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Conflits, coopération et systèmes de reconnaissance chez les fourmis du complexe d'espèces *Neoponera apicalis*

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INTRODUCTION

« Comme il naît beaucoup plus d'individus de chaque espèce qu'il n'en peut survivre ; et comme, en conséquence, il se produit fréquemment une lutte pour l'existence, il s'ensuit que tout être qui varie si légèrement que ce soit d'une façon qui lui soit profitable, sous des conditions de vie complexes qui varient parfois légèrement, aura la meilleure chance de survivre et sera ainsi naturellement sélectionné. A partir du principe fort de l'héritage, toute variété sélectionnée tendra à propager sa forme nouvelle et modifiée. »

C. Darwin, 1859

I. L'évolution sociale

L'apparition des sociétés animales est l'une des transitions majeures de l'évolution, au même titre que la formation des cellules par association de molécules réplicatrices, ou le regroupement de cellules pour constituer des organismes pluricellulaires (Maynard Smith & Szathmáry, 1995). Comme toute transition évolutive, elle est caractérisée par la coopération d'unités précédemment séparées (ici les individus solitaires) afin de former un niveau d'organisation supérieur (le groupe social) (Bourke, 2011). Il en résulte une augmentation de la complexité entre les niveaux d'organisation ; caractéristique à distinguer de la notion de progrès, absente dans l'évolution darwinienne (Maynard Smith & Szathmáry, 1995). La coopération est donc une des pierres angulaires de l'évolution sociale et une des caractéristiques majeures des groupes sociaux, mais comprendre comment ce processus a pu évoluer par sélection naturelle a en revanche intrigué les biologistes depuis Darwin (1859). Si l'on se place en effet dans le cadre de la théorie synthétique de l'évolution (Dobzhansky, 1937 ; Huxley, 1942 ; Mayr, 1942), on s'attend à observer une sélection des individus maximisant leur *fitness* (ou valeur sélective), c'est-à-dire ceux ayant la survie la plus longue leur permettant de produire le plus grand nombre de descendants. Les comportements d'aide, pourtant fréquemment observés, tels la coopération et plus encore l'altruisme où certains individus sacrifient leur propre reproduction au profit de celle de leurs congénères paraissent donc incompatibles avec la théorie néodarwinienne (West et al., 2007).

Les travaux fondateurs d'Hamilton (Hamilton, 1963, 1964a, b) ont fourni un cadre conceptuel permettant de comprendre comment la coopération et l'altruisme peuvent avoir été sélectionnés, et ceci grâce à la notion centrale de *fitness* indirecte (West et al., 2007). La solution consiste à se placer non plus au niveau des individus mais au niveau des gènes, et à considérer dans quelles conditions un gène responsable d'un comportement altruiste peut se répandre dans une population, c'est-à-dire augmenter sa représentation dans le pool génique à la génération suivante (Hamilton, 1963, 1964a). La formalisation qu'en fait Hamilton est souvent résumée par l'inégalité $r \cdot b - c > 0$, selon laquelle le gène responsable de l'altruisme sera sélectionné si le coût de l'acte altruiste (c) sur la *fitness* de l'individu donneur (mesuré par la réduction du nombre de ses descendants du fait de cet acte altruiste) est plus faible que le bénéfice issu de l'acte altruiste (b) sur la *fitness* de l'individu receveur (mesuré par l'augmentation du nombre

de ses descendants du fait de cet acte altruiste) pondéré par le coefficient d'apparentement (ou corrélation génétique, *r*) entre le donneur et le receveur. Cette règle stipule donc que l'altruisme ne peut évoluer qu'en cas d'apparentement positif. Elle suppose également que l'expression du gène responsable de l'altruisme est nécessairement facultative, de sorte que certains porteurs du gène se reproduisent quand même (Bourke & Franks, 1995 ; Queller & Strassmann, 1998). Si l'on se replace au niveau des individus, l'apport central de cette théorie est de considérer la *fitness* non plus seulement comme les opportunités directes de reproduction, mais aussi comme tous les gains issus de la reproduction des individus apparentés. Cette notion de *fitness* globale (ou *inclusive fitness*) est l'un des rares apports majeurs à la théorie de l'évolution depuis sa formulation initiale (West et al., 2007 ; Bourke, 2011). Malgré des critiques principalement issues du domaine de la génétique des populations (e.g. Nowak et al., 2010), la reconnaissance de la théorie de la sélection de parentèle (ou théorie de l'*inclusive fitness* ; Hamilton, 1963, 1964a, 1964b, 1972) comme essentielle pour expliquer l'évolution sociale fait largement consensus (Abbot et al., 2011 ; Boomsma et al., 2011 ; Bourke, 2011), du fait notamment de son caractère général et de son adéquation aux données expérimentales (Bourke & Franks, 1995 ; Crozier & Pamilo, 1996 ; Ratnieks et al., 2006 ; West et al., 2007 ; Bourke, 2011, 2014).

L'évolution des groupes sociaux et des comportements coopératifs dépendent donc *in fine* d'une coïncidence d'intérêts en termes d'*inclusive fitness* entre les membres du groupe. Ceci a permis d'atteindre le climax de l'évolution sociale : l'eusocialité.

II. La vie eusociale

L'eusocialité (socialité vraie) est caractérisée par une coopération dans l'élevage des jeunes, un chevauchement des générations entre parents et enfants, et une division du travail dans la reproduction de sorte que les tâches reproductives sont monopolisées par un (ou quelques) membre(s) du groupe seulement (Wilson, 1971). L'eusocialité représente l'état le plus dérivé dans l'évolution sociale, bien qu'on distingue souvent les espèces « primitivement » et « hautement » eusociales en relation avec la présence ou non d'une caste ouvrière perdant tout ou partie de son potentiel reproducteur (Crespi & Yanega, 1995 ; Bourke, 1999).

On compte de nombreuses évolutions indépendantes de l'eusocialité, et ce à différents niveaux de la phylogénie, que ce soit chez les crustacés (Decapoda : Duffy, 1996), les mammifères (Rodentia : Jarvis, 1981) ou encore et principalement chez les insectes (Blattodea : Thorne, 1997 ; Coleoptera : Kent & Simpson, 1992 ; Hemiptera : Ito, 1989 ; Hymenoptera : Wilson, 1971 ; Thysanoptera : Crespi, 1992). En outre, l'eusocialité a évolué au moins dix fois de manière indépendante chez les seuls hyménoptères aculéates regroupant les fourmis (Formicidae ; Moreau et al., 2006), les abeilles (nombreuses familles incluant les Apidae et Halictidae ; Thompson & Oldroyd, 2004 ; Brady et al., 2006b) et les guêpes avec dard (nombreuses familles incluant les Vespidae ; Hines et al., 2007). Les hyménoptères sociaux sont ainsi l'illustration typique de l'eusocialité et de la coopération dans le règne animal (Hölldobler & Wilson, 1990).

Les hyménoptères sont caractérisés par un déterminisme haplodiploïde du sexe, ce qui implique que les individus issus d'œufs fécondés et hétérozygotes pour le gène de détermination du sexe se développent en femelles, alors que ceux issus d'œufs non fécondés se développent en mâles par parthénogenèse arrhénotoque (Heimpel & de Boer, 2008). Il en résulte que les coefficients d'apparentement dans une colonie monogame (une reine accouplée avec un mâle) sont tels que les ouvrières sont plus apparentées à leurs sœurs (r = 0,75) qu'à leurs propres descendants (r = 0,5). Ceci a conduit à formuler l'hypothèse des trois-quarts d'apparentement selon laquelle l'haplodiploïdie pourrait avoir favorisé l'évolution répétée de l'eusocialité chez les hyménoptères (Hamilton, 1964a, b, 1972). Cette hypothèse a néanmoins été critiquée (e.g. Alpedrinha et al., 2013), notamment du fait de l'apparentement beaucoup plus faible qui relie les ouvrières avec leurs frères (r = 0,25), annulant tout bénéfice en *fitness* comparé à une reproduction directe (r_{moyen} = 0,5 dans les deux cas) (Trivers & Hare, 1976). En outre, il n'existe pas de lien simple entre le degré de ploïdie d'un taxon et son niveau de socialité. A l'inverse, la monogamie semble être un facteur commun à tous les groupes dans lesquels l'eusocialité a évolué (Hughes et al., 2008a). Ceci implique qu'une balance même marginalement favorable entre bénéfices retirés par l'aide apportée au nid maternel et coûts sur la reproduction directe peut avoir suffi à l'établissement de l'eusocialité de façon permanente (Boomsma, 2007, 2009). Cette hypothèse met donc encore une fois en exergue l'importance d'un apparentement élevé corolaire d'une structure familiale comme pré requis probable à toute évolution sociale (hypothèse de la route subsociale ; Michener, 1958 ; Lin & Michener, 1972). Une hypothèse non-

mutuellement exclusive considère les effets d'une manipulation maternelle contraignant les filles à adopter un rôle d'ouvrière (Alexander, 1974 ; Nonacs, 2014). Enfin certains facteurs écologiques et traits d'histoire de vie peuvent avoir facilité l'évolution de la socialité. Ils incluent notamment la protection des ressources via la construction du nid, ou encore le partage des soins parentaux relaxant les pressions de la mortalité lors de l'approvisionnement (Queller & Strassmann, 1998 ; Johnson et al., 2013). L'action au moins en partie commune de ces paramètres a sans doute favorisé la diminution des coûts associés à la vie sociale tout en en accroissant les principaux bénéfices (souvent indirects), à savoir l'augmentation de la productivité et de la survie des individus apparentés (Bourke, 2014).

III. Le maintien de la coopération

La vie en groupe procure de nombreux bénéfices mais elle est aussi une source de conflits. Les groupes sociaux représentent en effet des ressources importantes, en termes de nourriture, de nid ou de force de travail, qui peuvent être exploitées par des éléments à la fois internes et externes au groupe (Bourke, 2011).

1. Limitation de l'exploitation par des éléments externes

Un des critères majeurs permettant le maintien de l'intégrité des groupes sociaux est de prévenir les coûts associés à l'exploitation du groupe par les compétiteurs et les parasites. De tels exemples d'exploitation à la fois intra et interspécifique sont nombreux chez les insectes sociaux (Buschinger, 1986 ; Schmid-Hempel, 1998 ; Lenoir et al., 2001b ; Beekman & Oldroyd, 2008). Le principal mécanisme de défense consiste alors à pouvoir reconnaitre les membres du groupe, de façon à focaliser les actes altruistes envers les individus apparentés, et ainsi bénéficier des gains en *inclusive fitness* (Sherman et al., 1997 ; Queller & Strassmann, 2002).

Cette capacité à pouvoir discriminer les membres du groupe des individus étrangers est très largement répandue chez les insectes sociaux (Crozier & Pamilo, 1996 ; d'Ettorre & Lenoir, 2010). Dans ces taxons la communication est essentiellement chimique, et il est maintenant bien établi que les hydrocarbures sont les principales molécules impliquées

(Howard & Blomquist, 2005 ; Dani, 2006 ; Monnin, 2006 ; d'Ettorre & Lenoir, 2010). Ces molécules sont formées de longues chaines hydrocarbonées et constituent la majeure partie de la couche lipidique recouvrant la cuticule des insectes, où ils jouent le rôle de protection contre la dessiccation, les pathogènes et les prédateurs, mais agissent également comme les principaux indices de reconnaissance dans les processus de communication intra et intercoloniaux (Howard & Blomquist, 2005 ; Dani, 2006 ; Monnin, 2006 ; Martin & Drijfhout, 2009a ; d'Ettorre & Lenoir, 2010). Les hydrocarbures cuticulaires (HCC) sont influencés par des facteurs génétiques (Vander Meer & Morel, 1998 ; Lahav et al., 2001 ; van Zweden et al., 2010) et environnementaux (Heinze et al., 1996 ; Liang & Silverman, 2000 ; Couvillon et al., 2007), et ils peuvent ainsi présenter une importante variabilité temporelle (Lenoir et al., 2001a ; Suarez et al., 2002). Des processus d'homogénéisation via les comportements de trophallaxies (Boulay et al., 2000) et de toilettages mutuels (Soroker et al., 2003) permettent cependant la formation d'un visa colonial, sorte d'« odeur moyenne » partagée par tous les membres d'une même colonie (modèle de la *Gestalt* ; Crozier & Dix, 1979), et généralement acquise précocement dans la vie d'un individu (Isingrini et al., 1985). Ce visa sert ensuite de modèle de référence dans les processus de reconnaissance coloniale (Reeve, 1989 ; Crozier & Pamilo, 1996 ; d'Ettorre & Lenoir, 2010), bien que son niveau d'intégration central ou périphérique ne fasse pas consensus (Ozaki et al., 2005 ; Leonhardt et al., 2007 ; Brandstaetter et al., 2011). Ainsi la comparaison entre le profil chimique d'un individu rencontré et le modèle de référence déclenche un comportement allant de l'affiliation à l'agression manifeste (Crozier & Pamilo, 1996 ; d'Ettorre & Lenoir, 2010). L'acceptation ou le rejet dépend du degré de dissimilarité entre ces deux odeurs par rapport à un seuil de tolérance, lui même plastique et dépendant des coûts et bénéfices liés à l'acceptation d'un individu étranger et au rejet d'un membre du groupe (Reeve, 1989 ; Sherman et al., 1997).

2. Limitation de l'exploitation par des éléments internes

Une des forces de la théorie de l'*inclusive fitness* est de prédire à la fois les conditions dans lesquelles la coopération va apparaitre, mais également (et pour exactement les mêmes raisons) les situations où les conflits peuvent avoir lieu (Hamilton, 1964b ; Trivers & Hare 1976 ; Ratnieks et al., 2006). En effet, il est prédit que des conflits liés à la

reproduction peuvent survenir dès lors que les intérêts des différents membres du groupe ne sont pas parfaitement alignés, ce qui est le cas la grande majorité du temps. La raison principale de ces conflits d'intérêts est la structure non clonale (sauf rares exceptions) des colonies d'insectes qui entraine des asymétries d'apparentement entre les individus (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Ratnieks et al., 2006).

Ces phénomènes ont été les plus étudiés chez les hyménoptères, où le déterminisme haplodiploïde du sexe exacerbe encore davantage les asymétries d'apparentement et l'existence de conflits potentiels (Bourke & Franks, 1995 ; Ratnieks et al., 2006). Ces conflits potentiels concernent le sexe ratio des individus sexués (Trivers & Hare, 1976 ; Bourke & Franks, 1995 ; Crozier & Pamilo, 1996), la détermination des castes (Bourke & Ratnieks, 1999 ; Wenseleers et al., 2003 ; Wenseleers & Ratnieks, 2004), la production des mâles (Trivers & Hare, 1976 ; Ratnieks, 1988 ; Bourke, 1988b ; Hammond & Keller, 2004 ; Wenseleers et al., 2004), la production des femelles sexuées (Ratnieks & Reeve, 1992), ou encore l'accès à la reproduction chez les individus totipotents (Bourke & Franks, 1995 ; Monnin & Ratnieks, 2001).

Il convient néanmoins de faire une distinction entre la potentialité d'existence d'un conflit et son expression réelle (Ratnieks et al., 2006). En effet, si la théorie de l'*inclusive fitness* s'est révélée très pertinente pour prédire l'existence de conflits liés à la reproduction, il se peut également que certains de ces conflits ne soient jamais exprimés. Par exemple, la présence de plusieurs lignées maternelles (polygynie) et/ou paternelles (polyandrie) dans une même colonie entraine des différences d'apparentement entre les femelles des différentes lignées. La théorie prédit donc l'existence de népotisme, autrement dit que les ouvrières devraient favoriser les femelles de leur propre lignée avec qui l'apparentement est plus fort. Cependant les preuves de l'existence d'un tel biais sont très limitées (Hannonen & Sundström, 2003), et la plupart des études montrent que ce conflit pour la production des femelles sexuées ne s'exprime pas (Keller, 1997 ; Strassmann et al., 2000 ; Châline et al., 2005a ; Ratnieks et al., 2006 ; Monnin et al., 2009 ; Zinck et al., 2009 ; Kellner & Heinze, 2011). Il est probable que les coûts associés à la reconnaissance intracoloniale de la parentèle et les contraintes dans les indices de reconnaissance empêchent l'évolution du népotisme (Keller, 1997 ; Boomsma et al., 2003 ; Kellner & Heinze, 2011). Cet exemple illustre par ailleurs

l'importance de prendre en compte les coûts et bénéfices, en plus de l'apparentement, pour comprendre l'évolution des comportements sociaux (West et al., 2007).

D'une manière générale, la différence très majoritairement observée entre conflit potentiel et conflit exprimé révèle l'existence de mécanismes de régulation permettant de réduire les coûts associés à l'expression des conflits. Les principaux facteurs qui interviennent sont l'apparentement, la coercition et les contraintes (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Beekman & Ratnieks, 2003 ; Beekman et al., 2003 ; Ratnieks et al., 2006).

Pour les illustrer on peut prendre l'exemple du conflit pour la production des mâles (Trivers & Hare, 1976 ; Ratnieks, 1988 ; Bourke, 1988b ; Hammond & Keller, 2004 ; Wenseleers et al., 2004). Il s'agit d'un des conflits les plus largement exprimés chez les hyménoptères sociaux, car chez la majorité des espèces les ouvrières ont conservé des ovaires fonctionnels, et sont capables de produire des mâles par reproduction asexuée (parthénogenèse arrhénotoque) (Bourke, 1988b). Ce conflit oppose donc la reine et le collectif des ouvrières, mais également les ouvrières entre-elles. Quelque soit la structure génétique de la colonie, la reine est toujours plus apparentée à ses propres fils (r_{fils} = 0,5) qu'aux fils produits par les ouvrières ($r_{\text{petits-fils}}$ = 0,25), et les ouvrières sont également plus apparentées à leurs propres fils (r_{fils} = 0,5) qu'aux fils produits par la reine ($r_{\text{frères}}$ = 0,25). En revanche, l'apparentement moyen entre les ouvrières et les fils produits par les autres ouvrières dépend directement du degré de polyandrie de la reine, avec r_{neveux} = 0,375, 0,25 et <0,25 (tendant vers 0,125) pour respectivement un, deux et plus de deux accouplements (Trivers & Hare, 1976). Il apparait donc clairement que l'optimum reproductif des individus n'est pas le même, et que l'apparentement joue un rôle important sur les prédictions que l'on peut faire quant à l'expression du conflit (Wenseleers et al., 2004). En effet, alors qu'un conflit ouvert entre la reine et les ouvrières est prédit dans une structure monoandre, on s'attend à ce que les ouvrières ne se reproduisent pas si la reine s'accouple avec plus de deux mâles (Trivers & Hare, 1976), ou alternativement à l'existence de mécanismes de contrôle de la reproduction des ouvrières quand elle a lieu (étant donné que les ouvrières gagnent plus individuellement en cas de reproduction directe). Les données montrent effectivement que la proportion de mâles produits par les ouvrières est en général plus faible quand

les ouvrières sont plus apparentées à leurs frères qu'à leurs neveux (Wenseleers & Ratnieks, 2006).

Cependant, il est fréquent que la proportion de mâles produits par les ouvrières soit en fait plus faible que ce qui est prédit par la théorie de l'*inclusive fitness* (Trivers & Hare, 1976 ; Ratnieks & Reeve, 1992 ; Wenseleers et al., 2004). Ainsi par exemple chez l'abeille *Apis mellifera*, la théorie prédit que 54% des ouvrières devraient se reproduire (du fait du haut degré de polyandrie de la reine résultant dans un faible apparentement moyen entre ouvrières [Palmer & Oldroyd, 2000]), alors que la proportion observée d'ouvrières reproductrices est inférieure à 1% (Wenseleers et al., 2004 ; Ratnieks et al., 2006). En outre, alors que ces ouvrières pondent environ 7% des œufs mâles, la proportion de mâles adultes issus d'ouvrières est d'environ 0.1% (Visscher, 1996). Ces écarts illustrent les deux principaux mécanismes interconnectés responsables de la résolution des conflits liés à la reproduction, à savoir l'auto-restriction reproductive et la coercition (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Ratnieks et al., 2006).

L'auto-restriction reproductive correspond à une décision reproductive où les individus renoncent à toute reproduction directe. Elle est souvent en accord avec la théorie d'Hamilton, c'est-à-dire qu'elle représente la meilleure option permettant aux ouvrières d'augmenter leur *inclusive fitness* (Ratnieks & Wenseleers, 2008). Ceci peut s'expliquer tout d'abord par l'existence de coûts sur la productivité coloniale associés à la reproduction des ouvrières (Ratnieks & Reeve, 1992 ; Gobin et al., 2003 ; Hammond & Keller, 2004). En effet, les ouvrières reproductrices participent en général peu aux tâches ergonomiques, ce qui implique qu'une trop grande proportion d'ouvrières reproductrices est susceptible d'engendrer une « tragédie des biens communs » (Hardin, 1968), coûteuse à l'ensemble de la colonie. L'auto-restriction reproductive peut également être favorisée de part l'existence de mécanismes coercitifs limitant les opportunités de reproduction des ouvrières et/ou les bénéfices associés à cette reproduction (Wenseleers & Ratnieks, 2006 ; Ratnieks & Wenseleers, 2008). La coercition est une forme de pression sociale sous forme de *policing*, de punition ou de dominance dont la résultante est de diminuer les coûts associés à une reproduction incontrôlée des membres du groupe (Ratnieks & Wenseleers, 2008). Ces trois concepts sont en partie recouvrant, mais on peut en faire la distinction en fonction des bénéfices directs ou indirects retirés par l'individu responsable de l'acte coercitif, et des

conséquences de cet acte sur la possibilité de l'individu receveur d'agir égoïstement dans le futur (Monnin & Ratnieks, 2001).

Les mécanismes coercitifs les plus étudiés sont les comportements de *policing* (Ratnieks, 1988 ; Ratnieks & Visscher, 1989 ; Ratnieks et al., 2006 ; Ratnieks & Wenseleers, 2008), effectués par la reine ou par les ouvrières, et consistant à agresser directement les ouvrières reproductrices (Kikuta & Tsuji, 1999 ; Monnin & Ratnieks, 2001 ; Iwanishi et al., 2003), ou à détruire sélectivement leurs œufs (oophagie ; Ratnieks & Visscher, 1989 ; Foster & Ratnieks, 2000 ; Halling et al., 2001 ; d'Ettorre et al., 2004 ; Endler et al., 2004). L'occurrence de comportements de *policing* suit en général les prédictions découlant de la structure d'apparentement de la colonie (Wenseleers & Ratnieks, 2006), et il est probable que les fortes pressions engendrées par le *policing* sur la ponte des ouvrières influencent la propension des ouvrières à se reproduire, et donc favorisent en retour l'auto-restriction reproductive (Ratnieks & Wenseleers, 2008). On constate cependant l'existence de *policing* effectué par les ouvrières dans des situations non prédites par la théorie, comme par exemple chez des espèces avec accouplement unique ou clonales, ce qui semble correspondre soit à des moyens de réduire les coûts associés à la reproduction des ouvrières sur la productivité coloniale comme énoncé précédemment (Hartmann et al., 2003 ; Teseo et al., 2013), soit à des stratégies de compétition inter-individuelle (*policing* égoïste) où les ouvrières qui policent sont aussi celles qui pondent (Wenseleers et al., 2005), ce dernier cas étant finalement en accord avec la théorie puisqu'il est toujours préférable pour les ouvrières d'élever des fils que des neveux. On peut noter qu'en cas d'accouplements multiples les mâles contribuent rarement équitablement à la descendance, ce qui peut augmenter les valeurs d'apparentement entre les individus et contribuer aussi à expliquer les écarts entre les niveaux de reproduction des ouvrières prédits et observés. Plutôt que le nombre d'accouplements, il convient donc de prendre en compte la paternité effective des différents mâles (Boomsma & Ratnieks, 1996).

Enfin le dernier facteur influençant la résolution des conflits est l'existence de contraintes (Ratnieks et al., 2006). Ces contraintes peuvent être dues à des asymétries de pouvoir (comme des différences de force physique entre individus totipotents), à l'absence d'opportunités (comme par exemple chez les fourmis sans reine du genre *Diacamma* où la mutilation des appendices thoraciques des jeunes ouvrières par

l'ouvrière dominante les empêche irrémédiablement de pouvoir s'accoupler [Peeters & Higashi, 1989]), ou enfin à des contraintes d'information (Beekman et al., 2003 ; Beekman & Ratnieks, 2003). En effet, alors que les ouvrières dans une colonie monoandre bénéficient de l'élevage des femelles sexuées produites par la reine, elles gagnent en revanche davantage si elles élèvent leurs neveux plutôt que leurs frères. La destruction sélective des œufs mâles produits par la reine est donc pour les ouvrières la meilleure stratégie. Cependant si les œufs de reine portent une odeur différente (Endler et al., 2004), ceux-ci ne divergent pas suivant leur sexe mâle ou femelle. Dans ces conditions la destruction des œufs mâles est totalement aléatoire et contre-sélectionnée du fait des coûts très importants sur l'*inclusive fitness* associés à la destruction du couvain femelle très apparenté. La reine en revanche peut détruire tous les œufs des ouvrières car ce sont nécessairement des mâles. Ces contraintes d'information biaisées en faveur de la reine lui permettent donc dans ce cas de maintenir son monopole reproducteur (Beekman et al., 2003 ; Beekman & Ratnieks, 2003).

Ces mécanismes de régulation des conflits liés à la reproduction permettent de réduire les coûts associés à leur expression, et ainsi de maintenir la coopération entre les membres du groupe (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Beekman & Ratnieks, 2003 ; Ratnieks et al., 2006). Le résultat est l'apparente harmonie observée dans ces sociétés, puisque les manifestations visibles de conflits internes sont la plupart du temps excessivement rares. Une des exceptions les plus remarquables se produit lorsque les colonies contiennent simultanément plusieurs individus avec un potentiel reproducteur équivalent, et qui entrent en conflit pour accéder à la reproduction en établissant des hiérarchies reproductives (Heinze et al., 1994).

IV. Hiérarchies reproductives

1. Définitions et contextes

L'étude des hiérarchies sociales remonte aux observations d'un « ordre de becquetage » chez les poules lors du partage des ressources alimentaires (Schjelderup-Ebbe, 1922). Elles ont été par la suite principalement étudiées chez les vertébrés (Chase & Seitz, 2011), jusqu'à leur mise en évidence chez les invertébrés dans les études princeps de Pardi (1948) sur les guêpes polistes. Dès lors ce modèle a été l'objet de nombreuses

études sur les facteurs responsables de la mise en place et du maintien des hiérarchies de dominance (Gadagkar, 1980 ; Pfennig & Klahn, 1985 ; Röseler et al., 1985 ; Reeve, 1991 ; Röseler, 1991 ; Sledge et al., 2001a ; Tibbetts & Dale, 2004 ; Dapporto et al., 2007a ; Jandt et al., 2014). Cependant, l'existence de hiérarchies de dominance est également un phénomène fréquent chez les autres insectes sociaux (Heinze et al., 1994) : fourmis (Cole, 1981 ; Franks & Scovell, 1983 ; Bourke, 1988a ; Heinze, 1990 ; Ito & Higashi, 1991 ; Monnin & Peeters, 1999 ; Liebig et al., 2000 ; Heinze et al., 2002 ; Cuvillier-Hot et al., 2004b), abeilles (van Honk & Hogeweg, 1981 ; van Doorn & Heringa, 1986 ; Kukuk & May, 1988 ; Ayasse et al., 1995 ; Bull et al., 1998 ; Bloch & Hefetz, 1999), à l'exception notable des termites (Wilson, 1971).

Comme énoncé précédemment, la dominance est un mécanisme coercitif permettant de réguler le partage de la reproduction au sein d'un groupe social. Elle a été définie comme une priorité d'accès aux ressources qui résulte d'actes victorieux d'attaque, de combat, de poursuite ou d'évincement présents ou futurs (Morse, 1974). Les hiérarchies de dominance sont donc caractérisées par l'existence d'asymétries dans le partage des ressources, ici principalement en termes d'opportunités de reproduction directe. La confrontation répétée de paires d'individus par le biais d'interactions agonistiques définit la relation de dominance/subordination qui existe entre eux (Chase & Seitz, 2011). Il s'agit d'une forme de punition car les individus de haut rang, en agressant les autres membres du groupe, en retirent des bénéfices directs en *fitness*, tout en diminuant les opportunités de reproduction de leurs subordonnés (Monnin & Ratnieks, 2001). Au niveau du groupe émerge alors une structure hiérarchique où chaque individu (dans le cas d'une hiérarchie linéaire) possède un rang synonyme de statut social (Chase & Seitz, 2011). L'individu situé en haut de la hiérarchie (typiquement nommé alpha) domine ainsi tous les autres individus du groupe et n'est dominé par personne, l'individu beta domine tous les individus à l'exception d'alpha, et ainsi de suite jusqu'au dernier individu qui ne domine personne et est dominé par tout le monde. On comprend dès lors que la dominance est relative, puisque dans un groupe de n individus, et encore une fois dans le cadre d'une hiérarchie linéaire, il y a n-1 dominants, n-1 subordonnés, et n-2 individus à la fois dominants et subordonnés. Qualifier des individus de « dominants » ou « subordonnés » au niveau d'une hiérarchie n'a donc que peu de sens.

L'établissement des hiérarchies suit en général deux étapes successives : une période d'intenses interactions agonistiques qui définissent une hiérarchie de dominance, puis une diminution nette de ces comportements agonistiques conjointement à l'établissement d'une hiérarchie reproductive (Heinze et al., 1994). On se retrouve alors dans une situation classique dans les groupes sociaux avec la présence d'un fort biais reproducteur où l'individu (ou les quelques individus) situé(s) en haut de la hiérarchie monopolise(nt) la reproduction, alors que les autres individus largement majoritaires s'occupent des tâches ergonomiques (Reeve, 1991 ; Monnin & Peeters, 1999).

Ce modèle général s'observe dans trois contextes différents. Le premier concerne les cas de pléométrose, c'est-à-dire lorsque plusieurs reines, en général non-apparentées, s'associent pour fonder une nouvelle colonie (Cronin et al., 2013). L'établissement d'une hiérarchie détermine la division du travail dans la reproduction entre les fondatrices (Reeve, 1991 ; Gadagkar, 2001 ; Kolmer & Heinze, 2000). Ces associations sont le plus souvent temporaires, et se dissolvent lors de l'émergence des premières ouvrières par restauration de la monogynie via élimination des reines par les ouvrières ou lutte à mort des reines entre-elles. Dans ces épisodes, la chance d'hériter de la colonie est bien supérieure pour la reine de plus haut rang hiérarchique (Reeve, 1991 ; Sommer & Hölldobler, 1995 ; Bernasconi & Strassmann, 1999). Le deuxième contexte d'établissement de hiérarchies reproductives concerne les espèces primitivement eusociales (e.g. guêpes polistes et stenogastrines, abeilles halictes) dans lesquelles les individus sont totipotents et peuvent donc tous se reproduire sexuellement. C'est aussi le cas des fourmis sans reine, dans lesquelles la hiérarchie détermine en général une ouvrière alpha qui s'accouple (alors appelée gamergate ; Peeters & Crewe, 1984) et devient l'unique reproductrice de la colonie (Ito & Higashi, 1991 ; Monnin & Peeters, 1999 ; Cuvillier-Hot et al., 2004b). On observe enfin chez la majorité des espèces d'Hyménoptères la mise en place de hiérarchies reproductives entre ouvrières, en général après la mort de la reine, qui régulent le conflit pour la production des mâles (Cole, 1981 ; van Doorn & Heringa, 1986 ; Bourke, 1988a ; Oliveira & Hölldobler, 1990 ; Heinze et al., 2002).

Les interactions agonistiques, nous l'avons vu, sont un élément caractéristique de la formation des structures hiérarchiques puisque ce sont elles –entre autre– qui vont déterminer les relations de dominance/subordination de chaque dyade. Cependant les

comportements d'agression manifeste peuvent entrainer des coûts importants, en termes de dépenses de temps et d'énergie ou de blessures physiques (Hsu et al., 2006 ; Rutte et al., 2006), potentiellement supérieurs aux bénéfices liés à la formation de la hiérarchie. On constate en fait que les actes de dominance sont hautement ritualisés (Oliveira & Hölldobler, 1990 ; Reeve, 1991 ; Monnin & Peeters, 1999 ; Cuvillier-Hot et al., 2002, 2004b ; Heinze et al., 2002), voire même quasiment inexistants chez certaines espèces malgré une très nette hiérarchie reproductive (Gadagkar, 1980 ; Sledge et al., 2001b ; Lommelen et al., 2006).

2. Etablissement et maintien des hiérarchies

De nombreuses études, à la fois théoriques et empiriques, se sont intéressées aux mécanismes proximaux qui sous-tendent la formation et le maintien des hiérarchies (Dugatkin & Earley, 2004 ; Hsu et al., 2006 ; Hurd, 2006). Il en ressort une multitude de facteurs souvent non-mutuellement exclusifs qui peuvent être catégorisés si l'on se place à l'échelle de l'individu en facteurs intrinsèques et extrinsèques (Dugatkin & Earley, 2004 ; Hsu et al., 2006).

2. 1. Rôle des facteurs intrinsèques

Les facteurs intrinsèques se rapportent aux différences interindividuelles préexistantes avant une interaction agonistique. Ces traits correspondent notamment aux caractéristiques signalant la capacité d'un individu à monopoliser les ressources (*resource holding potential*, RHP) (Parker, 1974), souvent assimilée à l'aptitude au combat, telles que l'âge, la taille ou l'état hormonal (Hurd, 2006 ; Rutte et al., 2006 ; Chase & Seitz, 2011).

Si la taille peut parfois être corrélée à la dominance (Cervo et al., 2008), il semble qu'en général elle ne soit pas un facteur déterminant dans les interactions de dominance, au contraire de l'âge des individus (Hughes & Strassmann, 1988 ; Reeve, 1991 ; Monnin & Peeters, 1999 ; Cuvillier-Hot et al., 2001 ; Seppä et al., 2002 ; Ishikawa et al., 2010). L'influence de l'âge sur la dominance et la fertilité renvoie à des différences

physiologiques, les principaux paramètres étudiés étant l'hormone juvénile et les amines biogènes.

Le contexte hormonal est en effet fréquemment corrélé au statut hiérarchique et reproducteur (Röseler, 1991 ; Robinson & Vargo, 1997 ; Hartfelder, 2000). L'hormone juvénile, produite par les corps allates, est chez de nombreuses espèces primitivement eusociales une hormone gonadotrope influençant la vitellogenèse, c'est-à-dire la production du vitellus dans les oocytes en croissance (Robinson & Vargo, 1997 ; Hartfelder, 2000). Les taux d'hormone juvénile sont en effet positivement corrélés à la dominance et à la fertilité chez les guêpes polistes (Barth et al., 1975 ; Röseler, 1991 ; Giray et al., 2005 ; Tibbetts et al., 2011), et une augmentation expérimentale du taux d'hormone juvénile des fondatrices entraine une augmentation de leur fertilité et de leur dominance chez *Polistes dominula* (Tibbetts & Izzo, 2009) et *P. metricus* (Tibbetts & Sheehan, 2012). Un même lien entre hormone juvénile et fertilité/dominance se retrouve chez les abeilles primitivement eusociales *Bombus terrestris* (Bloch et al., 2000a) et *Megalopta genalis* (Smith AR et al., 2013).

Cependant ce lien est en fait inversé chez certaines espèces de fourmis (Sommer et al., 1993 ; Cuvillier-Hot et al., 2004a ; Penick et al., 2011), de termites (Brent et al., 2005) et chez l'abeille hautement eusociale *Apis mellifera* (Robinson et al., 1991 ; Corona et al., 2007). Il semble en effet chez ces espèces que la fonction gonadotrope ait été cooptée et que l'hormone juvénile serve à réguler la maturation et la division du travail chez les ouvrières (Robinson & Vargo, 1997 ; Sommer et al., 1993 ; Hartfelder, 2000 ; Cornette et al., 2008). On peut toutefois noter que chez les ouvrières de *P. dominula*, l'hormone juvénile influence à la fois la dominance et la division du travail (Tibbetts & Izzo, 2009). Enfin, si les ecdystéroïdes peuvent influencer la fertilité, il ne semble pas qu'ils interviennent dans la dominance, puisque chez *P. dominula* des fondatrices ovariectomisées ont des taux d'ecdystéroïdes très bas mais sont capables de maintenir leur statut hiérarchique une semaine plus tard (Röseler et al., 1985).

La dissociation fréquemment observée entre fertilité et hormone juvénile implique que d'autres facteurs régulent les changements physiologiques et comportementaux caractéristiques des hiérarchies reproductives. Les amines biogènes (jouant à la fois comme neuromodulateurs et neurohormones) sont ainsi supposées avoir un rôle clé dans la coordination entre dominance et fertilité (Kamhi & Traniello, 2013). La

dopamine et l'octopamine sont associées au niveau d'agressivité chez des insectes solitaires (Adamo et al., 1995 ; Baier et al., 2002 ; Stevenson et al., 2005), et l'octopamine a également été associée à la dominance chez *B. terrestris* (Bloch et al., 2000b) et *Streblognathus peetersi* (Cuvillier-Hot & Lenoir, 2006). La dopamine semble quant à elle avoir un rôle gonadotrope chez de nombreuses espèces (e.g. *A. mellifera* [Dombroski et al., 2003], *B. terrestris* [Bloch et al., 2000b], *Harpegnathos saltator* [Penick et al., 2014], *P. chinensis* [Sasaki et al., 2007], *Solenopsis invicta* [Boulay et al., 2001]), bien que ce ne soit pas le cas chez *Streblognathus peetersi* (Cuvillier-Hot & Lenoir, 2006). De plus, il a été montré chez l'abeille *A. mellifera* que des récepteurs à la dopamine sont exprimés au niveau des ovaires, en corrélation avec le statut reproducteur des individus (Vergoz et al., 2012).

Il apparait donc que le lien très fort qui existe entre dominance et fertilité soit eu moins en partie dû au fait que ces deux paramètres sont dépendants des mêmes facteurs neurophysiologiques. En outre si la dominance semble en général précéder la fertilité (Reeve, 1991 ; Jandt et al., 2014), cette dernière peut aussi influencer la dominance. En effet, chez *P. dominula* la dominance des fondatrices ovariectomisées est corrélée à la taille de leurs corps allates et à leur fertilité à l'émergence (Röseler et al., 1985). De plus chez certaines espèces les ouvrières pondent des œufs trophiques avant l'établissement d'une hiérarchie de dominance manifeste (Dietemann & Peeters, 2000). Ceci suggère donc qu'une variabilité dans l'activité des ovaires peut être présente avant même la formation de la hiérarchie, et influencer la mise en place des relations de dominance.

