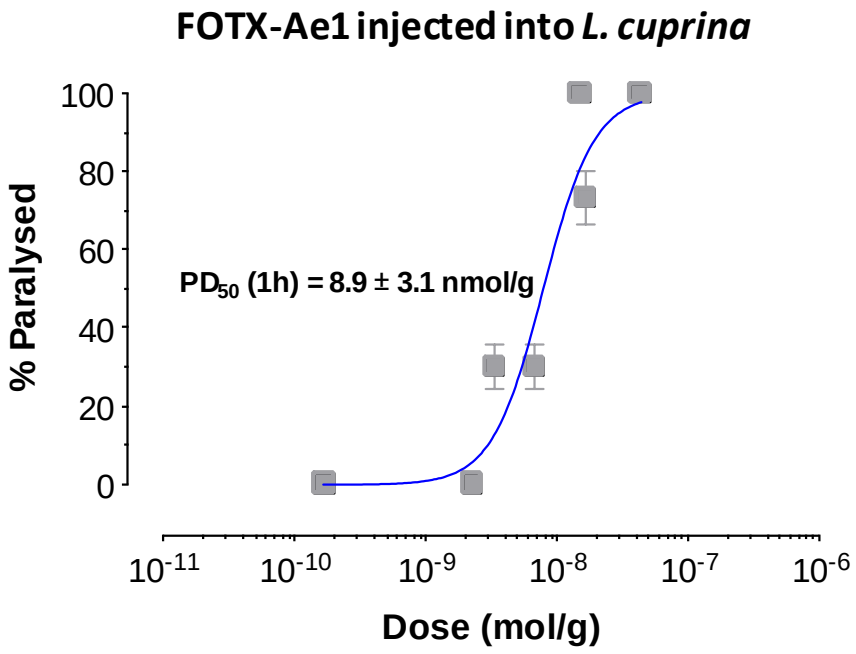

Figure 4. **Dose-response curve for *Lucilia cuprina* blowflies injected with FOTX-Ae1**. Seven doses of FOTX-Ae1 were injected and the percentage of paralyzed blowflies was determined after 1 h. The error bars indicate SEM values. The median paralytic dose (PD_{50}) was determined as the average ± SEM of three experiments.

Ion channel activity—Crude venom from *A. emarginatus* was screened in a FLIPR-based assay to identify its activity against voltage-gated calcium channels (Ca_v), voltage-gated sodium channels (Na_v) and the ligant-gated ion channel α7 nAChR using neuroblastoma SH-SY5Y cells. Neither activation nor inhibition was observed for Na_v and α7 nAChR (data not shown), but the crude venom inhibited the response of Ca_v1 channels (Figure 5A). Synthetic FOTX-Ae1 abolished the Ca_v1 response in neuroblastoma SH-SY5Y cells with an IC_{50} of 4.6 μM (Figure 5B). Interestingly, the activity of FOTX-Ae1 in Ca_v1 channels was detected only in the amidated peptide, suggesting that the C-terminal amidation plays a crucial role in the pharmacology of FOTX-Ae1 on this ion channel.

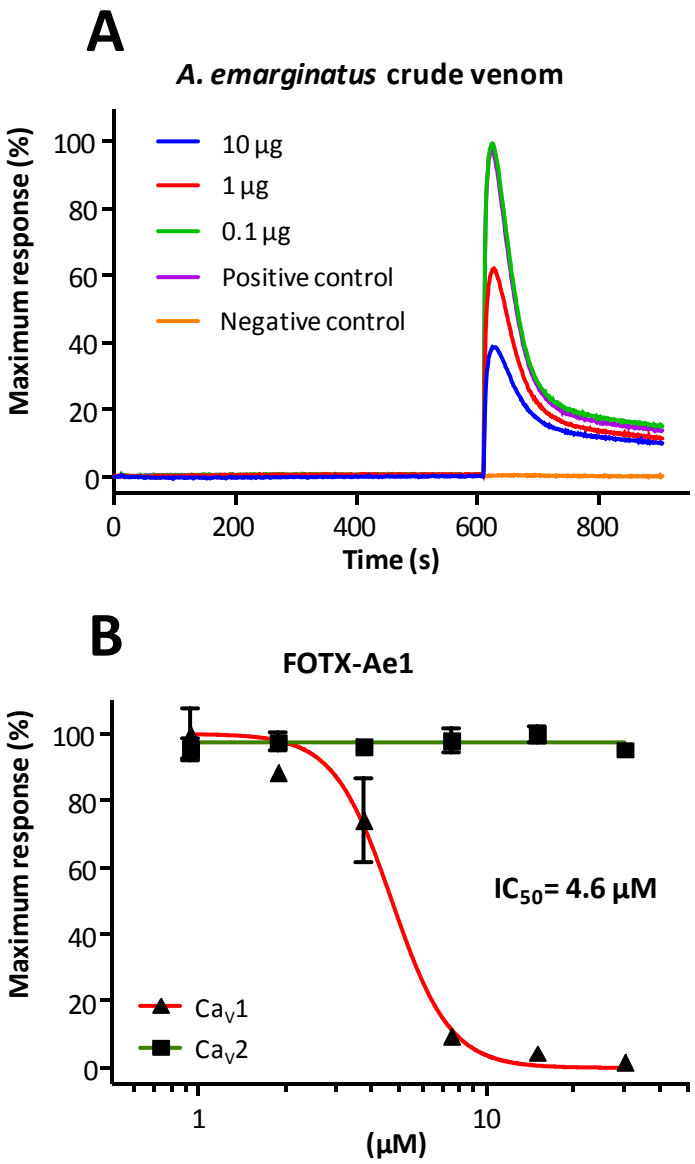

Figure 5. **Screening of *Anochetus emarginatus* crude venom and dose response of peptide** FOTX-Ae1 **in the neuroblastoma SH-SY5Y cell line**. (A) The *A. emarginatus* crude venom was reconstituted in an assay buffer and applied at 10, 1 and 0.1 µg/well in neuroblastoma SH-SY5Y cells loaded with Calcium 4 dye in the presence of 1µM CVID (Ca_v2 blocker). After 10 min incubation with the venom, the cells were activated with 90 mM KCl and 5 nM $CaCl_2$ and the intracellular Calcium responses recorded. This venom was able to reduce the Ca_v1 response to 60 and 40% at concentrations of 10 and 1 µg/well, respectively, and completely lost activity at 0.1 µg/well. (B) The peptide FOTX-Ae1 was evaluated in a Ca_v1 assay as described in Figure 4A. This peptide was able to inhibit the Ca_v1 responses with an IC_{50} of 4.6 µM. No Activity was detected in Ca_v2 (neuroblastoma SH-SY5Y cells were loaded with Calcium 4 dye in the presence of 10 µM Nifedipine).

NMR structure of FOTX-Ae1—An ensemble of 20 conformers representing the structure of FOTX-Ae1a (from a total of 30 structures calculated) is shown in Figure 6A. The disulfide connectivity for FOTX-Ae1 was determined by performing three separate structure calculations with the three possible disulfide isomers (*i.e.*, Cys^{2}–Cys^{14}/Cys^{6}–Cys^{16}; Cys^{2}–Cys^{6}/Cys^{14}–Cys^{16}; and Cys^{2}–Cys^{16}/Cys^{6}–Cys^{14}). Only the Cys^{2}–Cys^{14}/Cys^{6}–Cys^{16} connectivity was consistent with the pattern of NOEs seen in the NOESY spectrum and this connectivity was the only one to yield no restraint violations in the structure calculations. We therefore infer this to be the native disulfide connectivity for FOTX-Ae1. Structural statistics for the ensemble of FOTX-Ae1 are summarized in Table 2. The structure is highly precise, with a backbone rmsd of 0.17 ± 0.04 Å over residues 2–16. The stereochemical quality of the structure is also very high, with MobProbity analysis revealing a complete absence of steric clashes and 93.6% of the residues in the most favored region of the Ramachandran plot.

FOTX-Ae1 forms a compact structure in which a C-terminal β-hairpin is connected to the N-terminal region *via* the two disulfide bonds. This two-disulfide structure appears to be a novel fold that has not been previously reported for other toxins. A DALI comparison with the PDB was not possible because of the small size of the toxin (the cut-off size for DALI analysis is 20 residues). Mapping the electrostatic potential onto the surface of FOTX-Ae1 (Figure 6B) reveals that the C-terminal β-hairpin is highly cationic, with two positively charged residues emanating from β-strand 1 (Arg^{7}/Lys^{8}) and three from the β-hairpin loop (Lys^{9}/Arg^{10}/His^{11}). It remains to be determined whether this surface facilitates the interaction of the toxin with the target ion channels.

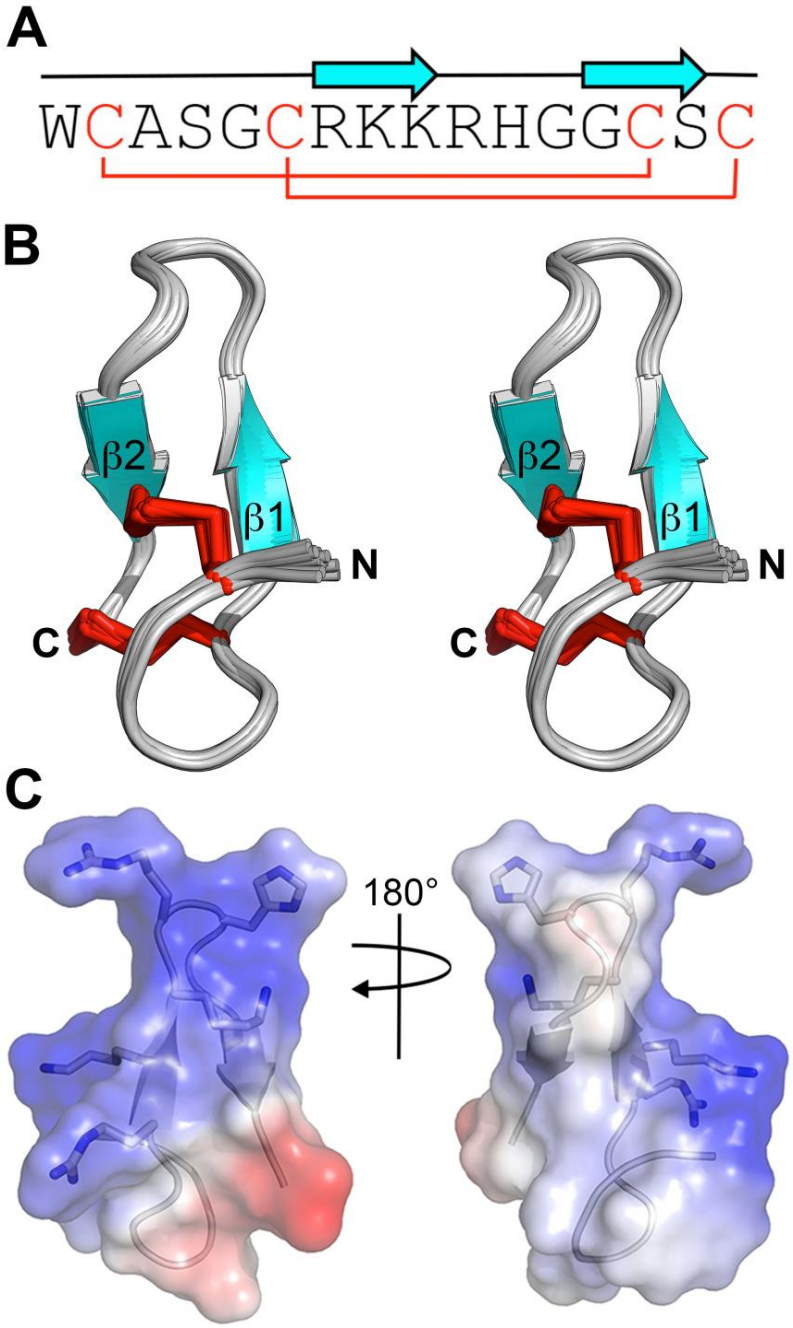

Figure 6**. Solution structure of FOTX-Ae1.** (A) Sequence of FOTX-Ae1. The secondary structure of FOTX-Ae1 mostly consists of random coils and two β-strands (cyan arrows). The cysteine and disulphide connectivity are highlighted in red. (B) Stereo view of an overlay of 20 structures of FOTX-Ae1 shown in cartoon representation. The β-strands are colored in cyan and the two disulfide bonds are shown as red sticks. (C) Electrostatic surface of FOTX-Ae1. The positively-charged regions are shown in blue and the negatively-charged regions are shown in red on the surface. Residues that contribute to the blue surface are highlighted as sticks.

Table 2

Statistical analysis of FOTX-Ae1 structures[1].

Experimental restraints[2]	
Interproton distance restraints-Total	190
Intraresidue (|i–j| < 0)	50
Sequential (|i–j| = <1)	64
Medium range (1 < |i–j| < 5)	35
Long range (|i–j| >= 5)	41
Disulfide-bond restraints	6
Dihedral-angle restraints (ϕ, ψ)	27
Total number of restraints per residue	11.9
R.m.s. deviation from mean coordinate structure (Å)	
Backbone atoms (residues 2–16)	0.17 ± 0.04
All heavy atoms (residues 2–16)	0.93 ± 0.14
Stereochemical quality[3]	
Residues in most favored Ramachandran region (%)	93.6 ± 2.2
Ramachandran outliers (%)	0 ± 0
Unfavorable sidechain rotamers (%)	4.2 ± 4.3
Clashscore, all atoms[4]	0 ± 0
Overall MolProbity score	1.3 ± 0.4

[1]All statistics are given as mean ± S.D.

[2]Only structurally relevant restraints, as defined by CYANA, are included.

[3]According to MolProbity (http://molprobity.biochem.duke.edu)

[4]Defined as the number of steric overlaps >0.4 Å per thousand atoms

DISCUSSION

Altogether our results showed that *A. emarginatus* venom and potentially the other *Anochetus* venoms have unique features compared to most animals and ants in particular. Besides being almost only composed of disulfide-linked peptides, *A. emarginatus* venom can be viewed as a more simple venom compared to other ant venoms as it contains only 35 peptides whereas more than 300 peptides were found in *Dinoponera quadriceps* venom [Cologna et al., 2013]. Moreover, it is mostly comprised of formicitoxins, which are a novel family of small toxins with a novel 3D structure stabilized by two disulfide bonds. The formicitoxins appear to be specific to the genus *Anochetus* as in *Odontomachus,* the sister genus, the venom peptidome is very different and only contains linear peptides as well as some peptides with one disulfide bond as minor components [Touchard et al., 2014a; Touchard et al., 2015]. These closely related ant genera share morphological and behavioral features: they both used their trap jaws to capture their prey, followed by a paralyzing sting. This suggests the rapid diversification of *Anochetus* venoms.

The formicitoxins characterized here present original sequences with no homology with any other ant venom peptides sequenced so far. Some formicitoxins are highly homologous with only a few differences in their sequences (FOTX-Ae3 and FOTX-Ae4 differ from only one residue) , while others have quite different amino acid compositions (*e.g.* FOTX-Ae5). Presumably, this will support a broad range of potency, selectivity or diverse modes of action among these toxins.