A ces différences peuvent s'ajouter des différences de structures cérébrales. Les individus dominants ont des corps pédonculés plus importants chez l'abeille *Megalopta genalis* (Smith et al., 2010) et les guêpes *P. dominula* (Ehmer et al., 2001), *P. instabilis* (Molina & O'Donnell, 2007) et *Mischocyttarus mastigophorus* (O'Donnell et al., 2007). Par ailleurs il a été mis en évidence que les patterns d'expression génique associés à l'agression étaient similaires chez *A. mellifera* (Alaux et al., 2009) et *P. metricus* (Toth et al., 2014). Les différences phénotypiques observées chez les individus fertiles et dominants semblent donc avoir trait à un ensemble de paramètres physiologiques et neuroendocrinologiques hautement interconnectés, bien que la question de savoir si ces paramètres sont la cause ou la conséquence d'un haut statut hiérarchique et/ou reproducteur ne soit pas entièrement résolue.

2.2 Rôle des facteurs extrinsèques

Les facteurs extrinsèques intervenants dans la mise en place des hiérarchies regroupent les influences des expériences passées et de l'environnement social. Ces influences correspondent principalement aux effets *winner* et *loser*, dans lesquels les victoires augmentent et les défaites diminuent la probabilité de gagner dans les rencontres futures (Chase et al., 1994 ; Dugatkin, 1997 ; Hsu et al., 2006). Ces effets sont répandus dans le règne animal (Hsu et al., 2006 ; Rutte et al., 2006) et leur impact sur la mise en place des hiérarchies peut être important (Dugatkin, 1997 ; Dugatkin & Earley, 2004), bien que leurs causes ultimes restent controversées (Rutte et al., 2006 ; Goubault & Decuignière, 2012). D'un point de vue proximal, les victoires et les défaites sont supposées induire des modifications dans le système neuroendocrinien, influençant ainsi le comportement et l'issue des rencontres futures (Dugatkin & Earley, 2004 ; Hsu et al., 2006), et on peut penser que les amines biogènes jouent à nouveau un rôle important dans ce processus.

L'influence de l'issue des rencontres passées sur la probabilité de gagner ou de perdre dans les rencontres futures a principalement été étudiée par des modèles théoriques (Landau, 1951 ; Dugatkin, 1997 ; Bonabeau et al., 1999 ; Mesterton-Gibbons, 1999 ; Hemelrijk, 2000 ; Dugatkin & Dugatkin, 2007 ; Fawcett & Johnstone, 2010). Ces modèles, inspirés pour la plupart des théories de l'optimalité (Parker & Smith, 1990), divergent quant à leurs hypothèses de travail regardant l'importance relative des facteurs intrinsèques et extrinsèques (Landau, 1951 ; Bonabeau et al., 1999), la nature additive ou multiplicative de ces facteurs (Dugatkin, 1997 ; Hemelrijk, 2000), ou encore leur niveau de complexité cognitive (Elwood & Arnott, 2012). Par conséquent les conclusions de ces modèles sont souvent divergentes (Hsu et al., 2006 ; Rutte et al., 2006). Elles s'accordent néanmoins à montrer que les effets *winner* et *loser* sont suffisants pour produire une structure hiérarchique linéaire (Landau, 1951 ; Dugatkin, 1997 ; Bonabeau et al., 1999 ; Mesterton-Gibbons, 1999), avec en général un effet *loser* plus important que l'effet *winner* (Mesterton-Gibbons, 1999 ; Hsu et al., 2006 ; Rutte et al., 2006 ; Fawcett & Johnstone, 2010). Les victoires et défaites passées peuvent influencer l'issue des rencontres futures par une action directe sur le RHP des individus, mais elles peuvent aussi affecter l'estimation (non nécessairement concordante) que les individus eux-

mêmes en font, ou encore la valeur de la ressource contestée (Hsu et al., 2006), parfois assimilée à la motivation (Maynard-Smith & Parker, 1976 ; Hurd, 2006).

Ces modèles insistent sur l'implication des processus d'auto-organisation, et donc du hasard, dans la détermination des rangs hiérarchiques. Cependant, la mise en place des hiérarchies peut également être affectée par les processus de reconnaissance, notamment la capacité des individus à estimer le RHP des autres membres du groupe. Lorsque cette estimation est possible, les modèles montrent une hiérarchie plus stable et un plus faible niveau d'agression (Dugatkin & Dugatkin, 2007). Ces résultats sont en accord avec la forte directionnalité des interactions agonistiques typiquement observée dans les hiérarchies de dominance (Hsu et al., 2006 ; Tibbetts & Dale, 2007 ; Chase & Seitz, 2011). Par ailleurs, lorsque les effets *winner* et *loser* sont multiplicatifs, la théorie prédit que les asymétries dans le RHP perçu sont bien plus importantes entre individus de haut rang qu'entre individus de bas rang, résultant dans une hiérarchie linéaire dans laquelle seuls les hauts rangs sont clairement définis (Landau, 1951 ; Dugatkin, 1997 ; Hsu et al., 2006), ce qui semble également correspondre aux observations où seuls les individus de haut rang sont effectivement impliqués dans la hiérarchie (Monnin & Peeters, 1999 ; Heinze et al., 2002 ; Cuvillier-Hot et al., 2004b).

L'estimation du RHP peut se faire soit de façon directe si les individus peuvent reconnaitre le statut des autres membres du groupe, soit de façon indirecte si les individus sont capables de reconnaissance individuelle (Hemelrijk, 2000 ; Tibbetts & Dale, 2007). La reconnaissance de statut est théoriquement suffisante pour faire émerger une structure hiérarchique linéaire, indépendamment du degré de familiarité entre les individus. Ce processus est cognitivement peu couteux, mais il suppose l'existence d'indices fiables permettant la discrimination des rangs proches, ce qui n'a pas encore été démontré (Liebig, 2010). A contrario, la reconnaissance individuelle est supposée avoir une grande importance dans la mise en place et la stabilisation des hiérarchies linéaires de part son haut niveau de précision (Barnard & Burk, 1979 ; Dale et al., 2001 ; Tibbetts, 2002 ; Thom & Hurst, 2004 ; d'Ettorre & Heinze, 2005 ; Tibbetts & Dale, 2007). Elle implique la mémorisation des caractéristiques propres à chaque individu et de l'historique de leurs rencontres passées (Dale et al., 2001 ; Tibbetts, 2002), et sa contrepartie est donc une forte complexité cognitive (Thom & Hurst, 2004 ; Wiley, 2013). Cependant l'existence de processus de reconnaissance individuelle a été

démontrée dans les hiérarchies de reines fondatrices chez la guêpe *P. fuscatus* (Tibbetts, 2002 ; Sheehan & Tibbetts, 2008) et les fourmis *Neoponera villosa* et *N. inversa* (d'Ettorre & Heinze, 2005 ; Dreier et al., 2007). Ces données apparaissent donc être en contradiction avec certains modèles montrant l'influence négative de la reconnaissance individuelle sur la formation des hiérarchies linéaires (Bonabeau et al., 1999 ; Hemelrijk, 2000 ; Dugatkin & Earley, 2004).

Au-delà des modèles, les observations montrent clairement que le contexte social a une influence non nulle sur la fertilité et la dominance (en plus du fait que la dominance n'existe par définition que dans un contexte social). Ainsi par exemple la mort de la gamergate chez les fourmis sans reine entraine un regain de comportements agonistiques définissant sa succession et donc l'accès à la reproduction (Monnin & Peeters, 1999 ; Ito & Higashi, 1991 ; Sommer et al., 1993 ; Cuvillier-Hot et al., 2004b). Les ouvrières de bas rangs, pourtant non-impliquées dans les interactions de dominance, ont également un rôle très important dans la stabilisation des hiérarchies en étant responsable de la punition par immobilisation, résultant dans la perte de leur statut, des individus de haut rang qui pourraient défier la gamergate (Monnin et al., 2002 ; Cuvillier-Hot et al., 2004a). Enfin, l'influence du contexte social apparait chez les espèces ou les reines peuvent fonder en solitaire et en groupe. Ainsi, chez la guêpe *Ropalidia marginata* et l'abeille *Megalopta genalis*, les reines dominantes qui fondent une colonie en groupe ont une fertilité supérieure aux reines qui fondent en solitaire, et l'inverse est observé chez les reines subordonnées (Lamba, 2007 ; Smith AR et al., 2009 ; Shukla et al., 2014). A ces facteurs sociaux peuvent se rajouter des facteurs environnementaux, telles des contraintes écologiques liées aux opportunités de fonder de nouveaux nids ou à la température (Jandt et al., 2014). L'ordre d'arrivée dans les groupes de fondatrices chez *P. dominula* est par exemple corrélé à leur statut hiérarchique futur (Reeve, 1991 ; Seppä et al., 2002 ; Zanette and Field, 2009).

Ce rapide exposé des faits montre l'importance potentielle des facteurs à la fois intrinsèques et extrinsèques dans la détermination des rangs hiérarchiques et la formation des hiérarchies sociales. Si leur importance relative est probablement variable en fonction des espèces, leur interaction parait toutefois indissociable et probablement nécessaire (Dugatkin & Earley, 2004 ; Hsu et al., 2006).

V. Signalement de la fertilité

Les hiérarchies reproductives, qui typiquement font suite aux hiérarchies comportementales, sont caractérisées par un faible niveau d'agression intra-coloniale (Gadagkar, 1980 ; Reeve, 1991 ; Heinze et al., 1994 ; Monnin & Peeters, 1999). Lors de cette seconde phase, les individus dominants sont donc capables de maintenir leur monopole reproducteur sans pour autant continuer à manifester comportementalement leur dominance, ce qui suggère l'existence de mécanismes de reconnaissance associés au statut hiérarchique et/ou reproducteur.

1. Importance des hydrocarbures cuticulaires

Les HCC, en plus de signaler l'appartenance coloniale, peuvent aussi servir à véhiculer des informations relatives à l'espèce, au sexe, à la caste ainsi qu'au statut hiérarchique et reproducteur (Greene & Gordon, 2003 ; Howard & Blomquist, 2005 ; Dani, 2006 ; Monnin, 2006 ; Martin & Drijfhout, 2009a ; d'Ettorre and Lenoir, 2010 ; Liebig, 2010). De nombreuses études ont en effet montré que les HCC étaient impliqués dans le signalement de la fertilité et/ou de la dominance, que ce soit chez les abeilles (Ayasse et al., 1995 ; Hoover et al., 2003), les fourmis (Monnin et al., 1998 ; Liebig et al., 2000 ; Heinze et al., 2002 ; Dietemann et al., 2003 ; Cuvillier-Hot et al., 2004b ; de Biseau et al., 2004 ; Endler et al., 2004 ; Smith AA et al., 2009 ; Holman et al., 2010), les guêpes (Sledge et al., 2001a ; Dapporto et al., 2007a ; Bhadra et al., 2010) ou les termites (Liebig et al., 2009 ; Weil et al., 2009). Il semble le plus souvent que les HCC signalent davantage le statut reproducteur que le statut hiérarchique, et jouent ainsi le rôle de signaux de fertilité (Monnin, 2006 ; Le Conte & Hefetz, 2008 ; Peeters & Liebig, 2009 ; Liebig, 2010). Cependant la forte corrélation généralement observée entre fertilité et dominance en fait aussi d'un point de vue fonctionnel des signaux de statut social. Les individus présentant des ovaires actifs ont un profil d'HCC spécifique, résultant de différences en général quantitatives entre un ou plusieurs composés et reflétant ainsi leur état de fertilité (Monnin et al., 1998 ; Liebig et al., 2000 ; Sledge et al., 2001a ; Dietemann et al., 2003 ; de Biseau et al., 2004 ; Hartmann et al., 2005). Il a été mis en évidence que ces signaux étaient à la base de la division du travail dans la reproduction caractéristique des sociétés d'insectes, en permettant le maintien d'un biais reproducteur très fort entre

les reproducteurs et les ouvrières résultant de l'auto-restriction et/ou de la coercition de ces dernières (Hoover et al., 2003 ; Endler et al., 2004 ; Dapporto et al., 2007b ; Smith AA et al., 2009 ; Bhadra et al., 2010 ; Holman et al., 2010 ; Smith et al., 2012 ; Van Oystaeyen et al., 2014). De plus, des études ont montré que l'information véhiculée par les HCC n'était pas une simple différence binaire entre fertiles et non fertiles, puisque les ouvrières sont capables de percevoir différents profils d'HCC associés à des niveaux variables d'activité ovarienne (Gobin et al., 1999 ; Liebig et al., 1999 ; Ortius & Heinze, 1999 ; Hannonen et al., 2002 ; Heinze et al., 2002 ; Cuvillier-Hot et al., 2004b).

L'implication des HCC dans le maintien des hiérarchies reproductives est également bien établie. La production des HCC associés à la fertilité commence en général suite au début de l'ovogenèse une fois les rangs hiérarchiques établis (Peeters et al., 1999 ; Liebig et al., 2000 ; Cuvillier-Hot et al., 2004b ; Hartmann et al., 2005). On observe alors typiquement une corrélation très précise entre le niveau de fertilité et le profil d'HCC, comme cela a été notamment montré chez les fourmis sans reine. Ainsi chez *Dinoponera quadriceps* une ouvrière accédant au rang d'alpha acquiert progressivement un profil typique de gamergate caractérisé notamment de fortes quantités relatives de 9-hentriacontène (Peeters et al., 1999). Le même phénomène s'observe chez *Harpegnathos saltator* avec entre autres le 13,23-diméthylheptatriacontane (Liebig et al., 2000), ou encore chez *Streblognathus peetersi* chez qui les quantités relatives d'un ensemble d'HCC sont modifiées conjointement à l'augmentation de la fertilité (Cuvillier-Hot et al., 2004b). Les comportements de courbure du gastre typique des gamergates décrits chez *Dinoponera quadriceps* et *Streblognathus peetersi* suggèrent aussi l'implication d'un signal chimique. Lors de ce comportement de dominance la gamergate expose les membranes inter-segmentaires de l'extrémité de son abdomen, région très concentrée en HCC (Monnin et al., 1998), aux antennes d'un individu subordonné, généralement de rang immédiatement inférieur dans la hiérarchie (Monnin & Peeters, 1999 ; Cuvillier-Hot et al., 2004b). Par ailleurs lorsque des individus alpha sont traités chez *Streblognathus peetersi* avec un analogue de l'hormone juvénile, leur fertilité diminue en même temps que leur profil d'HCC se modifie, ce qui entraine leur immobilisation par les ouvrières de bas rang et consécutivement leur exclusion de la hiérarchie (Cuvillier-Hot et al., 2004a). Le même résultat est observé chez *Dinoponera quadriceps* lorsque la gamergate marque une ouvrière défiant son statut (en général beta) avec des HC de sa glande de Dufour (Monnin & Peeters, 1999). Si un individu beta est expérimentalement traité avec le

contenu de la glande de Dufour d'une gamergate, il est également immobilisé par les ouvrières de bas rang (Monnin et al., 2002). Enfin l'implication d'un signal chimique comme indicateur du statut est reflétée dans la concordance entre le profil d'HCC des gamergates et les HC présents à la surface de leurs œufs. Ce signal intervient aussi dans le maintien du monopole reproducteur en permettant l'oophagie différentielle des œufs des individus de haut rang ne possédant pas ce signal spécifique (Monnin & Peeters, 1997).

Ces résultats indiquent donc l'implication des HCC comme signal de fertilité responsable du maintien des hiérarchies reproductives. Ils suggèrent par ailleurs que la biosynthèse des HCC et l'activité des ovaires seraient sous-tendues par des mécanismes communs, mettant notamment en jeu les hormones gonadotropes telle l'hormone juvénile (Cuvillier-Hot et al., 2004a ; Sledge et al., 2004 ; Peeters & Liebig, 2009 ; Dapporto et al., 2010 ; Izzo et al., 2010 ; Liebig, 2010). On peut noter que chez la guêpe *P. dominula*, un signal visuel (le motif de pigmentation du clypeus) est impliqué dans le maintien du statut hiérarchique au début du cycle colonial (Tibbetts & Dale, 2004), même si les HCC prennent le relais par la suite (Sledge et al., 2001a).

2. Signal honnête ou manipulation ?

Deux hypothèses ont été proposées pour expliquer l'existence de signaux de fertilité chez les insectes sociaux. Ces signaux ont tout d'abord été vus comme des phéromones de contrôle qui répriment activement la reproduction des ouvrières (hypothèse du contrôle royal) (Hölldobler & Wilson, 1983). Cette hypothèse s'inscrit dans le conflit qui oppose la reine et les ouvrières pour la production des mâles (Bourke, 1988b ; Ratnieks et al., 2006), et considère les signaux émis par la reine comme un moyen d'assurer son monopole reproducteur en contraignant les ouvrières à ne pas se reproduire. L'hypothèse alternative voit au contraire les signaux de fertilité comme des signaux honnêtes auxquels les ouvrières répondent dans leur propre intérêt (hypothèse du signalement honnête) (Keller & Nonacs, 1993 ; Heinze & d'Ettorre, 2009 ; Kocher & Grozinger, 2011). L'auto-restriction reproductive des ouvrières est ainsi considérée comme la résultante d'une prise de décision favorisant leur *inclusive fitness*. La balance entre coûts en *fitness* directe, bénéfices en *fitness* indirecte et apparentement peut en

effet favoriser la stérilité fonctionnelle des ouvrières (voir III. 2), à condition que l'individu reproducteur soit suffisamment fertile (Keller & Nonacs, 1993). Bien que différencier ces deux hypothèses de manière non équivoque soit probablement difficile, il est prédit que les pressions de sélection sous-jacentes à ce système de communication soient différentes. En effet, l'hypothèse du contrôle royal prédit l'existence d'une course à l'armement évolutive entre la reine et les ouvrières, favorisant la résistance au signal royal chez les ouvrières, et en conséquence une complexification rapide du bouquet royal au cours de l'évolution (Keller & Nonacs, 1993 ; Heinze & d'Ettorre, 2009). Les prédictions inverses sont faites pour l'hypothèse du signal honnête, selon laquelle les signaux associés à la fertilité devraient avoir une vitesse d'évolution beaucoup plus lente, et en conséquence devraient être largement conservés entre espèces proches (Keller & Nonacs, 1993 ; Heinze & d'Ettorre, 2009 ; Kocher & Grozinger, 2011 ; van Zweden et al., 2014).

La première hypothèse est principalement soutenue par des études sur l'abeille *Apis mellifera* et la fourmi de feu *Solenopsis invicta* qui ont montré la complexité chimique des signaux royaux originaires de multiples glandes (Vargo & Hulsey, 2000 ; Katzav-Gozansky, 2006), ainsi que leur effet direct sur la physiologie des ouvrières (Beggs et al., 2007). Les arguments en faveur de l'hypothèse du signalement honnête sont la forte association qui existe entre les HCC et la fertilité chez de nombreuses espèces (Monnin, 2006 ; Le Conte & Hefetz, 2008 ; Peeters & Liebig, 2009 ; Liebig, 2010), le fait que les ouvrières semblent ajuster leurs décisions reproductives selon le contexte social (Alaux et al., 2007 ; Malka et al., 2007 ; Yagound et al., 2012), ce qui favorise leurs intérêts en termes d'*inclusive fitness*, et les rares études comparatives montrant une conservation des signaux de fertilité chez des espèces proches (Brunner et al., 2011 ; Holman et al., 2013a ; van Zweden et al., 2014).

VI. Diversité des Ponerinae au sein des Formicidae

Les fourmis (Hymenoptera : Formicidae) sont parmi les organismes ayant le plus d'impact sur les écosystèmes, que ce soit en termes d'abondance, de biomasse, d'interactions écologiques (notamment symbiotiques), d'aération du sol ou de recyclage des nutriments (Wilson, 1971 ; Oster & Wilson, 1978 ; Hölldobler & Wilson, 1990 ;

Wilson & Hölldobler, 2005). Leur succès écologique est principalement dû à leur vie exclusivement eusociale, régie par une division du travail et un système de communication efficaces leur permettant l'exploitation de nouvelles niches et servant de support à leur radiation évolutive (Oster & Wilson, 1978 ; Hölldobler & Wilson, 1990). L'origine des fourmis remonte à il y a environ 140 millions d'années (Brady et al., 2006a ; Moreau et al., 2006 ; Moreau & Bell, 2013) à partir de guêpes prédatrices Sphéciformes (Apoidea), espèces solitaires mais qui construisaient déjà des nids et approvisionnaient leurs larves en nourriture (Johnson et al., 2013).

La famille des Formicidae regroupe une très importante diversité taxonomique, avec 16 sous-familles répertoriées pour près de 13 000 espèces décrites (Bolton, 2014 ; Brady et al., 2014), bien que la diversité réelle soit estimée à plus de 25 000 espèces (Ward, 2010). Les avancées récentes des phylogénies moléculaires ont permis de largement stabiliser la classification taxonomique des diverses sous-familles et de dresser leurs relations phylogénétiques (Brady et al., 2006a, 2014 ; Moreau et al., 2006 ; Ward, 2007 ; Rabeling et al., 2008 ; Ward et al., 2010, 2014 ; Moreau & Bell, 2013 ; Schmidt & Shattuck, 2014). Il apparait ainsi que la plupart des sous-familles soient monophylétiques, à l'exception notable des Amblyoponinae (Ward, 2007). Ces cas, manifestés également dans divers genres tels que *Aphaenogaster*, *Camponotus*, *Cerapachys* et *Pheidole* semblent être la manifestation d'évolutions indépendantes (convergence morphologique) ainsi que d'hétérogénéités dans les vitesses d'évolution morphologique (Ward, 2011).

Au sein des Formicidae, la majorité des espèces est comprise dans le clade des formicoides regroupant 9 sous-familles parmi lesquelles les Dolichoderinae, Formicinae et Myrmicinae totalisent plus de 10 200 espèces (Bolton, 2014). Les sept autres sous-familles forment le clade des ponéroides, à l'exception des Leptanillinae et Martialinae *incertae sedis* (Rabeling et al., 2008 ; Moreau & Bell, 2013). Les ponéroides sont constitués des Agroecomyrmecinae, Amblyoponinae, Paraponerinae, Ponerinae et Proceratiinae, ce qui correspond à l'ancien groupe polyphylétique des ponéromorphes (Bolton, 2003) sans les Ectatomminae et Heteroponerinae qui font maintenant partie des formicoides, et avec l'ajout des Agroecomyrmecinae comprenant le genre monotypique *Tatuidris* (Brady et al., 2006a ; Moreau et al., 2006 ; Ward, 2007).

La sous-famille des Ponerinae regroupe à elle-seule la grande majorité des ponéroides avec plus de 1 150 espèces décrites (Bolton, 2014), ce qui en fait en termes de diversité une des quatre plus grandes sous-familles de Formicidae. Les ponérines possèdent des traits typiquement « ancestraux » quant à leur structure coloniale et à leur organisation sociale (Peeters, 1997). On retrouve ainsi en général des tailles de colonie réduites (100–300 individus), un faible dimorphisme entre la reine et les ouvrières, une caste ouvrière monomorphe, un approvisionnement en solitaire, ainsi qu'un fort potentiel reproducteur des ouvrières (Hölldobler & Wilson, 1990 ; Peeters, 1997 ; Wilson & Hölldobler, 2005). Cependant ce taxon regroupe en fait une très importante diversité d'organisation sociale, de structure coloniale et de stratégies de reproduction (Peeters, 1993, 1997 ; Schmidt & Shattuck, 2014). Les ponérines sont donc un bon modèle pour étudier l'évolution de l'organisation sociale dans les sociétés d'insectes.

Cette sous-famille a récemment fait l'objet d'une importante révision taxonomique redéfinissant les genres et leurs relations phylétiques (Schmidt, 2013 ; Schmidt & Shattuck, 2014). Le résultat de cette révision soutien une monophylie très forte pour les Ponerinae, dont la diversité se regroupe en deux tribus, les Platythyreini qui comprennent le genre *Platythyrea*, et les Ponerini qui regroupent 46 autres genres (Figure 1). Ces genres sont regroupés en six taxons dont la monophylie reste pour certains équivoque, les groupes *Harpegnathos*, *Hypoponera*, *Odontomachus*, *Pachycondyla*, *Plectroctena* et *Ponera*. Le groupe *Pachycondyla* comprend 111 espèces réparties en 7 genres, parmi lesquels le genre *Neoponera* (Figure 1). Il est issu de l'éclatement de l'ancien genre *Pachycondyla* largement paraphylétique en 19 genres distincts. Les *Neoponera* sont parmi les ponérines les plus diverses, tant d'un point de vue morphologique, écologique, que comportemental (Schmidt & Shattuck, 2014). Ce genre abrite notamment plusieurs complexes d'espèces, tels les complexes *N. apicalis*, *N. crenata*, *N. emiliae* et *N. foetida* (Mackay & Mackay, 2010). Ces complexes regroupent en partie des espèces cryptiques, c'est-à-dire morphologiquement très similaires et consécutivement classées (à tort) sous un même nom (Bickford et al., 2007). Elles constituent un défi taxonomique, mais en même temps représentent des modèles de choix pour appréhender l'évolution de traits morphologiques, comportementaux ou écologiques entre des espèces proches.

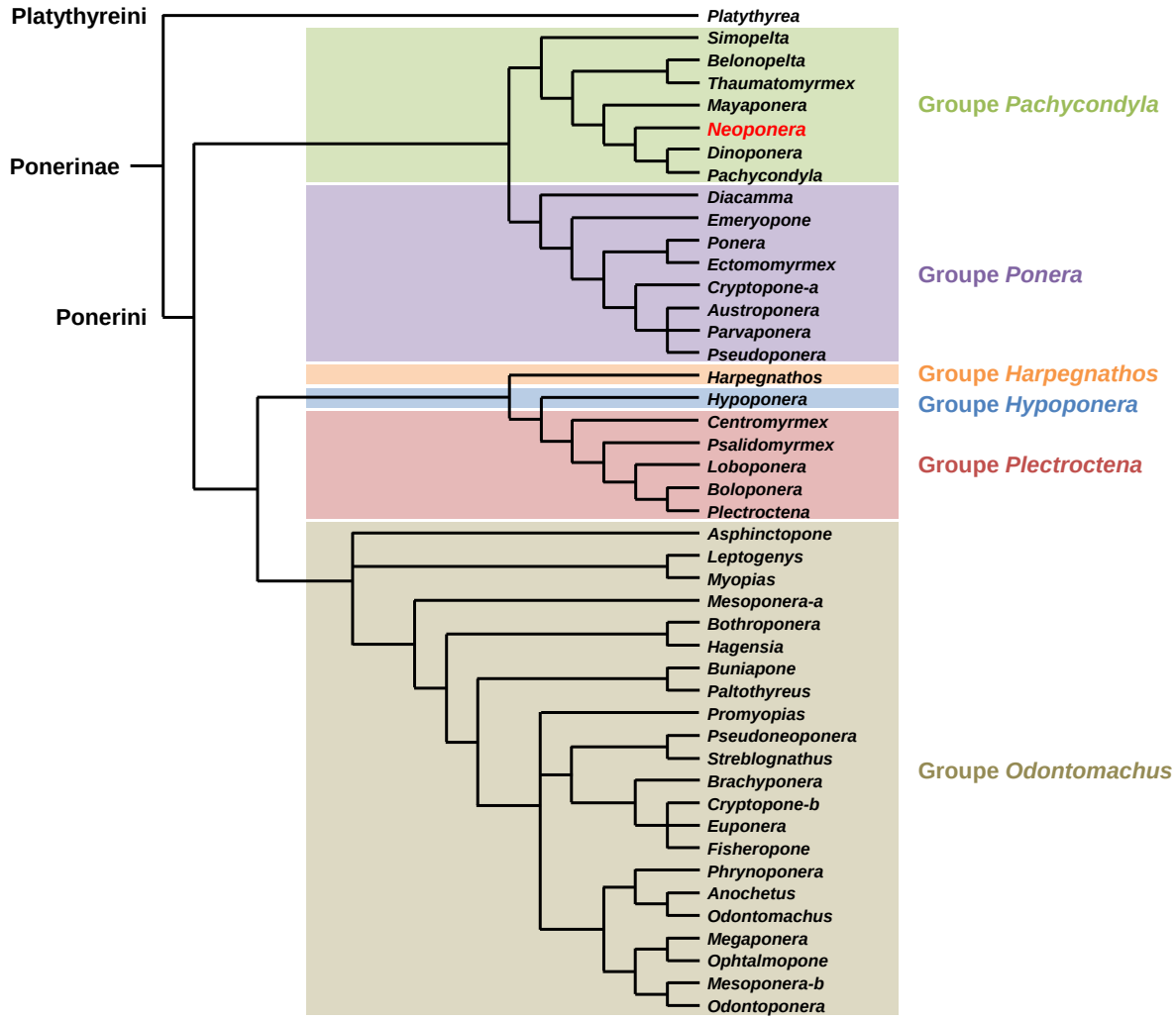

Figure 1. Relations entre les genres de Ponerinae. *Cryptopone-a* représente *C. hartwigi*, *Cryptopone-b* représente *C. gilva* et *C. testacea*, *Mesoponera-a* représente *M. melanaria* et *Mesoponera-b* représente *M. ambigua*. La position des taxons suivants n'est pas connue : *Dolioponera*, *Feroponera*, *Iroponera* et *Rasopone*. D'après Schmidt (2013) et Schmidt et Shattuck (2014), avec leur autorisation.

VII. Présentation du modèle : le complexe *Neoponera apicalis*

Le complexe d'espèces *Neoponera* (précédemment *Pachycondyla* ; Schmidt & Shattuck, 2014) *apicalis* regroupe quatre espèces décrites largement sympatriques, et dont la distribution Néotropicale s'étend du sud du Mexique au Paraguay (Kempf, 1972 ; Wild, 2005 ; Delabie et al., 2008) : *N. apicalis* (Latreille, 1802), *N. cooki* (Mackay & Mackay, 2010), *N. obscuricornis* (Emery, 1890) et *N. verenae* (Forel, 1922). Des études récentes pluridisciplinaires suggèrent une diversité bien plus importante au sein de ce complexe,

avec la présence de cinq « morphes » dans le clade *N. apicalis* et deux morphes dans le clade *N. verenae* (Delabie et al., 2008 ; Ferreira et al., 2010). Le statut taxonomique de ces morphes n'est pas encore défini étant donné qu'aucune description formelle n'en a était faite, mais la concordance au moins partielle des données morphologiques, génétiques, écologiques, bioacoustiques, chimiques, et cytogénétiques indique qu'il pourrait s'agir d'espèces valides (Delabie et al., 2008 ; Ferreira et al., 2010 ; Evison et al., 2012 ; Mariano et al., 2011).

Figure 2. Ouvrière de *Neoponera apicalis* morphe 6 en Guyane Française (photo Boris Yagound).

La majorité des études qui se sont intéressées à ce complexe ont été réalisées sur le clade *N. apicalis* (Figure 2) et certaines sur le clade *N. verenae*. Les informations sur *N. cooki* sont limitées du fait de sa description récente (Mackay & Mackay, 2010). Enfin, *N. obscuricornis* est plus rarement trouvée et demeure largement inconnue, bien que nombre de ses traits d'histoire de vie doivent être similaires à ceux du reste du complexe. On peut néanmoins dresser un état des lieux rapide des connaissances accumulées sur la biologie de ces fourmis. *N. apicalis* est supposée monogyne et monoandre (Fresneau, 1994), bien qu'il n'y ait pas de données moléculaires à ce sujet, alors que *N. verenae* est facultativement polygyne (Fresneau, 1984 ; Traniello &

Hölldobler, 1984 ; Evison et al., 2012). Ces fourmis montrent un polyéthisme d'âge classique (Fresneau, 1984 ; Fresneau & Dupuy, 1988). L'approvisionnement s'effectue en solitaire, avec un régime alimentaire mi-prédateur, mi-nécrophage. Les aires de fourragement sont distribuées en mosaïque et les fourrageuses montrent une fidélité individuelle au site d'approvisionnement et au trajet (Duelli & Duelli-Klein, 1976 ; Fresneau, 1985 ; Goss et al., 1989 ; Fresneau, 1994). Ce comportement d'approvisionnement a par ailleurs été utilisé pour développer un algorithme de recherche numérique (Monmarché et al., 2000). Il n'y a pas de comportement de construction et les colonies nichent préférentiellement dans des troncs d'arbres morts près du sol, dans d'anciennes galeries creusées par des Coléoptères saprophages (Fresneau, 1994), bien que *N. apicalis* morphe 6 niche préférentiellement dans les arbres et *N. verenae* morphe 2 dans le sol (Ferreira, 2010). Les colonies doivent déménager fréquemment et les émigrations se font par tandem running (Levings & Franks, 1982 ; Traniello & Hölldobler, 1984 ; Pezon et al., 2005). Ces fourmis montrent des comportements territoriaux, principalement à proximité du nid (Fresneau, 1994 ; Ferreira, 2010). Les colonies comprennent moins de 100 individus en moyenne, bien que de rares colonies puissent dépasser les 300 ouvrières. La durée de maturation du couvain est d'environ 2,5 mois. Les sexués sont produits toute l'année, mais il existe un pic de production à la fin de la saison sèche (Fresneau, 1994). L'appareil reproducteur des ouvrières est similaire à celui de la reine, bien que la spermathèque ne soit pas fonctionnelle (Fresneau, 1984 ; Gobin et al., 2006). Les ouvrières pondent des œufs trophiques en présence de la reine, mais établissent des hiérarchies reproductives quand celle-ci disparait (Oliveira & Hölldobler, 1990, 1991 ; Dietemann & Peeters, 2000 ; Gobin et al., 2003 ; Blacher et al., 2010). La présence d'intercastes fécondes à été observée chez *N. verenae* (Düssmann et al., 1996). Les HCC produits sont accumulés au niveau des brosses basitarsales situées sur les pattes avant, d'où ils sont distribués à tous le corps. La glande post-pharyngienne a été décrite comme l'organe de la *Gestalt* et l'homogénéisation de l'odeur coloniale se fait par contacts passifs et allotoilettage (Soroker et al., 1998, 2003 ; Hefetz et al., 2001). Il n'y a pas de trophallaxies vraies (Hölldobler, 1985). Ces fourmis utilisent aussi la communication acoustique au moyen de stridulations (Giovannotti, 1996 ; Pavan et al., 1997 ; Ferreira et al., 2010). On peut enfin préciser que des études se sont intéressées aux propriétés biochimiques du venin (Schmidt et al., 1980 ; Cruz-López & Morgan, 1997 ; Touchard et al., 2014) ainsi qu'à

l'ultrastructure des glandes tergale, sternale, metapleurale, labiale et pygidiale (Hölldobler & Engel-Siegel, 1982 ; Traniello & Hölldobler, 1984 ; Lommelen et al., 2002).

VIII. Objectifs de la thèse et organisation du manuscrit

L'eusocialité représente le plus haut degré de coopération observé dans le règne animal, et sa manifestation la plus évidente est l'altruisme reproducteur exprimé par la grande majorité des individus constituant ces sociétés. Cependant, cette forme extrême de coopération n'exempte pas ces groupes de conflits, puisqu'il existe nécessairement des individus égoïstes à la fois internes et externes au groupe social, qui peuvent exploiter ses ressources pour leur propre profit, et ainsi menacer la cohésion du groupe dans son ensemble. La compréhension des mécanismes permettant de limiter l'impact de ces sources de conflit externes et internes est donc un élément déterminant pour appréhender l'évolution sociale et le maintien de la coopération dans les groupes sociaux (Bourke, 2011). Elle constitue ainsi l'aspect central de cette thèse. Dans ce contexte, utiliser une approche comparative peut permettre de dégager les spécificités et les similitudes dans les traits étudiés, afin de comprendre quelles pressions et contraintes dans l'écologie et les traits d'histoire de vie des espèces peuvent avoir influencé leur évolution (West et al., 2007).

Dans un premier chapitre on s'est attaché à caractériser la diversité taxonomique présente dans le complexe d'espèces *Neoponera apicalis*. La position phylogénétique de ce taxon, ainsi que son mode d'organisation sociale en fait un modèle intéressant pour l'étude des mécanismes ayant trait au maintien de la coopération face aux pressions externes et internes (Fresneau, 1994). Ce complexe est supposé abriter une forte diversité spécifique, avec près d'une dizaine d'espèces potentielles largement sympatriques. Cependant leur statut taxonomique ainsi que leurs relations phylogénétiques restent équivoques (Wild, 2005 ; Delabie et al., 2008 ; Ferreira et al., 2010). Grace à une étude intégrative mêlant taxonomie chimique et phylogénie moléculaire, nous avons ainsi cherché à confirmer le statut des différents morphes de *N. apicalis* et *N. verenae* en tant qu'espèces distinctes, ainsi qu'à estimer la validité des HCC en tant qu'outil taxonomique dans la délimitation de ces espèces cryptiques (Bagnères & Wicker-Thomas, 2010 ; Kather & Martin, 2012).

La principale source de conflits internes à tout groupe social concerne les divergences d'optimum reproductif entre les différents membres du groupe, découlant directement de leurs fortes asymétries d'apparentement (Trivers & Hare, 1976). Etudier les conflits liés à la reproduction nécessite donc de connaitre la structure génétique des colonies, puisque c'est principalement ce facteur qui va influencer les niveaux d'apparentement entre les individus, et donc servir de base aux prédictions issues de la théorie de l'*inclusive fitness* quant à la survenue de conflits potentiels (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Ratnieks et al., 2006). Dans un deuxième chapitre, nous avons donc déterminé la structure génétique de plusieurs espèces du complexe *N. apicalis*, afin d'estimer le niveau de polygynie, de polyandrie effective, ainsi que l'apparentement moyen entre les principaux groupes d'intérêts présents dans ces colonies (Boomsma & Ratnieks, 1996 ; Heinze & Keller, 2000).

Dans un troisième chapitre, nous avons ensuite étudié chez une espèce du complexe en particulier, *N. apicalis* morphe 4, les modalités de régulation du conflit lié à la production des mâles et qui oppose la reine et le collectif des ouvrières, ainsi que les ouvrières entre-elles (Bourke, 1988b ; Ratnieks, 1988 ; Hammond & Keller, 2004 ; Wenseleers et al., 2004). Pour cela, nous avons étudié les mécanismes proximaux responsables de la mise en place et du maintien des hiérarchies reproductives dans des groupes d'ouvrières nouvellement isolées de la reine (Oliveira & Hölldobler, 1990). On s'est particulièrement intéressé à l'existence et à la nature des systèmes de reconnaissance sous-tendant les interactions de dominance/subordination lors de l'établissement de la structure hiérarchique, ainsi qu'à la nature des indices de reconnaissance sous-jacents, associés au statut hiérarchique et/ou reproducteur (Liebig, 2010). Nous avons également étudié l'expression de ces signaux putatifs chez les reines vierges et fécondées afin de voir s'ils peuvent aussi servir à établir le monopole reproducteur de la reine. Nous avons enfin testé le rôle actif des composés identifiés en réalisant des bioessais avec des individus dont les niveaux de signal sont expérimentalement manipulés.

Le quatrième chapitre vise à comparer les signaux associés à la dominance et surtout à la fertilité entre les différentes espèces du complexe *N. apicalis*. Cette étude du degré de conservation des signaux de fertilité entre ces espèces proches a ainsi pour objectif de caractériser leur mode d'action probable en tant que signal honnête ou manipulateur

(Keller & Nonacs, 1993 ; Heinze & d'Ettorre, 2009 ; Kocher & Grozinger, 2011), et donc *in fine* de mieux comprendre les mécanismes à l'œuvre dans la régulation des conflits liés à la reproduction et le maintien de la coopération dans ces sociétés.

Après avoir étudié dans les précédents chapitres les mécanismes permettant de réduire l'impact des conflits internes sur la cohésion du groupe social, nous avons étudié dans le cinquième chapitre les facteurs permettant de limiter l'exploitation du groupe par des éléments externes. Nous avons pour cela étudié les processus de reconnaissance coloniale (Crozier & Pamilo, 1996 ; d'Ettorre & Lenoir, 2010) et la possibilité de discrimination entre voisins et étrangers (Gordon, 1989 ; Heinze et al., 1996 ; Langen et al., 2000 ; Dimarco et al., 2010) à l'aide d'une approche combinant comportement, génétique et écologie chimique. En comparant trois espèces sympatriques du complexe ayant des traits d'histoire de vie très similaires mais des préférences de nidifications différentes, nous avons voulu étudier l'impact des facteurs écologiques sur la mise en place des processus de familiarisation et des relations intercoloniales.

CHAPITRE 1

TAXONOMIE CHIMIQUE ET PHYLOGÉNIE DU COMPLEXE *NEOPONERA APICALIS*

RÉSUMÉ

La caractérisation et la délimitation des espèces est un paramètre crucial pour les études en écologie, en éthologie et en biologie évolutive. Cette tâche peut cependant être particulièrement difficile lorsque leur morphologie est très similaire. Le complexe de fourmis *Neoponera apicalis* regroupe ainsi plusieurs espèces cryptiques largement sympatriques, mais dont le statut taxonomique est équivoque. En effet, si quatre espèces distinctes sont décrites actuellement, plusieurs études suggèrent l'existence d'une diversité supérieure avec la description de différents morphes au sein des espèces *N. apicalis* et *N. verenae*, mais sans conclure sur leur statut en tant qu'espèces valides.

Nous avons utilisé une approche intégrative mêlant taxonomie chimique (hydrocarbures cuticulaires) et phylogénie moléculaire (ADN mitochondrial COI) pour clarifier le statut taxonomique de ces morphes/espèces. Les résultats montrent que chaque morphe étudié possède un profil chimique spécifique, pouvant être discriminé à l'aide de seulement quelques composés diagnostiques, ce qui souligne la validité des hydrocarbures cuticulaires en tant qu'outil taxonomique. Ils révèlent de plus une forte variabilité intra-morphe, avec pour certains morphes la présence de plusieurs chémotypes, et parfois au sein de la même colonie. Ces chémotypes semblent être le reflet d'influences géographiques et possiblement d'évènements d'hybridation entre lignées divergentes.

Les résultats de la phylogénie moléculaire montrent des distances génétiques élevées aussi bien entre les clades qu'au sein des clades. Ils confirment la monophylie de tous les morphes identifiés dans les études précédentes et identifient un nouvel haplotype au sein de *N. verenae* (morphe 3). Si *N. cooki* semble être l'espèce sœur des groupes *N. verenae* et *N. apicalis*, les relations phylogénétiques entre ces groupes ne sont cependant que partiellement résolues avec la phylogénie actuelle, ce qui implique la nécessité d'inclure l'étude de nouveaux gènes, notamment nucléaires. La phylogénie révèle par ailleurs l'existence de plusieurs clades présentant une forte divergence dans la plupart des morphes de *N. apicalis*, et qui correspondent très probablement à des variations géographiques. Il n'y a en outre pas de correspondance entre la similarité chimique et la distance génétique entre les morphes.

Nos résultats montrent une forte divergence génétique entre les différentes espèces et morphes de ce complexe. Associés aux différences de profils chimiques et aux études précédentes, ils soutiennent fortement la présence de 11 espèces valides dans le complexe *N. apicalis*.

Article 1

Chemical taxonomy and phylogenetic relationships of the *Neoponera apicalis* species complex (Hymenoptera: Formicidae: Ponerinae)

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ABSTRACT

Inferring species boundaries is of crucial importance in the biological sciences. This task however often proves to be challenging because many species exhibit a very high morphological similarity, and their diversity remains cryptic. In this context, multidisciplinary studies can be useful to circumvent this issue and delimit species boundaries. Here we used an integrative approach combining chemical taxonomy based on cuticular hydrocarbon profiles and molecular phylogeny using mitochondrial DNA to clarify the taxonomic status of *Neoponera apicalis* ants, a complex of closely related and partly sympatric cryptic species. We found that each morph has a distinct chemical profile that can be discriminated using only a few morph-specific compounds. Within-morph variability can be high, reflecting geographic influences and possible hybridization between diverging lineages. These results were largely corroborated when integrated within the molecular phylogeography, which showed that all morphs represent distinct well-supported clades, whereas the intra-morph variability can be mostly explained by geographic differences. Phylogenetic relationships between morphs were however mostly unresolved. Overall this study highlights the usefulness of cuticular hydrocarbons as a taxonomic tool, and strongly suggests that the *N. apicalis* complex includes at least 11 distinct species.

Keywords: Ants, cryptic species, cuticular hydrocarbons, mitochondrial DNA, *Neoponera* (formerly *Pachycondyla*) *apicalis*, taxonomy.

INTRODUCTION

One of the major goals of the biological sciences is to characterise the diversity of life. Identifying species and delimiting species boundaries is therefore of crucial importance, because the species is the fundamental unit in biology (de Queiroz, 2007). Consequently, the defining criteria of this concept have been much debated (Hey, 2006), particularly since Mayr's (1942) introduction of the biological species concept. Indeed, the widely adopted view of species as populations composed of interbreeding individuals that are reproductively isolated from other populations (Mayr, 1942) has been challenged by more than 20 alternatives (e.g. the phenetic, evolutionary, ecological, or phylogenetic species concepts; Mayden, 1997), thus causing a great confusion since the same word is used to encompass different biological entities (de Queiroz, 2007; Hey, 2006). More recently, syntheses of all these approaches have been proposed. The unified species concept then sees species as separately evolving lineages (de Queiroz, 2007), without the necessity for the additional constraining criteria of previous definitions.

Investigating species boundaries (i.e. alpha-taxonomy) is crucial for studies in ecology, behaviour and evolution (Sites & Marshall, 2003). Morphology has traditionally been the principal mean to infer species boundaries (Schlick-Steiner et al., 2010; Seppä et al., 2011), but this approach may sometimes fail to distinguish different species, for example because of stabilizing selective pressures resulting in a morphological stasis. This is particularly true in the case of cryptic species, i.e. when two or more distinct but morphologically very similar species are erroneously classified, and hidden, under one species name (Bickford et al., 2007). Many methods have been proposed to infer species boundaries (Wiens & Servedio, 2000; Sites & Marshall, 2003; Leaché et al., 2009; Guillot et al., 2012; Puillandre et al., 2012), but a great emphasis has been put during the last decade –although their use in taxonomy was already common– on molecular characters, particularly mitochondrial DNA genes, to infer species boundaries and recreate their evolutionary history (Hebert et al., 2003; Ratnasingham & Hebert, 2007). The contribution of genetics in this field is indisputable, but molecular characters are, as many other traits, continuous, making the delimitation of species boundaries a challenge often epitomized by the question "How different is different enough?" In this context, a growing number of studies seek to combine multiple disciplines –including morphology, mitochondrial or nuclear DNA, enzymes, chemistry, cytogenetics, behaviour and

ecology– to cope with the potential drawbacks of each separate approach, and achieve a much better resolution of the status of potential or actual species (i.e. integrative taxonomy; Padial et al., 2010; Schlick-Steiner et al., 2010; Seppä et al., 2011; Yeates et al., 2011).

Chemical taxonomy in particular has proved to be successful in various taxa (e.g. Erpenbeck & van Soest, 2007; Reynolds, 2007; Bagnères & Wicker-Thomas, 2010). Insects have been the focus of many studies (Bagnères & Wicker-Thomas, 2010; Kather & Martin, 2012), because of the great importance of chemical communication in this taxon (Howard, 2003; Howard & Blomquist, 2005; d'Ettorre & Lenoir, 2010). Therefore, many groups of insects have been investigated, including aphids (Raboudi et al., 2005), beetles (Page et al., 1997), cockroaches (Saïd et al., 2005; Everaerts et al., 2008), fruit flies (Etges & Jackson, 2001; Rouault et al., 2001), grasshoppers (Broza et al., 2000), mosquitoes (Pappas et al., 1994), and the social insects: ants (Vander Meer & Lofgren, 1989; Bagnères et al., 1991; Akino et al., 2002; Elmes et al., 2002; Lucas et al., 2002; Steiner et al., 2002; Schlick-Steiner et al., 2006; Dahbi et al., 2008; Martin et al., 2008a; Drescher et al., 2010; Evison et al., 2012), bees (Carlson et al., 1991; Leonhardt et al., 2013), termites (Kaib et al., 1991; Clément et al., 2001; Dronnet et al., 2006; Haverty & Nelson, 2007) and wasps (Bruschini et al., 2007). Some studies have focused on venom compounds, such as alkaloids (Jones et al., 2003) or peptides (Touchard et al., 2014), but the vast majority has investigated cuticular hydrocarbons (CHCs) as the main chemotaxonomic tool (Bagnères & Wicker-Thomas, 2010; Kather & Martin, 2012). CHCs are long-chained molecules forming the major part of the lipid layer covering insects' cuticle, where they play both waterproofing and communicating functions (Howard & Blomquist, 2005; Richard & Hunt, 2013). CHCs are genetically heritable (Lahav et al., 2001; Shirangi et al., 2009; van Zweden et al., 2010) and are thus typically species-specific (Martin & Drijfhout, 2009a). These molecules are also influenced by physiological and environmental factors (Howard & Blomquist, 2005), and they may signal a range of features, including sex, colony, caste or reproductive status (Greene & Gordon, 2003; Howard & Blomquist, 2005; Dani, 2006; Monnin, 2006; d'Ettorre and Lenoir, 2010; Liebig, 2010). CHC-based alpha taxonomy has several potential strengths (Kather & Martin, 2012), including a close correspondence of chemical and genetic distances between species (Drescher et al., 2010), a high variation of chemical profiles allowing an easier discrimination of close species compared with morphological

characters (Seppä et al., 2011; Guillem et al., 2012), and consequently a high potential for identifying cryptic species (Page et al., 1997; Clément et al., 2001; Akino et al., 2002; Lucas et al., 2002; Schlick-Steiner et al., 2006; Martin et al., 2008a).

Cryptic species are supposed to be widespread in the Formicidae. Indeed, Seifert (2009) has estimated between 40% and 50% of *Cardiocondyla*, *Formica* and *Lasius* species to be cryptic, and predicted similar high ratios in other ant genera. In this context, integrative taxonomy combining CHCs with other –particularly molecular– characters may be highly relevant in inferring species boundaries in ants. Examples where CHCs have been used to distinguish between different species have been reported in the four major ant subfamilies: Formicinae, 13 species of *Formica* (Martin et al., 2008a), two species of *Lasius* (Cremer et al., 2008); Dolichoderinae, five species of *Tapinoma* (Berville et al., 2013); Myrmicinae, two species of *Solenopsis* and their hybrid (Vander Meer & Lofgren, 1989), two species in the *Temnothorax lichtensteini* complex (Csősz et al., 2014), seven species in the *Tetramorium caespitum*/*impurum* complex (Schlick-Steiner et al., 2006); Ponerinae, three species in the *Neoponera foetida* complex (Lucas et al., 2002).

The *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) *apicalis* complex (Hymenoptera: Formicidae: Ponerinae) regroups cryptic and largely sympatric species distributed from South Mexico to Paraguay (Kempf, 1972; Wild, 2005; Delabie et al., 2008). These ants have been the subject of many investigations related to colony social organization (Hölldobler, 1985; Fresneau & Dupuy, 1988), foraging (Duelli & Duelli-Klein, 1976; Fresneau, 1985; Goss et al., 1989), nesting preferences and emigration (Levings & Franks, 1982; Traniello & Hölldobler, 1984; Pezon et al., 2005), life-history traits (Fresneau, 1994), reproduction (Fresneau, 1984; Oliveira & Hölldobler, 1990, 1991; Düssmann et al., 1996; Dietemann & Peeters, 2000; Gobin et al., 2003, 2006; Blacher et al., 2010; Evison et al., 2012; Yagound et al., 2014), chemical ecology (Soroker et al., 1998, 2003; Hefetz et al., 2001), venom characteristics (Schmidt et al., 1980; Cruz-López & Morgan, 1997), glands structure (Hölldobler & Engel-Siegel, 1982; Traniello & Hölldobler, 1984; Lommelen et al., 2002), acoustic communication (Giovannotti, 1996; Pavan et al., 1997; Ferreira et al., 2010) and cytogenetics (Mariano et al., 2011), but their taxonomy remains unresolved.

Until recently, ants in this complex were divided in two species following Brown (1957): *N. apicalis* (Latreille, 1802) and *N. obscuricornis* (Emery, 1890). In his revision of the

complex, Wild (2005) established a third species, *N. verenae* (Forel, 1922), which in fact corresponds to what has previously been referred to as *N. obscuricornis*, with the true *N. obscuricornis* being rarely found. He further noted an important morphological variability within *N. apicalis* and *N. verenae* in almost all characters considered, but attributed this variance to geographical variation over a north-south cline, with no justification for further division of the complex (Wild, 2005). Later, Mackay and Mackay (2010) introduced *N. cooki* (Mackay & Mackay, 2010) based also on morphological characters, corresponding to a current state of four described species in the complex. Meanwhile, Delabie et al. (2008) reassessed this intraspecific variability based on morphological, cytogenetic and ecological characters and interpreted it as a mosaic of cryptic and partly sympatric species whose differences are consistent across their geographical range. They described four taxa within *N. apicalis* (morphs 1, 2, 3 and 4) and two within *N. verenae* (morphs 1 and 2), but without concluding concerning their status as valid species. Using bioacoustics and mitochondrial DNA variation, Ferreira et al. (2010) have since then corroborated these results for *N. verenae* morph 1 and *N. apicalis* morphs 3 and 4. These authors have further introduced a new morph, *N. apicalis* morph 6, and suggested that all *N. apicalis* and *N. verenae* morphs should be considered as valid species (Ferreira, 2010). Finally, Evison et al. (2012) have shown that the two geographically separated morphs of *N. verenae* 1 and 2 have distinct CHC profiles.

These multiple lines of evidence therefore strongly suggest that the *N. apicalis* complex is composed of many more species than the currently four recognised species, but the possibility that these are subspecies cannot be completely ruled out, given the fact that many ant species show considerable geographic variation (Ward, 2011). In this study we therefore sought to clarify the taxonomic status of the *N. apicalis* complex by combining chemical (CHCs) and molecular (mitochondrial DNA) characters in most of the species/morphs currently recognised in this complex. This was aimed at i) confirming the status of *N. apicalis* and *N. verenae* morphs as distinct species, and ii) assessing the validity of CHCs as a taxonomic tool to infer species boundaries in this complex.

METHODS

Ants

We used colonies or individual samples collected in Brazil (Belém [PA] 01°22'44''S, 48°17'25''W; Itabuna [BA] 14°46'28"S, 39°13'20"W; Una [BA] 15°16'42"S, 39°05'06"W; Viçosa [MG] 20°46'02"S, 42°52'01"W), Colombia (reserva El Caduceo en San Martín [Meta] 03°40'16"N, 73°39'38"W), French Guiana (Camp Patawa, 04°17'13"N, 52°11'12"W; Montagne des Singes, 05°04'26"N, 52°41'56"W; Petit Saut, 05°04'16"N, 53°02'36"W; Saut Sabbat, 05°23'48"N, 53°41'44"W), Mexico (Los Tuxtlas [VE], 18°28'13"N, 95°05'42"W; Miguel Hidalgo y Costilla [QR], 18°51'28"N, 88°22'07"W; Volcán Tacaná [CP], 15°04'32"N, 92°12'20"W), and Peru (Villa Carmen [MDD], 12°52'16"S, 71°24'36"W) between 2001 and 2013 (Table 1). Ants were classified in their respective species/morphs according to Delabie et al. (2008), Ferreira et al. (2010), Mackay and Mackay (2010), and previous observations on CHCs and genetic sequences that revealed the existence of a new morph, *N. apicalis* morph 7 (Yagound et al., unpublished data).

Experimental Design

Two sets of analyses were carried out. We first investigated the CHC profile of several species of the *N. apicalis* complex (five morphs of *N. apicalis*, *N. cooki* and one morph of *N. verenae*) and studied their chemical similarity. We then investigated the phylogenetic relationships between all but one species of the complex and compared the molecular phylogeny with the chemical taxonomy.

Cuticular Hydrocarbon Profiles

We determined the CHC profiles of 869 workers belonging to seven species/morphs of the *N. apicalis* complex (Table 1). All individuals were sampled from newly established queenless groups of workers (see Yagound et al., 2014). We additionally determined the CHC profiles of two workers from the related species *N. villosa* that was used as outgroup for the chemical clustering (see below).

Table 1. Colonies used in this study.

Species	Origin	Collection date	Sample size of colonies (individuals)		Colonies used in both analyses
			Chemical analyses	Genetic analyses	
N. apicalis morph 1	Itabuna, BA, Brazil	2008/2010	7 (226)	16 (20)	3
	Una, BA, Brazil	2008	1 (26)	n/a	n/a[a]
N. apicalis morph 2	Petit Saut, French Guiana	2007/2011	n/a	4 (4)	n/a
	Reserva El Caduceo en San Martín, Meta, Colombia	2012	n/a	2 (4)	n/a
	Villa Carmen, MDD, Peru	2013	n/a	2 (2)	n/a
N. apicalis morph 3	Volcán Tacaná, CP, Mexico	2011	2 (63)	3 (3)	2
	Miguel Hidalgo y Costilla, QR, Mexico	2011	n/a	1 (1)	n/a
	Los Tuxtlas, VE, Mexico	2001	n/a	1 (1)	n/a
N. apicalis morph 4	Petit Saut, French Guiana	2007/2011	6 (223)	10 (19)	1
	Saut Sabbat, French Guiana	2011	n/a	2 (3)	n/a
N. apicalis morph 6	Petit Saut, French Guiana	2007/2011	1 (29)	6 (12)	1
	Belém, PA, Brazil	2008	n/a	2 (1)	n/a
N. apicalis morph 7	Petit Saut, French Guiana	2011	3 (100)	6 (13)	3
	Montagne des Singes, French Guiana	2011	n/a	1 (1)	n/a
	Villa Carmen, MDD, Peru	2013	n/a	5 (5)	n/a
N. verenae morph 1	Camp Patawa, French Guiana	2007	1 (34)	n/a	n/a[a]
	Petit Saut, French Guiana	2007/2011	n/a	4 (6)	n/a
	Una, BA, Brazil	2008	1 (34)	n/a	n/a[a]
	Belém, PA, Brazil	2008	3 (96)	n/a	n/a
	Reserva El Caduceo en San Martín, Meta, Colombia	2012	n/a	2 (2)[b]	n/a
	Villa Carmen, MDD, Peru	2013	n/a	2 (2)	n/a
N. verenae morph 2	Viçosa, MG, Brazil	2009	n/a	1 (1)	n/a
N. cooki	Petit Saut, French Guiana	2007/2011	1 (38)	4 (4)	1
	Saut Sabbat, French Guiana	2011	n/a	1 (2)	n/a

n/a, not applicable.

[a] determined through additional analyses using mtDNA cytochrome *b*.

[b] one of these individuals corresponds to the later identified *N. verenae* morph 3.

Extraction was performed by placing an ant in 400 µl of pentane containing 8 ng/µl of an internal standard (*n*-C_{17}) for 20 min. We then transferred 100 µl into a 200 µl glass insert. Following evaporation, 20 µl of pentane were added to the 200 µl glass insert. We then manually injected 2 µl of the extract into a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an HP-5MS capillary column (30 m × 25 µm × 0.25 µm) and a split–splitless injector, coupled to a 5975c mass spectrometer (Agilent Technologies) with 70 eV electron impact ionization. The carrier gas was helium at 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 1 min, then 70–180°C at 30°C/min, then 180–320°C at 5°C/min, then hold at 320°C for 5min. The areas of the peaks in the chromatogram were integrated with the MSD ChemStation software E.02.01.1177 (Agilent Technologies). Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared with known standards. Several compounds eluted in the same peak and were considered as a single variable in subsequent analyses. We obtained a total of 122 peaks of which 48 were specific to *N. villosa*, which gives a total of 74 peaks for the *N. apicalis* complex (Figure S1 and Table S1).

Genetic Analyses

We sampled the DNA of 107 workers from 78 colonies of the *N. apicalis* complex (Table 1). This sample was thus much greater than the one used in the study of CHC profiles. The correspondence between colonies sampled in the chemical and molecular studies was unfortunately not always perfect, but in this case additional data (morphological characters, mtDNA cytochrome *b* and microsatellite loci variations, data not shown) confirmed the identification of the morph. We further added to the analysis the data of *N. apicalis* and *N. verenae* of Costa Rica from Smith et al. (2014) collected from GenBank, and 2 individuals of *N. villosa* were used as outgroup for the construction of the trees.

DNA was extracted from the head and thorax of workers preserved in ethanol in 500 µl of a 10% Chelex® 100 (Bio-Rad, Hercules, CA, USA) solution with 20 µl of proteinase K (Promega, Madison, WI, USA) at 10 mg/ml, incubated at 55°C for 40 min, then boiled at 100°C for 20 min. We analysed the mitochondrial DNA variation by amplifying a portion of the mtDNA cytochrome *c* oxidase 1 (COI, 710 base pairs) using the primers LCO1490

and HCO2198 (Folmer et al., 1994). Polymerase chain reactions (PCRs) were performed according to standard protocols in a Biometra TProfessional thermocycler (Labgene Scientific, Châtel-St-Denis, Switzerland). PCR conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing step at 50°C for 30 s and extension step at 72°C for 1 min, then a final extension step at 72°C for 10 min. Amplified products were sequenced with the same primers by Genoscreen (Lille, France) using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequences analyses were edited and aligned using the default settings of Clustal X (Thompson et al., 1997) and checked by eye. Ambiguous stretches at the 5' and 3' ends of the fragments were removed, resulting in fragments of 625 bp for further analyses. We checked the sequences for any problems (e.g inversions, deletions, or stop codons) visually and by translating with MEGA 6 (Tamura et al., 2013) a sequence of each morph to compare our amino acid sequences to published *Neoponera*'s ones (referred to as *Pachycondyla* in GenBank). The homology of our COI amino acid sequences reached 98% with published sequences from Smith et al. (2014).

Statistical Analyses

We calculated for each individual profile the relative quantities of each compound and the proportion of each major class of CHCs (i.e. saturated, methyl-branched and unsaturated) and compared them between all morphs using one-way ANOVAs. We also calculated the equivalent chain length of each compound according to Mjøs (2006). To investigate the existence of between-morphs differences in the CHC profiles, we first performed a non-metric multi-dimensional scaling (NMDS; Kruskal & Wish, 1978) using the Bray-Curtis coefficient of similarity (Bray & Curtis, 1957) of square-root transformed relative quantities of all CHCs (varying from 0% meaning complete dissimilarity to 100% meaning total similarity) using PRIMER 6.1.13 (Clarke & Gorley, 2006). The NMDS aimed at representing the best configuration of the closeness of the CHC profiles between groups by minimizing the degree of distortion (or stress) between the distance of the points in the NMDS plot and their Bray-Curtis coefficient of similarity (Clarke & Warwick, 2001). This ordination procedure was followed by an analysis of similarities (ANOSIM; Clarke, 1993) which uses a non-parametric randomisation test

contrasting the extent of within-group dissimilarity with the between-group dissimilarity (varying from $R = 0$ meaning that the groups investigated have no dissimilarity to $R = 1$ meaning that they are perfectly dissimilar). We then tested for between-groups statistical differences in the CHC profiles using permutational multivariate analysis of variance based on distances (PERMANOVA; Anderson, 2001) with PRIMER 6.1.13 and the PERMANOVA+ 1.0.3 add on package (Anderson et al., 2008). These analyses were also performed within each morph to test for between-colony differences and for investigating the existence of chemotypes (i.e. groups of individuals chemically similar at an intermediate level between the colony and the morph). Furthermore, we re-performed these analyses for each class of CHCs to test for their relative importance in between-group discrimination. We finally investigated the general chemical relationships between all morphs by constructing a chemotaxonomic tree diagram. We calculated for each colony the mean CHC profile by averaging the proportions of each peak between all nestmates. To further reduce the influence of major peaks on the analysis, we fourth-root transformed (Clarke & Warwick, 2001) the average colony profiles obtained and constructed a dendogram through hierarchical clustering (CLUSTER; Everitt, 1980) based on Bray-Curtis coefficient of similarity between all colonies of the *N. apicalis* complex and *N. villosa* as outgroup using PRIMER 6.1.13. This classification procedure allowed to form natural groups based on their relative similarities in CHC profiles (Clarke & Warwick, 2001).

The genetic analyses involved 125 nucleotide sequences and were conducted using the Kimura 2-parameter model (Kimura, 1980). We used pairwise distances (neighbour-joining algorithm, NJ; Saitou & Nei, 1987) to generate phylogenetic trees and tested the robustness of the tree with 1000 bootstrap replications. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 (Tamura et al., 2013). Moreover, we tested for each morph the correlation between the genetic distance (Kimura 2-parameter model) and the (ln-transformed) spatial distance using Mantel tests (Mantel, 1967) with 20 000 permutations as implemented in FSTAT 2.9.3 (Goudet, 2001).

Unless otherwise stated, statistical analyses were performed using R-3.2.0 (R Core Team, 2012). Post-hoc corrected *P*-values following the Bonferroni–Holm method (Holm, 1979) are denoted *P'*. Statistical significance was set at $P < 0.05$.

RESULTS

Cuticular Hydrocarbon Profiles

Between-group differences in CHC profiles

The CHC profiles of all morphs of the *N. apicalis* complex was composed of 19 to 41 peaks and comprised several series of *n*-alkanes, mono and dimethyl-branched alkanes, and alkenes and alkadienes with carbon number ranging from 19 to 37 (Figure S1 and Table S1). We found important differences in the chemical profiles between and sometimes within morphs. The compounds differed in their number of carbon between morphs (range of equivalent chain length: *N. apicalis* morph 1, 19.5–33.4; *N. apicalis* morph 3, 20.0–29.0; *N. apicalis* morph 4, 19.2–31.0; *N. apicalis* morph 6, 20.9–29.0; *N. apicalis* morph 7, 20.0–33.6; *N. verenae* morph 1, 21.0–29.0; *N. cooki*, 25.0–37.5; one-way ANOVA: $F_{6,202} = 14.85$, $P < 0.0001$), with *N. cooki* having the compounds with the longest chains (post-hoc all $P' < 0.0035$). Morphs also diverged in their proportions of saturated ($F_{6,865} = 180.87$, $P < 0.0001$, all $P' < 0.0094$ except for the comparison between *N. apicalis* morphs 3 and 7 $P' = 0.60$), methyl-branched ($F_{6,865} = 297.37$, $P < 0.0001$, all $P' < 0.0084$ except for the comparison between *N. apicalis* morph 1 and *N. verenae* morph 1 $P' = 0.13$) and unsaturated CHCs ($F_{6,865} = 130.56$, $P < 0.0001$, all $P' < 0.0010$; Figure 1). Depending on the morph, linear alkanes or alkenes constituted the major class of compounds in all *N. apicalis* and *N. verenae* morphs, and together they represented between 86.7% and 99.7% of the CHC profile. There was a marked difference in the chemical profile of *N. cooki*, with methyl-branched CHCs representing 76.3% of the profile (Figure 1). Noteworthy was the existence of important within-morph variations in the contribution of linear alkanes, methyl-branched alkanes and alkenes (between-colony gap of 23.2%, 16.2% and 24.6%, respectively) in *N. apicalis* morph 1. Likewise, one colony of *N. verenae* morph 1 from French Guiana had inversed proportions of methyl-branched and unsaturated CHCs (respectively 53.7% and 2.9%) compared with the other colonies from Brazil (2.6% and 48.7%). In contrast, these proportions were very similar (maximum between-colony gap of 3.1–7.9%) in *N. apicalis* morphs 3, 4 and 7. The extent of within-morph variation in *N. apicalis* morph 6 and *N. cooki* remains unknown due to smaller sample sizes. Linear alkanes, methyl-branched alkanes and alkenes had similar chain lengths in all morphs, except in *N. cooki* where linear alkanes

tended to have smaller chain lengths compared with methyl-branched and unsaturated CHCs.

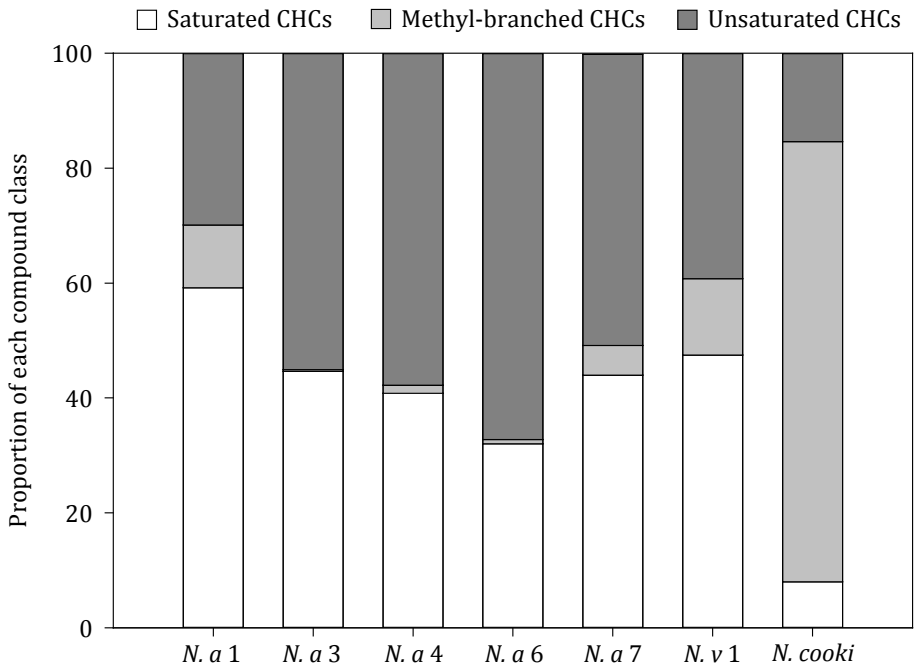

Figure 1. Mean proportion of each major class of CHCs in the chemical profiles of all morphs. *N. a*, *N. apicalis*; *N. v*, *N. verenae*.

All morphs could clearly be separated on the basis of their CHC profiles in a non-metric multi-dimensional scaling (Figure 2). *N. verenae* morph 1 showed a much higher within-morph variation than the other morphs, with three clusters relatively well separated corresponding to the three geographic areas (Bahia and Pará states in Brazil, and French Guiana) from where the colonies originated. All morphs had significantly different CHC profiles (PERMANOVA: Pseudo-$F_{7,871}$ = 398.89, P < 0.001; pairwise comparisons: all P < 0.003). Furthermore, there was a high and significant level of dissimilarity between all morphs (ANOSIM: global R = 0.89, P < 0.001; pairwise comparisons: all R > 0.35, P < 0.001; when excluding the comparisons with *N. verenae* morph 1, all R > 0.94, P < 0.001). This was also the case for *N. apicalis* morphs 3 and 7 (R = 0.95, p < 0.001) despite them being close in the NMDS analysis (Figure 2). Including the area of origin, and thus considering three different groups for *N. verenae* morph 1 further improved the separation of the taxa (ANOSIM: global R = 0.96, P < 0.001; pairwise comparisons: all R > 0.79, P < 0.001). Moreover, when we considered only the nine major peaks present in

the different morphs (*x*-$C_{21:1b}$, *n*-C_{21}, *x*-$C_{23:1a}$ + *x*,*y*-$C_{23:2a}$, *x*-$C_{23:1b}$ + *x*,*y*-$C_{23:2b}$, *x*-$C_{23:1c}$, *n*-C_{23}, *x*-$C_{25:1c}$, *n*-C_{25}, and *x*-$C_{27:1b}$ + *x*,*y*-$C_{27:2}$), recalculated the relative quantities accordingly and re-performed the analysis, we found that these few compounds were sufficient to trigger a very good discrimination between all morphs (PERMANOVA: Pseudo-$F_{8,869}$ = 963.50, P < 0.001; pairwise comparisons: all P < 0.001; ANOSIM: global R = 0.94, P < 0.001; pairwise comparisons: all R > 0.61, P < 0.001). Where it could be calculated, between-colony similarity was generally high within all morphs (*N. apicalis* morph 1: 73.4 ± 1.5%; *N. apicalis* morph 3: 86.5%; *N. apicalis* morph 4: 86.1 ± 1.5%; *N. apicalis* morph 7: 81.1 ± 0.4%), except in *N. verenae* morph 1 (56.6 ± 3.8%) unless the area of origin was taken into account (79.5 ± 1.5%).

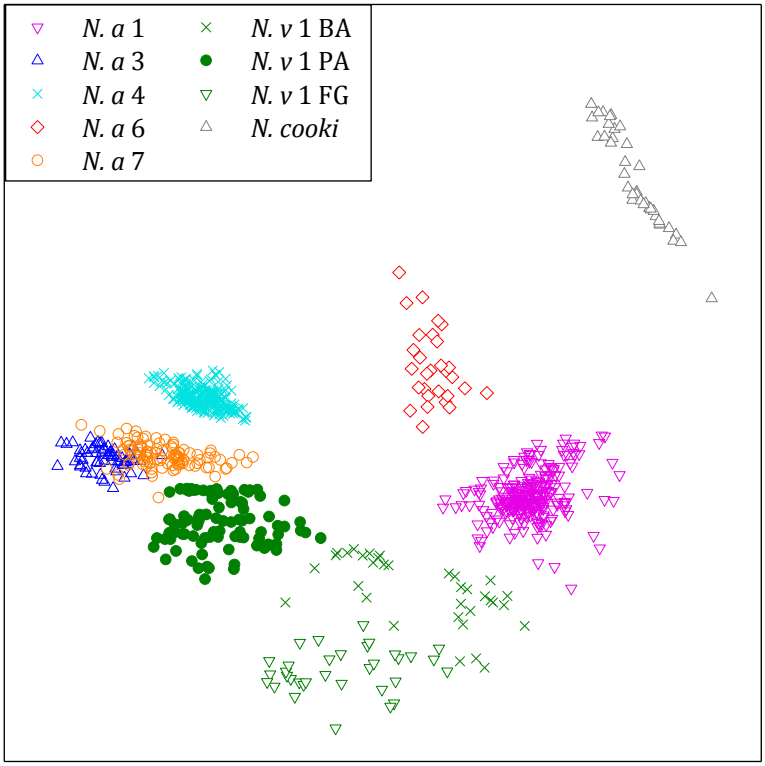

Figure 2. NMDS plot of the CHC profiles of all morphs and of geographic origin for *N. verenae* morph 1 (2D stress = 0.13). *N. a*, *N. apicalis*; *N. v*, *N. verenae*; BA, Bahia; PA, Pará; FG, French Guiana.

When looking at each morph separately, we found that all colonies had significantly different (PERMANOVA: *N. apicalis* morph 1, Pseudo-$F_{7,252}$ = 14.42, P < 0.001; pairwise comparisons: all P < 0.001; *N. apicalis* morph 3, Pseudo-$F_{1,63}$ = 33.42, P < 0.001; *N. apicalis* morph 4, Pseudo-$F_{4,223}$ = 42.74, P < 0.001; pairwise comparisons: all P < 0.001; *N. apicalis* morph 7, Pseudo-$F_{2,100}$ = 27.50, P < 0.001; pairwise comparisons: all P < 0.001; *N. verenae* morph 1, Pseudo-$F_{4,164}$ = 30.77, P < 0.001; pairwise comparisons: all P < 0.001) and dissimilar CHC profiles (ANOSIM: *N. apicalis* morph 1, global R = 0.66, P < 0.001, pairwise comparisons: all R > 0.12, P < 0.001; *N. apicalis* morph 3, global R = 0.59, P < 0.001; *N. apicalis* morph 4, global R = 0.68, P < 0.001, pairwise comparisons: all R > 0.46, P < 0.001; *N. apicalis* morph 7, global R = 0.59, P < 0.001, pairwise comparisons: all R > 0.49, P < 0.001; *N. verenae* morph 1, global R = 0.88, P < 0.001, pairwise comparisons: all R > 0.57, P < 0.001).

Within several morphs we also found the existence of significantly different chemotypes which were dissimilar from each other. Three chemotypes could be identified in *N. apicalis* morph 1 (PERMANOVA: Pseudo-$F_{2,252}$ = 88.20, P < 0.001; pairwise comparisons: all P < 0.001; ANOSIM: global R = 0.86, P < 0.001, pairwise comparisons: all R > 0.82, P < 0.001; Figure S2A). Interestingly one colony comprised individuals belonging to two different chemotypes, with an even distribution (57.1% of individuals in one chemotype and 42.9% in the other). Two chemotypes were found in *N. apicalis* morph 4 (PERMANOVA: Pseudo-$F_{1,223}$ = 59.72, P < 0.001; ANOSIM: global R = 0.90, P < 0.001; Figure S2B). As previously stated, three chemotypes corresponding to the geographic area were identified in *N. verenae* morph 1 (PERMANOVA: Pseudo-$F_{2,164}$ = 44.85, P < 0.001; pairwise comparisons: all P < 0.001; ANOSIM: global R = 0.93, P < 0.001, pairwise comparisons: all R > 0.83, P < 0.001; Figure S2C).