Except for cone snails venoms (*e.g.* α-Conotoxins [Terlau and Olivera, 2004]), small venom peptides (< 30 aa) structured by two disulfide bonds are quite rare in other venomous animals, although they have been occasionally noted in the venoms of some hymenopterans (*e.g.* apamine, tertiapin, MCDP [Argiolas et al., 1985; Gauldie et al., 1976; Hider and Ragnarsson, 1981; Palma, 2006]) and snake (*e.g.* Sarafotoxins [Takasaki et al., 1988]). Very few homologies can be observed between formicitoxins and these known small peptides reticulated by two disulfide bonds, making formicitotoxins quite unique among animal toxins (Table 3).

Table 3

Sequences of small peptide toxins folded by two disulfide bonds from venomous animals.
Percentage identity (% I) is relative to the first peptide (Formicitoxin-Ae1).

Peptide	Specie	Sequence	% I	Target
ω-Formicitoxin-Ae1	*Anochetus emarginatus*	WCASGCRKKRHGGCSC*	-	Ca_v channel
Apamin	*Apis mellifera*	CNCKAPETALCARRCQQH*	15	K_v channel
Tertiapin	*Apis mellifera*	ALCNCNRIIIPHMCWKKCGKK*	23.8	K_v channel
MCDP	*Apis mellifera*	IKCNCKRHVIKPHICRKICGKN*	22.7	K_v channel
Sarafotoxin 6b	*Atractaspis engaddensis*	CSCKDMTDKECLTFCHQDVIW	15	Endothelin receptor
α-Conotoxin AuIB	*Conus aulicus*	GCCSYPPCFATNPDC*	17.6	Nicotinic receptor
α-Conotoxin EI	*Conus ermineus*	RDOCCYHPTCNMSNPQIC*	17.6	Nicotinic receptor
α-Conotoxin GI	*Conus geographus*	ECCNPACGRHYSC*	18.8	Nicotinic receptor
α-Conotoxin IMI	*Conus imperialis*	GCCSDPCAWRC*	17.6	Nicotinic receptor
α-Conotoxin MII	*Conus magus*	GCCSNPVCHLEHSNLC*	17.6	Nicotinic receptor
α-Conotoxin PnIB	*Conus pennaceus*	GCCSLPPCAANNPDYC*	17.6	Nicotinic receptor
α-Conotoxin PIA	*Conus purpurascens*	RDPCCSNPVCTVHNPQIC*	17.6	Nicotinic receptor
α-Conotoxin SI	*Conus striatus*	ICCNPACGPKTSC*	25.1	Nicotinic receptor
ρ-Conotoxin TIA	*Conus tulipa*	FNWRCCLIPACRRNHKKFC*	23.5	Nicotinic receptor
χ -Conotoxin MrIA	*Conus marmoreus*	NGVCCGYKLCHOC	18.2	Nicotinic receptor

* Amidated C-terminus

Formicitoxin FOTX-Ae1 was investigated in details and showed medium to high insecticidal potency when compared to the range of potency spanning six orders of magnitude for the insecticidal activity of spider venom peptides [Windley et al., 2012]. Thus, the fact that FOTX-Ae1 caused fully reversible paralysis in blowflies would imply that other toxins in this venom are responsible for the irreversible paralysis of termite prey (A.T. pers. obs.). In accordance with the cases of cone snail and spider venoms [Herzig and King, 2013; Olivera and Cruz, 2001], FOTX-Ae1 could belong to a “toxin cabal” responsible for the quick paralysis of prey, whereas other toxins cause the lethal effects. Also, the venom compositions of snakes and cone snails are known to be dependent on prey specificity to ensure that they are efficiently captured and killed [Barlow et al., 2009; Bernardoni et al., 2014; Duda et al., 2009]. The prey preferences of most *Anochetus* are unknown, but they are likely a specialist predator of termites [Schatz et al., 1999], and dipterans are unlikely their natural prey, so the reversibility of the FOTX-Ae1 paralysis observed in blowflies could different in their natural prey.

Many toxins from the venoms have evolved to block or activate ion channels in order to subdue their prey. Among these ion channels, voltage-gated calcium channels are targeted by many toxin peptides with diverse selectivity on both vertebrate and invertebrate [de Weille et al., 1991; King, 2007; Olivera et al., 1987]. Here, we demonstrated that FOTX-Ae1 is a selective blocker of the L-type Ca_v channel (Ca_v1), a vertebrate ion channel, and this toxin should be named ω_1-FOTX-Ae1 following the peptide toxins nomenclature established by King et al. [King et al., 2008b]. Interestingly, only few peptides modulating the L-type calcium channels of vertebrates have been characterized so far. Calciseptine and calcicludine respectively isolated from the venoms of black mamba (*Dendroaspis polylepsis*) and green mamba (D*endroaspsis angusticeps*) block L-type Ca^{2+} channels of vertebrates [de Weille et al., 1991; Schweitz et al., 1994]. Among ants, the heterodimeric peptides Et-1 isolated from *Ectatomma tuberculatum* venom is capable of inhibiting whole-cell L-type calcium currents in isolated rat ventricular myocytes [Pluzhnikov et al., 1999]. Considering the level of identity between insect Ca_v channels and their closest human ortholog is only *ca.* 66 % [King et al., 2008a], FOTX-Ae1 could affect insect ion channels very differently. Extending pharmacological characterization of FOTX-Ae1 on insects ion channels would permit us to determine the molecular target in insects.

Few ant toxins have been described and only two three-dimensional structures of ant venom peptides are known [Nolde et al., 1995; Szolajska et al., 2004]. We examined the three-dimensional NMR structure of FOTX-Ae1 peptide and described this currently unique toxin scaffold in venoms. *Anochetus* seems to have evolved a novel biochemical strategy compared to other ants in order to interact with the ion channels of prey. These formicitoxins may have contributed to the evolutionary success of *Anochetus* which is abundant and widespread in the tropical and subtropical regions of the world. The exploration of the other formicitoxins isolated here and of the venoms of the 114 currently-described *Anochetus* species [AntWeb, 2014b] would indubitably lead to the discovery of novel ligands for ion channels. This highlights the immense potential of ant venom peptides as lead molecules in drug and bioinsecticides discovery and as pharmacological tools in the characterization of ion channel subtypes.

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2. Nocitoxines

Les propriétés algésiques de certaines toxines permettent d'assurer la fonction défensive des venins afin de repousser les prédateurs. Ainsi certaines toxines peptidiques sont capables d'activer les nocicepteurs des vertébrés, tels que les récepteurs TRP ou les récepteurs ASIC afin d'induire une sensation de douleur. On nomme de telles toxines des nocitoxines [Bohlen et al., 2011; Cromer and McIntyre, 2008]. Parmi les récepteurs impliqués dans la nociception, les TRP (Transient Receptor Potential) appartiennent à une grande famille de récepteurs dont de nombreux sous-types sont connus chez les mammifères. Ces récepteurs sont impliqués dans la thermoception, la chimioception et l'hyperalgésie et sont activés par une famille de molécules que l'on appelle les vanilloïdes [Julius, 2013]. Le canal TRPV1 est le premier à avoir été identifié chez les mammifères et il est notamment activé par la capsaïcine qui est la molécule active présente dans le piment. Récemment deux peptides, la vanillotoxine VaTx1-3 et le peptide DkTx (Double Knot Toxin) isolés à partir de deux venins de mygales ainsi que d'extraits de venins de cnidaires ont montré un effet agoniste sur ce récepteur et produisent une douleur inflammatoire [Bohlen et al., 2010; Cuypers et al., 2006; Siemens et al., 2006] (Figure 17).

A l'instar des guêpes, les fourmis sont réputées pour infliger des piqûres douloureuses [Schmidt, 2014]. On peut donc penser que certaines toxines présentes de leur venin pourraient activer des récepteurs tels que les TRP pour provoquer un effet algésique.

Nous avons donc testé l'effet de trois peptides synthétiques (Pc-1973, Mr-1435 et Ae-1733) sur les récepteurs humains hTRPV1 et hTRPV3 dans des ovocytes de Xénopes (voir le protocole en Annexe 2). Si aucun effet n'a été observé pour les peptides Mr-1435 et Ae-1733, le peptide Pc-1973 provoque une forte activation des deux canaux hTRPV1 et hTRPV3

(Figure 18). Le peptide Pc-1973 est la première toxine animale connue activant le récepteur hTRPV3. Il sera cependant indispensable de confirmer ces résultats dans le future, avec ces récepteurs exprimés dans des cellules de mammifères.

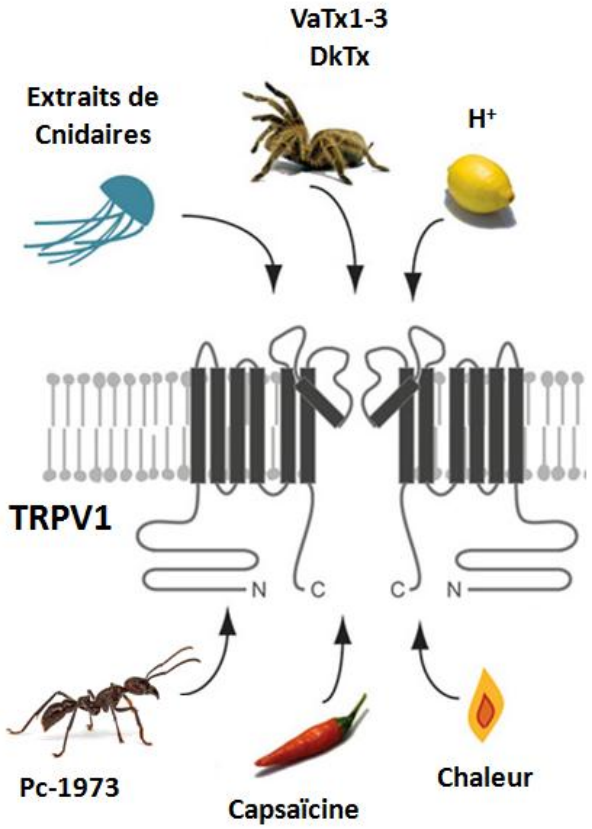

Figure 17- Les toxines agonistes du récepteur TRPV1. Ce récepteur détecte les signaux physiques et chimiques de l'environnement tels que le pH acide ou les températures élevées. Figure adaptée d'après [Siemens and Hanack, 2014].

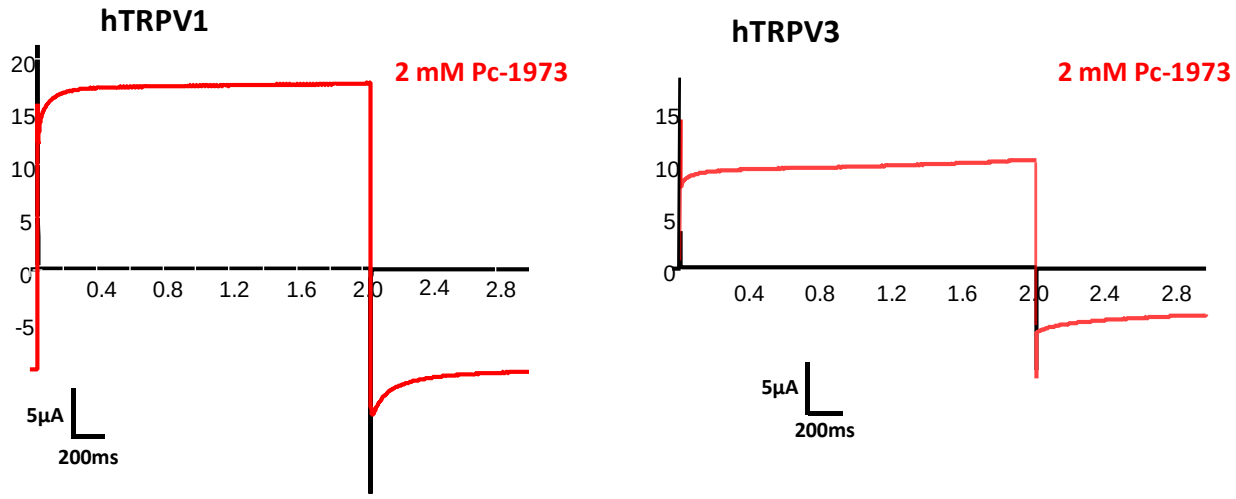

Figure 18- Activation des récepteurs hTRPV1 et hTRPV3 par le peptide Pc-1973 à 2mM.

Discussion générale

Jusqu'à une époque récente, les faibles quantités de venin disponible chez les fourmis ont grandement limité les études biochimiques et pharmacologiques de leurs composés peptidiques. Désormais, les avancées technologiques effectuées en spectrométrie de masse, chromatographie séparative, spectroscopie RMN ainsi que la miniaturisation des tests pharmacologiques offrent davantage de perspectives pour l'étude des toxines des petits organismes venimeux et particulièrement les fourmis qui sont en termes de biomasse et de distribution parmi les plus abondants. Afin d'explorer ces venins il est cependant nécessaire d'accumuler les venins de plusieurs individus de la même espèce. L'identification des espèces est donc essentielle. La diversité des toxines peptidiques dans les venins de fourmis est importante et chaque espèce venimeuse possède une panoplie de peptides qui lui est spécifique. Cette caractéristique nous a permis de mettre au point un outil de chimiotaxonomie basé sur les peptides des venins afin d'identifier facilement et rapidement les différentes espèces de fourmis. Cet outil taxonomique pourrait être applicable à des milliers d'espèces d'hyménoptères aculéates (guêpes, abeilles, bourdons, mutiles) puisque leur venin est essentiellement composé de peptides [Argiolas and Pisano, 1985; Baptista-Saidemberg et al., 2011; de Souza et al., 2004; Gauldie et al., 1976; Matysiak et al., 2011; Piek et al., 1989; Vincent et al., 2010] et contribuer ainsi à l'évaluation de leur diversité spécifique et biochimiques. Ce nouvel outil taxonomique permet d'identifier très rapidement les espèces de fourmis venimeuses et s'ajoute aux nombreuses autres méthodes de taxonomie des fourmis basée sur la génétique, la morphologie, la chimie cuticulaire, la cytogénétique ou encore l'acoustique des stridulations. Cette multiplicité des approches est utile dans un contexte de taxonomie intégrative afin de résoudre les problèmes posés par les

complexes d'espèces cryptiques. En associant cette méthode avec d'autres méthodes de taxonomie telle que le barcoding ADN, nous avons mis au jour une biodiversité cachée en révélant la présence d'espèces cryptiques dans nos échantillons.

Un autre défi majeur pour la recherche de molécules actives est la variation de la composition des venins au sein d'une même espèce, due à des facteurs liés à l'âge, au sexe, à la géographie ou aux variations génétiques. Nous avons montré qu'il n'y avait pas ou très peu de variation de la composition du venin de la fourmi *Odontomachus haematodus* lié au polyéthisme de la colonie (donc à l'ontogénie). Cependant il serait intéressant de tester l'effet du polyéthisme sur la composition des venins d'autres espèces de fourmis.

Les différences de composition des venins d'espèces phylogénétiquement proches suggèrent que les venins de fourmis se sont rapidement diversifiés après la spéciation. Cette évolution rapide des toxines des venins s'explique généralement par des mécanismes intenses de duplication des gènes au cours de l'évolution, suivis par une néo-fonctionnalisation et une sélection adaptative [Wong and Belov, 2012]. Les venins de fourmis se sont ainsi perfectionnés au cours de l'évolution grâce à un mécanisme d'évolution adaptative entre les fourmis et leurs proies mais aussi entre les fourmis et leurs prédateurs. Cette double course à l'armement a permis aux fourmis d'acquérir à la fois des toxines offensives et défensives originales afin de capturer efficacement leurs proies et de faire fuir leurs prédateurs.