Effect of CHC class on between-group discrimination

When considering each major class of CHCs separately, we found that all three saturated (ANOSIM: global R = 0.83, P < 0.001; pairwise comparisons: all R > 0.48, P < 0.001), methyl-branched (ANOSIM: global R = 0.95, P < 0.001; pairwise comparisons: all R > 0.79, P < 0.001) and unsaturated CHCs (ANOSIM: global R = 0.96, P < 0.001; pairwise comparisons: all R > 0.79, P < 0.001) were sufficient to accurately separate all the

morphs, with however a higher degree of between-morphs dissimilarity for methyl-branched and unsaturated CHCs. This was not surprising for alkenes, particularly with 23 (or occasionally 21 or 27) carbons, which represented a large part of the chemical profile (Figure 1). In contrast, methyl-branched CHCs were minority compounds (except in *N. cooki* and *N. verenae* morph 1 from French Guiana) but they nevertheless exhibited a high degree of dissimilarity between morphs. The level of between-colony dissimilarity was the highest for alkenes and the lowest for methyl-branched alkanes in all morphs (ANOSIM on linear alkanes, methyl-branched alkanes and alkenes, respectively: *N. apicalis* morph 1, $R = 0.53$, $R = 0.33$ and $R = 0.72$, all $P < 0.001$; *N. apicalis* morph 3, $R = 0.44$, $R = 0.08$ and $R = 0.56$, all $P < 0.001$; *N. apicalis* morph 4, $R = 0.38$, $R = 0.38$, $R = 0.68$, all $P < 0.001$; *N. apicalis* morph 7, $R = 0.39$, $R = 0.23$ and $R = 0.68$, all $P < 0.001$; *N. verenae* morph 1, $R = 0.74$, $R = 0.66$ and $R = 0.84$, all $P < 0.001$). In contrast, the effect of CHC class on between-chemotype dissimilarity was morph-specific (ANOSIM on linear alkanes, methyl-branched alkanes and alkenes, respectively: *N. apicalis* morph 1, $R = 0.66$, $R = 0.59$ and $R = 0.77$, all $P < 0.001$; *N. apicalis* morph 4, $R = 0.09$, $P = 0.020$, $R = 0.23$ and $R = 0.95$, both $P < 0.001$; *N. verenae* morph 1, $R = 0.74$, $R = 0.99$ and $R = 0.85$, all $P < 0.001$).

Chemotaxonomy

The CLUSTER analysis showed that all colonies of a particular morph systematically grouped together (Figure 3). In accordance with the NMDS analysis, *N. cooki* was highly different (only 30.4% of similarity) from all *N. apicalis* and *N. verenae* morphs which clustered in two groups. One cluster was composed of *N. apicalis* morphs 3, 4 and 7 (67.6% of similarity), and the other comprised a cluster composed of *N. apicalis* morphs 1 and 6 (61.4% of similarity) and a cluster comprising *N. verenae* morph 1. In this morph, the colony from French Guiana was highly dissimilar (55.6% of similarity) from the Brazilian colonies.

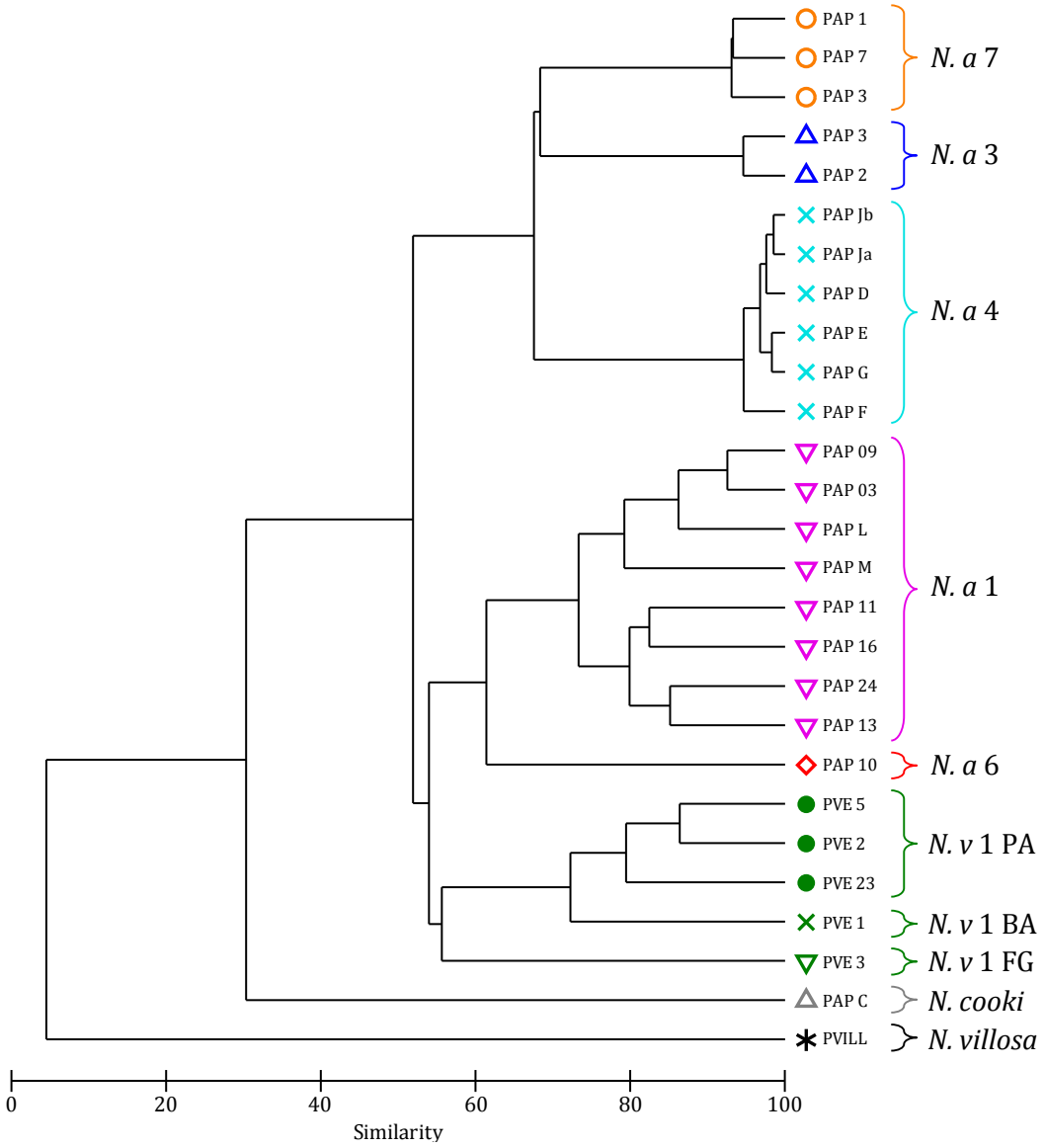

Figure 3. Chemical dendogram of the mean CHC profiles of all colonies. *N. a*, *N. apicalis*; *N. v*, *N. verenae*; BA, Bahia; PA, Pará; FG, French Guiana.

Mitochondrial Phylogeny

There were a total of 624 base pairs analysed for COI in the final dataset. We found a clear A-T bases compositional bias (respective average frequencies for A, T, C and G of 32.5%, 41.0%, 15.7% and 10.8%), as frequently observed in insect mitochondrial genomes (Cameron, 2014). The number of variable sites was 206, among which there were 167 parsimony-informative sites. The estimated transition/transversion bias was 2.74. There were three main clusters in the NJ phylogenetic tree, namely *N. apicalis* morphs, *N. verenae* morphs and *N. cooki* (Figure 4). These groups showed a very high genetic divergence (mean range 12.4–14.4%; Table 2). Though *N. cooki* was genetically the most distant species (Table 2), the phylogenetic relationships between these three taxa were not resolved (Figure 4). Within *N. verenae* morphs, three distinct clades were obtained, i.e. *N. verenae* morphs 1, 2 and a new haplotype named morph 3. This last new morph was found in Colombia in sympatry with *N. verenae* morph 1 (Figure 5), but it had a large genetic distance with the other two *N. verenae* morphs (Table 2). Overall, *N. verenae* morph 1 appeared the most largely distributed, with occurrences in Brazil, Costa Rica, Colombia, French Guiana and Peru, whereas *N. verenae* morph 2 and 3 were only found in Southeastern Brazil (MG) and Colombia, respectively (Figure 5). All *N. cooki* individuals were genetically very similar (Table 2), albeit originating from two distinct but close localities (c. 80 km) in French Guiana. Within *N. apicalis* morphs, we found six well-defined clades corresponding to *N. apicalis* morphs 1, 2, 3, 4, 6 and 7 (Figure 4). The position of *N. apicalis* morph 3 as the sister-group of all other *N. apicalis* morphs was well-supported. The genetic distance between this and the other *N. apicalis* morphs was also high (9.5 ± 0.1% of genetic divergence; Table 2). There were four genetically distinct clades (6.0 ± 0.4% of genetic divergence; Table 2) within this morph distributed in Mexico and Costa Rica (Figure 5). The relationships between the other *N. apicalis* morphs were not resolved by our phylogeny, except for *N. apicalis* morphs 4 and 6 that formed a relatively well-supported clade (Figure 4). *N. apicalis* morph 4 from French Guiana and *N. apicalis* morph 1 from Brazil (BA) were genetically homogeneous (respectively 0.6 ± 0.3% and 0.2 ± 0.1% of genetic divergence). In contrast, *N. apicalis* morphs 2, 6 and 7 comprised respectively three, two and two genetically distinct clades (3.6 ± 0.4%, 3.0 ± 0.1% and 4.7 ± 0.2% of genetic divergence, respectively; Figure 4 and Table 2). For each of these morphs, the different clades corresponded to distinct geographic areas (*N. apicalis* morph 7: French Guiana and Peru; *N. apicalis* morph 2:

French Guiana, Peru and Colombia; *N. apicalis* morph 6: French Guiana and Brazil [PA]; Figure 5).

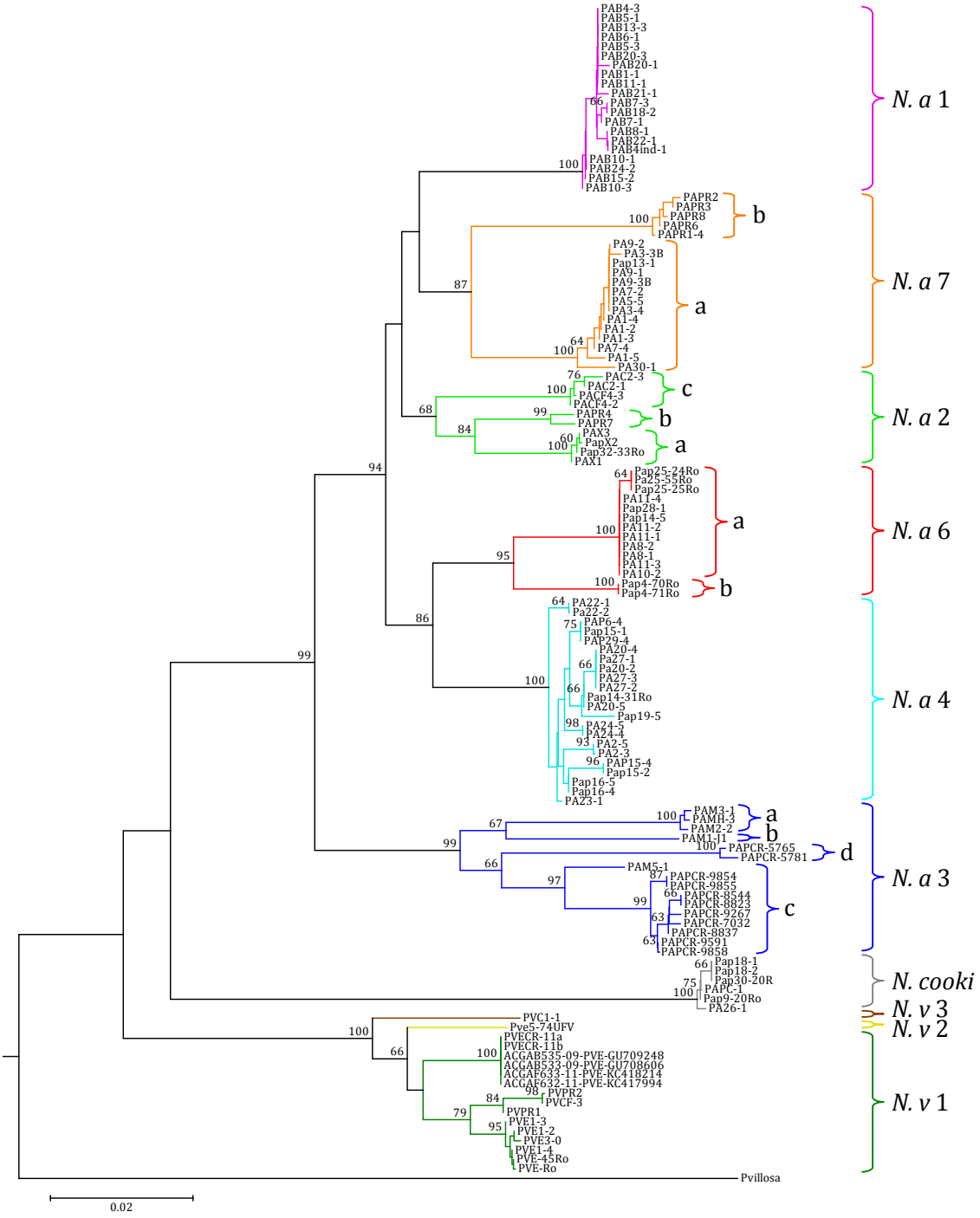

Figure 4. Neighbour-joining phylogenetic tree of the *N. apicalis* species complex based on 624 bp of the COI mtDNA gene. *N. villosa* is used as outgroup. Bootstrap percentages with values > 60 are shown at nodes. Scale bar shows the number of substitutions per site. *N. a*, *N. apicalis*; *N. v*, *N. verenae*.

Table 2. Divergence of COI sequences (mean ± SD) within and between all groups.

	N. a 1	*N. a* 2a	*N. a* 2b	*N. a* 2c	*N. a* 3a	*N. a* 3b	*N. a* 3c	*N. a* 3d	*N. a* 4	*N. a* 6a	*N. a* 6b	*N. a* 7a	*N. a* 7b	*N. v* 1	*N. v* 2	*N. v* 3	*N. cooki*
N. a 1	0.002 ± 0.001																
N. a 2a	0.050 ± 0.002	0.000 ± 0.000															
N. a 2b	0.052 ± 0.002	0.028 ± 0.002	0.007														
N. a 2c	0.053 ± 0.003	0.042 ± 0.003	0.037 ± 0.002	0.002 ± 0.001													
N. a 3a	0.093 ± 0.002	0.094 ± 0.001	0.094 ± 0.000	0.081 ± 0.003	0.002 ± 0.001												
N. a 3b	0.092 ± 0.002	0.100 ± 0.001	0.096 ± 0.000	0.089 ± 0.003	0.050 ± 0.001	n/a											
N. a 3c	0.092 ± 0.003	0.097 ± 0.003	0.094 ± 0.003	0.088 ± 0.004	0.066 ± 0.002	0.052 ± 0.002	0.007 ± 0.008										
N. a 3d	0.096 ± 0.001	0.096 ± 0.002	0.097 ± 0.002	0.094 ± 0.003	0.075 ± 0.001	0.062 ± 0.003	0.055 ± 0.002	0.003									
N. a 4	0.057 ± 0.003	0.056 ± 0.002	0.055 ± 0.002	0.057 ± 0.002	0.094 ± 0.002	0.089 ± 0.002	0.085 ± 0.003	0.098 ± 0.003	0.006 ± 0.003								
N. a 6a	0.063 ± 0.002	0.067 ± 0.001	0.064 ± 0.001	0.058 ± 0.002	0.091 ± 0.001	0.095 ± 0.001	0.086 ± 0.002	0.103 ± 0.002	0.046 ± 0.002	0.001 ± 0.001							
N. a 6b	0.063 ± 0.002	0.051 ± 0.001	0.051 ± 0.001	0.053 ± 0.003	0.093 ± 0.000	0.098 ± 0.000	0.089 ± 0.002	0.105 ± 0.002	0.047 ± 0.002	0.030 ± 0.001	0.000						
N. a 7a	0.049 ± 0.002	0.059 ± 0.002	0.052 ± 0.001	0.051 ± 0.002	0.095 ± 0.002	0.089 ± 0.001	0.095 ± 0.006	0.103 ± 0.001	0.066 ± 0.002	0.057 ± 0.002	0.063 ± 0.002	0.002 ± 0.002					
N. a 7b	0.061 ± 0.002	0.057 ± 0.002	0.067 ± 0.003	0.065 ± 0.003	0.104 ± 0.002	0.098 ± 0.003	0.099 ± 0.004	0.108 ± 0.002	0.070 ± 0.003	0.076 ± 0.002	0.066 ± 0.001	0.047 ± 0.002	0.001 ± 0.001				
N. v 1	0.118 ± 0.003	0.120 ± 0.003	0.108 ± 0.002	0.105 ± 0.002	0.131 ± 0.005	0.129 ± 0.003	0.140 ± 0.003	0.138 ± 0.003	0.126 ± 0.004	0.128 ± 0.003	0.124 ± 0.002	0.118 ± 0.003	0.128 ± 0.002	0.014 ± 0.010			
N. v 2	0.112 ± 0.001	0.117 ± 0.001	0.105 ± 0.003	0.108 ± 0.001	0.133 ± 0.001	0.124	0.145 ± 0.003	0.141 ± 0.003	0.123 ± 0.002	0.133 ± 0.001	0.120 ± 0.000	0.120 ± 0.001	0.125 ± 0.001	0.027 ± 0.003	n/a		
N. v 3	0.114 ± 0.002	0.117 ± 0.001	0.116 ± 0.000	0.111 ± 0.004	0.135 ± 0.003	0.128 ± 0.005	0.143 ± 0.003	0.141 ± 0.002	0.127 ± 0.002	0.129 ± 0.001	0.124 ± 0.000	0.122 ± 0.002	0.126 ± 0.002	0.036 ± 0.002	0.043	n/a	
N. cooki	0.137 ± 0.002	0.129 ± 0.002	0.132 ± 0.003	0.133 ± 0.003	0.139 ± 0.001	0.143 ± 0.001	0.151 ± 0.003	0.166 ± 0.001	0.137 ± 0.003	0.134 ± 0.001	0.127 ± 0.001	0.138 ± 0.002	0.146 ± 0.004	0.144 ± 0.003	0.143 ± 0.001	0.144 ± 0.001	0.001 ± 0.001

n/a, not applicable (these groups contain a single individual).

N. a, *N. apicalis*; *N. v*, *N. verenae*.

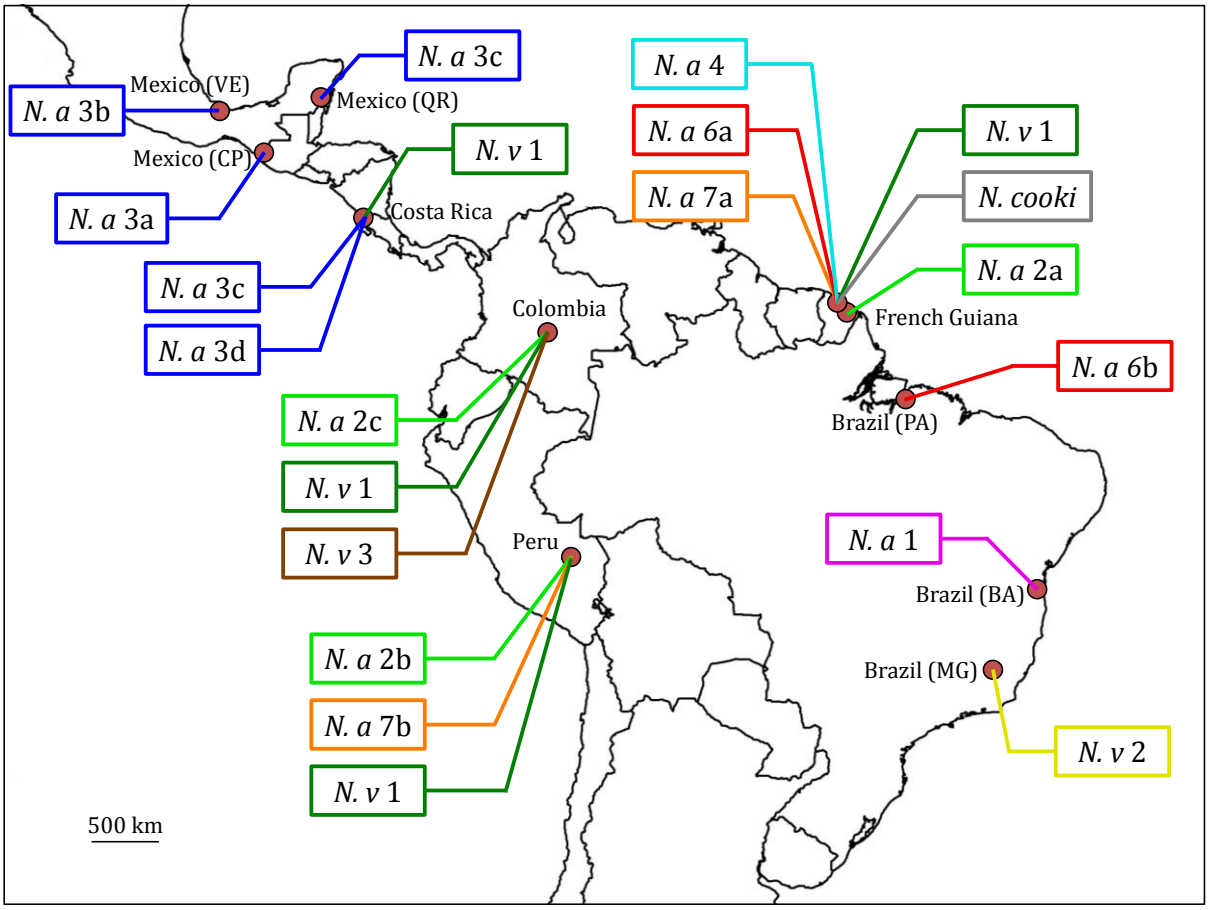

Figure 5. Geographical location of all morphs of the *N. apicalis* species complex sampled in this study.

There was a significant positive correlation between genetic and spatial distances in all morphs (Mantel tests: *N. apicalis* morph 2: $r = 0.93$, $P < 0.0001$; *N. apicalis* morph 3: $r = 0.61$, $P < 0.0001$; *N. apicalis* morph 4: $r = 0.18$, $P = 0.0071$; *N. apicalis* morph 6: $r = 0.99$, $P < 0.0001$; *N. apicalis* morph 7: $r = 0.95$, $P < 0.0001$; *N. verenae* morph 1: $r = 0.78$, $P < 0.0001$; *N. cooki*: $r = 0.68$, $P = 0.020$) except in *N. apicalis* morph 1 ($r = 0.10$, $P = 0.44$). It must however be noted the spatial distances are much smaller within *N. apicalis* morphs 1 and 4.

DISCUSSION

This study used a multidisciplinary approach combining CHC profiles and mitochondrial DNA to discriminate cryptic species in the *Neoponera apicalis* complex and clarify their taxonomic status. The chemical taxonomy showed that the seven taxa studied (*N. apicalis* morphs 1, 3, 4, 6 and 7, *N. cooki* and *N. verenae* morph 1) have clearly distinct CHC profiles. Variations in the chemical profiles are both quantitative (relative amounts of CHCs) and qualitative (chain length, number and position of unsaturations and methyl groups). Although the extent of qualitative differences could have been amplified by technical limitations in the detection of very small peaks, these results are compatible with reflecting CHC profiles belonging to different species (Martin & Drijfhout, 2009a). Linear CHCs represented most of the time the major part of the chemical profile, except in *N. cooki* which by far exhibit the most dissimilar CHC profile in the entire *N. apicalis* complex. Though each morph is characterised by a high level of within group similarity, all colonies could be well discriminated in each case. Thus CHCs appear particularly efficient to differentiate natural groups at various levels of organization (Howard & Blomquist, 2005).

These findings have implications for previous studies investigating the chemical ecology of these ants, but without taking into account the actual variability characterising this species complex. For example, Hefetz et al. (2001) studied the chemical profile of "*N. apicalis*" with two colonies collected in Northeast Brazil and one colony originating from French Guiana, thus almost certainly corresponding to two different morphs (i.e. morphs 1 and probably 4 which is the most abundant in French Guiana). Given the extent of dissimilarity between these two morphs, this implies that their resulting chemical analysis was partly artificial –though the focus of their study showing the role of the postpharyngeal gland and front basitarsal brushes in hydrocarbon circulation remains obviously valid.

Interestingly, we observed the existence of several chemotypes for three out of the seven morphs investigated (*N. apicalis* morphs 1 and 4, *N. verenae* morph 1). The high level of variability in CHC profiles in *N. verenae* morph 1 reflects geographical distance. Indeed, the three chemotypes were located in well separated geographic areas (c. 900–2700 km). Thus their dissimilarity, despite being high, is probably mostly influenced by environmental factors. This is in accordance with Evison et al. (2012) who also reported

geographical variations in CHCs in this morph. In contrast, the variability within *N. apicalis* morph 1 is found in sympatric colonies collected less than 300 metres apart in the same habitat. Environmentally-based differences are thus likely negligible, though we cannot completely rule out their influence. This is further indicated by the presence of two distinct chemotypes within the same colony. It thus seems that several partly diverging lineages coexist within *N. apicalis* morph 1 (and the same logic probably holds true for *N. apicalis* morph 4), each with its own distinct chemotype. Cytogenetic studies corroborate this hypothesis by showing a high variation of karyotypes in sympatric populations of *N. apicalis* morph 1, suggesting an evolution due to pericentric inversions (Delabie et al., 2008; Mariano et al., 2011). The occurrence of two chemotypes within one colony then suggests that either this colony contained two reproductive queens from different lineages, or alternatively that the queen has mated with two males from different lineages. In ants, differences in CHC profiles have already been reported between both matrilines (Helanterä et al., 2013) and patrilines (Nehring et al., 2011), and their occurrence in *N. apicalis* is thus well conceivable. The colony genetic structure of *N. apicalis* morph 1 remains undetermined, but investigations in several other *N. apicalis* morphs (i.e. morphs 4, 6 and 7) have revealed strict monogyny and facultative low polyandry (Yagound et al., unpublished data). Facultative polygyny is found in *N. verenae* morphs, but this likely follows the re-adoption of daughter queens (Evison et al., 2012). Thus, providing the same scenario occurs in *N. apicalis* morph 1, this would imply that matriline-associated differences are unlikely, and that either two queens have each mated with a male originating from a different lineage (either intra or intermorph), or that one queen has mated with two males from distinct lineages. Hybridization is probably not a rare phenomenon in ants (Seifert, 2009). In this case, its consequence could be a reduction of the divergence between these lineages. At this point a thorough investigation of the colony genetic structure in this morph appears necessary to eventually distinguish between the above hypotheses.

The various classes of CHCs contributed differently to between-group discrimination. Indeed, whereas all three classes are sufficient alone to distinguish between colonies, chemotypes and morphs, unsaturated CHCs have higher levels of dissimilarity compared with saturated CHCs, both at the colony and species level. Methyl-branched alkanes also show a very high dissimilarity level between morphs, but on the contrary are much more similar between colonies within each morph. This indicates that methyl-branched

and unsaturated CHCs have the best potential for being used as taxonomic tools in this species, which could be a general trend in ants (Dahbi et al., 2008; Martin et al., 2008a; Martin & Drijfhout, 2009a; Drescher et al., 2010; Guillem et al., 2012). Our results further show that restricting the analysis to only a handful of CHCs provides a very reliable way of distinguishing morphs. In this respect, it clearly seems that the proportions of isomers of $C_{23:1}$ and $C_{23:2}$ are morph-specific. An effort should now be made to precisely identify the position of the double bonds through dimethyl disulfide derivatization (Dunkelblum et al., 1985) in these compounds which have a great potential for being diagnostic taxonomic tools. This could allow an easier and quicker discrimination of these otherwise difficult to distinguish morphs. Instances of such a use of specific CHCs to differentiate similar species have already been reported, and highlight the validity of chemical taxonomy to resolve the challenge posed by their morphological identification. For example, two morphologically similar *Myrmica* species can perfectly be differentiated by just four characteristic CHCs, namely *Z*12-$C_{25:1}$ and 5-MeC_{25} for *M. sabuleti*, and 3-MeC_{23} and *Z*9-$C_{25:1}$ for *M. scabrinodis* (Guillem et al., 2012). Likewise, *Z*9-alkenes and dimethylalkanes can be used to discriminate between 13 species of *Formica* (Martin et al., 2008a).

Overall, these results thus indicate that CHCs are relevant to distinguish morphs/species in the *N. apicalis* complex. They add to the literature proving chemical taxonomy to be useful in discriminating species in ants (Vander Meer & Lofgren, 1989; Bagnères et al., 1991; Elmes et al., 2002; Lucas et al., 2002; Steiner et al., 2002; Dahbi et al., 2008; Martin et al., 2008a; Evison et al., 2012). Despite their numerous advantages, using CHCs to infer species boundaries can also have potential limitations. In particular, CHCs are known to be highly context-specific, varying with caste, age, task, or reproductive status (Greene & Gordon, 2003; Howard & Blomquist, 2005; Monnin, 2006), which, if not controlled, may create confounding effects by adding extra-variability between the groups investigated (Kather & Martin, 2012). In our case however, this intra-group variability could only have had a minor influence on our results, since the individuals in all morphs were sampled in the same conditions, i.e. in newly formed queenless groups with very similar intra-group variability. We are therefore confident that our results conclusively show the validity of CHCs as a taxonomic tool (Bagnères & Wicker-Thomas, 2010).

The mitochondrial phylogeny based on the COI gene shows that the different taxa of the *N. apicalis* complex have a high genetic divergence. Most of the clades identified were highly supported by the phylogeny, with low intra-clade genetic divergence. The only exception is *N. verenae* morph 1 which showed moderate levels of genetic divergence, possibly indicating the existence of a higher diversity within this morph. The phylogeny reveals three distinct morphs of *N. verenae*, i.e. the already described morphs 1 (from Costa Rica to Peru and French Guiana) and 2 (from Southeast Brazil), and the new morph 3 only found in Colombia together with morph 1. However, their high genetic divergence and sympatric occurrence strongly suggests that these are two distinct taxa (Sites & Marshall, 2003; Seifert, 2009). The *N. apicalis* group is clearly the most diverse, with six well-supported clades corresponding to the morphs 1, 2, 3, 4, 6 and 7. The geographic distribution of these morphs is largely sympatric for *N. apicalis* morphs 2, 4, 6 and 7, particularly in French Guiana. By contrast, *N. apicalis* morphs 1 and 3 were isolated in their area of origin, respectively in Northeast Brazil and Central America. Delabie et al. (2008) reported a larger distribution for *N. apicalis* morph 1, but this remains to be corroborated by genetic and/or chemical characters.

N. apicalis morphs 1 and 4 originated from a single location and are highly homogeneous. In contrast, the five other morphs are composed of several clades which have important levels of genetic divergence (2.8–7.5%). Barcoding studies typically consider 3% of sequence divergence as the threshold for distinguishing species (Song et al., 2008). Applying this rule would thus qualify most of the within-morph clades as distinct species, resulting in a potential number of 18 species in this complex. In most occasions however, these clades correspond to different geographic areas. Therefore it is likely that these results reflect the influence of geographic distance on genetic divergence between otherwise similar populations. These ants have a very large area of distribution (Kempf, 1972; Wild, 2005; Delabie et al., 2008), and thus the spatial distance separating two collection points for the same morph can be considerable (typically thousands of kilometres). Furthermore, there is a high correlation between spatial and genetic distance in all these morphs. We might thus expect that gradual divergence occurred through accumulated differences, e.g. because of mutations, genetic drift, and/or selection resulting from ecological pressures (Hartl & Clark, 1997). This implies that these within-morph clades do not actually represent distinct species. Blaimer and Fisher (2013) recently reported even higher levels of molecular divergence

within valid species of *Crematogaster*, also partly explained by geographic differences. Note that the situation in *N. apicalis* morph 3 may be more complicated, given the fact that one pair of within-morph clades occurs in sympatry in Costa Rica. Therefore, they may reflect true instances of lineage divergence (Fitzpatrick et al., 2008), although the sequencing of additional genes (e.g. nuclear genes) is necessary to resolve their status. More generally, these results call into question the relevance, both empirical and conceptual, of barcoding arbitrary thresholds for delimiting species (Virgilio et al., 2012; Zhang et al., 2012; Collins & Cruickshank, 2013), and stress the necessity to include additional characters (e.g. CHCs) within integrative taxonomic approaches (Seifert, 2009; Schlick-Steiner et al., 2010).

The relationships at deep levels are mostly unresolved in the phylogenetic tree, particularly between the three major clades *N. apicalis*, *N. cooki* and *N. verenae*. However, *N. cooki* has the highest genetic distance with all other morphs, together with presenting a really distinct CHC profile compared with the rest of the complex. This species has long been hidden inside the species *N. apicalis* until morphological characters recently proved its distinction (Mackay and Mackay, 2010). Ferreira et al. (2010) also showed that this species (then identified as *N. apicalis* morph 5) has a distinct stridulatory organ and sound production, and a well-supported basal position in a phylogenetic tree based on mtDNA cytochrome *b*. The position of *N. cooki* as basal within the *N. apicalis* complex tree is therefore, to our opinion, the most plausible. This species is in fact probably the easiest to distinguish in the complex.

Likewise, the basal position of *N. verenae* morph 3 within *N. verenae* and of *N. apicalis* morph 3 within *N. apicalis* are also well-supported. However, the relationships between the other *N. apicalis* morphs remain unresolved (except for morphs 4 and 6 that clustered together). Despite its massive use in phylogenies (Hebert et al., 2003; Ratnasingham & Hebert, 2007), mitochondrial DNA may be the source of errors when considered alone, due to high levels of inferred paraphyly (Seifert, 2009) or the amplification of nuclear-mitochondrial pseudogenes (Song et al., 2008). This also calls for the inclusion of additional molecular characters (e.g. nuclear genes), and additional samples along their vast geographical range to improve our understanding of the phylogenetic relationships within the *N. apicalis* clade and reconstruct its evolutionary history. Nonetheless, our study indicates that the degree of between-morphs divergence

is higher than the level of within-morphs divergence. Furthermore, all morphs investigated in their CHC profiles have a high degree of chemical dissimilarity, although there is no correspondence between the molecular phylogeny and the chemical dendogram (except for the high divergence of *N. cooki*), which indicates that CHCs are a valid taxonomic tool to delimit species, but not to infer their evolutionary relationships. These results, combined with their largely sympatric distribution and with previous investigations showing morphological, molecular, behavioural, cytogenetic and ecological differences (Delabie et al., 2008; Ferreira et al., 2010; Yagound et al., unpublished data) strongly suggest that the *N. apicalis* species complex is composed of at least 11 valid species: *N. apicalis* morphs 1, 2, 3, 4, 6 and 7, *N. cooki*, *N. obscuricornis* (not investigated here), and *N. verenae* morphs 1, 2 and 3.

Overall this study thus proves the usefulness of CHCs, a commonly investigated feature in social insects (Howard & Blomquist, 2005), as a taxonomic tool to identify species in the *N. apicalis* species complex. The very high morphological similarity of these species renders their identification with classical morphological taxonomy particularly challenging. However, using additional taxonomic tools such as chemical and molecular characters allows to much better infer species boundaries in these ants, and reveal a higher level of –until now hidden– diversity. Identifying cryptic species has important consequences in studies focusing on ecology, behaviour and evolution, as well as for conservation planning (Bickford et al., 2007). Efforts should thus be made to improve our knowledge of this fundamental, yet often neglected, level of biological organization.

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SUPPLEMENTARY MATERIAL

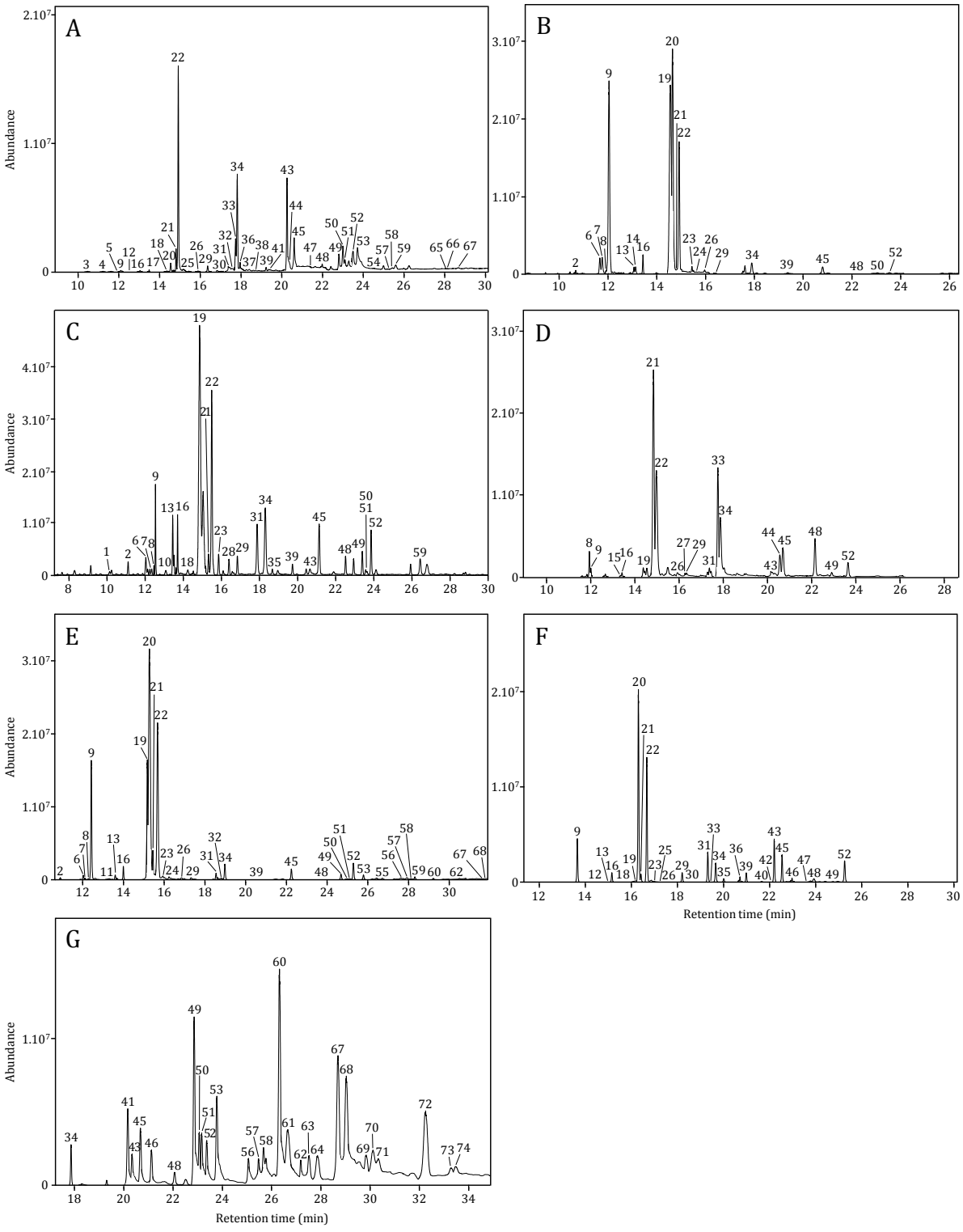

Figure S1. Chromatogram of the CHC profiles of moderately fertile workers in all morphs. A, *N. apicalis* morph 1. B, *N. apicalis* morph 3. C, *N. apicalis* morph 4. D, *N. apicalis* morph 6. E, *N. apicalis* morph 7. F, *N. verenae* morph 1 from Pará. G. *N. cooki*. Peaks used in the chemical analyses are indicated by numbers and refer to Table S1.

Table S1. Peaks used in the chemical analyses in all morphs of the *N. apicalis* complex.

Peak	Compound	Peak	Compound
1	10-MeC$_{19}$	38	*x,y*-C$_{26:2b}$
2	*n*-C$_{20}$	39	*n*-C$_{26}$
3	*x,y*-diMeC$_{20}$	40	11-MeC$_{26}$
4	3-MeC$_{20}$	41	3-MeC$_{26}$
5	*x*-C$_{21:1a}$	42	*x*-C$_{27:1a}$
6	*x*-C$_{21:1b}$	43	*x*-C$_{27:1b}$ + *x,y*-C$_{27:2}$
7	*x*-C$_{21:1c}$	44	*x*-C$_{27:1c}$
8	*x*-C$_{21:1d}$	45	*n*-C$_{27}$
9	*n*-C$_{21}$	46	11-,13-MeC$_{27}$
10	9-,11-MeC$_{21}$	47	3-MeC$_{27}$
11	5-MeC$_{21}$	48	*n*-C$_{28}$
12	3-MeC$_{21}$	49	2-MeC$_{28}$
13	*x*-C$_{22:1a}$ + *x,y*-C$_{22:2}$	50	*x*-C$_{29:1a}$ + *x,y*-C$_{29:2}$
14	*x*-C$_{22:1b}$	51	*x*-C$_{29:1b}$
15	*x*-C$_{22:1c}$	52	*n*-C$_{29}$
16	*n*-C$_{22}$	53	11-,13-MeC$_{29}$
17	*x,y*-diMeC$_{22}$	54	*x,y*-C$_{30:2}$
18	9-,11-MeC$_{22}$	55	*x*-MeC$_{30}$
19	*x*-C$_{23:1a}$ + *x,y*-C$_{23:2a}$	56	10-,12-MeC$_{30}$
20	*x*-C$_{23:1b}$ + *x,y*-C$_{23:2b}$	57	3-MeC$_{30}$
21	*x*-C$_{23:1c}$	58	*x*-C$_{31:1}$ + *x,y*-C$_{31:2}$
22	*n*-C$_{23}$	59	*n*-C$_{31}$
23	11-MeC$_{23}$	60	11-,13-,15-MeC$_{31}$
24	9-MeC$_{23}$	61	*x*-MeC$_{31}$
25	7-MeC$_{23}$	62	10-,12-MeC$_{32}$
26	3-,5-MeC$_{23}$	63	*x*-C$_{33:1a}$
27	*x*-C$_{24:1a}$	64	*x*-C$_{33:1b}$
28	*x*-C$_{24:1b}$ + *x,y*-C$_{24:2}$	65	*x*-C$_{33:1c}$
29	*n*-C$_{24}$	66	*x,y*-C$_{33:2}$
30	*x,y*-diMeC$_{24}$	67	11-,13-,15-MeC$_{33}$
31	*x*-C$_{25:1a}$ + *x,y*-C$_{25:2a}$	68	3-MeC$_{33}$
32	*x*-C$_{25:1b}$ + *x,y*-C$_{25:2b}$	69	12-MeC$_{34}$
33	*x*-C$_{25:1c}$	70	*x,y*-C$_{35:2}$
34	*n*-C$_{25}$	71	*x*-C$_{35:1}$
35	11-,13-MeC$_{25}$	72	11-,13-,15-MeC$_{36}$
36	5-MeC$_{25}$	73	*x*-MeC$_{37}$
37	*x*-C$_{26:1a}$	74	*y*-MeC$_{37}$

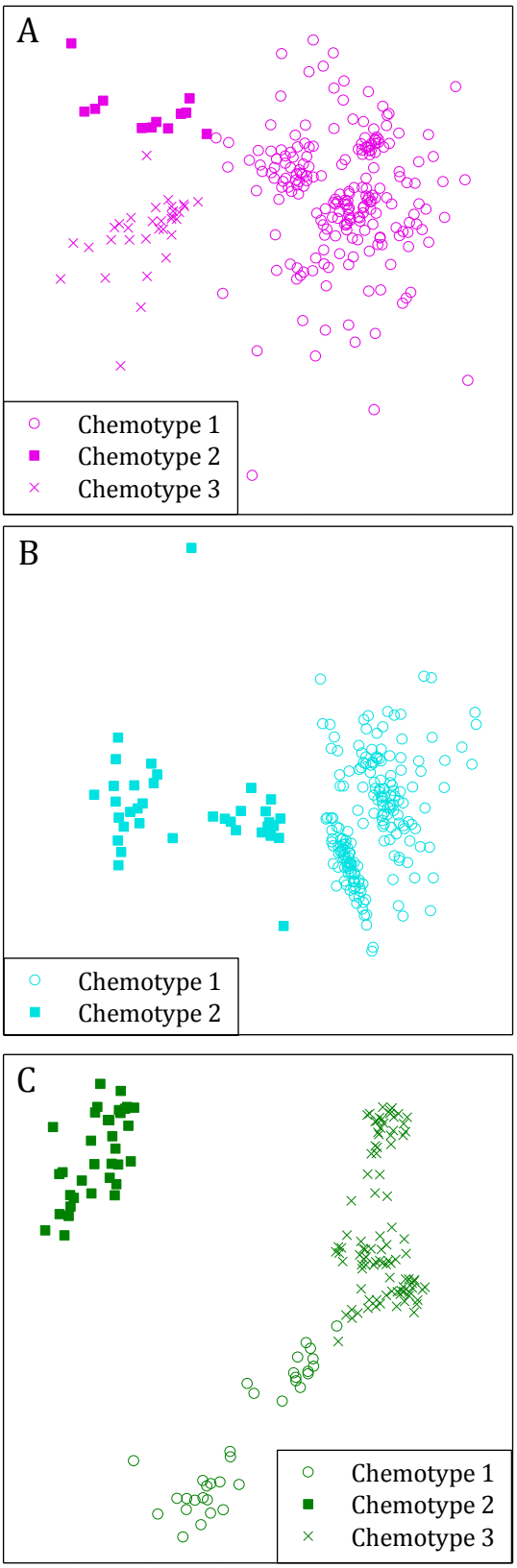

Figure S2. NMDS plot of the CHC profiles of chemotypes in (A) *N. apicalis* morph 1 (2D stress = 0.14), (B) *N. apicalis* morph 4 (2D stress = 0.10), and (C) *N. verenae* morph 1 (chemotype 1, Bahia; chemotype 2, French Guiana; chemotype 3, Pará; 2D stress = 0.08).

CHAPITRE 2

APPARENTEMENT ET STRUCTURE COLONIALE

RÉSUMÉ

Les colonies d'insectes sociaux sont caractérisées par leur coopération mais également par l'existence de conflits potentiels liés à la reproduction. Ces conflits sont dus à des asymétries d'apparentement entre les individus du groupe qui dépendent de la structure génétique de la colonie, elle-même affectée par la présence d'accouplements multiples de la reine et/ou de plusieurs reines. La connaissance de la structure génétique des colonies est donc cruciale pour appréhender leur organisation sociale et l'existence de conflits entre les différents groupes d'intérêts présents dans la colonie (i.e. la reine, le collectif des ouvrières et les ouvrières individuelles).

Dans cette optique, nous avons déterminé la structure génétique des colonies de plusieurs espèces du complexe *Neoponera apicalis* à l'aide de marqueurs microsatellites. Nous montrons que les colonies des différents morphes de *N. apicalis* sont strictement monogynes, et les résultats suggèrent qu'il en est de même pour *N. cooki*. La présence occasionnelle de plusieurs femelles désailées suggère ainsi des échecs d'accouplement pour certaines gynes qui sont ensuite ré-adoptées dans leur colonie mère où elles deviennent des ouvrières fonctionnelles. Les colonies de *N. verenae* présentent un apparentement moyen plus faible, influencé notamment par leur polygynie facultative, et les résultats indiquent là-aussi qu'il s'agit de polygynie secondaire par ré-adoption de filles fécondées, possiblement contrainte par des limitations dans la disponibilité des sites de nidification. Il n'y a cependant pas de structuration génétique des populations à faible échelle géographique. Enfin toutes les espèces étudiées sont facultativement polyandres, avec des paternités effectives supérieures pour *N. verenae* par rapport aux autres espèces qui sont fonctionnellement monoandres du fait du fort biais de paternité.

Ces résultats révèlent donc l'existence de variations dans la structure coloniale entre ces espèces proches. Ces relations d'apparentement montrent des différences d'optimums reproductifs entre la reine et les ouvrières, et prédisent ainsi l'existence de conflits liés à la reproduction entre ces groupes d'intérêts en ce qui concerne notamment le sexe ratio des sexués et la production des mâles.

Article 2

Colony genetic structure in the *Neoponera apicalis* species complex (Hymenoptera: Formicidae: Ponerinae)

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In preparation

ABSTRACT

One of the main characteristics of insect societies is the association of kin, where related individuals cooperate in reproductive and ergonomic tasks. The colony kin structure can however be complex, with the queen mating with multiple males or multiple queens being present in the same nest. Important variations in relatedness can thus occur, with significant consequences for the reproductive division of labour and the potential for selfishness versus altruism. Knowing the genetic structure of insect societies is therefore of crucial importance to understand their social organization. Here we studied the colony genetic structure in *Neoponera apicalis*, a species complex of ponerine ants. This group has the advantage of combining many ancestral traits with a great diversity in social organization, and thus constitutes a relevant model system to investigate the factors responsible for the observed complexity of family structure in social evolution. We found the average relatedness to be higher in *N. apicalis* morphs than in *N. verenae* morphs. Many queens mated with multiple males, but the effective paternity was commonly reduced to nearly one in all the species investigated. This facultative low polyandry involved related males and appeared not to increase the intra-nest genetic variability. Despite many nests containing multiple dealate females, we found that facultative polygyny was restricted to *N. verenae* morphs and involved related queens, which suggests a form of secondary polygyny through daughter re-adoption, possibly constrained by limitations in nesting sites or intraspecific competition. These results reveal variations in social structure among closely related species with similar life histories. We discuss the implication of these findings for the study of cooperation and reproductive conflicts in insect societies.

Keywords: Monandry, monogyny, *Neoponera* (formerly *Pachycondyla*) *apicalis*, social insects.

INTRODUCTION

Family life is a defining feature of insect societies. Group members are thus typically highly related, which is thought to have played an important role in the evolution of eusociality (Boomsma, 2007, 2009; Bourke, 2011). Hamilton's 1964 seminal papers have indeed clearly shown that positive relatedness is a prerequisite for altruism to be selected for, because it enables the compensation of direct fitness costs by indirect fitness benefits through the reproduction of relatives (Hamilton, 1964a, b). However, colonies of socials insects are –rare occasions excepted– non clonal, which gives rise to asymmetries in relatedness between group members. Reproductive interests of the queen and the workers may thus not be aligned, which creates potential reproductive conflicts (Ratnieks & Reeve, 1992; Bourke & Franks, 1995; Ratnieks et al., 2006). Relatedness, particularly between the workers and the brood they raise, is a critical factor affecting the potential for reproductive conflicts as predicted by inclusive fitness theory (Hamilton, 1964a, b). Investigating these conflicts and the mechanisms allowing their regulation therefore requires knowledge of the genetic structure of the colony.

Many social insects are characterised by a monogynous and monandrous (i.e. one single-mated queen) social structure (Wilson, 1971; Hölldobler & Wilson, 1990), which is thought to be ancestral in the evolution of sociality (Hughes et al., 2008a). However, departures from this simple colony structure are common (Crozier & Pamilo, 1996; Hughes et al., 2008a; Baer, 2011). These may be due to i) the queen mating with multiple males (polyandry), and/or ii) the colony containing multiple queens (polygyny). Polyandry and polygyny may seem puzzling regarding kin selection because these two factors can seriously dilute the intra-nest worker–brood genetic relatedness, and therefore decrease inclusive fitness benefits (Boomsma & Ratnieks, 1996; Heinze & Keller, 2000). Furthermore, multiple mating is costly in terms of time, energy, exposure to predators or disease transmission (Strassmann, 2001).

Many hypotheses have been proposed to explain the evolution of polyandry (Crozier & Fjerdingstad, 2001), including the avoidance of sperm limitation (Cole, 1983), the benefits linked with genetic variability (Crozier & Page, 1985) or the reduction of the potential for reproductive conflicts (Pamilo, 1991). For example, monogynous–monandrous colonies are expected to be subject to a queen–worker conflict over male parentage (Bourke, 1988b; Ratnieks, 1988; Hammond & Keller, 2004), because the

workers collectively are more highly related to the sons of their sisters (r = 0.375) than to their brothers (r = 0.25), while the queen should prefer her own sons (r = 0.5) over her grand-sons (r = 0.25). This situation however changes if the queen mates with more than two males, since the workers collectively become more related to their brothers than to their nephews (r < 0.25) (Hamilton, 1964a). Thus, the incentive for worker reproduction alleviates as the number of queen mating increases.

Polygyny can have even more dramatic effects on the genetic structure of the colony (Keller, 1995). If co-founding queens (primary polygyny in pleometrosis; Hölldobler & Wilson, 1990) are unrelated the average worker–brood relatedness can be zero. This may be why polygyny is often followed by secondary monogyny after the emergence of the first batch of workers (Steiner et al., 2010). However, some colonies can also remain permanently polygynous, with important interspecific variations in queen–queen relatedness, for example through queen re-adoption in secondary polygyny (Kellner et al., 2007; Evison et al., 2012). Polygyny may be adaptive due to the benefits of genetic diversity (Crozier & Page, 1985; Keller & Reeve, 1994), the protection against parasitism (Buschinger, 1986), or the more dynamic associated demography (Trunzer et al., 1998), but it is mostly thought to be influenced by ecological constraints such as the limitations in nesting sites rendering the solitary founding strategy unprofitable (Herbers, 1986; Bourke & Heinze, 1994; Bourke & Franks, 1995; Keller, 1995). Reproductive skew in polygynous nests can be highly variable, from even partitioning of reproduction (Kellner et al., 2007; Zinck et al., 2007) to functional monogyny (Ito, 2005). Both polygyny and polyandry have inherent costs and bring similar outcomes regarding the colony genetic structure. It has therefore been suggested that polygyny and polyandry are unlikely to co-occur (Keller & Reeve 1994), which is supported by some evidence (Hughes et al., 2008b).

Ants in the subfamily Ponerinae typically display ancestral traits relative to colony structure and organization (Peeters, 1997). Noteworthy is their small colony size, weak queen–worker dimorphism, monomorphic worker caste, solitary foraging and high potential for worker reproduction (Hölldobler & Wilson, 1990; Peeters, 1997; Wilson & Hölldobler, 2005). However, ponerines have evolved from this general pattern an important diversity in social organization, and with more than 1,100 species they are one of the four most diverse groups of ants (Brady et al., 2006a; Moreau & Bell, 2013;

Schmidt & Shattuck, 2014). For example, whereas colonies commonly comprise 100–300 workers, extreme colony sizes can be found from less than five individuals in some *Thaumatomyrmex* (Jahyny et al., 2002) to more than 50,000 workers in *Leptogenys processionalis* (Maschwitz et al., 1989). Likewise, ponerines exhibit a great diversity in reproductive strategies and colony structure. Apart from the well-represented and likely ancestral state of monogynous and monandrous colonies produced through independent semi-claustral foundation, some species may present multiple queens, multiple mating, ergatoid (i.e. permanently wingless) queens or gamergates (i.e. mated workers) (Peeters, 1993), with generally a shift to dependent colony founding (i.e. fission) in these last two cases (Cronin et al., 2013). Ponerines therefore represent a particularly relevant group to study questions related to the evolution of social organization in insect societies.

Here we studied the colony genetic structure of ants belonging to the *Neoponera apicalis* species complex. *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) is one of the most morphologically, ecologically and behaviourally diverse genus among the Ponerinae (Schmidt & Shattuck, 2014). The *N. apicalis* species complex is composed of several cryptic and largely undescribed Neotropical species (Delabie et al., 2008; Ferreira et al., 2010), most of them being currently grouped in the "*apicalis* morphs" and "*verenae* morphs". *N. verenae* morphs are facultatively polygynous (Fresneau, 1984; Traniello & Hölldobler, 1984), with queens being generally related (Evison et al., 2012). By contrast, the *N. apicalis* morphs are thought to be monogynous (Fresneau, 1994), but this has never been confirmed through genetic markers. In this study we therefore investigated the intra-nest relatedness and family structure in both *N. apicalis* and *N. verenae* morphs to better characterize one of the key aspects of colony life in this important group of ants.

METHODS

Ants

We collected colonies of the *Neoponera apicalis* complex in three distinct localities (Petit Saut, 5°04'15.8"N, 53°02'36.3"W; Saut Sabbat, 5°23'48.45"N, 53°41'44.54"W; Montagne des Singes, 5°04'25.76"N, 52°41'55.62"W) in French Guiana in November 2011. Colonies were attributed to their respective species by genetic analyses (Yagound et al., unpublished) and identified according to published studies (Delabie et al., 2008; Ferreira et al., 2010; Mackay & Mackay, 2010). A total of 35 colonies were collected from five different sympatric species as follows: 14 colonies of *N. apicalis* morph 4, 5 colonies of *N. apicalis* morph 6, 7 colonies of *N. apicalis* morph 7, 7 colonies of *N. verenae* morph 1, and 2 colonies of *N. cooki* (previously part of *N. apicalis*; Mackay & Mackay, 2010). Colony size were as follows: *N. apicalis* morph 4, 23.8 ± 3.6 (mean ± SE) workers, *N. apicalis* morph 6, 45.0 ± 21.8 workers, *N. apicalis* morph 7, 37.8 ± 8.5 workers, *N. verenae* morph 1, 33.1 ± 4.9 workers, *N. cooki*, 24.0 ± 4.0 workers. Part of the data used in this study for *N. apicalis* morph 4, 7 and *N. verenae* morph 1 has been presented elsewhere (Yagound et al., unpublished) but without a complete description of the colonies' genetic structure.

Colony Genetic Structure

A sample of 12 workers for each *N. apicalis* morphs colony (total number of workers analyzed: *N* = 166, 60, 63 and 19 for *N. apicalis* morph 4, 6, 7, and *N. cooki* respectively) and of 24 workers for each *N. verenae* morph 1 colony (*N* = 164) were genotyped at seven variable microsatellite loci: Pv1048, Pv1078, Pv2056, Pv2096, Pv2111, Pv4049, Pv4053 (Evison et al., 2010). We extracted DNA from the head and thorax of workers preserved in ethanol in 500 µl of a 10% Chelex® 100 (Bio-Rad, Hercules, CA, USA) solution with 20 µl of proteinase K (Promega, Madison, WI, USA) at 10 mg/ml, incubated at 55°C for 40 min, then boiled at 100°C for 20 min. Polymerase chain reactions (PCRs) were performed in a total volume of 10 µl containing 1 µl of each primer (10 µM), 5 µl of DreamTaq Master Mix (Fermentas, Vilnius, Lithuania), 2 µl of nuclease-free water and 1 µl of 1/5 diluted DNA. PCRs for loci Pv1078, Pv2096 and Pv4049 were carried out similarly, but with 1.5 µl of each primer and 1.5 µl of nuclease-free water. The DNA was

amplified in a Biometra TProfessional thermocycler (Labgene Scientific, Châtel-St-Denis, Switzerland) using an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing step at 56°C, 58°C, 60°C or 62°C depending on the primer (Evison et al., 2010) for 30 s and extension step at 72°C for 1 min, then a final extension step at 72°C for 10 min. PCR products were mixed in two sets (Pv1078, Pv2056, Pv3091, Pv4053, and Pv1048, Pv2111, Pv4049) with the internal size marker LIZ500™ (Applied Biosystems, Foster City, CA, USA) and analyzed by a 3130XL Genetic Analyser (Applied Biosystems). Fragment length was then scored using Peak Scanner™ Software 1.0 (Applied Biosystems).

Statistical Analyses

For all species we investigated the amount of genetic variation at each locus by calculating the number of alleles (A), the allelic richness (A_r; El Mousadik & Petit, 1996), the unbiased expected (H_e) and observed heterozygosities (H_o), and the inbreeding coefficient (F_{IT}; Weir & Cockerham, 1984) using FSTAT 2.9.3 (Goudet, 2001). The genotypes of all queens and their mates were deduced from their offspring genotypes using Matesoft 1.0 (Moilanen et al., 2004). This allowed us to estimate the number of reproductive queens and contributing fathers (k_{obs}) for each colony. We determined the average worker–worker relatedness within colonies (r_{ww}; Queller & Goodnight, 1989), and the average queen–worker relatedness (r_{qw}) and mate–mate relatedness (r_{mm}) within matrilines with GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). We compared r_{ww} and r_{qw} respectively with the expected 0.75 and 0.5 under monogyny using t-tests. We then estimated the effective mate number ($m_{e,p}$) corrected for sample size as in Nielsen et al. (2003) and calculated the pedigree worker–worker relatedness (pr_{ww}) as in Pamilo (1991). For each species we calculated the average weighted non-identification error (f') that takes into account errors due to non detection and non sampling, and estimated the proportion of double-mated queens (D_{est}) as implemented in Matesoft 1.0 following Pedersen and Boomsma (1999a). The arithmetic means r_{ww} and pr_{ww} were compared with paired t-tests. We also calculated the harmonic mean $m_{e,p}$ (Boomsma & Ratnieks, 1996) and the paternity skew index (S) as in Pamilo and Crozier (1996), which varies from 0 (equal paternity) to 1 (a single male monopolizes all paternity). The results for *N. cooki* are not reported in details because of the small sample size, but the main findings

are presented as a comparison with the other species of the *N. apicalis* complex. Unless otherwise stated, statistical tests were performed with R-3.2.0 (R Core Team, 2012). Statistical significance was set at $P < 0.05$.

RESULTS

The analyses showed that *N. apicalis* morph 6 had a generally lower genetic variability at all but one of the seven microsatellite loci compared with the other species (Supplementary Table S1). There was no sign of inbreeding in all species (*t*-test: *N. apicalis* morph 4, $F_{IT} = 0.19$, $t = 1.49$, $P = 0.25$; *N. apicalis* morph 6, $F_{IT} = 0.23$, $t = 1.15$, $P = 0.45$; *N. apicalis* morph 7, $F_{IT} = -0.07$, $t = -0.67$, $P = 0.57$; *N. verenae*, $F_{IT} = 0.05$, $t = 0.26$, $P = 0.81$). The power of correctly deducing all queen genotypes was high (>0.90) in all but one colony from *N. apicalis* morph 7 which was left out from the analysis (this value was in fact >0.98 in 91% of the colonies). There was systematically a single reproductive queen in all colonies of *N. apicalis* morphs, despite one colony containing multiple dealate females (i.e. putative queens) in *N. apicalis* morph 7 (Table 1). In contrast, one colony (14.3%) of *N. verenae* morph 1 was effectively polygynous with two contributing queens, but this is also less than the number of dealate queens (Table 1). This polygynous colony was split according to its matrilines which were subsequently treated as different groups in paternity analyses.

The majority of colonies (84%) of *N. apicalis* morphs were monandrous, with polyandrous colonies containing two to three contributing fathers (Table 1). Most colonies (71.4%) of *N. verenae* morph 1 were polyandrous and contained up to eight contributing fathers (Table 1). Accordingly, the estimated proportion of double-mated queens was low and similar in all *N. apicalis* morphs, whereas it was much higher in *N. verenae* morph 1 (Table 1). The non-identification error was low in all species (*N. apicalis* morph 4, $f' = 0.002$; *N. apicalis* morph 7, $f' = 0.092$; *N. verenae* morph 1, $f' = 0.003$), but it was relatively higher in *N. apicalis* morph 6 due to low polymorphism ($f' = 0.136$). The estimated effective mate number was lower than two in all species, and very close to one in all *N. apicalis* morphs (Table 1). In addition, the paternity skew index indicated unequal contributions of the different fathers in all species (Table 1). Both

colonies of *N. cooki* investigated were monogynous and monandrous, and thus had a similar colony structure than the *N. apicalis* morphs.

Table 1. Colony genetic structure in all species.

Variable	*N. apicalis* morph 4 *N* = 14	*N. apicalis* morph 6 *N* = 5	*N. apicalis* morph 7 *N* = 7	*N. verenae* morph 1 *N* = 7
Dealate females	0.9 ± 0.1*	1.0 ± 0.0	1.2 ± 0.2 (1–2)	4.9 ± 1.6 (1–12)
Reproductive queens	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.1 (1–2)
Monogynous colonies	100.0%	100.0%	100.0%	85.7%
D_{est}	14.3%	23.2%	18.3%	75.2%
k_{obs}	1.2 ± 0.2 (1–3)	1.2 ± 0.2 (1–2)	1.3 ± 0.3 (1–3)	3.5 ± 0.9 (1–8)
Monandrous colonies	85.7%	80.0%	83.3%	28.6%
$m_{e,p}$	1.09 ± 0.13	1.03 ± 0.03	1.10 ± 0.20	1.74 ± 1.05
S	0.26 ± 0.04	0.83	0.40	0.55 ± 0.08

Data are presented as arithmetic means ± SE, except for $m_{e,p}$ (harmonic means ± SE). Range is presented between brackets when appropriate. Sample sizes refer to number of colonies.

*One colony was queenless at the time of collection.

D_{est}, double-mated queens; k_{obs}, contributing fathers; $m_{e,p}$, effective mate number; S, paternity skew index.

Average intra-nest relatedness was not significantly different from r_{ww} = 0.75 for *N. apicalis* morphs 4, 6 and 7 (*t*-test: respectively r_{ww} = 0.73 ± 0.02, t = −1.44, P = 0.16; r_{ww} = 0.72 ± 0.04, t = −0.61, P = 0.44; r_{ww} = 0.72 ± 0.04, t = −0.68, P = 0.42). This also appears to be the case for *N. cooki* (r_{ww} = 0.72 ± 0.28). However, it was significantly lower for *N. verenae* (r_{ww} = 0.61 ± 0.06, t = −2.32, P = 0.015), indicating moderate levels of polygyny. Furthermore there was no significant difference between this average worker–worker relatedness and the expected pedigree worker–worker relatedness in all species (paired *t*-tests: *N. apicalis* morph 4, pr_{ww} = 0.71 ± 0.03, t = 0.48, P = 0.71; *N. apicalis* morph 6, pr_{ww} = 0.74 ± 0.01, t = −0.23, P = 0.99; *N. apicalis* morph 7, pr_{ww} = 0.70 ± 0.05, t = −0.01, P = 0.97; *N. verenae* morph 1, pr_{ww} = 0.54 ± 0.06, t = 2.08, P = 0.11). The average queen–worker relatedness within matrilines was similar to the expected r_{qw} = 0.5 in all species (*t*-tests: *N. apicalis* morph 4, r_{qw} = 0.51 ± 0.03, t = 0.22, P = 0.83; *N. apicalis* morph 6, r_{qw} = 0.43 ± 0.15, t = −0.44, P = 0.76; *N. apicalis* morph 7, r_{qw} = 0.47 ± 0.10, t = −0.30, P = 0.79; *N. verenae* morph 1, r_{qw} = 0.45 ± 0.05, t = −1.00, P = 0.36). In the polygynous *N. verenae* colony, although the average worker–worker relatedness was higher within than between matrilines (respectively r_{ww} = 0.53 and 0.33), the two reproductive queens were related (r_{qq} = 0.33). In colonies with multiple mating, the different males mated with the same female tended also to be related in all cases (*t*-tests compared with 0: *N. apicalis* morph 4, r_{mm} = 0.38 ± 0.04, t = 18.59, P = 0.020; *N. apicalis* morph 6, r_{mm} = 0.31 ± 0.02, t = 7.88, P = 0.023; *N. apicalis* morph 7, r_{mm} = 0.16 ± 0.05, t = 3.59, P = 0.111; *N. verenae* morph 1, r_{mm} = 0.19 ± 0.04, t = 4.85, P = 0.004). Queens and their mates were however systematically unrelated (*t*-tests compared with 0: *N. apicalis* morph 4, r_{qm} = 0.08 ± 0.07, t = 1.12, P = 0.28; *N. apicalis* morph 6, r_{qm} = 0.15 ± 0.14, t = 1.02, P = 0.30; *N. apicalis* morph 7, r_{qm} = −0.10 ± 0.17, t = −0.58, P = 0.61; *N. verenae* morph 1, r_{qm} = 0.01 ± 0.10, t = 0.11, P = 0.92).

DISCUSSION

Our results show that most colonies of the *Neoponera apicalis* species complex have a monogynous and monandrous genetic structure. Multiple mating can be present in all species investigated, but most of the time the effective paternity is virtually indistinguishable from a single-mating structure. Significant paternity skew is indeed present in all species, thus explaining the discrepancy between observed and effective mating frequencies. This phenomenon of reduced effective paternity is common across the social insects (Boomsma & Ratnieks, 1996). Its underlying mechanisms include sperm competition between the different mates and cryptic female choice (Baer, 2011). Consequently the effective mate number rarely exceeds two (Strassmann, 2001), as observed here. Notable exceptions can be found in highly derived taxa such as army ants (Kronauer et al., 2004), leaf-cutting ants (Villesen et al., 2002) or honeybees (Palmer & Oldroyd, 2000). Species of the *N. apicalis* complex thus all exhibit a facultative low polyandry (Hughes et al., 2008b), although *N. verenae* morphs appear to have higher effective mate numbers than *N. apicalis* morphs (this study; Evison et al., 2012).

Several hypotheses have been proposed to explain the occurrence of polyandry (Crozier & Fjerdingstad, 2001), many of which stating that the induced higher genetic variability can provide benefits though a greater behavioural flexibility or a better resistance to pathogens or parasites (Crozier & Page, 1985). Here this hypothesis does not seem to hold because of the very low effective paternity having only negligible effects on intranest genetic variability. Furthermore, when multiple mating occurred the different mates were always related, which suggests that they probably originated from the same colony. Sexuals are produced year-round in these tropical species, but are typically released in batches (Fresneau, 1994). This implies that colonies are likely to have either several or no males at one particular time. Multiple mating could thus be an unselected by-product (Pedersen & Boomsma, 1999b) arising because of an absence of male rejection from the queen during her period of receptivity, allowing several males to copulate with her.

Genetic analyses clearly showed that all *N. apicalis* morphs were strictly monogynous, despite having sometimes multiple dealate females in a same nest. This confirms that colony reproduction occurs through independent semi-claustral foundation, i.e. where single newly-mated females start new colonies alone and frequently leave the nest to

forage until the first workers emerge (Hölldobler & Wilson, 1990). The presence of additional dealate females can mostly be explained by gynes failing to mate and returning to their mother nest where they assume a worker-like role (Fresneau & Dupuy, 1988; Fresneau, 1994; Nehring et al., 2012). This also appears to be the case for *N. cooki* which was until recently still considered as *N. apicalis* (Mackay & Mackay, 2010). In contrast, *N. verenae* was found to be facultatively polygynous, as has been previously reported (Fresneau, 1984; Traniello & Hölldobler, 1984; Evison et al., 2012). The number of dealate females was much more important than in the *N. apicalis* morphs, but most of them were again unmated. Interestingly, Evison et al. (2012) reported the queens in polygynous *N. verenae* colonies to be related, suggesting a form of secondary polygyny through daughter re-adoption. Our results are compatible with this hypothesis, which could be the sign of a greater plasticity in reproductive strategies in these morphs. This also points to a possible switch towards dependent colony foundation in *N. verenae* (Delabie et al., 2008).

A monogamous colony structure is thought to be an ancestral trait in social insects (Hughes et al., 2008a; Boomsma, 2009). This is exemplified in ponerine ants which have retained many behavioural and ecological plesiomorphic characters (Hölldobler & Wilson, 1990; Peeters, 1997; Wilson & Hölldobler, 2005). Nevertheless, some ponerines have significant levels of polyandry (e.g. *Hypoponera opacior* [Foitzik et al., 2002], *N. villosa* [Kellner et al., 2007], *Paltothyreus tarsatus* [Villet et al., 1989]) and/or polygyny (e.g. *N. inversa* [d'Ettorre et al., 2006], *N. marginata* [Leal & Oliveira, 1995], *N. villosa* [Trunzer et al., 1998; Kellner et al., 2007]), which makes them interesting models to study the possible factors influencing the evolution of multiple mating and intra-nest queens co-occurrence. Our results show that variations –to some degree– in colony structure can be found even between closely related sympatric species with very similar life histories.

The reasons explaining the difference between *N. apicalis* and *N. verenae* morphs in levels of polygyny and to some extent polyandry are not fully resolved. They might be due to differences in habitat saturation constraining daughters to return to their mother nest after mating (Keller, 1995). Indeed, nest densities can be higher in *N. verenae* which can be found in more open and disturbed habitats (N. Châline, personal observation). Furthermore, this species seems to have higher rates of emigration due more ephemeral

nesting sites (Ferreira, 2010; Yagound et al., unpublished data), which increases intraspecific competition and might promote colony fusion. This form of facultative secondary polygyny is different from the facultative primary polygyny found in the *N. foetida* complex (Fernandes et al., 2014). Indeed, in *N. villosa* and *N. inversa*, polygynous colonies are formed through pleometrosis involving unrelated queens (Trunzer et al., 1998; Kellner et al., 2007), and where dominance relationships regulate the reproductive division of labour between queens (Kolmer & Heinze, 2000). Reproductive hierarchies between workers in hopelessly queenless nests are common in *N. verenae* (Oliveira & Hölldobler, 1991), but whether dominance behaviours also occur between queens is unknown. It must be noted that the context is very different compared to species of the *N. foetida* complex, since queens are related, and mostly because workers are present in the colony at the time of daughter re-adoption (i.e. secondary polygyny). Thus the pressures relative to the partitioning of inside and outside tasks between queens are completely relaxed. In any case, polygyny in *N. verenae* may be beneficial under some circumstances, notably if it enhances colony productivity and provides inclusive fitness returns due to the high relatedness between co-occurring queens. Indeed, all individuals in the adopting colony have an interest in the successful reproduction of young related queens (Bourke & Heinze, 1994).

Overall, this study shows that ants of the *N. apicalis* species complex are typically monogynous and monandrous, as probably most ponerines. Facultative low polyandry is however common across the complex, whereas facultative secondary polygyny is restricted to *N. verenae* morphs. Most colonies are characterized by important differences between observed mating frequencies and effective paternity, as well as between observed number of dealate females and effective number of reproductive queens. This stresses the importance of using molecular tools to investigate the levels of polygyny and polyandry in social insects. The known genetic structure of *N. apicalis* ants can now be used to make kin-selected predictions regarding the potential for reproductive conflicts in these species and to study the factors underlying their expression (Ratnieks & Reeve, 1992; Bourke & Franks, 1995; Ratnieks et al., 2006). For example, a queen–worker conflict over male parentage is predicted under these kin structures (see above) (Bourke, 1988b; Ratnieks, 1988; Hammond & Keller, 2004), and workers indeed have high reproductive capabilities (Fresneau, 1994). However, this reproductive conflict is typically only expressed when the queen lost her fecundity or

dies (Yagound et al., 2014). Beyond relatedness, this highlights the importance of other factors such as coercion and constraints (Ratnieks et al., 2006) in influencing the balance between costs and benefits of worker reproduction, and therefore the occurrence and magnitude of reproductive conflicts in eusocial insects. The *N. apicalis* species complex thus seems to be a promising model system to investigate social evolution.

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SUPPLEMENTARY MATERIAL

Table S1. Genetic variation at seven microsatellite loci in all species.