Dans les autres groupes d'animaux venimeux intensivement étudiés, les venins sont composés de nombreux peptides structurés par des ponts disulfure leur conférant une grande stabilité chimique et une importante résistance aux dégradations enzymatiques [King and Hardy, 2013]. Ce n'est pas le cas dans les venins de fourmis qui possèdent une majorité

de petits peptides linéaires. Cependant, bien que minoritaires, des peptides structurés par des ponts disulfure sont présents dans de nombreux venins appartenant à différentes sous-familles de fourmis à aiguillon. Ces peptides à ponts disulfure n'ont presque jamais été étudiés et leur rôle biologique reste méconnu. L'Ectatomine Et-1 est le seul peptide à ponts disulfure dont la pharmacologie et l'activité biologique ont été élucidées [Arseniev et al., 1994; Pluzhnikov et al., 2000]. Les dix peptides sélectionnés durant cette étude sont des peptides monomériques qui possèdent un (Pc-1973, Mr-1435 et Oh-1777) et deux (Ae-1733, Ae-1689, Ae-1790, Ae-1852, Ae-1866, Ae-1975 et Ae-1917) ponts disulfure ce qui constitue deux nouvelles classes structurales de peptides. Les peptides à un pont disulfure (Pc-1973, Mr-1435 et Oh-1777) possèdent des séquences très différentes et promettent des activités biologiques très variées.

Le peptide Ae-1733 appartient à un groupe de peptides que nous avons nommés « formicitoxines ». Ils sont structurés par deux ponts disulfure et possèdent une amidation C-terminale. Remarquablement, la quasi intégralité du peptidome du venin de la fourmi *Anochetus emarginatus* est constituée par ces formicitoxines alors que les peptidomes des autres espèces de fourmis ne contiennent qu'une minorité de peptides à ponts disulfure. La formicitoxine Ae-1733 est un peptide neurotoxique capable de bloquer les canaux calciques humain de type L. En suivant la nomenclature utilisée pour la dénomination des toxines animales [King et al., 2008b], ce peptide a été nommé ω_1-formicitoxine-Ae1733. Etant donné que les proies naturelles des *Anochetus* sont de petits arthropodes, il serait intéressant dans un travail futur de tester l'activité de cette toxine sur les canaux ioniques d'insectes.

L'émergence des formicitoxines dans les venins a probablement apporté un avantage sélectif important pour les fourmis dans la capture des proies contribuant ainsi au succès

évolutif des *Anochetus*. L'exploration biochimique des venins d'autres espèces d'*Anochetus* à mis en évidence la présence d'autres formicitoxines. Cette famille structurale de peptides semble donc être une caractéristique spécifique des venins du genre *Anochetus*. En effet, les fourmis du genre *Odontomachus* sont morphologiquement et phylogénétiquement très proches des *Anochetus*, mais possèdent des venins dont la composition est très différente. Il semble donc que les fourmis du genre *Anochetus* aient adopté une stratégie biochimique différente des autres fourmis (Figure 19).

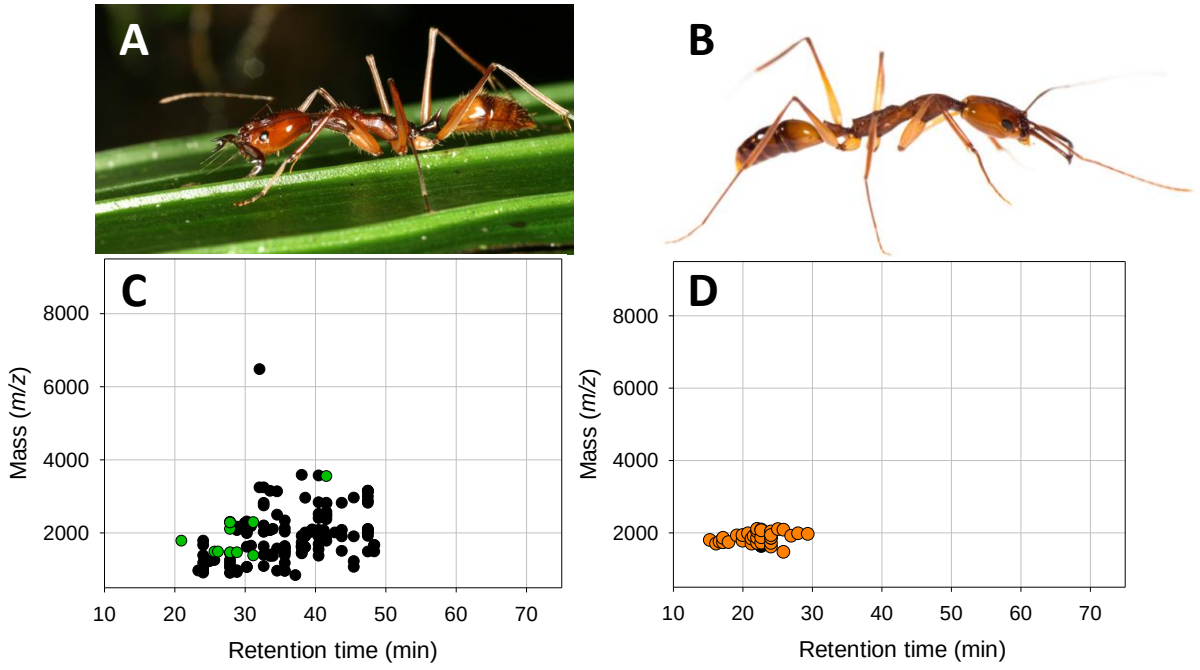

Figure 19- Les genres *Odontomachus* et *Anochetus* sont très proches morphologiquement et phylogénétiquement mais possèdent des venins dont la composition est très différente. A) *Odontomachus hastatus*. B) *Anochetus emarginatus* (photo P. Escoubas). C) Peptidome du venin d'*Odontomachus hastatus*. D) Peptidome du venin d'*Anochetus emarginatus*. Les points noirs représentent les peptides linéaires, les points verts sont les peptides avec un pont disulfure et les points oranges représentent les peptides structurés par deux ponts disulfure.

La vie en société impose aux fourmis, à l'instar des autres hyménoptères sociaux, la protection de leur nid et de ses habitants contre une grande variété de prédateurs souvent bien plus volumineux qu'elles. Il s'agit là d'une différence importante avec les animaux venimeux solitaires qui le plus souvent privilégient la fuite et utilisent leur venin seulement en dernier recours. Le rôle défensif des venins, bien que peu étudié, est supposé avoir un rôle mineur dans l'évolution des venins et la diversification des toxines [Casewell et al., 2013]. Cependant chez les fourmis, ce rôle défensif pourrait avoir joué un rôle majeur dans la sélection de toxines répulsives. De nombreuses fourmis sont en effet réputées pour infliger des piqûres très douloureuses (e.g. *Paraponera clavata*, *Neoponera* spp., *Solenopsis* spp., *Manica rubida*, *Pogonomyrmex* spp., *Pseudomyrmex* spp., *Tetraponera* spp., *Myrmecia* spp.) [Schmidt, 2014; Starr, 1985]. La piqûre de la fourmi *Neoponera commutata* quant à elle, provoque rapidement une vive douleur et est même exploitée par une tribu amérindienne comme une épreuve initiatique dans laquelle les jeunes filles sont piquées par une soixantaine de fourmis de cette espèce [Balée, 2000; Schmidt and Overal, 2009]. Le peptide Pc-1973 qui a été isolé au cours de cette étude à partir du venin de *Neoponera commutata* a été nommé commutatoxine. Il s'agit d'une toxine agoniste des récepteurs hTRPV1 et hTRPV3. Chez les vertébrés ces récepteurs sont impliqués dans la sensation de chaleur et leur activation provoquent une douleur inflammatoire. Si plusieurs toxines activatrices du récepteur TRPV1 avaient déjà été découvertes dans des venins de mygales [Bohlen et al., 2010; Siemens et al., 2006], la commutatoxine est la première nocitoxine agoniste du récepteur TRPV3 découverte à ce jour. La découverte d'une telle toxine témoigne de l'importance du rôle défensif dans l'évolution des venins de fourmis.

La penetratine est une autre toxine défensive que nous avons isolée à partir du venin de la fourmi *Pseudomyrmex penetrator*. Ce peptide hétérodimérique structuré par deux

ponts disulfure est très cytotoxique sur les cellules d'insectes (*Aedes albopictus*). Cette fourmi qui est associée avec un myrmécophyte utilise son venin uniquement pour protéger la plante contre les attaques des herbivores. Il serait donc intéressant d'une part, de tester l'effet de la penetratine sur les mammifères et notamment au niveau de leurs nocicepteurs et d'autre part, de rechercher la présence de peptides dimériques dans les venins des autres espèces de *Pseudomyrmex* vivant dans les myrmécophytes. Cela permettrait de savoir si ce mode de vie a permis la sélection de ces toxines défensives dimériques.

Conclusion et perspectives

Pour la recherche de molécules d'intérêt, la stratégie classiquement utilisé est un fractionnement des venins guidé par des tests d'activités biologiques et pharmacologiques. Cependant cette stratégie est peu applicable aux petits organismes venimeux car elle requiert une quantité importante de venin. Dans cette thèse, nous avons favorisé une stratégie alternative pour la découverte de molécules originales mieux adaptée pour les venins de fourmis. Nous avons effectué une sélection de toxines basée sur des critères structuraux avec notamment la recherche de ponts disulfure. En effet, les peptides à ponts disulfure, en plus d'être largement inexplorés dans les venins de fourmis, possèdent des caractéristiques intéressantes pour le développement de molécules bioactives (résistance enzymatiques, stabilité chimique et diversité de structures). Le travail effectué durant cette thèse étude constitue l'une des plus importante explorations des peptidomes des venins de fourmis et a permis de révéler la grande richesse structurale des toxines. Cela nous a permis d'isoler et de caractériser de nouvelles molécules bioactives aux structures innovantes.

La formicitoxine. Le peptide Ae-1733 est une neurotoxine qui possède deux ponts disulfure. Cette toxine a montré un effet antagoniste sur les canaux calciques humain Ca_v1. L'exploration des venins des 114 espèces actuellement décrites d'*Anochetus* [AntWeb, 2014b] pourrait permettre la découverte de nouveaux ligands aux canaux ioniques, en particulier ceux impliqués dans les pathologies humaines .

La commutatoxine. Pc-1973 est un peptide structuré par un pont disulfure agoniste des récepteurs hTRPV1 et hTRPV3. Il s'agit d'un peptide défensif qui pourrait être utilisé comme un outil pharmacologique pour comprendre les processus impliqués dans l'induction de la douleur chez l'Homme.

Mr-1435. Ce peptide structuré par un pont disulfure a été isolé du venin de *Manica rubida*. Nous n'avons trouvé aucune activité biologique contre les récepteurs que nous avons testés. Cependant de nombreux autres tests pourraient être envisagés pour évaluer l'activité antimicrobienne, antifongique, analgésique/algésique (récepteur ASIC), ou cytotoxique.

Oh-1777. Ce peptide n'a fait l'objet d'aucune investigation cours de cette thèse et son rôle biologique reste inconnu. De nombreux tests pourraient donc être réalisé afin d'étudier son potentiel insecticide, antimicrobien, algésique et cytotoxique.

Il ne faut pas non plus négliger les peptides linéaires dans les études futures sur les venins de fourmis. La présence en grand nombre de ces peptides bioactifs dans presque tous les venins analysés témoigne de l'importance fonctionnelle de cette classe de toxines. Les innovations attendues dans le domaine de la vectorisation des peptides laisse présager un avenir radieux à ces petits peptides bioactifs [Pimenta and De Lima, 2005].

Au vu des rôles biologiques et des caractéristiques pharmacologiques affichés par les peptides, les venins de fourmis apparaissent comme une source de candidats potentiels pour le développement d'antimicrobiens (antibiotiques et antifongiques), d'analgésiques (antagonistes des canaux ioniques) ou encore d'anticancéreux (activité cytotoxique). Les fourmis sont également de redoutables prédateurs qui utilisent leur venin pour paralyser et tuer de nombreux invertébrés. Les toxines produites par leurs glandes venimeuses pourraient cibler sélectivement les récepteurs des insectes sans aucune toxicité pour les vertébrés. De telles toxines seraient des candidats idéals pour le développement de bioinsecticides.

A ce jour, seule une infime partie de la composition des venins de fourmis est connue. Les études futures des autres espèces de fourmis conduiront sans nul doute à de nouvelles découvertes. Les 9.100 espèces actuellement décrites de fourmis à aiguillon associées aux variations intraspécifiques, ainsi qu'aux espèces encore non décrites (illustré par les espèces cryptiques) promettent une source immense de peptides bioactifs. De plus, les peptides des venins de fourmis sont de petites tailles et possèdent peu de ponts disulfure, ce qui rend leur séquençage ainsi que leur synthèse relativement simples et rapides encourageant la caractérisation biochimique et pharmacologique de nouveaux peptides. Pour l'exploration future des venins de fourmis, il sera nécessaire d'appliquer une stratégie de séquençage intégrant les technologies de pointe en matière de transcriptomique et de protéomique. Ceci permettra de construire une banque de toxines bioactives mimant la diversité structurale naturelle présente dans les venins de fourmis. Ensuite, La production de ces toxines par synthèse chimique ou par voie recombinante permettrait de réaliser un screening de cette banque de molécules sur les cibles d'intérêt thérapeutique humaines.

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Annexe 1

Article 1

Diversity of peptide toxins from stinging ant venoms

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Diversity of peptide toxins from stinging ant venoms

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Running head: Ant venom peptide toxins

Key words: Ant venom, peptides, venom biochemistry, disulfide linkage, chemotaxonomy

Abstract

Ants (Hymenoptera: Formicidae) represent a taxonomically diverse group of arthropods comprising nearly 13,000 extant species, and are among the most abundant venomous animals. Sixteen ant subfamilies, representing over 9,100 species, have individuals that possess a venom apparatus including a stinger for injecting venom making them a vast source of potentially unique bioactive toxins. Ants use their venom for several purposes such as a defense against predators, competitors and microbial pathogens, for predation, as well as for social communication. As a result, they exhibit a wide range of activities including antimicrobial, hemolytic, cytolytic, paralytic, insecticidal and pain-producing pharmacologies. While ant venoms are known to be rich in alkaloids and hydrocarbons, ant venoms rich in peptides are becoming more common, yet remain highly understudied. Recent advances in analytical techniques especially mass spectrometry have begun to reveal the true complexity of peptide toxin composition from ant venoms. In the few venoms explored so far, most of these peptide toxins appear to occur as small polycationic linear toxins, with antibacterial properties and insecticidal activity. Unlike other venomous animals, a number of ant venoms also contain a range of unique homodimeric and heterodimeric peptides with one or two interchain disulfide bonds possessing pore-forming, allergenic and paralytic actions. However, ant venoms, contrary to arachnid and cone venoms, seem to have a very small number of disulfide-linked peptides, with only one peptide with an inhibitor cystine knot structural motif, the *Dinoponera* ICK-like peptide, described to date. The present review details the structure and pharmacology of ant venom peptide toxins and their potential as a source of novel bioinsecticides and therapeutic agents.

1. Stinging ant biodiversity

Hymenopterans are among the most speciose group of venomous animals. With approximately 120,000 currently described species [van Emden, 2013], they are significantly more diverse than the major venomous phyla including spiders (44,906 species), snakes (3,496 species), cone snails (3,253 species), sea anemones (3,248 species) and scorpions (1,454 species) [Hallan, 2005; Platnick, 2013; Uetz et al., 2013]. Among the stinging aculeate Hymenoptera, ants and wasps (superfamily Vespoidea) and bees together with sphecoid wasps (superfamily Apoidea) are sister groups [Johnson et al., 2013]. Ants (family Formicidae) evolved from wasp-like ancestors between 115 and 135 million years ago [Brady et al., 2006] and became a diverse taxonomical group with ~13,000 extant species belonging to 21 subfamilies [Agosti and Johnson, 2005; AntWeb, 2014a]. Due to their ubiquitous nature in terrestrial environments, and the fact that they constitute 15–20% of the animal biomass in tropical rainforests [Hölldobler and Wilson, 1990; Wilson, 1990], ants are arguably amongst the most abundant venomous animals.

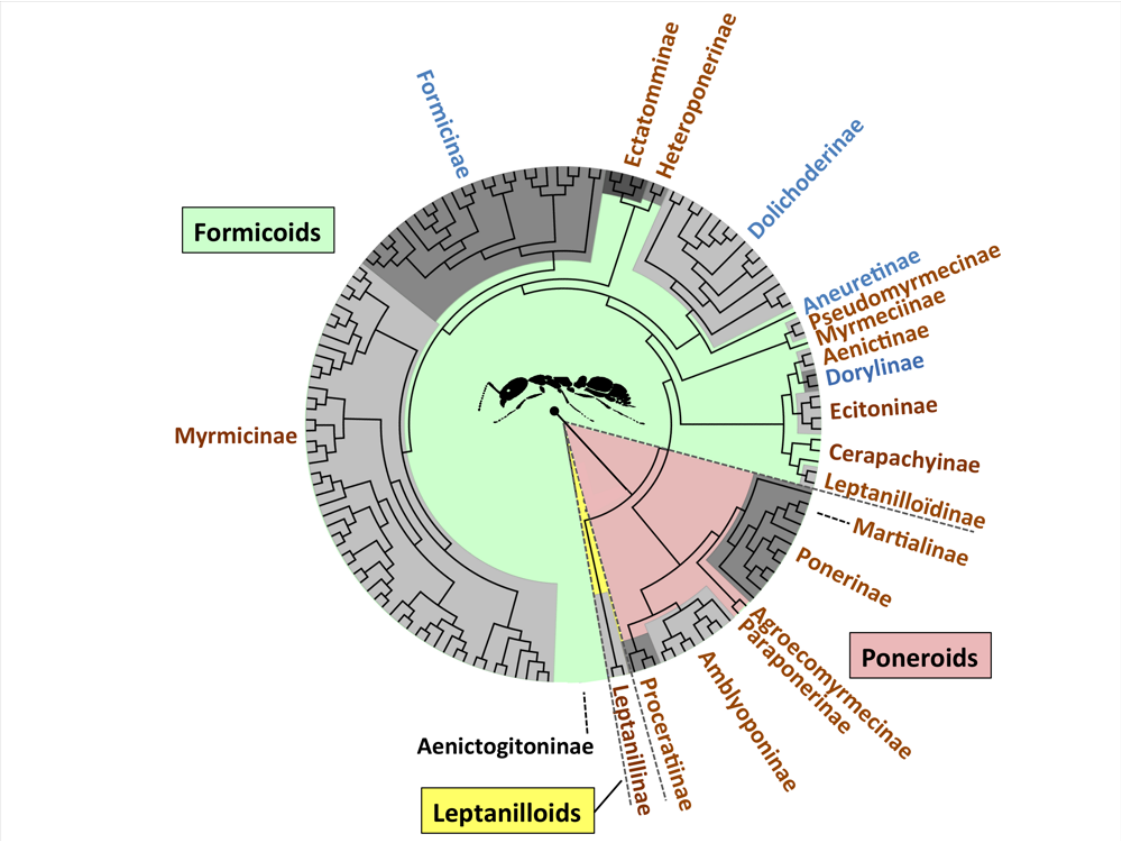


Fig. 1. Ant subfamily relationships as inferred from molecular phylogenetic. Phylogenetic relationships were generated from the S1573 TreeBASE data file [Moreau et al., 2006] using the FigTree v1.4.2 software package (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic relationships for the subfamilies Aenictogitoninae and Martialinae are currently unavailable. During evolution, four subfamilies lost their capacity to sting (blue text). Remaining subfamilies represent stinging ants (brown text). Ant clades are shaded green (Formicoids), red (Poneroids) and yellow (Leptanilloids). Females of subfamily Aenictogitoninae (black text) remain undiscovered and so this subfamily connot be classified as either stinging or non stinging. For clarification of colours in this figure, refer to the web version of this article. Note added in proof: Recently, the ant subfamilies Leptanilloïdinae, Cerapachyinae, Ecitoninae, Dorylinae, Aenictinae and Aenictogitoninae have been regrouped into one subfamily; Dorylinae [Brady et al., 2014].

Ants that belong to the subfamilies Formicinae, Dolichoderinae, Aneuretinae and Dorylinae lost their ability to sting during evolution (Fig. 1). Instead, they usually spray their venoms or have a residual, but non-functional, abdominal stinger. Also, it is unclear if the recently discovered subfamily Aenictogitoninae is venomous or not, as only male castes have been seen and females (workers and queens) are yet to be described [Brady et al., 2006]. The remaining 16 subfamilies are all stinging ants (Fig. 1) and comprise of ~9,100 extant

species. This makes ants taxonomically more diverse than scorpions, snakes and cone snails. However, this biodiversity is not equally distributed within stinging ant subfamilies (Fig. 2). For example, Myrmicinae is the most speciose ant subfamily, with ~6,500 extant species, with a widespread distribution throughout the world. However, ponerine ants that belong to the subfamily with the second highest number of ants, Ponerinae (~1,200 species), are mainly confined to tropical rainforests [AntWeb, 2014b; Johnson et al., 2013]. Furthermore, the subfamilies Paraponerinae and Martialinae only contain a single ant species both of which are found in Neotropical areas. Thus, taxonomic diversity varies within each ant subfamily however there is little doubt that ant venoms likely constitute a vast source of unique bioactive toxins.

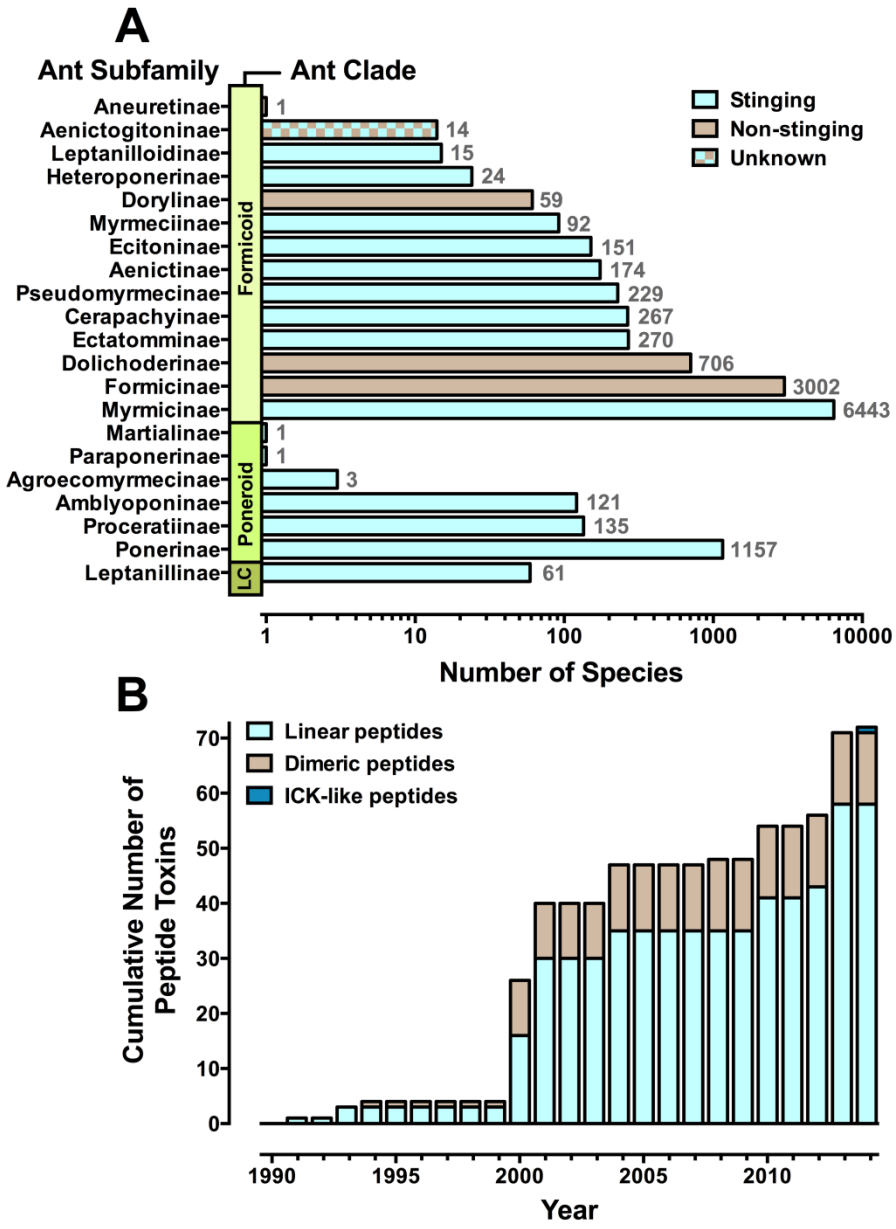


Fig. 2. (A) Species richness of ant subfamilies. (A) Ants have been grouped according to three clades, where LC represents the single genus Leptanilloid clade. Stinging ants are represented by cyan bars and comprise around 70 % of all ant species. Non-stinging ant subfamilies are depicted by brown bars. The total number of species in each subfamily is noted at right of each bar. The Aenictogitoninae subfamily is currently unclassified. Note added in proof: Recently, the ant subfamilies Leptanilloïdinae, Cerapachyinae, Ecitoninae, Dorylinae, Aenictinae and Aenictogitoninae have been regrouped into one subfamily; Dorylinae [Brady et al., 2014]. (B) Cumulative total number of peptide-toxin sequences reported from ant venom studies since the first described venom peptide (poneratoxin) in 1991, showing the three main structural classes: cyan, linear peptides; brown, dimeric peptides; teal, ICK-like peptides. Ant venom peptides remain barely investigated with only 72 peptides sequenced to date. For clarification of colours in this figure, refer to the web version of this article.

2. Ant venom functions

Ant venom is composed of a complex mixture of chemicals such as proteins, enzymes, biogenic amines, peptides, hydrocarbons, formic acid and alkaloids [Davies et al., 2004; Kem et al., 2004]. All these compounds are produced by the venom gland, which consists of two free cylindrical elongated and convoluted tubes, linked to a venom reservoir [Ortiz and Mathias, 2006]. The venom secreted by the tubular glands is stored in the reservoir, linked to the delivery apparatus and, for example, can deliver up to 130 micrograms of venom after each sting [Schmidt, 1990]. The stinger itself is a modified ovipositor located at the distal base of the abdomen. Ants use their venom for several purposes such as a defense against predators/competitors and microbial pathogens, for predation, as well as for social communication [Orivel et al., 2001; Schmidt, 1982]. Hence, ant venoms have evolved to carry out many different functions.

2.1. Offensive venoms

Ants are one of the leading predators of invertebrates in most ecosystems [Brady et al., 2006]. They have developed, through natural selection, a vast arsenal of behavioural adaptations and weapons to subdue their prey including trap-mandibles and potent venoms [Casewell et al., 2013]. Ant venom has paralytic and lethal effects on many arthropods [Maschwitz et al., 1979; Orivel and Dejean, 2001] and many ants are generalist predators, preying on numerous classes of invertebrates. Nevertheless, many ants are specialised predators and only feed on a restricted group of species. Such specialized hunters prey exclusively on earthworms, isopods, centipedes, millipedes, polyxena, collembolan, termites, other ants or even spider eggs [Cerdá and Dejean, 2011]. Solitary hunting is the most common hunting behavior employed by primitive ants such as ponerines. However, many

ants have also developed a cooperative hunting behaviour such as army ants exhibiting extreme group hunting behaviour.

The ecological diversity of ants is also revealed in their preference for various nesting habitats. Predatory ants are primarily ground, or litter-dwelling, predators. However, some ants have evolved predatory behaviors adapted to foraging in trees (arboreal ants) and exhibit adaptions to prevent their prey from escaping by flying away, jumping or dropping. Accordingly, venoms of solitary-foraging, arboreal predatory ants are believed to be more efficient than ground-dwelling species at rapidly immobilising prey [Orivel and Dejean, 2001]. Thus, the use of venom as an offensive weapon is likely to be the major driver of the venom composition during evolution. This has been shown with the differing composition and toxicity of venoms from arboreal *versus* ground-dwelling species of *Pseudomyrmex* and *Pachycondyla* [Dejean et al., 2014; Orivel and Dejean, 2001; Touchard et al., 2014b]. The wide ranging diet and hunting behaviours of ants are therefore likely to drive major differences among ant venom toxins.

2.2. Defensive venoms

Eusociality within hymenopteran colonies offers a range of evolutionary advantages including the capability of a mounting a collective defence against vertebrate and other arthropod predators, the ability to gather and store food and nutrients more efficiently, and to specialize in specific tasks, such as to care cooperatively for offspring [Wilson, 1971]. Nevertheless, these benefits can only be realized if the colony can defend against large predators who find the large biomass of the colony a potential food source worth their effort, in contrast to preying upon solitary hymenopterans. The evolution of venom in hymenopterans therefore provided a mechanism of defense against large intelligent

vertebrate predators and enabled them to develop complex societies. The combination of algesic and lethal actions of ant venom is therefore thought to be critical in the long term evolutionary success of insect stings to deter large predators [Schmidt, 2014]. For example, some ant stings are known to be extremely painful for humans. These include stings by fire ants (*Solenopsis* spp.), ponerine ants (*Pachycondyla* spp.) or the bullet ant (*Paraponera clavata*). In particular, bullet ants have been classified as producing the most painful sting among all hymenoptera and the third most painful sting of all venomous animals [Schmidt et al., 1983; Starr, 1985].

It is also clear that some ants, such as the *Pogonomyrmex* group of harvester ants, have developed venoms primarily for defence against vertebrates [Schmidt and Snelling, 2009]. For example, the venom of *Pogonomyrmex badius* is highly toxic towards mice, but not very toxic towards insects. Therefore, *Pogonomyrmex* ants do not appear to employ their venom to hunt, but use it exclusively as a deterrent against vertebrate predators [Schmidt and Blum, 1978b, c], akin to the defensive role of bee venom against vertebrates. Some Pseudomyrmecine ants have also evolved a defensive venom as part of a mutualistic relationship with myrmecophytes. Myrmecophytes are plants that provide a nesting place for a limited number of ant species, whilst the ants protect the myrmecophyte from defoliating arthropods and browsing mammals by stinging them. Natural selection has allowed ants that are known to have a painful sting to survive in such a habitat to the extent that some ants from the genera *Pseudomyrmex* and *Tetraponera* are obligate inhabitants of myrmecophytes. In some cases, ants use their venom in unusual ways. For example, *Pachycondyla tridentata* ants produce a foaming venom when disturbed and use their venom to paralyze their prey. This release of foam is a defense mechanism which is very effective against other small ants [Maschwitz et al., 1981].