Locus	*N. apicalis* morph 4				*N. apicalis* morph 6				*N. apicalis* morph 7				*N. verenae* morph 1			
	A	A_r	H_e	H_0	A	A_r	H_e	H_0	A	A_r	H_e	H_0	A	A_r	H_e	H_0
Pv1048	16	7.20	0.88	0.89	2	1.14	0.02	0.02	6	2.89	0.82	0.66	12	10.19	0.93	0.86
Pv1078	2	1.90	0.26	0.14	1	1.00	0.00	0.00	3	2.02	0.62	0.40	1	1.00	0.00	0.00
Pv2056	10	5.50	0.79	0.71	1	1.00	0.00	0.00	5	2.62	0.69	0.78	8	7.57	0.85	0.82
Pv2111	14	7.15	0.90	0.84	2	1.27	0.03	0.00	2	1.83	0.47	0.71	11	8.64	0.89	0.89
Pv3091	10	5.41	0.79	0.85	2	1.25	0.03	0.03	4	1.94	0.52	0.37	2	1.69	0.05	0.05
Pv4049	4	2.41	0.31	0.09	3	1.97	0.15	0.16	3	2.03	0.54	0.65	3	3.00	0.60	0.77
Pv4053	8	5.33	0.79	0.85	8	6.75	0.94	0.77	3	1.91	0.41	0.44	3	2.03	0.09	0.08
Mean ± SE	9.14 ± 1.90	4.99 ± 0.79	0.67 ± 0.10	0.62 ± 0.13	2.71 ± 0.92	2.05 ± 0.79	0.17 ± 0.13	0.14 ± 0.11	3.71 ± 0.52	2.18 ± 0.15	0.58 ± 0.05	0.57 ± 0.06	5.71 ± 1.71	4.87 ± 1.43	0.49 ± 0.16	0.50 ± 0.16

A, number of alleles; A_r, allelic richness; H_e, unbiased expected heterozygosity; H_o, observed heterozygosity.

CHAPITRE 3

RÉGULATION DU PARTAGE DE LA REPRODUCTION

RÉSUMÉ

Le partage de la reproduction est une source de conflits importants chez les insectes sociaux du fait des asymétries d'apparentement entre les individus créant des conflits d'intérêt, et de la possibilité pour les ouvrières de produire des descendants mâles chez la plupart des espèces. Des mécanismes de régulation permettent de réduire l'impact de ces conflits, et ils sont souvent basés sur des indices de reconnaissance à la base des décisions reproductives des individus. Dans ce contexte, le signalement de la fertilité est supposé avoir une influence primordiale. Chez *Neoponera apicalis* morphe 4, les ouvrières ne se reproduisent pas en présence de la reine, mais quand celle-ci disparait elles entrent en conflit entre-elles en établissant une hiérarchie reproductive. Dans cette étude nous avons déterminé les mécanismes proximaux sous-tendant le partage de la reproduction chez cette espèce, et particulièrement le rôle des signaux liés à la fertilité.

Nous avons montré que les interactions agonistiques ritualisées font émerger une hiérarchie linéaire où les statuts hiérarchiques et reproducteurs sont très fortement corrélés. Nous avons étudié les processus de reconnaissance responsables de la détermination des relations de dominance/subordination dans une tâche de discrimination comportementale et nous avons montré que ces processus jouent un rôle capital dans la formation et la stabilisation de la structure hiérarchique. En effet, tous les individus dans la colonie sont capables de discriminer les individus de haut rang, impliqués dans la compétition reproductive, des individus de bas rang. De plus, les individus de haut rang sont également capables de discriminer individuellement, d'un point de vue fonctionnel, les autres hauts rangs, alors que les bas rangs n'ont pas montré de telles capacités. Nos résultats montrent par ailleurs que les individus de statuts différents ont des profils d'hydrocarbures cuticulaires spécifiques, et qu'un composé en particulier, le 13-méthylpentacosane (13-MeC_{25}), pourrait jouer le rôle de signal de fertilité à la base de la discrimination du statut des individus.

Nous avons ensuite voulu savoir si ce signal de fertilité putatif pouvait jouer un rôle plus large dans la régulation du partage de la reproduction. Nous avons alors montré qu'il existe déjà des différences de fertilité entre les ouvrières en présence de la reine et qu'elles sont déjà associées à des profils chimiques différents, notamment dans les proportions de 13-MeC_{25}. Par ailleurs, ce composé est également retrouvé dans les profils des reines dans des proportions encore plus importantes que chez les ouvrières

reproductrices, alors que ses quantités relatives sont beaucoup plus faibles chez les gynes. L'association de ce composé avec le statut reproducteur est donc présente quelle que soit la caste et le niveau de développement ovarien. En outre nous avons réalisé des bioessais où les quantités de 13-MeC_{25} sont expérimentalement augmentées, ce qui entraine une diminution de la réponse agonistique des individus de haut rang, et corrobore l'implication de ce composé dans la perception du statut des individus.

Cette étude révèle donc un système de reconnaissance de la fertilité pouvant servir de base à la régulation permanente du partage de la reproduction chez cette espèce. L'association probablement contrainte entre la production du 13-MeC_{25} et l'activité des ovaires permet en effet le signalement du statut reproducteur de tous les membres de la colonie. Cette information parait ainsi être un des facteurs principaux sous-jacents à la prise de décision reproductive des ouvrières et leur permettant d'accorder leurs intérêts en termes d'*inclusive fitness*.

Article 3

Status discrimination through fertility signalling allows ants to regulate reproductive conflicts

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ABSTRACT

Dominance hierarchies allow group-living animals to regulate the partitioning of reproduction, but the recognition systems underlying dominance interactions remain equivocal. Individual recognition, a cognitively complex recognition system, is often posited as an important mechanism for the regulation of linear dominance hierarchies because of its high level of precision. However, providing it actually allows a fine-scale discrimination of the individuals' statuses, status discrimination may offer an alternative, simpler, recognition system allowing the same level of precision while saving the memory-related costs associated with individual recognition. With the aim of disentangling the cognitive mechanisms underlying the formation and maintenance of hierarchies, we here studied the within-group recognition systems in the ant *Neoponera apicalis*, where orphaned workers compete over male parentage in a linear hierarchical structure. Overall, we found that status discrimination abilities were in fact sufficient for the establishment and stabilization of linear hierarchies. The observed level of accuracy allowed fine-scale discrimination of all top rankers' hierarchical status, and thus translated into a functional individual discrimination of all competing workers at the top of the hierarchy. Low-ranking workers did not exhibit such fine-scale status discrimination. We moreover showed that a putative signal of fertility, 13-methylpentacosane, precisely labelled the workers' position in the hierarchy, thereby providing the recognition cue likely to explain the individuals' discrimination abilities. This signal could therefore play a key role in the regulation of the reproductive conflict in this species. In contrast with the traditional view, our study shows the implication of a cognitively simple but equivalently efficient recognition system during the emergence and stabilization of a linear dominance hierarchy.

Keywords: Cuticular hydrocarbons, dominance hierarchy, individual recognition, *Neoponera* (formerly *Pachycondyla*) *apicalis*, recognition system, status discrimination.

INTRODUCTION

The existence of recognition systems is a central feature of group living. Recognition is used in a wide range of social interactions, thereby allowing group members to adapt their behaviour according to the age, sex, kinship, group membership, hierarchical status, reproductive status, species and neighbourhood of the individuals with which they interact (Sherman et al., 1997; Thom & Hurst, 2004; Tibbetts & Dale, 2007). Understanding the exact nature of the recognition mechanisms across taxa, their contexts and associated costs and benefits is therefore a major challenge in the biological sciences (Wiley, 2013).

Dominance hierarchies are widespread throughout the animal kingdom. These hierarchies are characterized by asymmetries among group members in the partitioning of resources (Zanette & Field, 2009), and can induce important fitness consequences by mediating access to reproduction, food resources or susceptibility to diseases (Ellis, 1995). Nevertheless, the overt aggression often associated with these hierarchies can also bear important costs in terms of time, energy, physical injuries or vulnerability to predators (Hsu et al., 2006; Rutte et al., 2006). Reducing these costs may imply the use of ritualization mechanisms, as is frequently observed in hierarchical contests (Hemelrijk, 2000; Hsu et al., 2006; Tibbetts & Dale, 2007). These mechanisms allow the individuals to adapt their behaviour towards encountered nestmates without the need for overt aggressive interactions, and therefore play a key role in the stabilization of dominance hierarchies.

Numerous empirical and theoretical studies have proposed a variety of intrinsic and extrinsic factors that may be responsible for the formation and maintaining of dominance hierarchies (Dugatkin & Earley, 2004; Hsu et al., 2006). These factors include pre-existing differences between competing individuals (Parker, 1974), the value of the contested resource (Maynard Smith & Parker, 1976) and the influence of previous experiences on the outcome of future encounters (Dugatkin & Earley, 2004; Hsu et al., 2006; Rutte et al., 2006). Game-theoretical studies have shown that hierarchy formation could rely on self-organizing processes, such as winner and loser effects (Dugatkin & Earley, 2004; Hsu et al., 2006; Rutte et al., 2006), without the need for any particular recognition mechanism. In this case the outcome of past encounters influences the chance of winning or losing in future interactions in a self-reinforcing manner, i.e.

regardless of the identity or rank of the opponent. However, dominance interactions are often highly directed (Hsu et al., 2006; Tibbetts & Dale, 2007; Chase & Seitz, 2011), indicating that individuals actually recognize the status of their opponents, through either direct or indirect (i.e. memory-based) rank perception (Hemelrijk, 2000; Tibbetts & Dale, 2007). Recognition systems are therefore an important feature of dominance interactions, although not mutually exclusive with self-organizing processes. However, the recognition systems underlying dominance interactions remain equivocal (Hsu et al., 2006), particularly since they very often translate into a linear hierarchical structure.

Individual recognition has often been posited as an important mechanism for the regulation and stabilization of linear dominance hierarchies (Dale et al., 2001; Tibbetts, 2002; Thom & Hurst, 2004; d'Ettorre & Heinze, 2005; Tibbetts & Dale, 2007). In this indirect rank perception system (Hemelrijk, 2000), individuals remember earlier interactions with specific group members and adjust their dominance behaviour in subsequent encounters with these same individuals (Dale et al., 2001; Tibbetts, 2002). Despite the complexity of this cognitive mechanism, recognizing individual identity is therefore supposed to provide high benefits by matching the level of precision required for the maintenance of linear hierarchies (Thom & Hurst, 2004).

However, linear hierarchies can also theoretically emerge and be maintained through direct rank perception (i.e. status recognition; Hemelrijk, 2000). Individuals in this case base their decisions on the characteristics signalling an opponent's absolute fighting abilities (resource-holding potential; Parker, 1974), such as age, size, weight or dominance badge (Chase & Seitz, 2011). In contrast to individual recognition, there is thus no need for the opponents to be familiar (Tibbetts & Dale, 2007). Status recognition could therefore save the costs of memory characterizing individual recognition (Thom & Hurst, 2004). However, a critical assumption for the involvement of such a recognition system in the formation and stabilization of linear hierarchies is that it allows a fine-scale discrimination of ranks, but this has never been demonstrated.

Dominance hierarchies are commonly found in social insects (e.g. ants: Heinze et al., 1994; Monnin & Peeters, 1999; Liebig et al., 2000; Heinze et al., 2002; Cuvillier-Hot et al., 2004b; bees: Ayasse et al., 1995; Bull et al., 1998; wasps: Sledge et al., 2001a; Tibbetts, 2002), and this is particularly true when the colonies comprise several individuals with equivalent reproductive potentials competing to gain access to reproduction. Workers in

hopelessly queenless colonies thus typically compete with one another over male parentage (Bourke, 1988b; Ratnieks et al., 2006), with a resulting linear or near-linear hierarchical structure of dominance relationships regulating the partitioning of reproduction (Heinze et al., 1994; Heinze et al., 2002; Peeters & Liebig, 2009), as in the Neotropical ant *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) *apicalis* (Oliveira & Hölldobler, 1990). This species shares all the traits typically characterizing Ponerinae ants, i.e. small societies, a limited queeneworker dimorphism and a high potential for worker reproduction (Fresneau, 1994), and is therefore a good model system for studying the recognition mechanisms involved in the formation and maintenance of dominance hierarchies.

A previous study has shown that low-ranking individuals are able to discriminate top-ranking from low-ranking workers, suggesting a capacity to recognize the social status of their nestmates (Blacher et al., 2010). However, these recognition abilities have never been investigated in top-ranking workers. Since they are the individuals actually involved in the reproductive competition, the costs of mistaking ranks for those of adjacent-ranking nestmates are, in contrast to low rankers, potentially high. We could hypothesize that a more precise recognition system (e.g. individual recognition) is necessary for an efficient discrimination among top-ranking individuals (Tibbetts & Dale, 2007), but this could also be achieved without a necessarily greater level of cognitive complexity in the eventuality of fine-scale status discrimination. Assessing top rankers' cognitive abilities therefore remains a crucial step in understanding the recognition mechanisms underlying the formation and stabilization of the hierarchical structure in these social groups (Elwood & Arnott, 2012; Wiley, 2013). Here we tested the possibility of fine-scale status discrimination without the need for individual recognition by studying the cognitive abilities of *N. apicalis* top- and low-ranking workers. Furthermore, the nature of the recognition cues involved in these dominance interactions remains unknown, but they probably involve chemical communication. Chemical signals, mainly cuticular hydrocarbons, are widely acknowledged to be of primary importance in the communication of dominance and especially reproductive status in social insects (Monnin, 2006; Liebig, 2010). We therefore also analysed the individuals' chemical profile to investigate the nature of the putative recognition cues at the basis of these dominance interactions.

METHODS

Ants

Colonies of *N. apicalis* were collected in the Kérenroch forest, Petit Saut (5°04'15.8"N, 53°02'36.3"W), French Guiana in March 2007 and have been kept in the laboratory in France ever since. Ants were housed in plaster nests (18 × 14 cm) connected to a foraging area of the same dimensions, where food (crickets and honey/apple mixture) was provided twice a week and water ad libitum. Each colony had a queen, more than 70 workers and brood at every developmental stage. Nests were maintained at a temperature of 27 ± 2°C, a relative humidity of 60 ± 5% and a 12:12 h light:dark cycle. Ant collection, husbandry and experimental procedures used in this study fulfilled all the legal requirements concerning insect experimentation of France.

Dominance Hierarchy

From our stock colonies, we created six experimental colonies by isolating 40 randomly chosen workers and placing them in a new nest. Taking the workers away from the influence of the queen induces the formation of a dominance hierarchy by means of ritualized agonistic behaviours (Oliveira & Hölldobler, 1990; Blacher et al., 2010). All ants were individually labelled with numbered tags and dots of paint to allow the individual monitoring of their behaviour. Housing and feeding conditions were the same as above.

Each experimental colony was then observed 1 h a day during a 14-day period, during which we recorded all behavioural acts linked to the establishment of the dominance hierarchy, i.e. ritualized biting and antennal boxing (antennal strokes on another ant's body) (Oliveira & Hölldobler, 1990; Heinze et al., 2002; Cuvillier-Hot et al., 2004b). All observations started the same day the workers were isolated from the queen, and were performed through a red plastic film to avoid disturbances that may affect the ants' behaviour. All agonistic interactions (performed and received) were then compiled in a matrix and arranged in an order minimizing the number of inconsistencies (i.e. when an individual is given a lower rank than an individual it dominates). This allowed us to reconstruct the dominance hierarchy and to assign a hierarchical rank to each individual

(see Blacher et al. (2010) and references therein for a detailed description of the method used). Ants at the top of the hierarchy that collectively performed more than 75% of the agonistic acts (mean ± SE: 11.5 ± 0.2 individuals, N = 6 colonies; Table 1) were considered high-ranking individuals. Two other classes of individuals were additionally determined, namely middle-ranking individuals (remaining ants performing up to 95% of the agonistic acts with the exclusion of high-ranking workers, 11.8 ± 1.6 individuals) and low-ranking individuals (remaining ants at the bottom of the hierarchy, 13.8 ± 1.6 individuals).

Table 1. Dominance hierarchy characteristics

Colony	Number of top rankers	Linearity*	Correlation with dominance index	Correlation with ovarian index
A	11	$K = 0.85$ $P < 0.0001$	$r_s = -0.68$ $P < 0.0001$	$r_s = -0.75$ $P < 0.0001$
B	12	$K = 0.91$ $P < 0.0001$	$r_s = -0.80$ $P < 0.0001$	$r_s = -0.85$ $P < 0.0001$
C	11	$K = 0.45$ $P = 0.031$	$r_s = -0.40$ $P = 0.014$	$r_s = -0.69$ $P < 0.0001$
D	12	$K = 0.69$ $P < 0.0001$	$r_s = -0.69$ $P < 0.0001$	$r_s = -0.75$ $P < 0.0001$
E	12	$K = 0.94$ $P < 0.0001$	$r_s = -0.89$ $P < 0.0001$	$r_s = -0.61$ $P < 0.0001$
F	11	$K = 0.92$ $P < 0.0001$	$r_s = -0.86$ $P < 0.0001$	$r_s = -0.81$ $P < 0.0001$

* See Appleby (1983). Note that the smaller values of K for colony C and to a lesser extent colony D are mainly due to more missing values for some dyads (11 and 3 for colonies C and D respectively) compared with the other colonies (0 or 1) (de Vries, 1995).

Habituation–Discrimination Procedure

To test the cognitive abilities of high- and low-ranking workers, we used a habituation–discrimination paradigm, a classical procedure in cognitive studies (Ferguson et al., 2002) consisting of two consecutive phases. The habituation phase consists of four consecutive exposures (4 min each with a 5 min interval) of a stimulus (a nestmate ant) to a tested individual. The tested ant thus becomes familiar with the proposed stimulus. The stimulus ant is CO_2-anaesthetized to avoid any influence of its behaviour on the tested ant's response. Following a 5 min interval, the discrimination phase consists of a single test (4 min) in which the tested ant is confronted with two stimuli: a familiar stimulus (the anaesthetized ant previously used during the habituation phase) and an unfamiliar stimulus (another anaesthetized nestmate). Discrimination is typically manifested by a longer duration of the behavioural response towards the unfamiliar stimulus compared with the familiar stimulus (Wiley, 2013). Note that both stimuli are nestmates of the tested ant, and therefore both have potentially already interacted with it. The use of 'familiar' and 'unfamiliar' thus refers to the habituation–discrimination procedure only, since these terms are classically used in these experiments.

Each test was performed in a neutral arena (diameter = 5.3 cm) with externally black-covered walls and Fluon-coated sides to prevent the ant from escaping. To enable familiarization with the device, the tested ant was gently placed in it 30 s before the test began. The stimulus ant was then introduced into the centre of the arena, in which a filter paper had been placed as a substrate (the paper was replaced between each test to avoid any odour residues). All tests were videorecorded for subsequent analyses of the tested ants' behaviour. During these analyses, we measured the duration of antennal contacts with the stimulus (commonly taken as a measure of an ant's interest towards a social stimulus; Boulay et al., 2000) with EthoLog 2.2 software (Ottoni, 2000). In addition, we investigated the possibility that the behavioural response of high- and low-ranking workers could be influenced by differences in their overall reaction or motivation in the experimental device by quantifying their mobility pattern in 58 randomly selected habituation tests derived from all four habituation tests and all colonies using EthoVision 3.1.16 (Noldus Information Technology, Wageningen, Netherlands). Three behavioural variables were quantified: total distance moved, mean angular velocity and duration of mobility. We also quantified the duration close to the

stimulus ant, which reflects the general attraction/avoidance of the tested ant towards the proposed stimulus. Observations were performed twice and blind.

On day 15, four different experiments were carried out using this procedure in which tested and stimuli ants were either low- or high-ranking individuals as previously defined. All tested and stimuli ants were only used in a single habituation–discrimination test.

Experiment 1: status discrimination by high rankers

In the first experiment, the tested ant (N = 21, two for colony B, three for colony A, four for colonies C, D, E and F) was a high ranker and was confronted during the discrimination phase with stimuli belonging to different rank classes, i.e. a low- and a high-ranking individual (mean gap in their respective rank of 22.5 ± 0.6). This experiment was aimed at verifying whether high-ranking workers are capable of status discrimination, an ability that has already been shown in low-ranking workers (Blacher et al., 2010). To avoid the stimulus rank class affecting the tested ant's behaviour, the familiar stimulus was a low-ranking individual for half of the tested ants (N = 11) and a high-ranking individual for the other half (N = 10).

Experiment 2: fine-scale discrimination of high-ranking individuals by high rankers

The second experiment was aimed at testing the possibility of fine-scale discrimination among high-ranking workers. High rankers (tested ants, N = 19, two for colony E, three for colonies B, C and D, four for colonies A and F) were confronted during the discrimination phase with two high-ranking workers (stimuli ants) located higher in the hierarchy and separated by a single rank (mean gap in their respective rank of 1.0 ± 0.0).

Experiment 3: fine-scale discrimination of low-ranking individuals by high rankers

In the third experiment, high rankers (tested ants, N = 21, three for colonies B, E and F, four for colonies A, C and D) were confronted during the discrimination phase with two

low-ranking workers (stimuli ants) with again very similar ranks (mean gap in their respective rank of 1.7 ± 0.3), but this time located at the bottom of the hierarchy.

Experiment 4: fine-scale discrimination of high-ranking individuals by low rankers

Finally, the fourth experiment was done in order to compare the behavioural discrimination of high- and low-ranking workers. Low rankers have already been shown to discriminate high- and low-ranking individuals, but not low-ranking individuals (Blacher et al., 2010). However, their ability to discriminate high-ranking workers has never been investigated. Low rankers (tested ants, N = 24, four in each colony) were therefore confronted with two high-ranking workers (stimuli ants, mean gap in their respective rank of 1.1 ± 0.1) in the discrimination phase.

Fertility Measurement

To link the ants' hierarchical rank with their reproductive dominance, all workers were frozen for dissection at the end of the habituation–discrimination procedure, and their fertility was determined. As an ovarian index, we measured the total size of the six basal oocytes. We then determined three classes of individuals depending on their number of developed oocytes (i.e. size > 0.5 mm; Fresneau, 1994): highly fertile individuals (five to six developed oocytes, 11.8 ± 2.4 individuals, N = 6 colonies), moderately fertile individuals (one to four developed oocytes, 13.3 ± 2.7 individuals) and infertile individuals (no developed oocytes, 12.0 ± 2.3 individuals). Some individuals (two in colonies A, D and F, three in colonies C and E, five in colony B) died before the experiments and were therefore not included in the analyses.

Chemical Profiles

We finally investigated the nature of the putative recognition cues involved in the dominance interactions of the reproductive hierarchy by analysing the cuticular hydrocarbon profile of all individuals. This procedure allowed us to study how ants diverged in their chemical signature according to their social rank and fertility state. We

sampled the cuticular hydrocarbons of a total of 223 ants. Extraction was performed by placing an ant in 400 µl of pentane containing 8 ng/µl of an internal standard (n-C_{17}) for 20 min. We then transferred 100 µl into a 200 µl glass insert. Following evaporation, 20 µl of pentane were added to the 200 µl glass insert. We then manually injected 2 µl of the extract into an Agilent 7890A gas chromatograph, equipped with an HP- 5MS capillary column (30 m × 25 µm × 0.25 µm) and a split–splitless injector, coupled to an Agilent 5975c mass spectrometer with 70 eV electron impact ionization. The carrier gas was helium at 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 1 min, then 70–180°C at 30°C/min, then 180–320°C at 5°C/min, then hold at 320°C for 5 min. The areas of 34 peaks present in all ant cuticular extracts (Appendix Figure A1) were integrated with the Agilent ChemStation software. Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared with known standards.

Statistical Analyses

Following hierarchy reconstruction, we calculated the K index of linearity varying from 0 (no linearity) to 1 (linear hierarchy) and tested the statistical significance of linearity according to Appleby (1983). The test of linearity was performed on high-ranking workers only, because middle- and particularly low-ranking workers performed far fewer agonistic interactions, thus creating incomplete information, which is known to underestimate the values of linearity (de Vries, 1995). Using the Spearman rank correlation test, we calculated the correlation between the hierarchical rank and (1) the dominance index (proportion of agonistic acts performed) and (2) the ovarian index. We compared the proportion of agonistic acts that high rankers performed towards other high-ranking workers of consecutive ranks with the Friedman test followed by post hoc exact permutation tests with the Bonferroni–Holm method (Holm, 1979).

For each experiment, we compared the duration of antennal contacts with the stimulus between the first and the fourth habituation test, and the antennation duration towards the familiar and the unfamiliar stimulus in the discrimination test using exact permutation tests for paired samples. Each mobility variable was compared between high and low rankers confronted with high-ranking nestmates, and between high

rankers confronted with high- and low-ranking nestmates using exact permutation tests for independent samples.

For chemical analyses, we arcsine-transformed (Sokal & Rohlf, 2012) the relative quantities of 34 compounds common to all individuals. We then performed a discriminant function analysis to investigate how individuals diverge in their chemical profile according to their hierarchical rank (i.e. high-, middle- and low-ranking individuals), and their fertility level (i.e. highly fertile, moderately fertile and infertile individuals). We finally investigated the existence of a putative fertility signal (Monnin, 2006) by comparing the absolute quantity of all compounds between the three fertility groups using one-way ANOVAs followed by post hoc exact permutation tests with the Bonferroni–Holm method. Absolute quantities of the compounds with the highest contribution to the discrimination were also correlated with the individuals' fertility and social rank for all individuals using the Spearman rank correlation test. The Monte Carlo procedure was used when appropriate to deal with large sample sizes (Metropolis & Ulam, 1949).

All statistical analyses were performed with StatXact 8.0 (Cytel Software Corporation, Cambridge, MA, U.S.A.) and Statistica 8.0 (StatSoft, Tulsa, OK, U.S.A.). Statistical significance was set at $P < 0.05$.

RESULTS

Dominance Hierarchy

During the 14-day observation period of dominance/subordinate relationships, we recorded a total of 11 808 agonistic acts (1968.0 ± 248.2 per colony), which allowed us to determine the hierarchical rank of the ants successfully. The linearity or near-linearity of the hierarchy was significant in all colonies (Table 1). Furthermore, the individuals' hierarchical rank was highly correlated with their dominance index (Table 1). Among high rankers, agonistic interactions were not randomly directed towards other high-ranking workers, but instead were highly biased towards the individuals with the closest lower ranks in the hierarchy (Friedman test on the proportion of agonistic acts that high rankers performed towards individuals with immediate consecutive ranks: $T_{F,4} = 53.73$, $N = 69$, $P < 0.0001$; Figure 1).

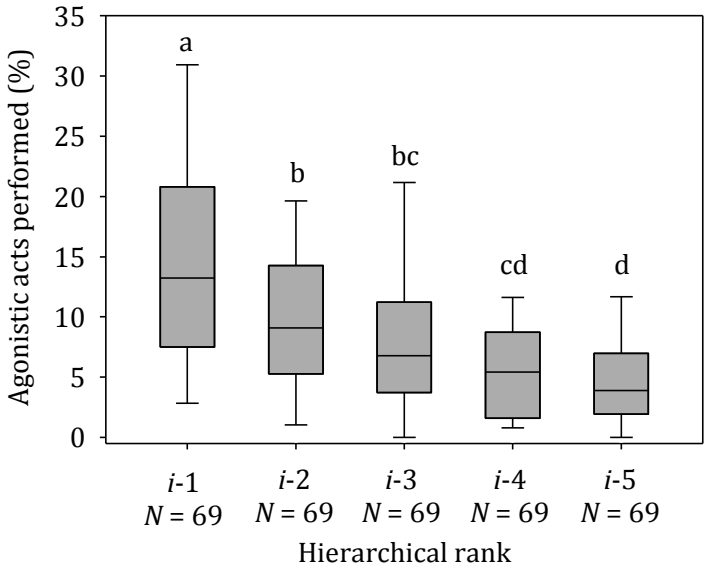

Figure 1. Percentage of agonistic acts performed by high rankers (rank *i*) towards the individuals with the closest lower ranks in the hierarchy (rank *i*−1 to rank *i*−5). Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of individuals are indicated below each box plot. Different letters denote statistical differences.

Agonistic interactions directed towards dominant individuals (inconsistencies) were very rare among high rankers (2.56 ± 0.84% per colony). In addition, the formation of a linear hierarchy was very quick, since the proportion of inconsistencies was already small during the first 24 h of isolation (12.76 ± 8.09% per colony).

Habituation–Discrimination Procedure

Habituation, manifested by a decrease in the duration of antennal contacts with the stimulus ant between the first and the last habituation test (Wiley, 2013), occurred in the four experiments (permutation tests: all $P < 0.021$; Appendix Figure A2). In the first experiment, habituation occurred whether the tested ant was familiarized with a high- or a low-ranking nestmate ($N = 10$, $P = 0.041$ and $N = 11$, $P = 0.003$, respectively; Appendix Figure A2a). High and low rankers showed similar mobility patterns in the experimental device ($N = 39$, all $P > 0.18$; Appendix Table A1), as did high rankers towards high- and low-ranking individuals ($N = 39$, all $P > 0.18$; Appendix Table A1). Furthermore, duration of contacts with the stimulus ant was similar for each of the four habituation tests and all tests combined whether the stimulus was a high- or a low-ranking individual ($N = 21$, all $P > 0.22$). Tested ants were thus able to familiarize themselves with a nestmate, the social status of this nestmate having no influence on this process.

In the discrimination test of the first experiment, tested ants spent more time antennating unfamiliar than familiar ants ($N = 21$, $P = 0.0004$; Figure 2), irrespective of the status of the habituation stimulus (high-ranking nestmates: $N = 10$, $P = 0.020$; low-ranking nestmates: $N = 11$, $P = 0.018$). There was no significant difference in the duration of antennal contacts between high- and low-ranking familiar stimuli ($N = 21$, $P = 0.96$) and between high- and low-ranking unfamiliar stimuli ($N = 21$, $P = 0.97$). High rankers were thus well able to discriminate the social status of their nestmates, as has already been shown in low rankers (Blacher et al., 2010).

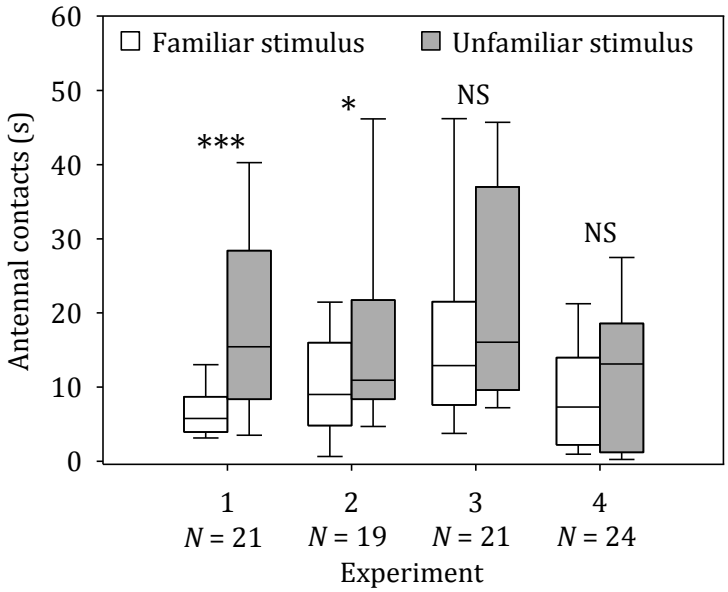

Figure 2. Duration of antennal contacts (s) towards the familiar and unfamiliar stimuli during discrimination tests in all experiments. In experiments 1–3, tested ants were high rankers and were confronted with a low- and a high-ranking nestmate (experiment 1), two high-ranking nestmates (experiment 2) or two low-ranking nestmates (experiment 3). In experiment 4, tested ants were low rankers and both stimuli were high-ranking nestmates. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of individuals are indicated below each box plot. ***$P < 0.001$; *$P < 0.05$.

In the second experiment, duration of antennal contacts towards unfamiliar ants was higher than towards familiar ants ($N = 19$, $P = 0.037$; Figure 2). Since stimuli ants were separated by a single rank, this clearly demonstrates that high rankers were capable of fine-scale status discrimination of other high-ranking workers.

In the third experiment, duration of antennation towards unfamiliar and familiar nestmates was not significantly different ($N = 21$, $P = 0.42$; Figure 2). High rankers therefore did not show a behavioural discrimination of low-ranking nestmates with very similar statuses. This result further confirms that the differential response of the tested ants towards unfamiliar stimuli compared with familiar stimuli in the first and second experiments was based on status discrimination.

In the fourth experiment, low-ranking tested ants did not spend significantly more time antennating unfamiliar or familiar nestmates ($N = 24$, $P = 0.15$; Figure 2). Furthermore,

there was no significant difference between low- (experiment 4) and high-ranking tested ants (experiment 2) in the duration of antennal contacts towards high-ranking familiar stimuli (N = 43, P = 0. 79) and in the duration of antennal contacts towards high-ranking unfamiliar stimuli (N = 43, P = 0.36). In contrast to high rankers, low rankers thus did not exhibit fine-scale status discrimination of high-ranking nestmates.

Fertility Measurement

Hierarchical rank was strongly correlated with the ovarian index in all colonies (Table 1). This corroborates the well-known relationship between fertility and social status in insect societies (Monnin & Peeters, 1999; Heinze et al., 2002; Cuvillier-Hot et al., 2004b; Blacher et al., 2010).

Chemical Profiles

Workers could clearly be separated on the basis of their cuticular hydrocarbon profiles according to their hierarchical rank (Wilks's λ = 0.304, $F_{40,402}$ = 8.19, P < 0.0001; Appendix Figure A3a) and even more strongly to their ovarian development (Wilks's λ = 0.130, $F_{52,390}$ = 13.33, P < 0.0001; Appendix Figure A3b). This indicates that the chemical signature provides a reliable cue for an ant to discriminate the fertility and social rank of its nestmates.

From the 34 compounds constituting the shared chemical profile of *N. apicalis* workers (Appendix Figure A1), 14 displayed significant differences in their amounts between individuals of varying fertility (Appendix Table A2). Among these compounds, 13-methylpentacosane (13-MeC_{25}) had the highest contribution to the discrimination of all individuals according to their ovarian development (partial Wilks's λ = 0.799, $F_{2,195}$ = 24.39, P < 0.0001) and hierarchical rank (partial Wilks's λ = 0.867, $F_{2,201}$ = 15.47, P < 0.0001) in the discriminant function analyses. Quantity of 13-MeC_{25} was further strongly correlated with both fertility (Spearman rank correlation: r_s = 0.77, N = 223, P < 0.0001; Figure 3a; Appendix Table A2), and hierarchical rank (r_s = –0.73, N = 223, P < 0.0001; Figure 3b).

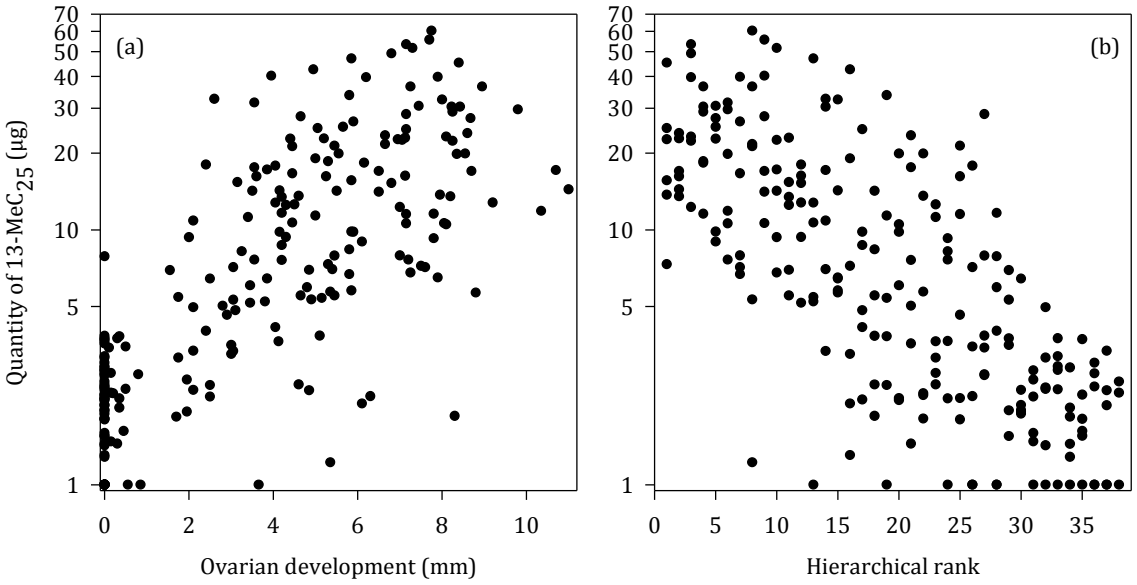

Figure 3. Relationship between quantity of 13-MeC_{25} (mg) and (a) ovarian development (mm) and (b) hierarchical rank. Note log scale on ordinates.

DISCUSSION

Our results clearly show that *N. apicalis* workers in hopelessly queenless colonies establish a linear dominance hierarchy in which agonistic behaviour, fertility, cuticular hydrocarbon profile and social rank are all closely correlated. Dominance interactions were highly directed, with top-ranking individuals performing most agonistic acts towards other high rankers with immediate consecutive ranks. The vast majority of hierarchical relationships were clearly and quickly established. Furthermore, it has been shown in *N. apicalis* and several other species that once the hierarchy has emerged, dominance relationships can be maintained for extended periods of time, but with a dramatic decrease in agonistic behaviour (Monnin & Peeters, 1999; Cuvillier-Hot et al., 2004b; Blacher et al., 2010). Although this does not exclude the influence of self-organizing processes, at least in the beginning (Dugatkin & Earley, 2004; Hsu et al., 2006; Rutte et al., 2006), it unambiguously indicates the implication of recognition mechanisms in the formation and maintenance of the hierarchical structure.

All ants were able to discriminate top-ranking from low-ranking nestmates, thus confirming and expanding the existence of status discrimination abilities in this species

(Blacher et al., 2010). Furthermore, top rankers were able to discriminate two high-ranking nestmates separated by a single rank in the hierarchy. This ability of fine-scale status discrimination among top rankers is beneficial for the regulation of reproductive dominance. It allows each competing ant to adapt its behaviour according to the social status of all encountered nestmates, i.e. dominantly towards a lower ranker and submissively towards a higher ranker. Avoiding recognition errors decreases the costs associated with dominance interactions in terms of colony productivity, and therefore enhances the individuals' inclusive fitness (Gobin et al., 2003). Note that both stimuli in the discrimination phase were dominant for the tested ant, meaning that individuals did not merely discriminate an ant higher than itself from a lower ant in the hierarchy. This emphasizes that recognizing all the competing individuals' statuses can be adaptive in the eventuality of a hierarchy disruption (Hart & Monnin, 2006), as has been shown to occur (Oliveira & Hölldobler, 1990).

It has been shown in two related species, *N. villosa* and *N. inversa*, that unrelated co-founding queens establishing dominance hierarchies seem capable of individual recognition (d'Ettorre & Heinze, 2005; Dreier et al., 2007). In our study, top rankers did not behaviourally discriminate low rankers of virtually identical status. This could partly stem from a low motivation for accomplishing this task, as low-ranking nestmates are not involved in the reproductive competition. The habituation–discrimination procedure was, however, used to reduce the influence of motivation on the tested ants' response. Although the context of hierarchy formation is different in *N. apicalis*, and unambiguously demonstrating individual recognition abilities is particularly challenging (Wiley, 2013), our results nevertheless suggest an absence of identity-based discrimination in workers. Within the scope of status discrimination abilities, and as we discuss in more depth below, this absence of behavioural discrimination between two low rankers could most likely be explained by an absence of recognition cues allowing an unequivocal discrimination.

If our results fail to provide any strong evidence in favour of individual recognition, they, however, clearly indicate that linear hierarchies can arise without such a recognition system. Status discrimination, a cognitively simpler mechanism (Wiley, 2013), has in this species at least the level of accuracy enabling a fine-scale discrimination of the individuals' statuses without the necessity of recognizing their identity. This recognition

system thus appears suitable for precise regulation of dominance interactions without the need for aggressive behaviours, and more importantly avoids the cognitive costs linked to individual recognition. Indeed, individual recognition relies on the memory of each opponent's distinctive features, and of their history of encounters in the context of dominance interactions. It thus requires active learning and an accurate memory, both of which are costly processes in terms of time and energy expenditure (Dukas, 2008; Burns et al., 2011). By contrast, status discrimination is a cognitively less demanding task, as it relies on the direct perception of each encountered individual's rank. Carefully investigating alternative hypotheses thus remains a crucial step when studying animal cognition (Elwood & Arnott, 2012). Overall, these results show that direct rank perception is probably a critical factor in the establishment and stabilization of the hierarchical structure.

Whereas high and low rankers showed very similar behavioural reactions in the experimental device in terms of their proximity and duration of antennal contacts towards stimuli in both the habituation and the discrimination phase, low rankers failed to discriminate at a fine scale ants belonging to the same rank class, be they high- or low-ranking nestmates (this study; Blacher et al., 2010). This difference with top rankers could be due to an absence of motivation since all high-ranking nestmates are by definition much higher in the hierarchy and could induce the same submissive behaviours from low rankers. In contrast to other species (Hart & Monnin, 2006), low rankers indeed play no role in top ranker replacements (Oliveira & Hölldobler, 1990), and thus in this context they have no benefits from discriminating two high rankers. Alternatively, the difference between high- and low-ranking workers could also be due to differences in their cognitive abilities. Indeed, top and low rankers can differ in a number of physiological characteristics. For example, neuroendocrine activities (levels of brain biogenic amines) can vary depending on the dominance and reproductive status. Top rankers have higher levels of octopamine than low rankers in the bumble bee *Bombus terrestris* (Bloch et al., 2000a), and octopamine levels are correlated with reproductive activity in the queenless ant *Streblognathus peetersi* (Cuvillier-Hot & Lenoir, 2006). Octopamine further acts as a neuromodulator which is known to affect cognitive processes such as learning and memory (Farooqui, 2007; Verlinden et al., 2010). Different internal states according to the individuals' hierarchical status could thus theoretically mediate various levels of recognition abilities. Intraspecific variation

in recognition abilities has recently begun to be explored (e.g. Injaian & Tibbetts, 2014), and future investigations are therefore required to examine the existence of actual differences in cognitive abilities depending on the individual's social status.

Cuticular hydrocarbon profiles diverged between reproductive and nonreproductive individuals, and probably constitute the recognition cues used in dominance interactions. The chemical nature of cuticular hydrocarbons signalling fertility can differ markedly according to species, but the occurrence of fertility signals seems to be a general phenomenon in social insects (Liebig et al., 2000; Sledge et al., 2001a; Heinze et al., 2002; Cuvillier-Hot et al., 2004b; Monnin, 2006; Liebig, 2010). This can be explained by the fact that being permanently informed about the fertility state of the egg-layer(s) provides inclusive fitness benefits to all colony members (Keller & Nonacs, 1993). Here, amounts of 13-methylpentacosane (13-MeC_{25}) were highly correlated with the individuals' ovarian activity. This compound may therefore have the role of a putative fertility signal (Monnin, 2006; Liebig, 2010). Hydrocarbons are synthesized in the oenocytes, cells associated with the epidermis and the fat bodies (Martins & Ramalho-Ortigão, 2012). It is generally assumed that common endocrinological mechanisms (e.g. gonadotropic hormones) underlie the biosynthesis of cuticular hydrocarbons and the activity of the ovaries (Cuvillier-Hot et al., 2004b; Peeters & Liebig, 2009; Liebig, 2010), thus explaining the close link between reproductive activity and chemical signals. Such an intrinsic causal link would mean the recognition system could not be faked, and fertility signals would be evolutionarily stable (i.e. honest; Maynard Smith & Harper, 1995; Laidre & Johnstone, 2013).

The quantity of the putative fertility signal was also highly correlated with the individuals' social rank, because of the close link between fertility and hierarchical status. The exponential form of the relationship between amounts of 13-MeC_{25} and hierarchical rank means that two individuals with very different social statuses (i.e. a high- and a low-ranking worker) would have a large difference in their amounts of 13-MeC_{25}, therefore allowing status discrimination. Two nestmates having close but none the less different hierarchical statuses (i.e. two top rankers in this case) would have a lower but yet significant difference in their amounts of 13-MeC_{25}. In this case, status discrimination becomes precise enough to allow a fine-scale discrimination of their ranks, corresponding to a functional individual discrimination. In contrast, two

individuals having a very similar social status (i.e. two low rankers) would have a very small difference in their amounts of 13-MeC_{25}, thus making any discrimination probably difficult, possibly beyond the workers' sensory and information-processing capabilities. According to this hypothesis, 13-MeC_{25} is likely to form the proximate signal at the basis of the individuals' status discrimination, and could in this respect constitute a chemical badge of status (Guilford & Dawkins, 1995). Such signals, traditionally linked with the individuals' resource-holding potential (Johnstone & Norris, 1993), could allow individuals in this context to select the best egg-layer in the colony. Similar mechanisms have already been suggested (Cuvillier-Hot et al., 2004b), and could form a general rule in the regulation of dominance hierarchies in insect societies. The dynamics of fertility signalling during the establishment of the hierarchical structure remains unknown, but it is conceivable that prefertility differences at the onset of orphaning could strongly influence dominance interactions and therefore the determination of hierarchical ranks. Such investigations should thus be conducted in future studies.

In conclusion, we have shown here that status discrimination based on a putative fertility signal is able to generate a linear dominance hierarchy in *N. apicalis* ants. This single cuticular hydrocarbon appears to act as a badge of status by precisely labelling the individual's position in the hierarchy, and therefore regulates the conflict over male parentage in this species. Whereas the formation of linear hierarchies is often assumed to rely on complex cognitive processes such as individual recognition, our results suggest on the contrary that simpler recognition mechanisms can be sufficient to regulate dominance interactions efficiently. By mutually benefiting all members of the nest, this recognition system is thus very likely to have been selected for by both individual- and colony-level selection pressures.

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APPENDIX

Table S1. Mobility pattern of tested ants in the experimental device.

	Total distance moved (cm)	Mean angular velocity (rad/s)	Duration of mobility (s)	Duration close to the stimulus ant (s)
High-ranking tested ants confronted with high-ranking stimuli (1, *N* = 20)	28.72 ± 5.48	18.14 ± 3.62	28.14 ± 5.28	8.12 ± 1.73
Low-ranking tested ants confronted with high-ranking stimuli (2, *N* = 19)	39.67 ± 5.78	14.62 ± 2.22	35.86 ± 4.97	11.87 ± 3.01
High-ranking tested ants confronted with low-ranking stimuli (3, *N* = 19)	22.21 ± 4.76	12.19 ± 2.22	22.09 ± 4.29	8.80 ± 1.89
P (1) vs (2)	0.18	0.42	0.29	0.31
P (1) vs (3)	0.38	0.18	0.38	0.79

Data are presented as mean ± SE.

Table S2. Chemical differences between workers of varying fertility.

Compound	Highly fertile individuals (*N* = 72)	Moderately fertile individuals (*N* = 80)	Infertile individuals (*N* = 71)
Unidentified	23.57 ± 6.38	24.70 ± 8.11	28.03 ± 3.82
10-MeC_{19}	2.68 ± 0.23 (a)	2.70 ± 0.20 (a)	3.65 ± 0.29 (b)
n-C_{20}	24.17 ± 1.50	20.10 ± 1.21	23.27 ± 1.57
Unidentified	3.10 ± 1.04	2.97 ± 1.26	1.87 ± 0.23
$C_{21:1}$	132.14 ± 34.10	45.90 ± 11.84	108.62 ± 47.33
$C_{21:1}$	42.12 ± 33.76 (a)	183.59 ± 79.26 (a)	802.62 ± 178.15 (b)
$C_{21:1}$	12.50 ± 0.63	10.75 ± 0.57	69.30 ± 59.80
$C_{21:1}$	79.31 ± 9.56	67.66 ± 8.42	67.56 ± 9.76
n-C_{21}	2400.67 ± 106.70 (a)	2215.76 ± 79.44 (ab)	2023.28 ± 113.08 (b)
11-MeC_{21}	3.21 ± 0.50 (a)	4.64 ± 2.15 (a)	0.65 ± 0.12 (b)
9-MeC_{21}	3.92 ± 0.52 (a)	2.77 ± 0.33 (a)	1.56 ± 0.28 (b)
$C_{22:2}$	189.62 ± 14.08	184.60 ± 10.75	202.48 ± 14.92
n-C_{22}	214.87 ± 9.78	203.73 ± 6.85	199.56 ± 8.37
11-MeC_{22}	10.76 ± 1.09	9.18 ± 0.74	8.53 ± 0.97
9-MeC_{22}	1.80 ± 0.19	2.02 ± 0.23	2.42 ± 0.46
$C_{23:2}$	7333.15 ± 388.67 (ab)	7548.17 ± 286.85 (a)	6265.83 ± 444.56 (b)
$C_{23:1}$	261.48 ± 33.84	220.02 ± 27.47	277.65 ± 65.43
n-C_{23}	2300.92 ± 72.74	2272.40 ± 70.29	2409.52 ± 82.63
11-MeC_{23}	75.45 ± 6.59 (a)	61.35 ± 4.79 (a)	28.63 ± 3.98 (b)
$C_{24:2}$	38.40 ± 3.12	37.92 ± 1.81	36.29 ± 2.38
n-C_{24}	19.02 ± 0.62	17.73 ± 0.56	19.24 ± 0.62
$C_{25:2}$	485.87 ± 41.50 (a)	395.56 ± 27.64 (a)	219.15 ± 27.79 (b)
n-C_{25}	288.14 ± 12.40	258.25 ± 9.96	263.71 ± 13.81
13-MeC_{25}	19.50 ± 1.61 (a)	9.96 ± 1.06 (b)	1.25 ± 0.14 (c)
11-MeC_{25}	4.42 ± 0.55 (a)	4.12 ± 0.42 (a)	2.67 ± 0.32 (b)
n-C_{26}	22.49 ± 1.13	20.13 ± 0.93	19.13 ± 1.23
$C_{27:2}$	31.19 ± 4.13 (a)	20.19 ± 2.05 (b)	5.90 ± 0.60 (c)
n-C_{27}	444.30 ± 25.12 (a)	378.11 ± 18.45 (ab)	354.58 ± 21.98 (b)
n-C_{28}	27.25 ± 1.51	26.61 ± 1.10	29.15 ± 1.87
2-MeC_{28}	88.35 ± 4.64	87.93 ± 3.86	98.24 ± 6.52
$C_{29:2}$	18.55 ± 1.47 (a)	14.27 ± 0.85 (b)	11.13 ± 0.72 (c)
$C_{29:1}$	12.91 ± 0.71	12.17 ± 0.62	11.47 ± 0.73
n-C_{29}	338.79 ± 23.86	340.40 ± 17.10	380.66 ± 22.18
n-C_{31}	43.92 ± 3.82 (a)	59.54 ± 4.64 (b)	86.39 ± 5.70 (c)

Data correspond to absolute quantities (μg) of 34 cuticular hydrocarbons common to all individuals, and are presented as mean ± SE. Different letters (a, b, c) denote statistical differences.

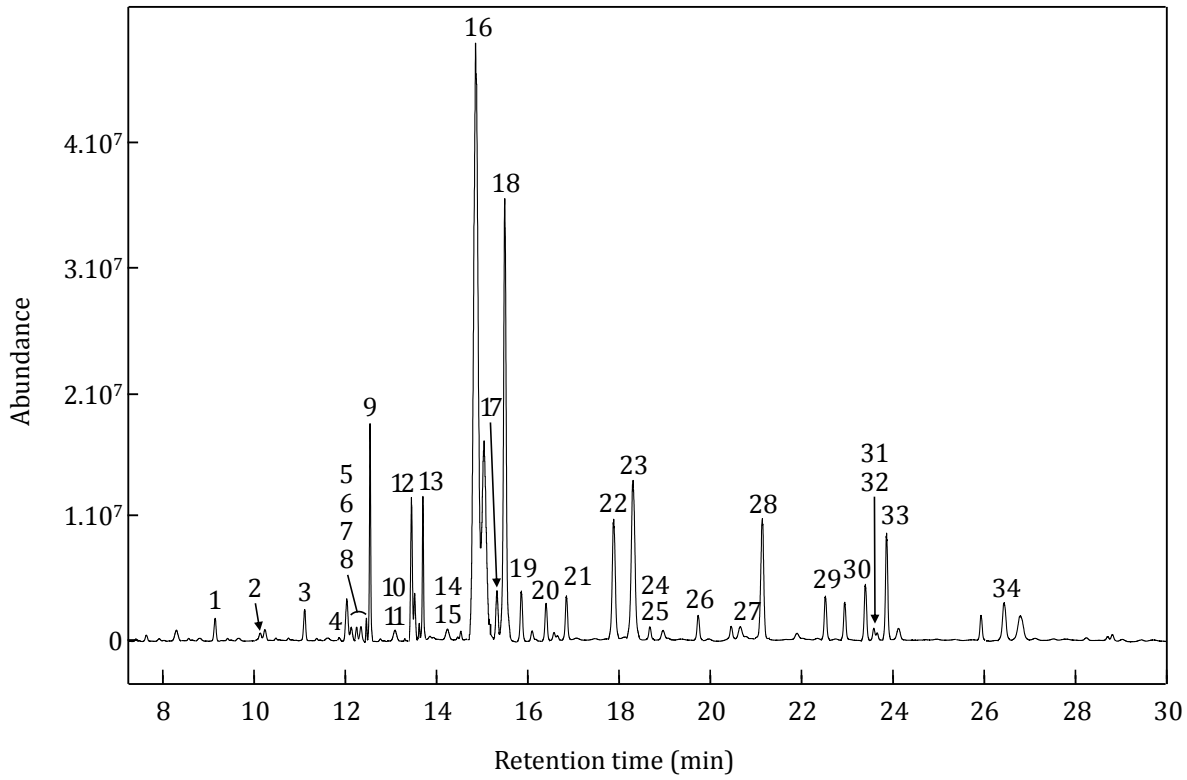

Figure A1. Chromatogram of the cuticular hydrocarbon profile of a moderately fertile *N. apicalis* worker. Peaks used in the statistical analysis are indicated by numbers: 1 = unidentified; 2 = 10-MeC_{19}; 3 = *n*-C_{20}; 4 = unidentified; 5 = $C_{21:1}$; 6 = $C_{21:1}$; 7 = $C_{21:1}$; 8 = $C_{21:1}$; 9 = *n*-C_{21}; 10 = 11-MeC_{21}; 11 = 9-MeC_{21}; 12 = $C_{22:2}$; 13 = *n*-C_{22}; 14 = 11-MeC_{22}; 15 = 9-MeC_{22}; 16 = $C_{23:2}$; 17 = $C_{23:1}$; 18 = *n*-C_{23}; 19 = 11-MeC_{23}; 20 = $C_{24:2}$; 21 = *n*-C_{24}; 22 = $C_{25:2}$; 23 = *n*-C_{25}; 24 = 13-MeC_{25}; 25 = 11-MeC_{25}; 26 = *n*-C_{26}; 27 = $C_{27:2}$; 28 = *n*-C_{27}; 29 = *n*-C_{28}; 30 = 2-MeC_{28}; 31 = $C_{29:2}$; 32 = $C_{29:1}$; 33 = *n*-C_{29}; 34 = *n*-C_{31}.

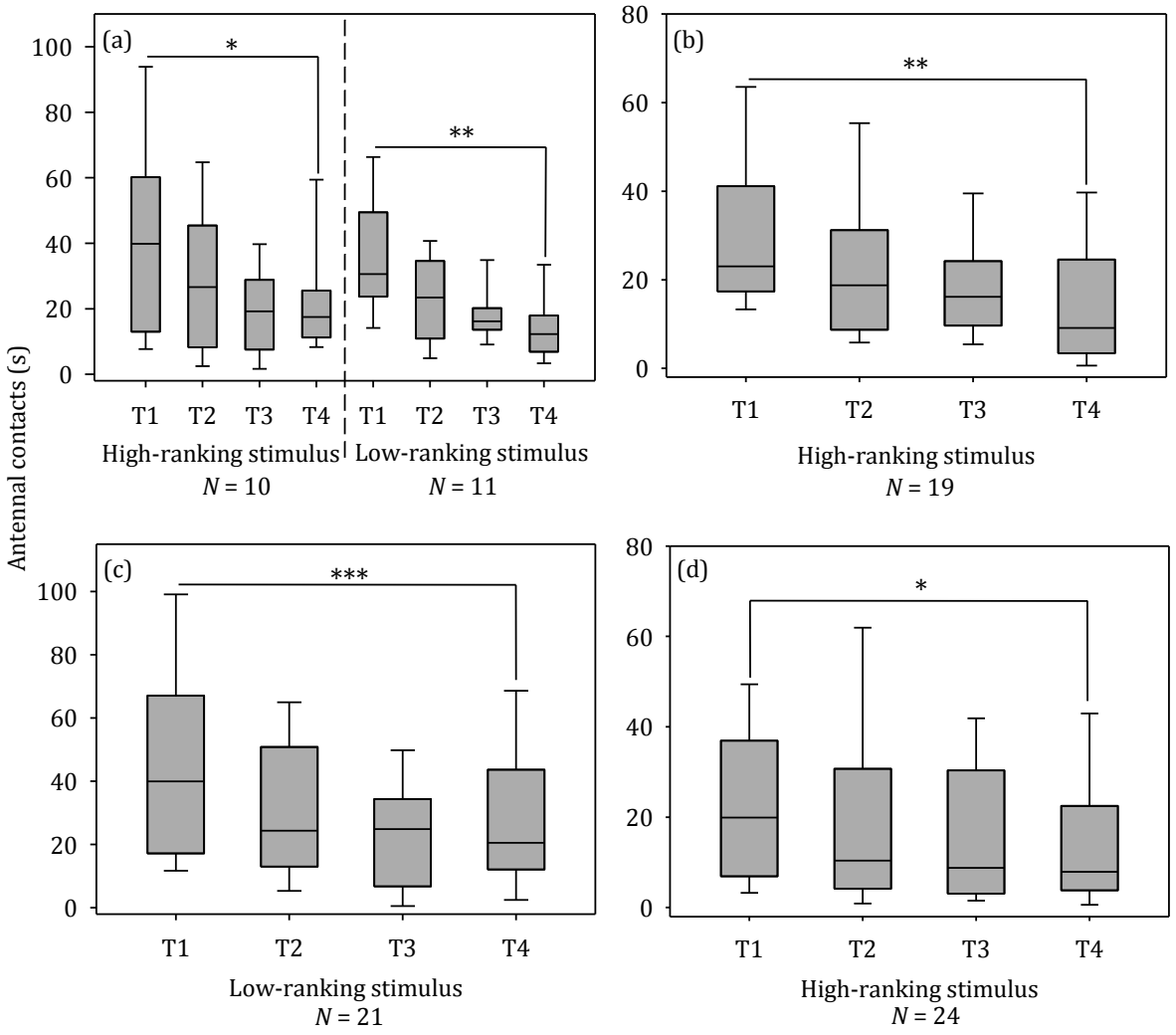

Figure A2. Duration of antennal contacts (s) towards the stimulus nestmate during habituation tests 1–4 (4 min each with a 5 min interval) in all experiments (see text). (a) Experiment 1: a high ranker was confronted with either a high- or a low-ranking nestmate. (b) Experiment 2: a high ranker was confronted with a high-ranking nestmate. (c) Experiment 3: a high ranker was confronted with a low-ranking nestmate. (d) Experiment 4: a low ranker was confronted with a high-ranking nestmate. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of individuals are indicated for each experiment. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

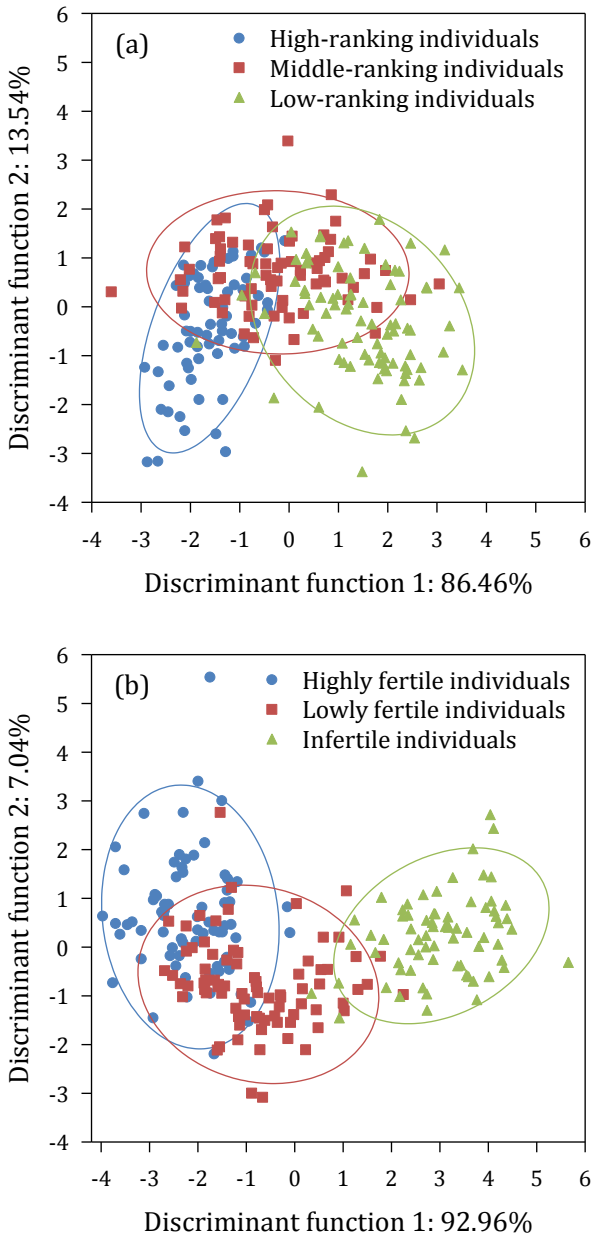

Figure A3. Discriminant function analyses showing the differences in the chemical profiles of 223 workers according to their (a) hierarchical rank and (b) ovarian development. Ellipses represent 90% confidence intervals around centroids. The percentage of variance explained is depicted on each axe.

Article 4

Fertility signalling and partitioning of reproduction in the ant *Neoponera apicalis*

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In preparation

ABSTRACT

All individuals in social insect colonies benefit from being permanently informed about the presence and fertility state of reproducers. This allows the established reproductive individuals to maintain their reproductive monopoly without the need for physical control, and the non-reproductive individuals to make appropriate reproductive decisions. The existence of reliable recognition –fertility-associated– cues is therefore critical, and cuticular hydrocarbons are good candidates as honest fertility signals. Here we studied whether fertility signalling is responsible for the partitioning of reproduction in the ant *Neoponera apicalis*. This species form small colonies from one single-mated queen, and workers establish reproductive hierarchies when hopelessly queenless. Previous studies have identified putative fertility signals, particularly the hydrocarbon 13-methylpentacosane (13-MeC_{25}), and shown that precise status discrimination based on these signals could be involved in the regulation of reproductive activities. Here, we extend these findings and reveal that all individuals, be they queens or workers, differ in their cuticular hydrocarbon profile according to their fertility state. Proportions of 13-MeC_{25} were a strong predictor of the individuals' ovarian activity, and could thus advertise the established reproducer(s) in both queenright and queenless conditions. Furthermore, this compound could play a key role in the establishment of the reproductive hierarchy, since workers with low fertility at the onset of hierarchy formation already exhibit relatively high amounts of 13-MeC_{25}. Dyadic encounters showed that individuals with experimentally increased amounts of 13-MeC_{25} triggered less agonistic interactions from top rankers, in line with them advertising a higher status. These bioassays thus proved the use of 13-MeC_{25} by the competing ants. We discuss the different selection pressures that could have favoured the evolution of this simple recognition system potentially allowing to permanently regulate the partitioning of reproduction in this species. We argue that our results supports the index hypothesis of honest signalling, according to which there is a mechanistic connection between ovary activation and the production of fertility-associated chemicals, thus providing a mechanism guaranteeing the stability of this recognition system.

Keywords: Cuticular hydrocarbons, dominance, honest signalling, *Neoponera* (formerly *Pachycondyla*) *apicalis*, recognition system, reproductive hierarchy.

INTRODUCTION

The ecological dominance of social insects mostly relies on their reproductive division of labour, where only one or a few individuals (usually queens) invest in egg-laying activities, whereas the vast majority of the colony foregoes any direct reproduction (Wilson, 1971). However, in most eusocial Hymenoptera (ants, some bees and wasps), non-reproductive individuals (workers) have maintained functional ovaries, and thus have the potential to lay their own eggs (Bourke, 1988b). The non-clonal kin structure of insect societies creates potential reproductive conflicts where, under some circumstances, workers are predicted to favour their own over the colony's reproducer offspring (Ratnieks & Reeve, 1992; Ratnieks et al., 2006). For example under single mating of the queen, colonies are subject to a queen–worker conflict over male parentage (Trivers & Hare, 1976; Bourke, 1988b; Ratnieks et al., 2006). Levels of worker reproduction then depend on the kin structure of the colony and on the constraints and costs to colony-level productivity preventing or limiting the benefits of direct reproduction (Ratnieks & Reeve, 1992; Hammond & Keller, 2004; Wenseleers et al., 2004), with self-restraint and coercion being the two main mechanisms regulating the extent of worker reproduction (Bourke, 1988b; Wenseleers & Ratnieks, 2006; Ratnieks et al., 2006). In this context, the presence and fertility state of a reproducer in the colony has a crucial influence on the propensity of worker reproduction, and theoretical models and empirical observations indeed show that levels of worker reproduction are commonly much higher in queenless than in queenright colonies (Bourke, 1988b; Ratnieks, 1988; Wenseleers et al., 2004; Ratnieks et al., 2006). The often extreme reproductive skew characteristic of insect societies therefore necessitates that workers are correctly informed about the presence of a fertile reproducer inside the colony.

Fertility-associated chemical signals are present in virtually all insect societies (Heinze, 2004; Monnin, 2006; Le Conte & Hefetz, 2008; Peeters & Liebig, 2009; Liebig, 2010). They mostly consist of long-chained hydrocarbons, a major part of the lipid layer covering insects' cuticle, which primarily act as a barrier preventing desiccation, but are also highly involved in communication (Howard & Blomquist, 2005; Richard & Hunt, 2013). Specific (saturated, methyl-branched, or unsaturated) hydrocarbons in the chemical profile are then typically over-expressed in fertile individuals, thereby informing their nestmates about their presence and fertility state (Monnin et al., 1998;

Liebig et al., 2000; Sledge et al., 2001a; Dietemann et al., 2003; de Biseau et al., 2004; Hartmann et al., 2005). Numerous studies indicate that social insects not only detect the presence of a reproducer in the colony and react accordingly by reproductive self-restraint and/or coercion towards would-be egg-layers (Hoover et al., 2003; Endler et al., 2004; Dapporto et al., 2007b; Smith AA et al., 2009; Bhadra et al., 2010; Holman et al., 2010; Smith et al., 2012; Van Oystaeyen et al., 2014), but that they can also perceive various levels of ovarian activity among fertile individuals (Gobin et al., 1999; Liebig et al., 1999; Ortius & Heinze, 1999; Hannonen et al., 2002; Heinze et al., 2002; Cuvillier-Hot et al., 2004b; Yagound et al., 2014).

Therefore the traditional view of queen-produced fertility chemicals as inhibiting pheromones actively suppressing worker reproduction (Wilson, 1971) has now been largely replaced by considering fertility-associated cuticular hydrocarbons as honest signals (Keller & Nonacs, 1993; Monnin, 2006; Heinze & d'Ettorre, 2009; Peeters & Liebig, 2009). Workers reproductive decisions thus follow their own interest, since responding to these signals by refraining or preventing other workers from reproducing ultimately results in an increase in the workers' inclusive fitness as long as the fertility state of the established reproducer is high enough (Keller & Nonacs, 1993).

Here we studied whether honest fertility signalling is responsible for the regulation of reproduction in the ant *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) *apicalis* (Hymenoptera: Formicidae: Ponerinae). This monogynous and monandrous species (Yagound et al., unpublished data) form small colonies of typically less than one hundred individuals, characterized by a small queen–worker dimorphism and where workers have a high reproductive potential (Fresneau, 1994). As in many social hymenopterans, the partitioning of reproduction is characterised by two distinct phases, namely a queen reproductive monopoly during most of the colony's life, and then when the queen fecundity declines a final worker reproductive competition over male parentage resulting in the establishment of a reproductive hierarchy (Oliveira & Hölldobler, 1990; Blacher et al., 2010, Yagound et al., 2014).

Previous studies in hopelessly queenless colonies (colonies which have lost their queen and where no replacement can occur; Châline et al., 2004) have shown that status discrimination based on putative fertility signals is involved in the establishment and maintenance of the hierarchical structure (Blacher et al., 2010, Yagound et al., 2014). A

cuticular hydrocarbon in particular, 13-methylpentacosane (13-MeC_{25}), critically differentiates workers according to their level of ovarian activity (Yagound et al., 2014). As worker reproductive restraint in queenright nests is supposed to depend on non-volatile queen pheromones (Dietemann & Peeters, 2000) and because of the general similarity between queen and worker fertility signals in other species (Heinze et al., 2002; Dietemann et al., 2003; Smith et al., 2008; Liebig, 2010), we might expect 13-MeC_{25} to be also present in queens, and to vary according to their fertility state. It is therefore possible that this signal mediates the regulation of the reproductive division of labour in both queenright and queenless situations.

Furthermore, the production of fertility signals in hopelessly queenless colonies is usually thought to begin once the hierarchical ranks have been determined and the individuals have started their oogenesis (Peeters et al., 1999; Liebig et al., 2000; Cuvillier-Hot et al., 2004b; Hartmann et al., 2005; Monnin, 2006; Peeters & Liebig, 2009). However, workers have been reported to occasionally lay trophic eggs in the presence of a queen (Oliveira & Hölldobler, 1990; Fresneau, 1994; Dietemann & Peeters, 2000). Therefore the possibility remains that workers with low fertility in queenright nests already possess relatively high amounts of 13-MeC_{25}, and that this signal could be involved during the very first steps of hierarchy formation to settle the dominance/subordinate interactions.

Fertility signals could thus play a crucial role in the regulation of reproduction in this species (Yagound et al., 2014). In this study we extend previous findings by investigating the chemical profiles of queens and workers of various fertility levels in both queenright and queenless conditions to verify the above predictions and provide additional evidence supporting the honest fertility signalling hypothesis. To corroborate the correlative chemical evidence and confirm the role of 13-MeC_{25} as a chemical badge of status within hierarchies, we finally conducted behavioural bioassays where we monitored the behavioural response of high- and low-ranking individuals towards nestmates of various ranks whose fertility-associated compounds were manipulated.

METHODS

Ants and Rearing Conditions

We used 10 colonies of *Neoponera apicalis* morph 4 (Delabie et al., 2008; Ferreira et al., 2010) collected in Petit Saut (5°04'15.8"N, 53°02'36.3"W), French Guiana, in 2007 and 2011. Each colony comprised a queen, 64.1 ± 8.7 (mean ± SE) workers, and brood. They were reared in plaster nests (18 × 14 cm) connected to a foraging area. Ants were provided twice a week with crickets and honey/apple mixture, and water ad libitum. Housing conditions were as follows: relative humidity of 60 ± 5%, temperature of 27 ± 2°C, 12:12 h light:dark cycle. Ant collection, husbandry and experimental procedures used in this study fulfilled all the legal requirements concerning insect experimentation of France.

Experimental Procedures

Two consecutive experiments were carried out to study fertility signals in *N. apicalis*. In the first experiment, we compared the chemical profiles of fertile and unmated queens and of workers of varying fertility both at the onset of hierarchy formation (orphaning point) and in well-established reproductive hierarchies (hopelessly queenless situation). This was aimed at (i) confirming the honest fertility signalling hypothesis, according to which amounts of 13-MeC_{25} should be higher in fertile queens compared to fertile workers, whereas those of unmated queens should be similar to those of unfertile workers, and (ii) studying the link between signal expression and ovarian activity both before and after the formation of the dominance hierarchy. In the second experiment, we manipulated the amounts of cuticular 13-MeC_{25} of top and low rankers and monitored their subsequent behavioural interactions with high- and low-ranking nestmates during dyadic encounters to confirm the use of this cuticular hydrocarbon as a badge of status.

Experiment 1

Between-caste fertility signal comparison

Fertile queens (N = 8) and gynes (young unmated queens, N = 12) were collected from our stock colonies for sampling of their cuticular hydrocarbon profiles, using the non-destructive method of solid-phase microextraction (SPME). A 100 µm polydimethylsiloxane fiber (Supelco, Bellefonte, PA, U.S.A.) was carefully rubbed against the third and fourth abdominal segments of live ants for 2 min. The fiber was then desorbed in the injection port of a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, U.S.A.), equipped with an HP-5MS capillary column (30 m × 25 µm × 0.25 µm) and a split–splitless injector, coupled to a 5975c mass spectrometer (Agilent Technologies) with 70 eV electron impact ionization. The carrier gas was helium at 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 5 min, then 70–250°C at 30°C/min, then 250–260°C at 1°C/min, then 260–320°C at 20°C/min, then hold at 320°C for 5 min. Peaks areas were integrated with the MSD ChemStation software E.02.01.1177 (Agilent Technologies). Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared to known standards.

To link the individuals' chemical profile with their level of fertility, gynes were frozen for dissection and the mean size of the six basal oocytes was taken as an ovarian index. Head width as an index of body size was also measured for each gyne to account for the possible effects of this factor on levels of ovarian activity or amounts of fertility-associated cuticular hydrocarbons. The fertility level of queens was not determined to avoid sacrificing stock colonies. However, as is usual in social insects, queen fertility in *N. apicalis* is known to be higher than reproductive workers' fertility (Fresneau, 1994), despite their limited dimorphism. Observations of stock colonies before and long after this experiment clearly showed that all sampled queens were fertile (i.e. laying female-destined eggs). The chemical profiles of queens and gynes and the ovarian activity of the latter were then compared to the data obtained for the worker caste in both queenright and queenless conditions (see the following section).

Dynamics of signal expression

Three groups of 20 workers were isolated from our stock colonies and placed in a new nest (same dimensions). This dequeening procedure typically induces the formation of a reproductive hierarchy manifested by ritualised agonistic behaviours (Oliveira & Hölldobler, 1990; Yagound et al., 2014). Age is known to critically affect the reproductive and dominance status of the workers (Dietemann & Peeters, 2000), with mainly relatively young individuals being involved in the reproductive competition, whereas callow and old workers hardly ever engage in dominance interactions. To control for the presence of all age classes (and their associated physiological characteristics) in our groups we waited for the first unambiguous dominance/subordinate interactions to be clearly visible, which occurred within two hours in each case. By that time, all workers were sacrificed for subsequent analyses (same as described in the previous section). Since this situation is virtually identical to a queenright situation (in terms of workers' chemical profile and ovarian activity), these individuals are hereafter considered as queenright workers and represent the physiological state of workers at the onset of hierarchy formation. As dissections showed that some individuals already exhibited activated ovaries, workers were classified according to their number of developed oocytes: moderately fertile workers (MFW, two to four developed oocytes, N = 26), infertile workers (IW, zero to one developed oocytes, N = 34).

Six additional groups of 20 workers were also isolated following the same procedure, but this time they stayed in these queenless groups for 15 days. This duration is sufficient for a clear near-linear reproductive hierarchy to be established through ritualised agonistic behaviours and fertility signalling (Yagound et al., 2014). At day 16 the same analyses as for queenright workers were performed on all individuals. Three groups of varying fertility (adapted from Yagound et al., 2014) were determined: highly fertile workers (HFW, five to six developed oocytes, N = 38), moderately fertile workers (MFW, two to four developed oocytes, N = 37), and infertile workers (IW, zero to one developed oocytes, N = 41). Some workers (one in two groups, two in one group) died before day 16 and were therefore not included in the analyses.

Experiment 2

Reproductive hierarchies

Six orphaned colonies of 41 workers were created following the same procedure as in the first experiment. All workers were individually labelled with numbered tags glued in their thorax and dots of paint on their abdomen. Housing and feeding conditions were the same as above, but this time the nest was connected through a 5-cm tunnel to a circular chamber (diameter = 3.5 cm) freely accessible to the workers and subsequently used as the test arena in the bioassays. Chamber and tunnel walls were black-covered so that ants experience no discontinuity (namely light stimulation) with the rest of the nest.

All orphaned colonies were observed one hour a day for 15 days, with orphaning as day one. We recorded all agonistic behaviours (antennal boxing and biting) typical of dominance/subordinate interactions (Oliveira & Hölldobler, 1990; Yagound et al., 2014). This allowed the reconstruction of the matrix of hierarchical ranks for all individuals (see Blacher et al., 2010; Yagound et al., 2014). Ants with ranks 1–12 were considered top rankers (following Yagound et al., 2014), whereas the last 18 ranks (i.e. ranks 20–37 due to some mortality) were considered low rankers.

Bioassays

At day 16, behavioural bioassays were conducted to confirm the use of 13-MeC_{25} as a badge of status. The test arena was disconnected from the nest and subsequently used during the tests, thereby providing the ants with a "colonial context", at least considering the olfactory modality. Bioassays consisted in dyadic encounters between a focal ant and a treated stimulus nestmate whose fertility-associated hydrocarbons had been manipulated. Three treatments were used: increasing amounts of 13-MeC_{25} (putative badge of status), increasing amounts of eicosane (*n*-C_{20}, control compound naturally found on *N. apicalis* cuticles but whose quantities are not correlated to fertility or rank; Yagound et al., 2014), and solvent (hexane, i.e. manipulation control).