It is therefore clear that ants have evolved venoms containing numerous toxins to induce pain, discomfort, paralysis and/or death in vertebrate and arthropod predators or prey. This is because protection of the nest, particularly protection of the brood and the queen, is a major concern for worker ants.

2.3. Antimicrobial properties of ant venoms

Ants are eusocial insects that typically live in colonies of relatives with a high population density. This increases the risk of introduction and spread of microbial pathogens. Consequently, ants have evolved strategies to inhibit microbial infections including the development and use of antimicrobial peptides. Firstly, predatory ant species may use their venom to inhibit internal pathogens present in captured prey that are brought back to the colony. In this way, the venom may protect the colony from infections following consumption of the prey species. In the ant venoms studied so far, this activity has been attributed to abundant linear, polycationic cytolytic peptides (see Section 3.1) that demonstrate potent antibacterial activity against both Gram-positive and Gram-negative bacteria [Cologna et al., 2013; Davies et al., 2004; Inagaki et al., 2004; Johnson et al., 2010; Kuhn-Nentwig, 2003; Mackintosh et al., 1998; Orivel et al., 2001; Rifflet et al., 2012; von Sicard et al., 1989; Zelezetsky et al., 2005]. More recently, similarity searches of ant genomes have revealed a number of tachystatins (antimicrobial chitin-binding peptides) with an inhibitor cystine knot (ICK) fold, as well as proline-rich abaecin-like, glycine-rich hymenoptaecin-like, insect defensin-like, and crustin-like antimicrobial peptides [Zhang and Zhu, 2012]. These peptides may be part of the uncharacterized antimicrobial secretions from the thoracic metapleural glands that are spread over certain ants and the nest [Mackintosh

et al., 1999; Yek and Mueller, 2011]. Nevertheless, there is no evidence that these peptides are present in ant venoms.

3. Ant venom peptides

Alkaloid-rich ant venoms have been well-studied, particularly among the genera *Solenopsis* [Brand, 1978; Jones et al., 1996b] and *Monomorium* [Jones et al., 2009; Jones et al., 1982; Jones et al., 1988; Jones et al., 2003]. However, proteinaceous venoms remain highly understudied despite the fact that they appear to be very common in both the Poneroid and Formicoid clades of ant venoms. Thus, venoms from Poneroid ants have been shown to be rich in peptides especially venoms from the subfamilies Ponerinae [Cologna et al., 2013; Johnson et al., 2010; Orivel et al., 2001; Torres et al., 2014; Touchard et al., 2014a] and Paraponerinae [Piek, 1991; Piek et al., 1991a; Piek et al., 1991b]. Peptides have also been characterized from the venoms of Formicoid ants belonging to the subfamilies Myrmicinae [Bouzid et al., 2013b; Rifflet et al., 2012], Myrmeciinae [Davies et al., 2004; Inagaki et al., 2004; Inagaki et al., 2008a; Lewis et al., 1968; Mackintosh et al., 1998; Wiese et al., 2006; Wu et al., 1998], Pseudomyrmecinae [Touchard et al., 2014b] and Ectatomminae [Arseniev et al., 1994; Nolde et al., 1995; Pluzhnikov et al., 1999].

Peptides are the dominant compounds in most animal venoms and they represent a huge source of structurally diverse and biologically active toxins with high potency and selectivity for a range of targets [King and Hardy, 2013]. Despite the clear potential that ant venom peptides represent, their investigation and characterisation remains highly underexplored. To date, only 72 ant venom peptides, from 11 ant species, have been fully sequenced (Fig. 3). This is a very small number in comparison to snakes, cone snails, scorpions or spiders. For example, 922 spider peptide toxins have currently been sequenced

from 86 spider species and are available in the ArachnoServer 2.0 [Herzig et al., 2011]. Therefore, it has been estimated that more than 98% of arachnid venoms remain completely uncharacterized [Quintero-Hernández et al., 2011], and with ant venoms this figure would be closer to 99.9%.

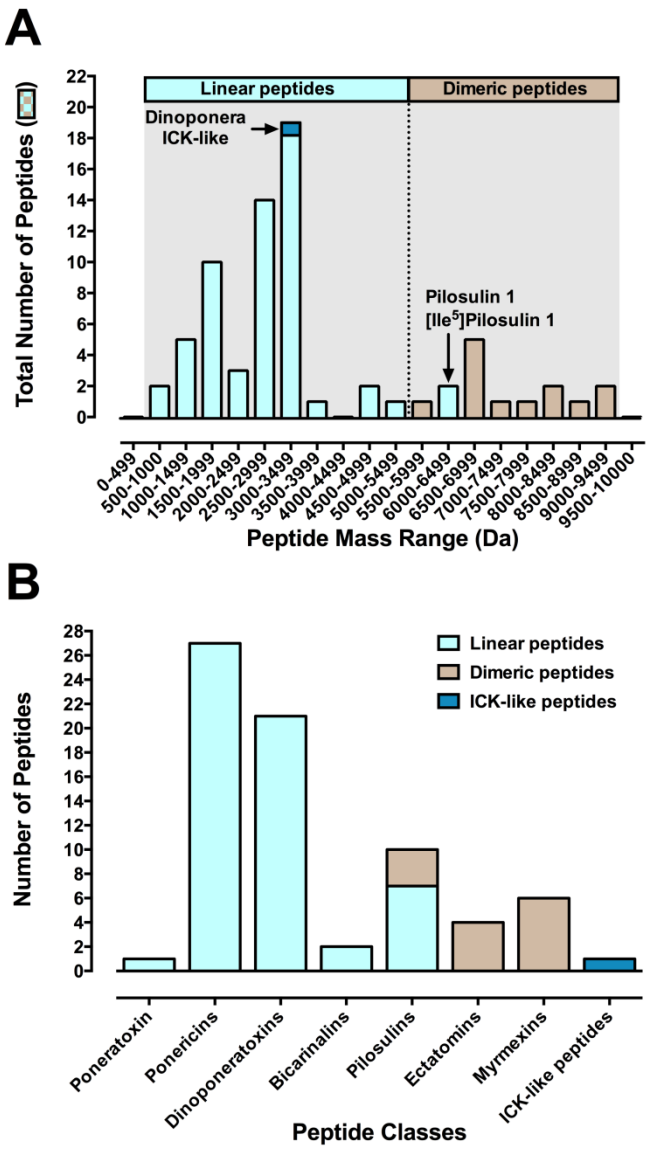


Fig. 3. (A) Bimodal mass distribution of the 71 characterized peptide toxins from ant venoms. Linear peptides range in mass from 761 to 5275 Da (except pilosulin 1, 6048 Da and [Ile^5]pilosulin 1, 6062 Da), while dimeric peptides range from 5603 to 9419 Da. (B) Ant peptide toxin classes. In both panels: cyan, linear peptides; beige, dimeric peptides; teal, ICK-like peptides.

Until recently, the main reason for the limited number of studies on ant venoms is the small size of ants, and hence the small yield of venom. However, advancements in analytical

techniques, particularly in mass spectrometric technologies, has resulted in higher sensitivity and resolving power, allowing for a more extensive exploration of the ant venom peptidome. This review summarizes the current knowledge on the biochemical and pharmacological properties of all peptide toxins sequenced from ant venoms to date. For the purposes of this review, these peptides have been classified based on their structure and classified into three main groups; (i) linear, (ii) dimeric and (iii) inhibitor cystine knot (ICK)-like peptides.

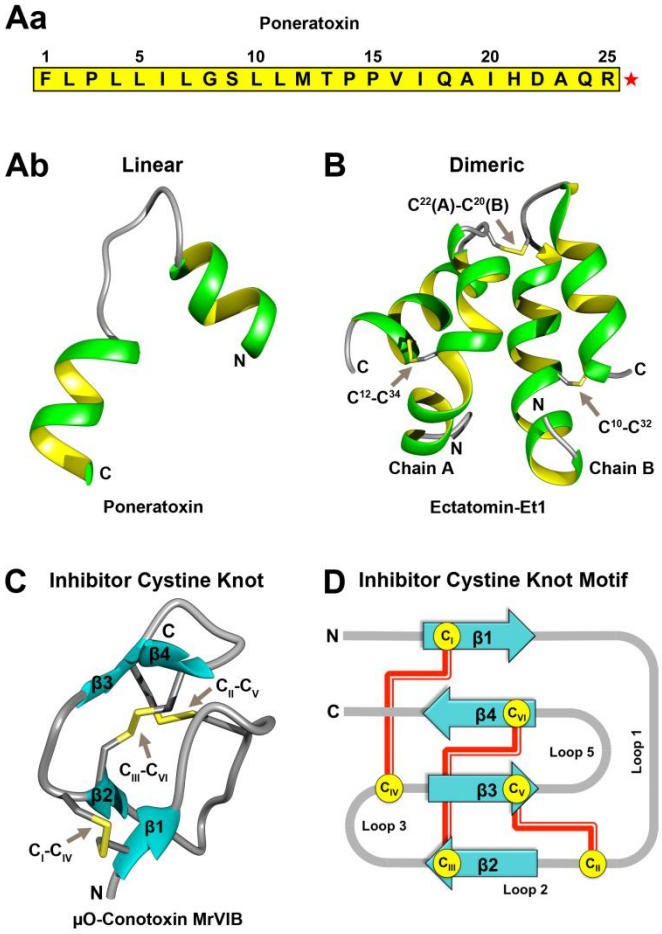


Fig. 4. Structures of ant peptide toxins. (Aa) Poneratoxin is a 2754.60 Da linear peptide (UniProtKB Accession POTX_PARCV) with no sequence homology to other peptides. The red star represents C-terminal amidation. (Ab) NMR structure of poneratoxin (PDB Accession 1G92) shows it comprises of two α-helices. (B) NMR structure of ectatomin (PDB Accession 1ECI), a heterodimeric peptide that forms a four-α-helical bundle structure. The intra- and interchain disulfide bonds are labeled for clarity. (C) Homology model of *Dinoponera* ICK-like peptide modeled on μO-conotoxin MrVIB from the venom of the cone snail *Conus marmoreus* (PDB Accession 1RMK; UniProtKB Accession CO16B_CONMR). In all panels, the peptide backbone is shown as a gray tube; β-sheets are represented by cyan arrows, α-helices are depicted as green/yellow spirals and disulfide bonds are shown as yellow tubes. The N-terminus (N) and C-terminus (C) of each peptide are also labeled. (D) Schematic representation of an ICK-like peptide. The pseudo knot is formed when one disulfide bridge (C_{III}-C_{VI}) crosses through a ring formed by two other disulfides (C_I-C_{IV} and C_{II}-C_V) and the intervening backbone. For clarification of colours in this figure, refer to the web version of this article.

3.1. Linear peptides

Most of the proteomic studies on ant venoms have so far revealed that the majority of the proteinaceous component of ant venoms are small, polycationic linear peptides with masses below 5 kDa [Cologna et al., 2013; Johnson et al., 2010; Orivel et al., 2001; Rifflet et al., 2012]. This is consistent with studies performed on other hymenopteran (wasp and bee) venoms [Argiolas and Pisano, 1985; Baptista-Saidemberg et al., 2011; de Souza et al., 2004; Dias et al., 2014; Favreau et al., 2006; Gomes et al., 2014; Mendes et al., 2004; Qiu et al., 2012]. Many of these linear peptides have antimicrobial properties and some possess additional insecticidal activity. Examples include ponericins from the neotropical ant *Pachycondyla goeldii* [Orivel et al., 2001], certain dinoponeratoxins (from *Dinoponera australis*) [Cologna et al., 2013] and pilosulins from the Australian jack jumper ant *Myrmecia pilosula*, which have been shown to have antimicrobial activity [Inagaki et al., 2004; Zelezetsky et al., 2005]. These antimicrobial peptides demonstrate broad spectrum antibacterial activity and include α-helix antimicrobial peptides, and peptides with homology to the antimicrobial mucroporins, cecropins, brevinins, gaegurins, temporins and demaseptins [Cologna et al., 2013; Davies et al., 2004; Inagaki et al., 2004; Johnson et al., 2010; Kuhn-Nentwig, 2003; Mackintosh et al., 1998; Orivel et al., 2001; Rifflet et al., 2012; von Sicard et al., 1989; Zelezetsky et al., 2005].

3.1.1. Poneratoxin

In 1991, the first ant venom peptide toxin, poneratoxin, was isolated and sequenced [Piek et al., 1991b]. Poneratoxin is a 25-residue peptide neurotoxin derived from the bullet ant *Paraponera clavata* (subfamily Paraponerinae) with no apparent homology to other known peptides (Fig. 4Aa) [Piek et al., 1991a]. The 3D NMR structure of poneratoxin has also been determined [Szolajska et al., 2004] and revealed a 'V'-shaped peptide with two α-

helices connected by a β-turn (Fig. 4Ab). It has been shown to modulate voltage-gated sodium (Na_V) channels of both vertebrates and invertebrates and blocks synaptic transmission in the insect CNS. Poneratoxin induces long-lasting plateau action potentials and repetitive firing due to the presence of a slow developing inward sodium current that activates at hyperpolarising potentials. This results from a potential toxin-induced interconversion between a fast and a slow conducting state of the Na_V channel [Duval et al., 1992; Hendrich et al., 2002; Szolajska et al., 2004].

3.1.2. Ponericins

Ponericins are a group of 27 peptides characterised from the venom of the ponerine ants, *Pachycondyla goeldii* [Orivel et al., 2001], *P. apicalis*, *P. inversa* [Orivel, 2000] and *P. commutata* (Touchard and Aili, unpublished data). Ponericins possess amphipathic α-helical structures in polar environments, and have been shown to exhibit haemolysis, antibacterial activity against both Gram-positive and Gram-negative bacteria as well as insecticidal activity [Orivel et al., 2001]. Ponericins have been classified into three different families ('G', 'W' and 'L') based on sequence homology (Fig. 5). The ponericins show considerable sequence homology with other previously characterised peptides. For example, ponericin G peptides show homology to cecropin-like peptides from moths, flies, beetles and butterflies [Lee et al., 2013]; ponericin W peptides have homology with the cytolytic peptide bee peptide melittin and gaegurins from frogs [Lee et al., 2011; Palma, 2013]; and ponericin L peptides with dermaseptins isolated from the skin of *Phasmahyla* and *Phyllomedusa* frogs [Amiche and Galanth, 2011; Nicolas and Amiche, 2013]. Given the known actions of these other peptides as cytolytic agents, ponericins may also form amphipathic α-helical structures in cell membranes, although only ponericin W peptides appear to have additional haemolytic

actions. This function maybe important in preventing the spread of microbial pathogens in ant colonies following ingestion of contaminated prey or their introduction into their colony following paralysis and subsequent transport of the prey into the colony [Lai et al., 2012].

3.1.3. Dinoponeratoxins

The giant Neotropical hunting ant *Dinoponera australis* (subfamily Ponerinae) is a solitary foraging, predatory ant whose venom paralyses invertebrates and causes a range of systemic effects in vertebrates [Haddad Junior et al., 2005]. Envenomation in humans is rare, although stings have been reported to produce rapid and excruciating pain, diaphoresis, nausea, vomiting, tachycardia and lymphadenopathy [Haddad Junior et al., 2005]. Liquid chromatography–mass spectrometry (LC-MS) analysis of the venom identified over 75 proteinaceous components with numerous small mass peptides (429–3214 Da) and a wide range of hydrophobicity and abundance. The six most abundant peptides were sequenced by tandem MS and Edman degradation and named dinoponeratoxins (‘Da’ toxins) [Johnson et al., 2010]. Subsequently similar dinoponeratoxin peptides have been isolated and sequenced from the related ant *Dinoponera quadriceps* - henceforth known as ‘Dq’ toxins [Cologna et al., 2013]. All 21 Dq and Da dinoponeratoxins show various degrees of homology with existing linear peptides and can be separated into six groups (Fig. 6).

Group I are short 7–9 residue Dq peptides forming a three-member orphan peptide family with no homology to existing peptides (Fig. 6A), and no known biological activity [Cologna et al., 2013]. Group II has only one member, Da-1039 (Fig. 6B), with only very limited homology to the uperin family of antibacterial frog skin secretions [Bradford et al., 1996; Steinborner et al., 1997]. Group III comprise three 9–11 residue Dq toxins with moderate homology with the temporin family of antibacterial frog skin secretions [Abbassi

et al., 2008; Rinaldi and Conlon, 2013; Simmaco et al., 1996]. Temporins are one of the largest groups of antimicrobial peptides within the cationic host defence peptide family. They were originally isolated from skin secretions of the frog *Rana temporaria*, and are amphipathic α-helical peptides of 8–19 residues with a low net positive charge (0 to +3) and C-terminal amidation [Mangoni et al., 2007; Suzuki et al., 2007]. The reasonably high homology of the temporin-like Dq toxins would suggest potentially similar biological activity, especially given the conservation of the common Pro and Leu residues found in temporin peptide families (Simmaco et al., 1996; residues in green boxes in Fig 6C). This leucine-rich tail has previously been shown to be important for membrane interaction [Avitabile et al., 2013]. The antimicrobial activity of temporins is associated with an alteration of the cytoplasmic membrane permeability, without destruction of cell integrity [Mangoni et al., 2004]. Temporins are particularly active against Gram-positive bacteria but most do not affect eukaryotic cells. However, they may act in a more complex way to inhibit various metabolic functions of the cell [Epand and Vogel, 1999; Park et al., 1998].

Group IV is the largest group of dinoponeratoxins and have masses between 1837 and 1984 Da with 17–19 residues. These have significant homology (53–63% similarity) with the antibacterial cationic host defense peptides BmKb (caerin-like) and mucroporin originally isolated from the venom of the scorpions *Mesobuthus martensii* and *Lychas mucronatus*, respectively [Dai et al., 2008; Zeng et al., 2004]. These antimicrobial peptides are now found in a range of scorpion species and are being investigated as novel anti-infective drugs or lead compounds, for treating antibiotic-resistant microbial infections [Harrison et al., 2014].

Group V is a recently discovered collection of 15 dinoponeratoxins from the venom of *Dinoponera quadriceps* sequenced from a total of 354 peptides found in this venom [Cologna et al., 2013]. These were found to share homology with the ponericin W family,

dinoponeratoxins (from *D. australis*) and poneratoxin. These peptides also revealed both antimicrobial and antifungal activities [Cologna et al., 2013].

Group VI comprises of the ant venom peptides Da-3105 and Da-3177, from the giant Neotropical hunting ant *D. australis*, which show considerable homology to ponericin G2 and may possess similar bioactivity.

3.1.4. Bicarinalins

Two novel peptides, bicarinalin 1 and P17 (bicarinalin 2) have been isolated and characterised from the venom of the ant *Tetramorium bicarinatum* (Myrmicinae) from a total of 31 peptides identified in this venom [Rifflet et al., 2012]. Interestingly, these peptides show very low homology with known peptide toxins (Fig. 5). Bicarinalin 1 exhibits all the characteristics of an amphipathic helical peptide and has broad and potent antibacterial activity similar to melittin, pilosulin and defensin but with weaker hemolytic activity [Rifflet et al., 2012]. Accordingly, it is being investigated as an anti-infective agent for use against emerging antibiotic-resistant pathogens. Recently the venom gland transcriptome of *Tetramorium bicarinatum*, one of the world's most broadly distributed ant species, has also been published [Bouzid et al., 2013a]. Transcribed *T. bicarinatum* venom gland ESTs revealed allergenic/cytotoxic peptides, with homology to pilosulins 1, 3 and 5, and paralytic peptide toxins, one of which possesses homology with the insect cytokine precursor uENF2. These allergenic/cytotoxic and paralytic toxins contributed close to 70% of the total EST cDNAs.

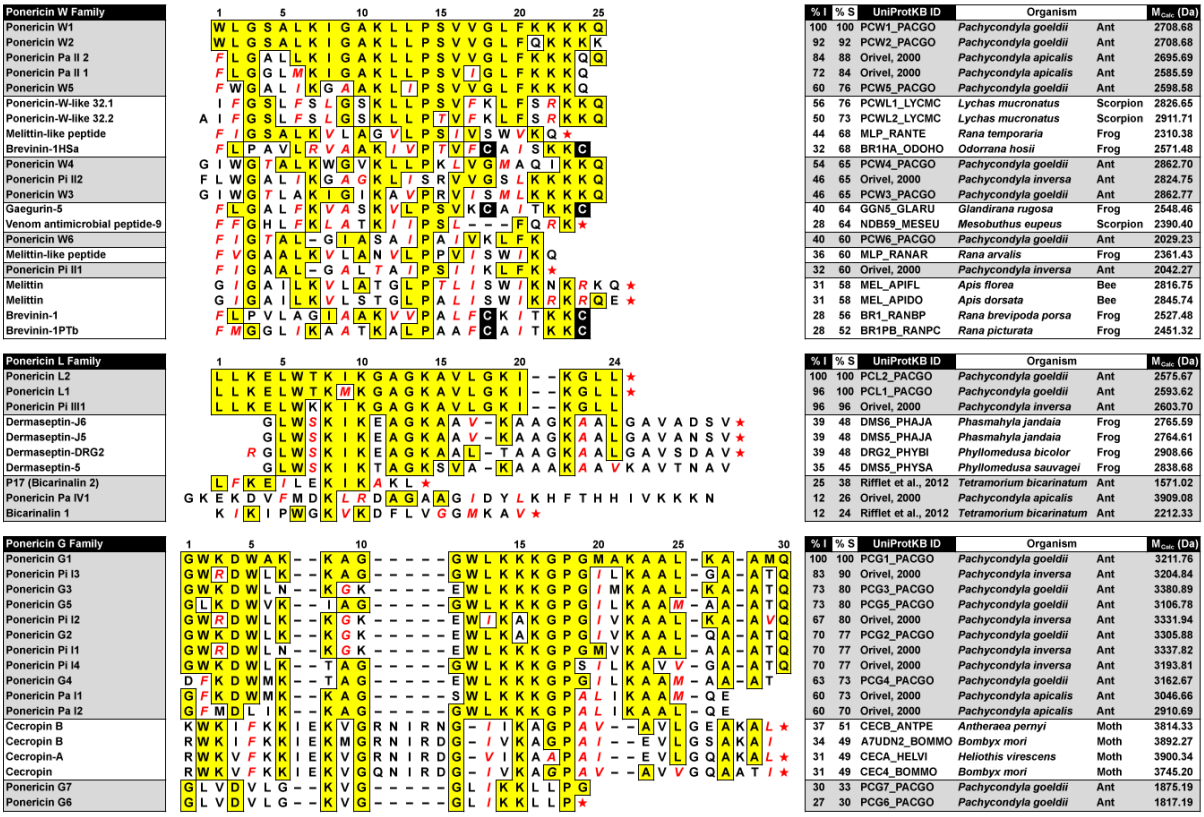


Fig. 5. Sequence alignment of bicarinalin and three ponericin families of linear peptides. Toxin names boxed in light gray are derived from ants. Identical residues in the peptide sequences are boxed in yellow while conservative substitutions are shown in red italic text. Cysteines are highlighted in black while red stars represent C-terminal amidation. Gaps were introduced to optimize the alignments. Percentage identity (%I) is relative to the first peptide of each family, while percentage similarity (%S) includes conservatively substituted residues. M_{calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. For clarification of colours in this figure, refer to the web version of this article.

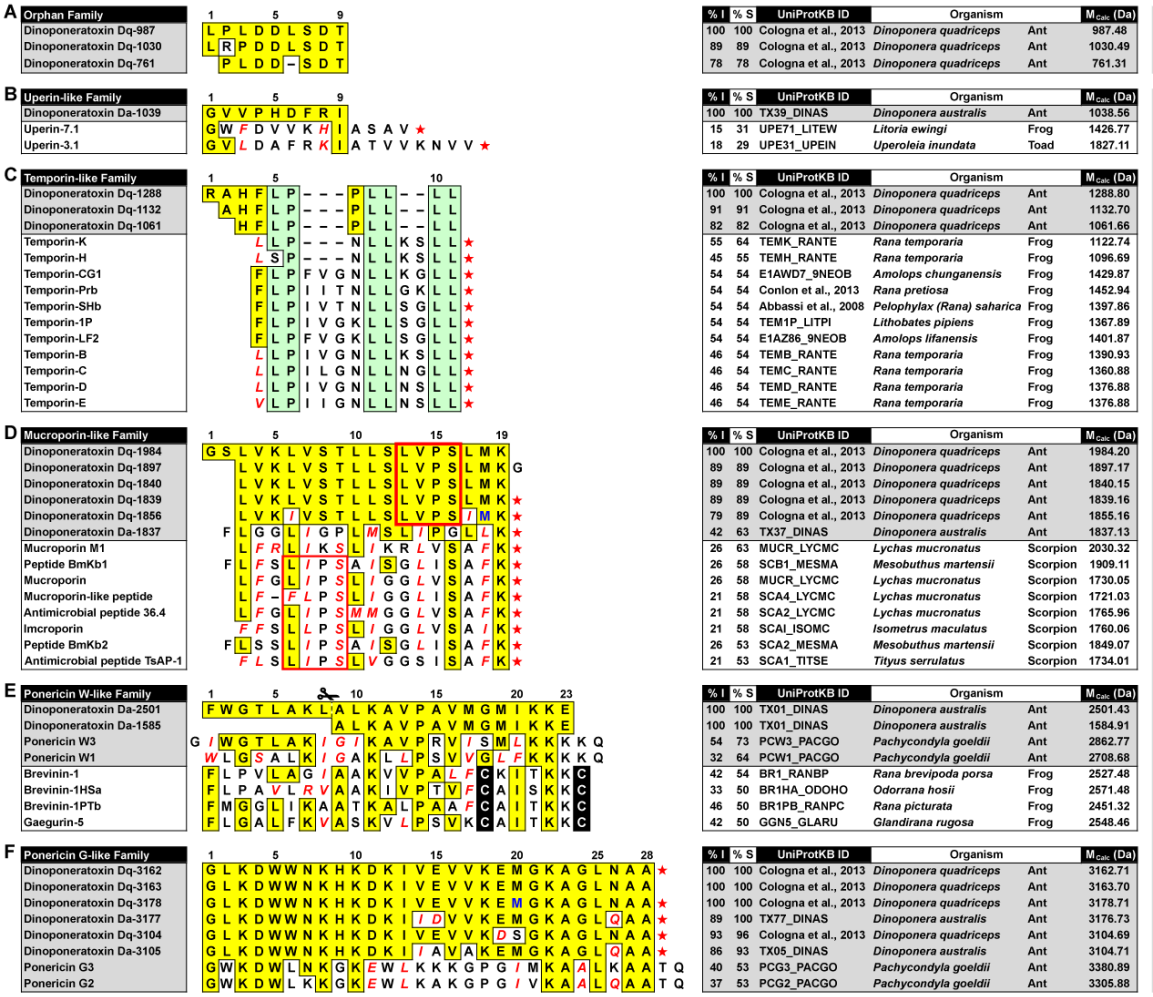


Fig. 6. Alignment of the dinoponeratoxin families of linear peptides. Toxin names boxed in light grey are derived from ants. Identical residues are boxed in yellow while conservative substitutions are shown in red italic text. Numbering is according to the first peptide in each family. Gaps were introduced to optimize the alignments. Red stars indicate an amidated C-terminus and cysteines are highlighted in black. Percentage identity (%I) is relative to the first peptide in each alignment while percentage similarity (%S) includes conservatively substituted residues. Apart from uperin peptides in panel B, only homologies greater than 50% are displayed. M_{Calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. (C) Residues Leu[5], Pro[6], and Leu[8-11] (numbering from Dq-1288), a common motif within temporin peptide families, are highlighted in green. (D) The residues K(V/L/I)IPS within the red boxes are thought to be critical for function in the scorpion antimicrobial peptide pandinin 2 and the scorpion peptides in panel D [Harrison et al., 2014]. (E) Dinaponeratoxin Da-2501 is cleaved at the position marked to generate Da-1585. The blue Met[18] in Dinoponeratoxin Dq-1837 (panel D) and Met[20] in Dinoponeratoxin Dq-3178 (panel F) indicate residues that are probably oxidized. For clarification of colours in this figure legend, refer to the web version of this article.

3.2. Dimeric peptides

Dimeric peptides are peptides with two subunits that are linked covalently with a disulfide bond [Sarray et al., 2013] and peptide dimerization is currently being investigated as a potential way to increase the activity of certain peptide toxins [Vizzavona et al., 2009]. Except for snake venoms [Osipov et al., 2008], a dimeric scaffold in peptides is quite rare in venomous animals, although it has occasionally been reported in the venoms of some scorpions [Zamudio et al., 1997], spiders [Santos et al., 1992] and marine cone snails [Loughnan et al., 2006]. In the case of ant venoms, dimeric peptides seem to be common in the subfamilies Ectatomminae, Myrmeciinae and Pseudomyrmecinae (see below), but have not yet been described in other subfamilies. The amino acid sequences and disulfide connectivity of the known dimeric peptide-toxins are reported in Figs. 7–9.

3.2.1. Ectatomins

One of the most potent neurotoxic peptides isolated from ant venoms, is ectatomin (Et-1), from the venom of the ant *Ectatomma tuberculatum* (Ectatomminae) [Pluzhnikov et al., 1994] and its homologue, ectatomin Et-2 [Pluzhnikov et al., 2000]. These peptides are highly basic heterodimeric complexes consisting of two highly homologous amphiphilic polypeptide chains linked together by one inter-chain disulfide bond [Arseniev et al., 1994]. Each chain also possesses an intra-chain disulfide bond (Fig. 7A-B). Disulfide bonds render venom peptides resistant to a number of different proteases and environmental extremes resulting in stable peptide toxins [King and Hardy, 2013]. The three dimensional structure of Et-1 was determined by NMR and revealed that each ectatomin chain comprises two anti-parallel α-helices linked by a hinge region of four amino acid residues and a disulfide bridge (Fig. 4B) [Nolde et al., 1995]. Two other ectatomins (Eq-1 and Eq-2) have also been isolated from the venom of *Ectatomma brunneum* (previously *E. quadridens*). These novel Eq ectatomins are

also dimeric and linked by one inter-chain disulfide bond. However, they lack the intra-chain disulfide bond present in Et-1 and -2 [Pluzhnikov et al., 2000].

Et-1 appears to account for the major toxic effect of *Ectatomma tuberculatum* venom causing toxic effects in both mammals and insects [Pluzhnikov et al., 1999]. At high concentrations (0.50–1 μM), Et-1 is a pore-forming peptide that inserts into cellular and artificial membranes but is not internalized. It produces hemolytic and cytolytic effects on rabbit erythrocytes, *Xenopus laevis* oocytes, rat cardiomyocytes and both insect and vertebrate cell lines. In *X. laevis* oocyte membranes, this arises due to the formation of nonselective cationic channels by two Et-1 molecules and appears to involve binding to lipids rather than a specific receptor. The increase in cell permeability with resultant ion leakage results in cell death [Pluzhnikov et al., 1999; Pluzhnikov et al., 1994]. At much lower concentrations (1–10 nM), Et-1 is capable of inhibiting whole-cell L-type calcium currents in isolated rat ventricular myocytes. Importantly, it prevents β-adrenoceptor or adenylate cyclase mediated activation of calcium currents suggesting that Et-1 interacts directly or allosterically with agonist-bound β-adrenoceptors preventing activation of calcium channels further down the signal transduction cascade. The modulation of calcium channels and possibly β-adrenoceptors by Et-1 may underlie its potent toxicity by interfering with the process of muscle contraction, neurotransmitter release and neuromodulation [Pluzhnikov et al., 1999].

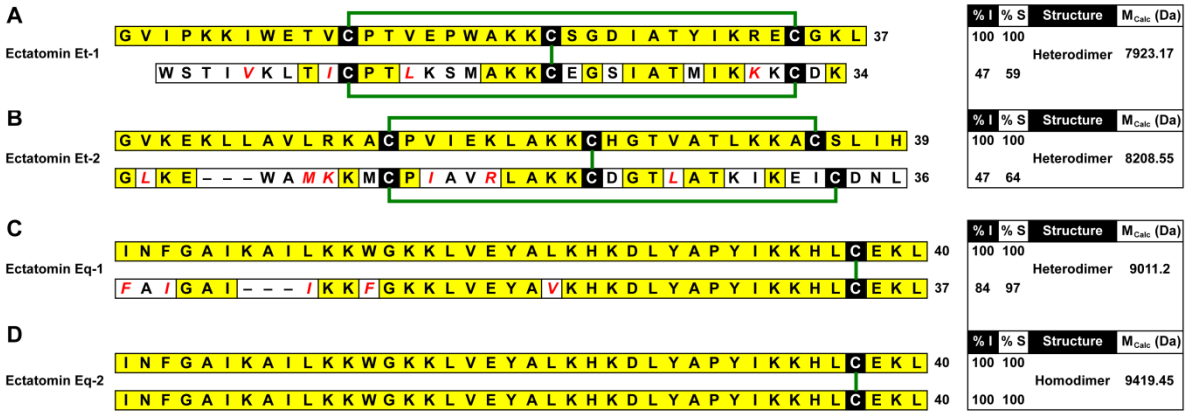


Fig. 7. Sequences and structures of the ectatomin family of dimeric ant peptides. Identical residues are boxed in yellow while conservative substitutions are shown in red italic text. Cysteines are highlighted in black and the predicted disulfide-bonding pattern is shown in green between the sequences. Gaps were introduced to optimize the alignments. Percentage identity (%I) is relative to the longer (upper) chain for each peptide while percentage similarity (%S) includes conservatively substituted residues. M_{Calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. The heterodimeric ectatomin Et peptides (A-B) are from *Ectatomma tuberculatum* while ectatomin Eq peptides (C-D) are from *Ectatomma brunneum* (formerly *E. quadridens*). (A) Sequences for ectatomin Et-1 toxins are from UniProtKB Accessions ECAA_ECTTU and ECAB_ECTTU. (B-D) Remaining sequences are from [Pluzhnikov et al., 2000]. For clarification of colours in this figure, refer to the web version of this article.

3.2.2. Myrmexins

In vitro and clinical studies have shown that *Pseudomyrmex triplarinus* (Pseudomyrmecinae) ant venom decreases pain and inflammation in patients with rheumatoid arthritis and reduces swelling in animal models of inflammation [Altman et al., 1984; Hink and Butz, 1985; Schultz and Arnold, 1984]. Myrmexins are a family of six related polypeptides (myrmexins I-VI) that have been purified from the venom of *Pseudomyrmex triplarinus*. These peptides are heterodimeric complexes comprising a combination of a short subunit of 29 residues (SS1, SS2 or SS3) and a long subunit of 33 residues (LS1 or LS2) stabilized by two inter-chain disulfide bonds [Pan and Hink, 2000] (Fig. 8). Unfortunately, it is not known at present which of the myrmexin peptides are associated with the anti-inflammatory activity observed with whole venom. Three additional myrmexin-like

polypeptides from the venom of the related ant *Pseudomyrmex penetrator* (one heterodimeric and two homodimeric) have also been identified, however, they are yet to be sequenced [Touchard et al., 2014b]. These myrmexins may represent a new class of toxins present in Pseudomyrmecine ants.

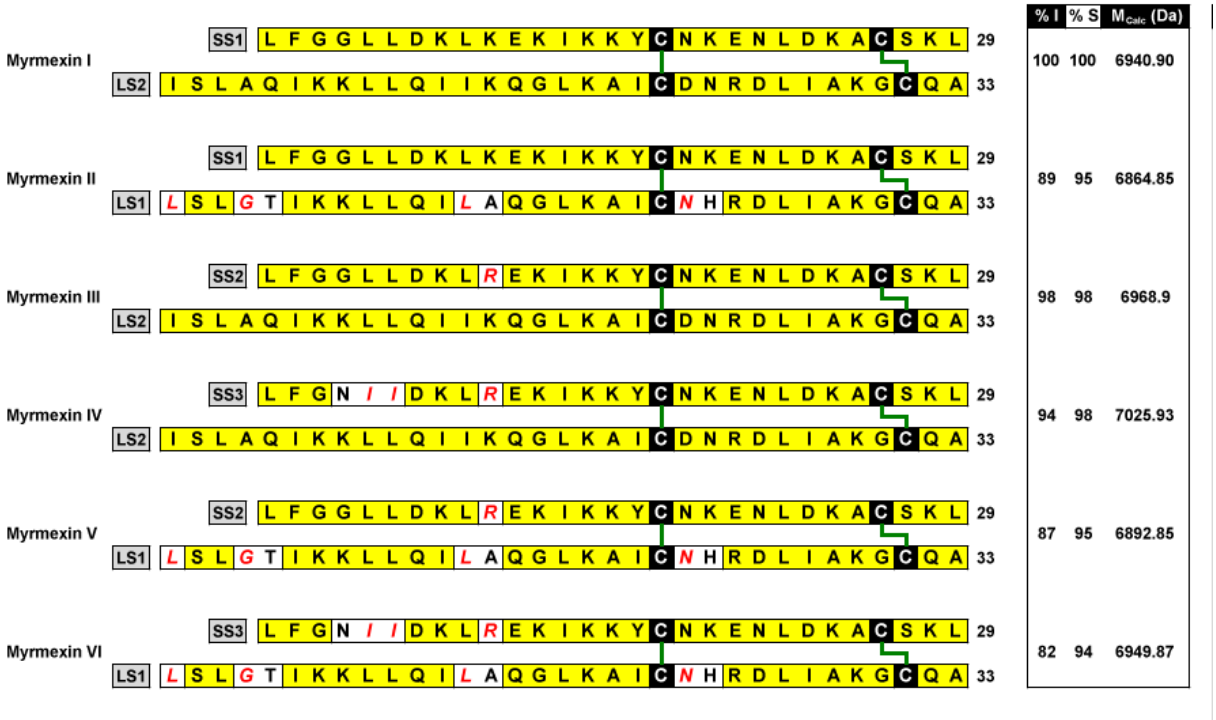


Fig. 8. Sequences and structures of the myrmexin family of heterodimeric peptides from the venom of the ant *Pseudomyrmex triplarinus*. Each myrmexin is composed of a short subunit (SS1, SS2 or SS3; grey boxes) and a long subunit (LS1 or LS2; grey boxes) linked by two disulfide bonds. Identical residues are boxed in yellow while conservative substitutions are shown in red italic text. Cysteines are highlighted in black and the predicted inter-chain disulfide-bonding pattern is shown in green between the sequences. Gaps were introduced to optimize the alignments. Percentage identity (%I) is relative to myrmexin I while percentage similarity (%S) includes conservatively substituted residues. M_{Calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. Sequences are taken from Pan and Hink (2000). For clarification of colours in this figure, refer to the web version of this article.

3.2.3. Pilosulins

Australian ants of the *Myrmecia pilosula* species complex (Myrmeciinae), also known as jack jumper ants, have a painful sting that is responsible for around 90% of life-threatening ant sting allergies in Australia [Brown et al., 2003; Douglas et al., 1998; Street et al., 1994]. In

South Eastern Australia around 2.7% of the population are allergic to *Myrmecia pilosula* venom, with approximately 50% of allergic people experiencing life-threatening reactions [Brown et al., 2003]. The toxicity of the venom appears to result from the presence of a variety of histamine-like, hemolytic and eicosanoid-releasing factors, peptides such as pilosulins, and enzymes including phospholipases, hyaluronidase, and phosphatases [Matuszek et al., 1994a; Matuszek et al., 1992, 1994b; McGain and Winkel, 2002].

Using cDNA sequencing, two major protein allergens from *Myrmecia pilosula* sharing a common leader sequence have been identified [Donovan et al., 1993; Donovan et al., 1995; Donovan et al., 1994; Street et al., 1996]. They encode the 112 and 75 amino acid prepropeptides Myr p 1 and Myr p 2, respectively (Fig. 9A-B). Pilosulin 1, the mature peptide product from residue 57 to 112 of Myr p 1 (Myr p 1 57 → 112), is a 6048 Da linear allergenic basic peptide that exhibits hemolytic and cytotoxic activity and is one of the major allergens that have been identified in this venom [Donovan et al., 1993; Donovan et al., 1994; Wu et al., 1998]. However, pilosulin 1 exists mainly, and sometimes exclusively, as a Val5Ile substituted isoform known as [Ile^{5}]pilosulin 1 [Davies et al., 2004] (Fig. 9A). Pilosulin 1 is also cleaved to form four additional N-terminally truncated isoforms with varying degrees of cytotoxic activity (Fig. 9A).

Pilosulin 2 (Myr p 2 49 → 75) has never been detected in whole venom in its monomeric form [Donovan and Baldo, 1997]. However, a des-Gly^{27} pilosulin 2 peptide (renamed pilosulin 3a) has been found as part of the 5603 Da heterodimeric peptide pilosulin 3. The additional subunit of pilosulin 3 from *Myrmecia pilosula* is the 23 residue pilosulin 3b [Davies et al., 2004], or the variant pilosulin 3.1b from *Myrmecia banksi* [Inagaki et al., 2004], thought to be part of the *Myrmecia pilosula* species complex [Imai et al., 1994] (Fig. 9C). Pilosulin 3 displays antimicrobial activity, and is the major allergen in *M. pilosula* venom,

along with [Ile5]pilosulin 1 accounting for 80% of the total venom peptide content. Pilosulin 4a peptide was originally identified via cDNA cloning [Inagaki et al., 2004] but was not detected in venom, while its Asp31Glu variant pilosulin 4.1a was found to be present only as a homodimeric peptide, pilosulin 4.1 [Wiese et al., 2006] (Fig. 9D). cDNA cloning also revealed the presence of a novel bioactive dimeric peptide pilosulin 5 connected by a single disulfide bond. Synthetic pilosulin 5 dimer causes significant histamine release that maybe related to the weak homology of the peptide to the wasp peptide mastoparan [Inagaki et al., 2008a].

Although the monomeric pilosulin peptides (pilosulin 2, 3.2b, 4 and 5) all show antibacterial and histamine-releasing activities [Inagaki et al., 2004; Inagaki et al., 2008a] and some pilosulins, particularly 3a and to a lesser extent 4.1 and [Ile^5]pilosulin 1, are known to be highly allergenic [Wiese et al., 2007], the biological activities of these peptides have not been fully investigated.

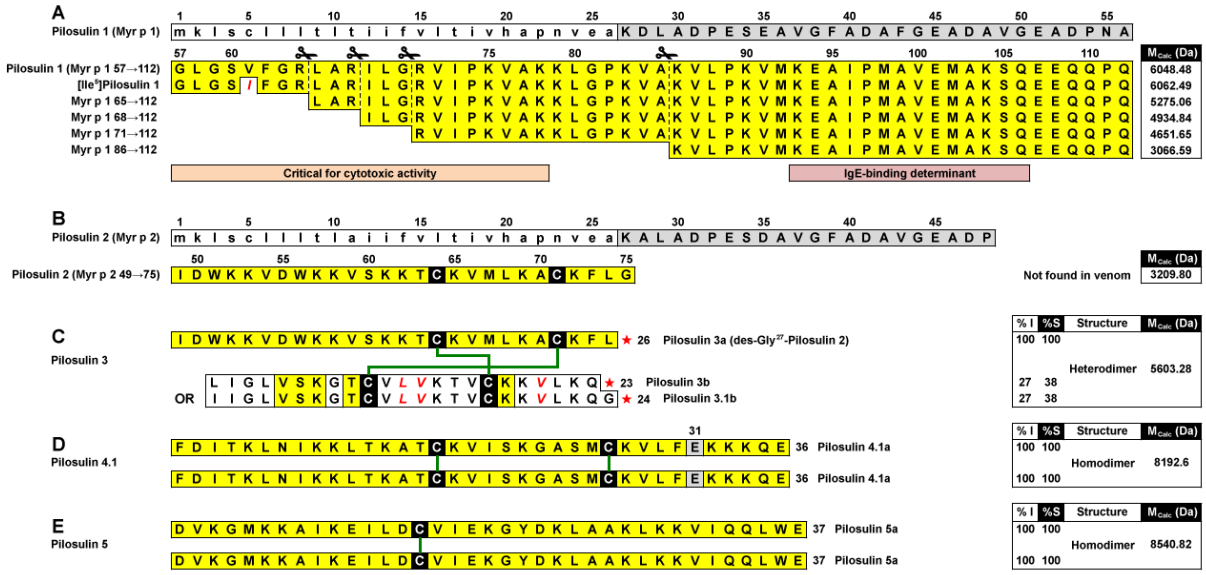


Fig. 9. Sequences and structures of the pilosulin family of linear and dimeric ant peptides. (A-B) Translated sequences of the linear peptides pilosulin 1 (A; from *Myrmecia pilosula*; UniProtKB accession MYR1_MYRPI) and pilosulin 2 (B; from *Myrmecia pilosula*; UniProtKB accession MYR3A_MYRPI). Sequences represent complete prepropeptides, where signal peptides are boxed in white and in lowercase, propeptide sequences are boxed in gray and the mature peptides are boxed in yellow. (A) In addition to the natural variant [Ile^{5}]pilosulin 1, pilosulin 1 undergoes cleavage at the sites marked above the mature peptide sequence to yield four additional peptides, while the residues important for cytotoxic activity and IgE binding are highlighted beneath the sequences. (B) Pilosulin 2 does not appear to be found in venom but undergoes post-translational modification to yield the monomer pilosulin 3a (des-Gly^{27}-Pilosulin 2) that forms the heterodimer pilosulin 3 (C) with the monomer pilosulin 3b (MYR3B_MYRPI). A natural variant, pilosulin 3.1b, can be found in the venom of *Myrmecia banksi* (MYR3_MYRBA). (D-E) Pilosulin 4.1 [Wiese et al., 2006] and pilosulin 5 (MYR5_MYRBA) are homodimers from *Myrmecia banksi*. In the case of pilosulin 4.1, cDNA cloning predicted a homodimer of pilosulin 4a (MYR4_MYRBA), but this was not detected in venom and the [Glu^{31}]pilosulin 4 variant (pilosulin 4.1) found in venom is shown. For the dimeric peptides (C-E), cysteines are highlighted in black and the predicted disulfide-bonding pattern is shown in green between the sequences. Red stars indicate an amidated C-terminus. M_{Calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. For clarification of colours in this figure, refer to the web version of this article.

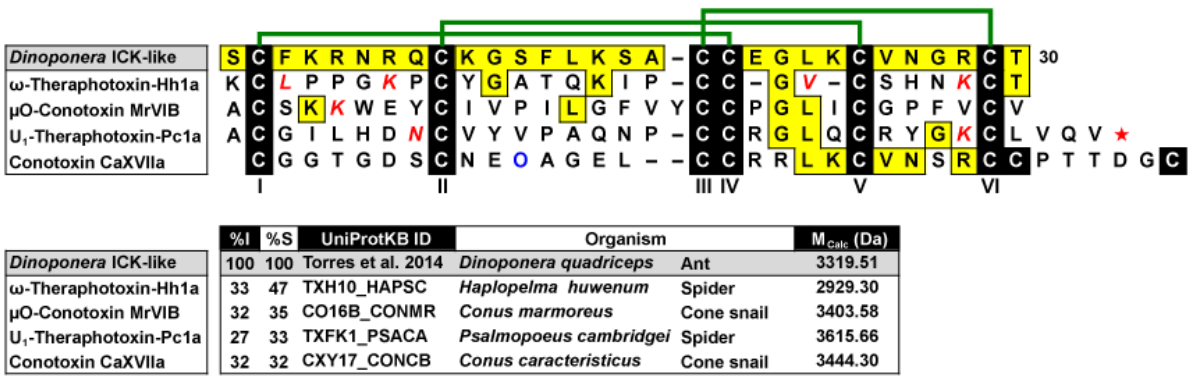
3.3. ICK-like peptides

The inhibitor cystine knot (ICK) structural motif is an evolutionary conserved structure that has been found in plants, fungi, viruses, antimicrobial peptides from horseshoe crabs

(tachystatins) and the venoms of many organisms such as spiders, scorpions, cone snails, insects (bees) and sea anemones [Gilly et al., 2011; Pallaghy et al., 1994; Rodríguez et al., 2014; Zhu et al., 2003]. The ICK motif is defined as an embedded ring formed by two disulfide bonds Cys(I-IV) and Cys(II-V) and their connecting backbone segments through which is threaded a third disulfide bond Cys(III-VI), forming a cystine knot. It is invariably associated with a nearby anti-parallel β-sheet and appears to be a highly effective motif for stabilizing peptide structures (Fig. 4D). Peptides with an ICK motif represent attractive scaffolds in drug design because of their inherent chemical stability and resistance to proteases provided by the fold and the wide range of amino acid sequences that can be accommodated in the structure [Craik et al., 2001; Pallaghy et al., 1994; Zhu et al., 2003]. While large numbers of ICK peptide toxins have been reported from other arthropod venoms such as spiders and scorpions, only two types of ant venom peptides displaying this structural motif are currently known – dinoponeratoxin and SKTXs.

3.3.1. Dinoponera ICK-like toxin

The recent transcriptome analysis of the venom glands of the ant *Dinoponera quadriceps* (Ponerinae) has confirmed the presence and sequence of the first ICK-like peptide in ant venoms [Torres et al., 2014]. This *Dinoponera* ICK-like peptide is a minor component of the venom of *Dinoponera quadriceps* but has a VI/VII cysteine framework (–C–C–CC–C–C–) consistent with other ICK toxins (Fig. 4C). This peptide shows limited homology to the ICK toxins ω-theraphotoxin-Hh1a and μO-conotoxin MrVIB peptides found in tarantula and cone snail venoms, respectively, both of which exhibit neurotoxic activity via activity on voltage-gated ion channels [Liu et al., 2006; McIntosh et al., 1995] (Fig. 10).



	%I	%S	UniProtKB ID	Organism		M_{Calc} (Da)
Dinoponera ICK-like	100	100	Torres et al. 2014	*Dinoponera quadriceps*	Ant	3319.51
ω-Theraphotoxin-Hh1a	33	47	TXH10_HAPSC	*Haplopelma huwenum*	Spider	2929.30
μO-Conotoxin MrVIB	32	35	CO16B_CONMR	*Conus marmoreus*	Cone snail	3403.58
U_1-Theraphotoxin-Pc1a	27	33	TXFK1_PSACA	*Psalmopoeus cambridgei*	Spider	3615.66
Conotoxin CaXVIIa	32	32	CXY17_CONCB	*Conus caracteristicus*	Cone snail	3444.30

Fig. 10. Structure and sequence alignment of the *Dinoponera* ICK-like peptide. The upper panel shows the disulfide bonding connectivity and alignment with homologous peptides. Cysteines are highlighted in black and the predicted disulfide-bonding pattern, similar to other ICK peptides, is shown in green above the sequences. Identical residues are boxed in yellow while conservative substitutions are shown in red italic text. Gaps were introduced to optimize the alignments. Red stars indicate an amidated C-terminus while the blue O^{11} in conotoxin CaXVIIa indicates a hydroxyproline residue. The lower panel shows the percentage identity (%I) relative to *Dinoponera* ICK-like peptide while percentage similarity (%S) includes conservatively substituted residues. M_{Calc}, Theoretical monoisotopic mass calculated using GPMAW 9.20 software. For clarification of colours in this figure, refer to the web version of this article.

3.3.2. SKTXs

The venom of the ant *Strumigenys kumadori* (Myrmicinae) also possesses ICK-like peptides which have been named SKTXs [Inagaki et al., 2008b]. SKTXs are thought to modulate Na_V channels of *Drosophila*, however, this study remains unpublished and sequences of these peptides are still unknown.

4. Conclusion and perspectives

Until recently, the low yield of venom from ant species has severely restricted the biochemical and pharmacological characterization of ant venom peptides. However, advances in the development of miniaturized bioassays and improvements in the sensitivity of mass spectrometry and NMR spectroscopy now allow broader investigations of the small

quantities of venom peptides provided by small animals, especially ants. Indeed, mass spectrometry has been used as a method to improve the accuracy of taxonomic findings to reveal cryptic ant species within species complexes [Touchard et al., 2014a]. This chemotaxonomic tool can therefore contribute to more rapid species identification and more accurate taxonomies.

The limited number of studies to date has revealed a number of unique structures across a broad range of ant subfamilies that differ from those described in other animal venoms. Given the diversity in ant species and distribution, ant venoms therefore represent vast sources of potentially novel bioactive toxins that could be exploited in drug and bioinsecticide discovery programs. For example, there is increasing awareness that peptides represent an under-utilized source of lead compounds for new therapeutics. Arguably, the largest source of chemical diversity comes from peptides derived from animal venoms. In animal venoms the evolutionary pressure for improved prey capture and/or defence has resulted in complex preoptimised combinatorial peptide libraries with extremely diverse pharmacologies that interact with a wide range of molecular targets. The discovery that these peptides bind to their cognate receptors and ion channels with high affinity and selectivity means that many are now being investigated as sources of lead compounds in therapeutic discovery pipelines [Bosmans et al., 2009; Escoubas and King, 2009; Lewis and Garcia, 2003; Vetter et al., 2011; Vetter and Lewis, 2012]. Hence, there is a growing number of novel peptide or peptidomimetic therapeutics appearing on the drug market, or in clinical trials, which are derived from toxins from the venoms of cone snails, snakes, Gila monster, scorpions, spiders and sea anemones. Ants could also provide a unique source of potential therapeutic leads, especially antimicrobials and neuroactive compounds.

Since some venomous animals, particularly arachnids and ants, prey upon insects their venom contains large numbers of insecticidal peptide toxins that have evolved to kill or paralyze insect prey. These toxins often modulate the function of their targets with high insect selectivity, lacking any overt toxicity against their vertebrate counterparts [Bende et al., 2013; Windley et al., 2012], which can even extend to unique insect family selectivity [Bende et al., 2014]. Hence, many of these toxins are being explored as novel insecticides in biopesticide discovery programs [King and Hardy, 2013; Smith et al., 2013; Windley et al., 2012]. The limited number of studies on ant venoms would indicate that potential insect-selective peptide neurotoxins are present in their venoms and could be exploited as novel insecticides leads.

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Annexe 2. Matériel et méthode pour les tests d'électrophysiologie des récepteurs TRPV.

Oocyte Electrophysiology

Channel function was assessed using two-electrode voltage-clamp experiments performed on *Xenopus laevis* oocytes expressing homomeric hTRPV1 channels. WT hTRPV1 cRNA was synthesized using an mMessage mMachine *in vitro* transcription kit (Ambion Inc., Austin, TX, USA) and injected at 5 ng per oocyte. Oocytes were incubated at 17°C in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, 5 mM pyruvate, 50 µg/ml gentamicin (pH 7.4), and horse serum (2.5%). Experiments were performed at room temperature (21–22°C) in ND96 solution (as defined above but minus serum, pyruvate and antibiotics) with or without Ca^{2+} 48 h after cRNA injection. Oocytes were clamped at -60 mV (Axoclamp 900A) using electrodes of resistance 0.5–0.8 MΩ when filled with 3 M KCl solution. The bath was constantly perfused and 3 M KCl agar bridges were used to ground the bath. To test the activation of TRPV1 channels, 2-s pulses at 60 mV was applied from holding potential, followed by 1-s pulse at -60 mV. Background subtracted currents elicited by the test pulse were plotted against recording time. Data acquisition and analysis were performed using pCLAMP10 software (Molecular Devices).

Annexe 3. Abstract

Abstract

Venoms are sophisticated weapons employed by venomous organisms to ward off predators, as well as to subdue and kill prey. However, in nature, good is never far from bad and venom toxins may prove to be efficient therapeutic agents. Ant venom peptides were investigated in the course of this thesis to evaluate their potential in the discovery of novel drugs. Like other insect venoms, ant venoms remain understudied, mainly due to the small size of individual ants and, so, the limited amount of venom available. However, ants are social insects and very abundant in terrestrial environments. By collecting venom from several individual ants, it is possible to obtain enough material for both biochemical and pharmacological analyses.

To ensure the reproducibility of those analyses, proper taxonomic identification is required. To that end, a chemotaxonomic tool was developed during the first stage of this thesis (permitting then to pool venom from several ant colonies in order to offset the limited amount of venom obtainable per individual or from a single colony).

Then, we examined the ecological factors involved in the diversification of ant venom. To do so, we analyzed the toxicity and composition of ant venoms in relation to polyethism, diet and defensive specialization.

The ecological diversity of ants has largely contributed to venom diversification. By studying the venom peptidomes from 82 ant species, we have revealed the great structural diversity of the toxins. Although the majority of the peptidomes are comprised of small and linear peptides, peptides structured by disulfide bonds were also brought to light in numerous venoms and constitute novel structural classes of toxins.

The purification of some of these disulfided peptides permitted their biochemical characterization and the assessment of their biological functions. We thus described a group of neurotoxic peptides, called ‘formicitoxins’, which are able to block human L-type calcium channels. Commutatoxine is a peptide reticulated by one disulfide bond and seems to be a strong activator of the human receptors TRPV1 and TRPV3, suggesting that this peptide is involved in the induction of pain in mammals.

The enormous peptide diversity revealed among venoms combined with the great ecological and taxonomical diversity of ants suggests that ant venoms constitute a promising new source in the search for both novel drugs and insecticides. Ant venom augments the vast bioactive molecules library represented by venoms from other venomous animals.

Keywords: Ant venoms, Mass spectrometry, MALDI-TOF, Chemotaxonomy, Polyethism, Peptides, Peptidomes, Disulfide bonds, Neurotoxins

Résumé

Les venins sont des armes sophistiquées, utilisées par les organismes venimeux pour se défendre des prédateurs, ainsi que pour paralyser et tuer leurs proies. Mais dans la nature, le bien n'est jamais très loin du mal, les toxines venimeuses pouvant se révéler être des agents thérapeutiques efficaces. Les peptides de venins de fourmis ont donc été étudiés dans cette thèse afin de déterminer le potentiel de ces toxines pour la découverte de molécules thérapeutiques innovantes. A l'instar des autres venins d'insectes, les venins de fourmis restent peu étudiés, principalement en raison de la petite taille de ces insectes et des quantités limitées de venins disponibles. Cependant, les fourmis offrent l'avantage d'être des insectes sociaux très abondants dans tous les milieux terrestres. En collectant les venins de plusieurs individus, il est donc possible d'obtenir des quantités suffisantes de venin pour les analyses biochimiques et pharmacologiques.

Afin d'assurer la reproductibilité des analyses, une identification taxonomique correcte est nécessaire. Dans cette optique, un outil de chimiotaxonomie a été développé durant cette thèse (permettant ainsi de regrouper les venins provenant de plusieurs colonies afin de compenser les faibles quantités de matériel biologique par individu ou par colonie).

Ensuite, nous nous sommes intéressés aux facteurs écologiques impliqués dans la diversification des venins de fourmis. Pour cela, la toxicité et la composition des venins de fourmis ont été analysés en relation avec le polyéthisme, la spécialisation alimentaire et la spécialisation défensive.

La diversité écologique des fourmis a amplement contribuée à la diversification des venins. En étudiant les venins de 82 espèces de fourmis, nous avons révélé la grande diversité structurale des toxines. Bien que la majorité des peptidomes sont composés par de petits peptides linéaires, des peptides structurés par des ponts disulfure ont été révélés dans de nombreux venins et constituent de nouvelles familles structurales de toxines.

La purification de certains de ces peptides à ponts disulfure a permis leur caractérisation biochimique et l'évaluation de leur rôle biologique. Ainsi nous avons décrit un groupe de peptides neurotoxiques, baptisés les formicitoxines qui sont capables de bloquer les canaux calcium humains de type L. La commutatoxine est, quant à elle, un peptide avec un pont disulfure qui semble activer les récepteurs humains TRPV1 et TRPV3 et laisse supposer une implication dans l'induction de la douleur chez les mammifères.

La grande diversité des peptides mise en évidence dans les venins, associée à la grande diversité écologique et taxonomique des fourmis, suggère que les venins de fourmis constituent un nouveau champ d'exploration prometteur pour la recherche de molécules thérapeutiques et insecticides. Les venins de fourmis s'ajoutent à la chimiothèque conséquente déjà représentée par les venins des autres animaux venimeux.

Mots-clés : Venins de fourmis, Spectrométrie de masse, MALDI-TOF, Chimiotaxonomie, Polyéthisme, Peptides, Peptidomes, Ponts disulfure, Neurotoxines