13-MeC_{25} was synthesised according to published methods (Guédot et al., 2009; see the Supplementary Material). To manipulate the stimuli's odour, we followed the protocol of Smith et al. (2012). Stock solutions of 8.4 mg of synthetic 13-MeC_{25} or *n*-C_{20} (Sigma

Aldrich, St. Louis, MO, U.S.A.) per 14 ml of hexane were prepared. For each treatment, 25 µl of stock solution were added onto the surface of a 10-ml glass beaker filled with deionised water. Following hexane evaporation, a stimulus ant briefly anesthetised through freezing temperatures for 30 s was immersed at the surface of the deionised water and gently swirled, thereby transferring the surface hydrocarbon film on its cuticle (Smith et al., 2012). The treated stimulus was then allowed to dry in a box for 10 min.

To verify the effectiveness of the odour manipulation, a subset of individuals was sampled with SPME both before and after chemical treatment. The protocol was the same as in the first experiment, except for the temperature program: an initial hold at 70°C for 1 min, then 70–250°C at 40°C/min, then 250–258°C at 1°C/min, then 258–320°C at 40°C/min, then hold at 320°C for 3 min. There was a 47.9 ± 11.2% increase in 13-MeC_{25} (N = 16), which is within the natural range of variation separating top and low rankers (33.6–103.9%, N = 206, data not shown). The increase in n-C_{20} was slightly but not significantly lower compared to 13-MeC_{25} (28.3 ± 5.8%, N = 19; permutation test: P = 0.12), and was necessarily beyond its normal range of variation (0.3–1.3%, N = 206, data not shown).

The treated stimulus and focal ant were then introduced in the test arena, temporarily separated by a microscope slide for 30 s to allow them to become accustomed to the device. Tests began following the microscope slide removal and lasted 10 min. Each test was video-recorded, and the ants' behaviour was subsequently analysed with EthoLog 2.2 software (Ottoni, 2000). We recorded the duration of antennal contacts and the number of all agonistic acts (antennal boxing, mandible opening, biting). Observers were systematically blind to the treatment.

Three conditions were used for each treatment: a top ranker confronted to a low ranker (mean gap in their respective rank of 21.7 ± 0.7, N = 36), a top ranker confronted to another top ranker (mean gap in their respective rank of 2.0 ± 0.0, N = 36), and a low ranker confronted to another low ranker (mean gap in their respective rank of 1.3 ± 0.3, N = 36). Two tests per treatment per colony were realised for each condition. Each ant was only involved in a single test. Focal ants always had a higher actual rank compared to treated stimuli, except in the case of low rankers whose ranks were very similar. All tests for each experimental colony were realised within a few hours to avoid any

changes in hierarchical order due to the consecutive removal of tested individuals. After the completion of the tests all workers were frozen for measurements of their ovarian activity.

Statistical Analysis

Experiment 1

We compared the relative quantities of 27 peaks common to all individuals. To avoid problems arising from multicollinearity, compounds highly correlated ($r^2 > 0.8$) were treated as a single variable (Martin & Drijfhout, 2009b). This resulted in a final high ratio (7.8) of observations to independent variables. A discriminant analysis was then performed on geomean-log transformed proportions according to Reyment (1989): $Z_{i,j} = \ln[Y_{i,j}/g(Y_j)]$, where $Y_{i,j}$ is the area of peak i for the individual j, and $g(Y_j)$ is the geometric mean of all peak areas for individual j. We studied the general relationship between fertility, proportion of compounds and size using Spearman rank correlation tests. One-way ANOVAs with the Monte Carlo procedure (Metropolis & Ulam, 1949) were also performed on relative amounts of cuticular hydrocarbons between all groups, and on ovarian and size indexes between all groups except queens.

Experiment 2

Following hierarchy reconstruction, we calculated the K index of linearity varying from 0 (no linearity) to 1 (linear hierarchy) and tested the statistical significance of linearity with the Appleby (1983) method. We further verified if the hierarchical rank was correlated with both dominance (proportion of agonistic acts performed) and fertility with the Spearman rank correlation test.

We used generalized linear mixed-effects models (GLMM) to test the effect of treatment (13-MeC_{25}, n-C_{20}, solvent) on the tested ants' behavioural response inside each condition (top ranker vs low ranker, top ranker vs top ranker, low ranker vs low ranker) with R-3.2.0 (R Core Team, 2012), using the package lme4 (Bates et al., 2013). The total number of agonistic behaviours (response variable) was compared using GLMMs with a Poisson

error distribution and a log link function. We compared the duration of antennal contacts (response variable) using GLMMs with a Gaussian error distribution and an identity link function. Treatment as a fixed factor and colony as a random factor were each time included in the models.

Statistical analyses were performed using R-3.2.0 (R Core Team, 2012) and Statistica 8.0 (StatSoft, Tulsa, OK, U.S.A.). Post-hoc corrected *P*-values following the Bonferroni–Holm method (Holm, 1979) are denoted *P'*. Statistical significance was set at $P < 0.05$.

RESULTS

Experiment 1

All groups diverged in their level of fertility (one-way ANOVA: $F_{5,188} = 189.70$, $P = 0.001$; Figure 1A). As expected, gynes' ovarian activity was very low and similar to that of infertile workers (Figure 1A).

Each group of queens and workers bore a distinct chemical profile as revealed by the discriminant function analysis (Wilks's $\lambda = 0.003$, $F_{138,981} = 11.87$, $P < 0.0001$; between-groups comparisons: all $P' < 0.037$). Compared with workers, queens had significantly higher relative amounts of 10-MeC_{19}, *x,y*-$C_{22:2}$, *n*-C_{22}, 11-MeC_{23}, 13-MeC_{25}, and there was a strong trend for *x,y*-$C_{29:2}$, whereas workers had higher proportions of *n*-C_{29} than queens (Table 1). The trend was similar for gynes compared with workers for 10-MeC_{19}, *x,y*-$C_{22:2}$, *x,y*-$C_{29:2}$ and *n*-C_{29}, thus making these compounds potentially caste signals, but not for *n*-C_{22}, 11-MeC_{23} and 13-MeC_{25}. Relative amounts of *x,y*-$C_{22:2}$, *n*-C_{22}, *x,y*-$C_{29:2}$ and *n*-C_{29} were not significantly different between queens and gynes, whereas 10-MeC_{19} was surprisingly over-expressed in gynes compared with queens, and may be involved in courtship or mating. By contrast, queens had higher relative quantities of 11-MeC_{23} and 13-MeC_{25} than gynes (Table 1).

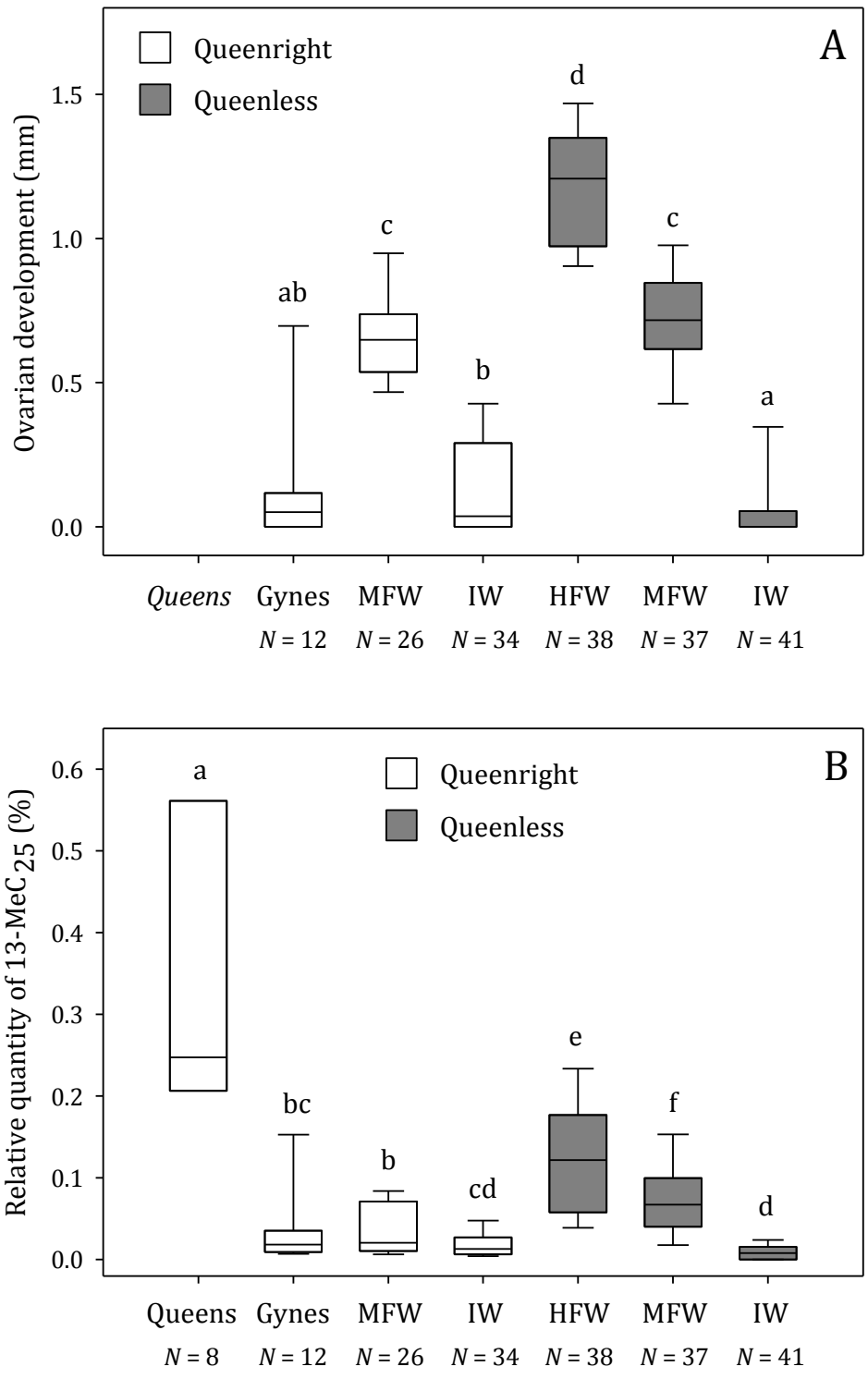

Figure 1. Between-group differences in (A) ovarian development (mm) and (B) relative quantity of 13-MeC_{25} (%). Queens are indicated in (A) even if not dissected for reading convenience. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of individuals are indicated below each box plot. Different letters denote statistical differences. HFW, highly fertile workers; MFW, moderately fertile workers; IW, infertile workers.

The correlation between relative amount of cuticular hydrocarbons and level of fertility proved to be significant for 10-MeC_{19}, 11-MeC_{23}, 13-MeC_{25} and *x*,*y*-$C_{29:2}$ (Table 1). As expected, this correlation was much higher for 13-MeC_{25} (Spearman rank correlation: r_s = 0.67, N = 188, P' < 0.0001). Proportion of 13-MeC_{25} and fertility level diverged between groups in a very close relationship (Table 1 and Figures 1A and B), thus being in accordance with the fertility signal hypothesis. Queens indeed had the highest relative amounts of 13-MeC_{25} (Figure 1B), and arguably the highest fertility levels (Fresneau, 1994). By contrast, gynes whose ovarian activity was similar to infertile workers had much reduced relative amounts of 13-MeC_{25}, lying between moderately fertile and infertile workers (Figures 1A and B).

Highly fertile, moderately fertile and infertile workers in hopelessly queenless situation showed very marked differences in their amounts of 13-MeC_{25} and levels of fertility, with a very close relationship between these two variables (Figures 1A and B), as has already been described (Yagound et al., 2014). Interestingly, moderately fertile workers at the onset of orphaning had by that time higher relative amounts of 13-MeC_{25} compared with queenright infertile workers (P' = 0.022; Figure 1B).

Although individuals exhibited actual differences in their index of body size (range 1.75–2.24 mm, N = 188), this index was correlated neither with fertility (r_s = −0.04, N = 188, P' = 0.60), nor with relative amounts of 13-MeC_{25} (r_s = 0.06, N = 188, P' = 0.42). No difference in size was found between all groups of individuals, even when including gynes ($F_{5,188}$ = 1.06, P = 0.36).

Table 1. Caste- and fertility-related differences in relative amounts of cuticular hydrocarbons.

Compound	One-way ANOVA all groups	Between-caste comparison			Correlation with fertility level
		Queens (Q) vs workers[a] (W)	Gynes (G) vs workers (W)	Queens (Q) vs gynes (G)	
10-MeC$_{19}$	$F_{6,196}$ = 27.08 **P < 0.001**	Q > W all **P' < 0.011**	G > W all **P' < 0.003**	Q < G **P' = 0.003**	r_s = −0.23, N = 188 **P' = 0.0016**
x,y-C$_{22:2}$	$F_{6,196}$ = 5.39 **P < 0.001**	Q > W all **P' < 0.016**	G > W[b] all P' < 0.065	P' = 0.70	r_s = −0.07, N = 188 P' = 0.37
n-C$_{22}$	$F_{6,196}$ = 4.96 **P < 0.001**	Q > W all **P' < 0.037**	all P' > 0.56	P' = 0.61	r_s = 0.01, N = 188 P' = 0.87
11-MeC$_{23}$	$F_{6,196}$ = 9.74 **P < 0.001**	Q > W all **P' < 0.026**	all P' > 0.26	Q > G **P' = 0.030**	r_s = 0.41, N = 188 **P' < 0.0001**
13-MeC$_{25}$	$F_{6,196}$ = 24.02 **P < 0.001**	Q > W all **P' < 0.0018**	MFW > G > IW[c]	Q > G **P' = 0.0018**	r_s = 0.67, N = 188 **P' < 0.0001**
x,y-C$_{29:2}$	$F_{6,196}$ = 5.47 **P < 0.001**	Q > W[b] all P' < 0.052	G > W[b] all P' < 0.052	P' = 0.74	r_s = 0.23, N = 188 **P' = 0.0016**
n-C$_{29}$	$F_{6,196}$ = 31.40 **P < 0.001**	Q < W all **P' < 0.0018**	G < W all **P' < 0.0018**	P' = 0.17	r_s = 0.06, N = 188 P' = 0.43

Only cuticular hydrocarbons with clear between-caste differences are presented. See Yagound et al. (2014) for a comprehensive description of the cuticular hydrocarbon profile of *N. apicalis*. Significant values are highlighted in bold.

[a] Comparisons are made for highly fertile, moderately fertile and infertile workers.

[b] Some comparisons are not significantly different but there is a strong trend.

[c] MFW, moderately fertile workers; IW, infertile workers. See Figure 1B.

Experiment 2

The linearity or near-linearity of the hierarchy was significant in all colonies (*K* index of linearity ranging from 0.60 to 1, $N = 6$, all $P < 0.0029$). The hierarchical rank was further highly correlated with both dominance (r_s ranging from −0.64 to −0.92, $N = 6$, all $P < 0.0001$) and fertility (r_s ranging from −0.70 to −0.84, $N = 6$, all $P < 0.0001$). All colonies at the time of bioassays thus exhibited a clear near-linear reproductive hierarchy.

The agonistic response of focal high rankers towards low-ranking stimuli varied among treatments (GLMM: $\chi^2_2 = 19.19$, $P < 0.0001$, $N = 36$; Figure 2A). The number of agonistic acts performed by focal high rankers towards low rankers with increased amounts of 13-MeC_{25} and *n*-C_{20} was smaller compared to focal high rankers encountering hexane-treated low-ranking stimuli (post-hoc tests: both $P' < 0.0049$), but there was no significant difference between focal high rankers confronted to 13-MeC_{25}- and *n*-C_{20}-treated low-ranking stimuli ($P' = 0.29$). Focal high-ranking ants significantly decreased their agonistic response towards 13-MeC_{25}-treated high-ranking stimuli compared with both *n*-C_{20}- and hexane-treated high-ranking stimuli (GLMM: $\chi^2_2 = 26.69$, $P < 0.0001$, $N = 36$; both $P' < 0.016$; Figure 2B). Focal individuals confronted to *n*-C_{20}-treated high rankers also decreased their agonistic response compared to individuals encountering hexane-treated high rankers ($P' = 0.0063$). There was no significant difference among treatments in the number of agonistic acts of focal low rankers confronted to low-ranking stimuli (GLMM: $\chi^2_2 = 0.98$, $P = 0.61$, $N = 36$; Figure 2C).

The duration of antennal contacts from focal ants towards treated stimuli was not affected by the treatments in top rankers confronted to low-ranking stimuli (13-MeC_{25}: 86.20 ± 13.20 s, *n*-C_{20}: 76.20 ± 14.66 s, hexane: 79.48 ± 11.56 s; GLMM: $\chi^2_2 = 1.77$, $P = 0.41$, $N = 36$) and in top rankers confronted to top-ranking stimuli (13-MeC_{25}: 85.86 ± 15.08 s, *n*-C_{20}: 71.88 ± 17.91 s, hexane: 95.21 ± 18.85 s; GLMM: $\chi^2_2 = 0.65$, $P = 0.73$, $N = 36$). Antennal contacts performed by focal low rankers confronted to low-ranking stimuli were however significantly longer towards 13-MeC_{25}-treated stimuli than towards *n*-C_{20}- but not hexane-treated stimuli (13-MeC_{25}: 83.43 ± 15.27 s, *n*-C_{20}: 50.14 ± 10.43 s, hexane: 55.49 ± 8.03 s; GLMM: $\chi^2_2 = 7.47$, $P = 0.024$, $N = 36$, $P' = 0.036$ and $P' = 0.12$ respectively).

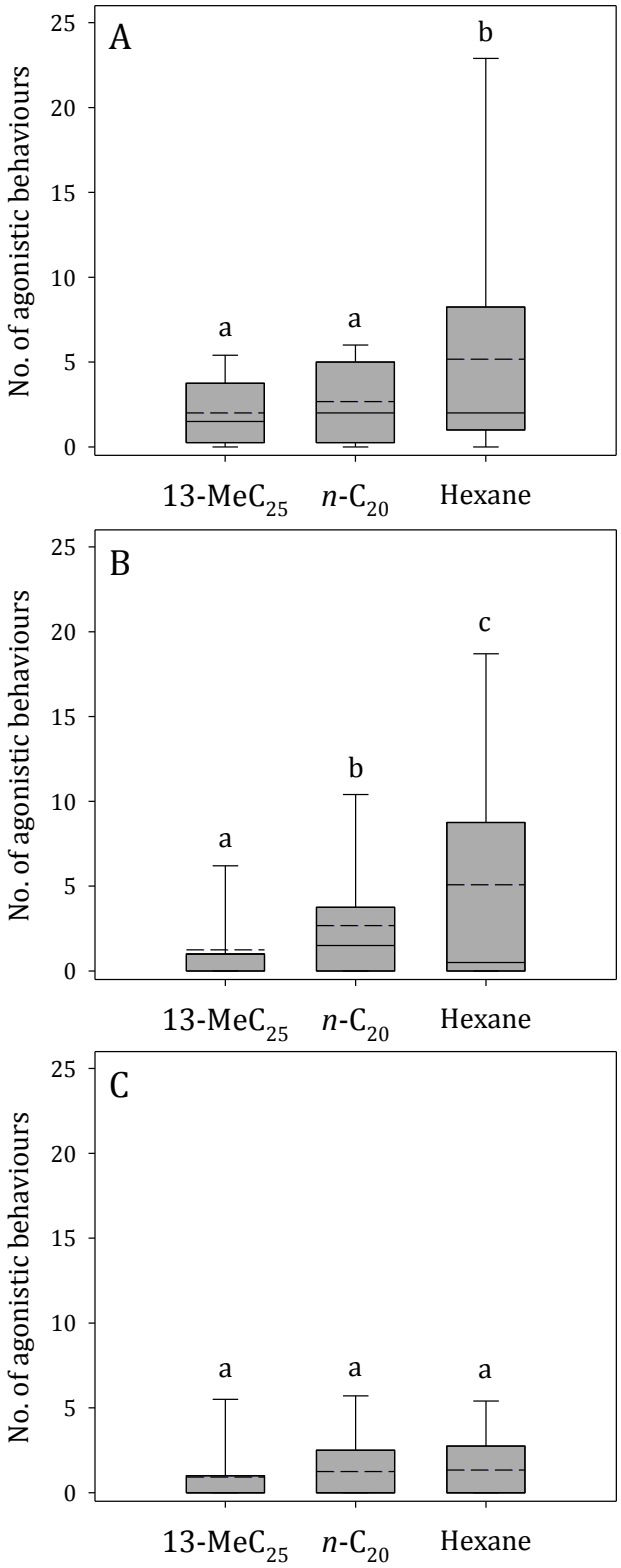

Figure 2. Total number of agonistic behaviours performed by focal workers towards nestmates with different treatments (13-MeC_{25}, *n*-C_{20} and solvent) in each condition. (A) High-ranking focal ants are confronted to low-ranking treated stimuli. (B) High-ranking focal ants are confronted to high-ranking treated stimuli. (C) Low-ranking focal ants are confronted to low-ranking treated stimuli. Box plots represent 10th, 25th, 50th (median), mean (dotted line), 75th and 90th percentiles. Sample sizes of individuals are *N* = 12 for each box plot. Different letters denote statistical differences.

DISCUSSION

Our study provides new evidence supporting the honest fertility signalling hypothesis. Whereas queens and workers differed in their chemical profile, fertile individuals had distinct cuticular hydrocarbon profiles compared with their infertile nestmates, regardless of their caste. No clear pattern emerges when looking at which specific compounds or group of compounds may signal fertility in social Hymenoptera (Liebig, 2010; but see Van Oystaeyen et al., 2014). Indeed, saturated (Smith et al., 2008), methyl-branched (Hannonen et al., 2002; Heinze et al., 2002; Holman et al., 2010) and unsaturated (Monnin et al., 1998; Smith et al., 2012) hydrocarbons have all been shown to correlate with ovarian activity depending on the species. Nevertheless, it appears that alkenes and particularly methyl-alkanes are the predominant compound classes advertising fertility, possibly because of their higher communicative potential (Monnin, 2006). We found the same pattern in our analysis, with only one alkene (*x*,*y*-$C_{29:2}$) and three monomethyl-branched alkanes (10-MeC_{19}, 11-MeC_{23} and to a higher extent 13-MeC_{25}) being linked with fertility.

13-MeC_{25} was closely correlated with fertility in workers in hopelessly queenless condition, thus confirming previous results (Yagound et al., 2014). Furthermore, the same relationship was found in reproductive and non-reproductive queens. Concomitantly with their high ovarian activity (Fresneau, 1994), reproductive queens exhibited the highest proportions of 13-MeC_{25} compared with all other groups, including highly fertile workers. By contrast, non-reproductive unmated queens were similar to infertile workers in their proportions of 13-MeC_{25} and their fertility. Similarity in the chemical profiles of queens and reproductive workers has already been reported (Heinze et al., 2002; Dietemann et al., 2003; Smith et al., 2008), and seems particularly common in species where, like in *Neoponera apicalis*, there is no strong queen–worker dimorphism and workers have a high reproductive potential (Liebig, 2010).

Another important result in favour of the honest fertility signalling hypothesis comes from the study of the dynamics of signal expression before and after the formation of the reproductive hierarchy. Workers in queenright colonies already differed in their ovarian development, with moderately fertile individuals already exhibiting higher amounts of 13-MeC_{25} compared to their infertile nestmates. It has often been shown that a behavioural hierarchy precedes with sometimes a long time interval the reproductive

hierarchy and therefore the expression of fertility signals (Peeters et al., 1999; Liebig et al., 2000; Cuvillier-Hot et al., 2004b; Hartmann et al., 2005). Our results show on the contrary that the expression of fertility signals is not delayed in *N. apicalis*.

First, this suggests that dominance interactions may not be necessary for the activation of the ovaries and the production of their associated chemical signals. Indeed, no obvious signs of dominance interactions where recorded in the queen's presence (Yagound, personal observation). However, Oliveira and Hölldobler (1990) found a dominance order to be present in one queenright colony, but with a much weaker intensity than in our queenless groups. Though our results indicate that fertility may precede dominance, we cannot rule out the hypothesis that some dominance/subordinate interactions were already established before orphaning and could have induced the activation of the ovaries for these individuals. Disentangling the relative cause and consequence of these inter-related phenomena thus remains challenging (Izzo et al., 2010).

The formation of social hierarchies is known to depend on a variety of factors including intrinsic differences between competing individuals and self-organizing processes (Hsu et al., 2006; Rutte et al., 2006). Here size was uncorrelated with both fertility and amounts of 13-MeC_{25}, whereas these latter two factors were closely linked, even in queenright condition. This suggests that the signalled fertility state could have a non-negligible influence on the outcome of agonistic interactions, while reducing the injury- and time-related costs of overt aggressions (Hsu et al., 2006; Rutte et al., 2006). Queenright workers with low fertility already exhibiting higher amounts of fertility signal could be recognized as such through the workers' fine-scale status discrimination abilities (Yagound et al., 2014). The workers' physiological divergence at the onset of hierarchy formation, probably resulting from their heterogeneous –age-depending– hormonal states, could be amplified during the subsequent agonistic interactions. We could thus hypothesise these moderately-fertile individuals to eventually gain the top ranks of the reproductive hierarchy through this self-sustaining process. Physiological –aggression-mediated– changes, potentially implicating the juvenile hormone (Hartfelder, 2000), could furthermore participate to the activation of the ovaries (Lamba et al., 2007), thus reinforcing their reproductive status. These results thus suggest that

fertility signalling is not restricted to the maintenance of the hierarchy and could also be involved in the establishment of the hierarchical structure.

Finally, this apparent inseparable association between the production of 13-MeC_{25} and the activation of the ovaries adds to the evidence in favour of the index hypothesis (Maynard Smith & Harper, 1995) as the mechanism maintaining the honesty of fertility signals. A variety of mechanisms can potentially explain the widespread occurrence of reliability in animal communication (Searcy & Nowicki, 2005; Számadó, 2011). Honesty has long been supposed to depend on costs preventing or reducing the benefits of cheating for low-quality individuals (the handicap principle; Zahavi, 1975; Grafen, 1990). As production costs are likely to be low in the case of fertility signals (Wyatt, 2014), honesty-guaranteeing costs may be linked to the maintenance of such signals, particularly if cheaters suffer social punishment (Monnin et al., 2002; Smith AA et al., 2009; Smith et al., 2012). The fundamental assumption that honest signals need to be costly has however been recently refuted (Getty, 2006; Számadó, 2011), showing that handicaps are neither necessary nor sufficient for signals to be honest. Alternatively, the index hypothesis assumes that physiological constraints cause an unfakeable connection between a signal and the trait it advertises (Maynard Smith & Harper, 1995). Such causal relationship therefore guarantees honesty simply because cheating is impossible. Numerous studies in insects point towards the implication of common endocrinological mechanisms involving gonadotropic hormones (juvenile hormone and ecdysteroids) that may be responsible for such a connection (Cuvillier-Hot et al., 2004b; Monnin, 2006; Peeters & Liebig, 2009; Blomquist, 2010; Izzo et al., 2010; Holman, 2012). As has been previously suggested (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009; Smith AA et al., 2009), this index hypothesis of honest fertility signalling is thus likely to have a general significance across various taxa of social insects.

The short time-window available for successful male production in hopelessly queenless nests and the unpredictability of the onset of the worker reproductive competition in this tropical species exert strong ecological pressures on workers reproductive strategies (Fresneau, 1994; Dietemann & Peeters, 2000). Indeed, whereas most high-ranking workers may eventually lay at least one viable egg, the vast majority (~95%; Dietemann & Peeters, 2000) will not develop into adult males because of policing from the top rankers and severe food supply limitations due to the death of the foraging

workers. Therefore the only few eggs having an actual chance of developing into adult males are most likely among the very first eggs to be laid. This would explain why some moderately-fertile workers laying trophic eggs are found in queenright nests (Dietemann & Peeters, 2000), and the rapidity with which the first reproductive eggs are laid in queenless conditions (~10 days after orphaning; Dietemann & Peeters, 2000; Blacher et al., 2010). These strong selective pressures could have favoured the very close link between ovary activation and the biosynthesis of cuticular hydrocarbons signalling fertility. This index-based honest signalling system would thus allow a quick resolution of the reproductive competition and the possibility for all nestmates to gain inclusive fitness benefits by raising a few males before the colony eventually collapses.

Corroborating chemical correlations with behavioural bioassays is important but rarely performed due to its inherent difficulties (Howard, 1993; Monnin, 2006; Martin & Drijfhout, 2009b; Peeters & Liebig, 2009; Liebig, 2010). Here, manipulating the amounts of 13-MeC_{25} induced a modification of the behavioural response of high rankers towards high- and low-ranking treated nestmates, as expected. Indeed, the agonistic response of focal ants decreased towards 13-MeC_{25}-treated stimuli, in accordance with these treated stimuli advertising a higher status compared to sham-treated controls. This effect appeared stronger for high- than for low-ranking treated stimuli, possibly because of their higher endogenous levels of 13-MeC_{25} resulting in a higher total amount of fertility signal. Low rankers are seldom involved in agonistic interactions (Yagound et al., 2014). Their similar behavioural response in all conditions is thus probably the consequence of a bottom effect, although they increased their duration of antennal contacts towards 13-MeC_{25}- compared with *n*-C_{20}-treated stimuli, possibly indicating an increased interest towards a high-ranking signal. Surprisingly, *n*-C_{20}-treated high- and low-ranking individuals also induced a reduction of the agonistic response of high rankers, though not with the same magnitude than 13-MeC_{25}-treated individuals. This result seems puzzling since this compound is not linked with fertility. It is possible that manipulating the proportions of *n*-C_{20} perturbed the recognition system (Howard & Blomquist, 2005), although the treatments had no effect on the duration of antennation towards treated stimuli. Alternatively, a decrease in the agonistic response would be expected if *n*-C_{20}-treated stimuli were perceived as advertising a lower rank compared with sham-treated controls, as low rankers are not involved in the reproductive competition and receive very few aggressions from high rankers (Yagound et al., 2014). Hydrocarbons

specifically interact with each other in the lipid layer covering the cuticle (Gibbs, 1995). Increasing the proportions of *n*-C_{20} could thus have influenced the relative availability of the other compounds and their perception. It is thus well possible that the similar behavioural response towards 13-MeC_{25}- and *n*-C_{20}-treated individuals was paradoxically triggered by two opposite causes. Bioassays heavily depend on the motivation of the tested ants and are context-dependent (Howard, 1993; Peeters et al., 1999; Buczkowski & Silverman, 2005). Although we tried to provide the ants with a realistic context, and despite the relative long duration of the tests, the very few agonistic acts observed could also have influenced our results. This methodological limitation is mainly due to the late moment when the tests were performed relative to orphaning, i.e. at a time where the intensity of agonistic acts has already decreased (Blacher et al., 2010). This was however necessary because of the minimum period of observation needed to correctly assess the individuals' rank. Additional studies would therefore be necessary to fully confirm the results of these bioassays. For example it is possible that 13-MeC_{25} advertise fertility together with other cuticular hydrocarbons (e.g. 11-MeC_{23}). Whereas single-compound fertility signals have been shown or suggested in some species (Monnin et al., 1998; Heinze et al., 2002; Smith AA et al., 2009; Smith et al., 2012), it has been shown in others that reproductive and non-reproductive individuals diverge in a large part of their chemical profile (Endler et al., 2004; de Biseau et al., 2004).

Overall, this study reinforces the hypothesis that 13-MeC_{25} constitutes, or at least contributes to, an honest signal of fertility in *N. apicalis*. Interestingly, our results indicate that 13-MeC_{25} could be involved in the partitioning of reproduction in both queenless and queenright conditions, and therefore without the necessity for specific caste signals advertising the queen's presence. The honest signalling hypothesis predicts that all individuals should respond to a strong signal (advertising a fully fertile reproducer), because the benefits of helping raising the reproducer's highly related offspring exceed the costs of refraining or preventing others from reproducing (Keller & Nonacs, 1993). Monitoring the amounts of 13-MeC_{25} could allow the workers to make appropriate reproductive decisions, because it identifies the best egg-layer in the colony, namely the mother queen during the vast majority of the colony's life, and then the worker(s) with the highest reproductive potential when the colony becomes hopelessly queenless. This signal could be sufficient for establishing the reproducers' reproductive

monopoly without the need for physical control because of shared fitness interests between all nestmates (Reeve & Jeanne, 2003). The queen is indeed never aggressive towards the workers (Dietemann & Peeters, 2000) and she maintains her reproductive monopoly through fertility signalling, which is expected on relatedness grounds. The workers' fitness interests in hopelessly queenless colonies are however not aligned, and agonistic behaviours are frequently expressed during the establishment of the reproductive hierarchy (Oliveira & Hölldobler, 1990; Yagound et al., 2014). However, the short time-window available for worker reproduction strongly constrains a quick resolution of the reproductive conflict (Dietemann & Peeters, 2000), and therefore the switch to fertility signalling and status recognition. The fine tuning of relatedness-mediated benefits, productivity-associated costs and life-history constraints seems to have resulted in a simple yet efficient recognition system in *N. apicalis*, where a single cuticular hydrocarbon is theoretically sufficient to permanently regulate the reproductive division of labour.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Preparation of 13-methylpentacos-3-ene

At room temperature under nitrogen atmosphere, a solution of butyllithium (2.6 M in hexane, 0.14 µl, 1.51 mmol) was added dropwise to a stirring solution of dodecyltriphenylphosphonium bromide (0.72 g, 1.41 mmol) in 10 ml of anhydrous ether. After 30 min, a solution of 2-tetradecanone (0.15 g, 0.71 mmol) dissolved in 2.5 ml of anhydrous ether was added dropwise under nitrogen atmosphere. The reaction mixture was allowed to stir at room temperature and followed by TLC eluting with cyclohexane (Rf dodecyltriphenylphosphonium bromide = 0.37; Rf 2-tetradecanone = 0.49; Rf 13-methylpentacos-3-ene = 0.90). After 30 min, the resulting slurry was acidified with aqueous hydrochloric acid (HCl) 1M until pH = 1-2. The reaction mixture was extracted with 3 × 10 ml cyclohexane. The combined hexane phases were washed with saturated aqueous $NaHCO_3$ and brine, dried over anhydrous sodium sulfate (Na_2SO_4) and filtered. The residue on evaporation was purified by flash chromatography on silica gel eluting with cyclohexane which gave 270 mg of alkene 13-methylpentacos-3-ene as colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 0.88 (t, 6H, *J* = 6.8 Hz, 2 × C<u>H$_3$</u>-CH_2), 1.25 (m, 38H, 19 × CH_2, 1.93-2.00 (m, 4H, 2 × C<u>H$_2$</u>-C=C), 1.66 (s, 3H, C<u>H$_3$</u>-C=C), 5.10 (t, 1H, *J* = 6.4 Hz, C<u>H</u>=C). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 14.26, 15.99, 22.84, 23.57, 27.94, 28.03, 28.12, 28.20, 29.42, 29.51, 29.56, 29.72, 29.76, 29.81, 29.84, 30.05, 30.28, 31.07, 31.87, 32.07, 39.86, 124.69, 125.42, 135.24, 135.52. Anal. Calcd for $C_{26}H_{52}$: C, 85.63; H, 14.37. Found: C, 83.12; H, 13.73.

Preparation of 13-methylpentacosane

Catalytic hydrogenation is carried out using hydrogen gas from an external supply and 5% palladium on charcoal catalyst (10 mg) for 3 h to hydrogenate the mixture of purified alkene 13-methylpentacos-3-ene (181 mg) in hexane. The reaction was complete as determined by ^{1}H NMR, which showed the disappearance of peaks corresponding to alkene group at 5.10 ppm. After filtration, 193 mg of the corresponding alkane 13-methylpentacosane was obtained as a low-melting white solid, mp 20-22°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 0.83 (d, 3H, *J* = 6.5 Hz, C<u>H$_3$</u>-CH), 0.87 (t, 6H, *J* = 6.5 Hz, C<u>H$_3$</u>-CH_2), 1.25 (m, 45H, 22 × C<u>H$_2$</u>, C<u>H</u>. ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 14.25, 19.86, 22.83, 27.23, 29.51, 29.81, 29.85, 29.88, 30.18, 31.04, 32.07, 32.89, 37.24. Anal. Calcd for $C_{26}H_{52}$: C, 85.63; H, 14.37. Found: C, 84.25; H, 13.97.

CHAPITRE 4

ÉVOLUTION DES SIGNAUX DE FERTILITÉ

RÉSUMÉ

La division du travail dans la reproduction caractéristique des sociétés d'insectes dépend en grande partie de signaux reflétant l'état de fertilité des individus reproducteurs. Le mode d'action de ces signaux de fertilité fait cependant encore débat. En effet, selon l'hypothèse du contrôle royal ces signaux agiraient en inhibant activement la reproduction des ouvrières, alors que l'hypothèse du signalement honnête considère l'absence de ponte des ouvrières comme résultant d'une auto-restriction reproductive favorisant leurs intérêts en présence d'un individu reproducteur apparenté et fertile. La première hypothèse prédit une course à l'armement évolutive entre les reproducteurs et les ouvrières résultant dans des signaux de plus en plus complexes et divergeant rapidement. Au contraire, l'hypothèse du signalement honnête prédit une conservation des signaux associés à la fertilité entre des espèces proches.

Afin de tester ces hypothèses, nous avons comparé les hydrocarbures cuticulaires liés à la fertilité chez plusieurs espèces fortement apparentées du complexe *Neoponera apicalis*. Les résultats montrent une forte diversité dans la nature des signaux de fertilité putatifs, ceux-ci pouvant être des composés linéaires, insaturés ou présentant des groupements méthyl. Ils révèlent de plus des différences probables dans le potentiel de reproduction des ouvrières entre ces espèces, qui pourraient être associées à leurs opportunités différentes d'accéder à la reproduction directe. Malgré cette diversité, les signaux associés à la fertilité sont fortement conservés entre toutes ces espèces. Ces résultats soutiennent ainsi l'hypothèse du signalement honnête de la fertilité. Ces signaux seraient donc évolutivement stables, ce qui permettrait d'expliquer leur rôle très général dans la régulation de la reproduction chez les insectes sociaux.

Article 5

A comparative study of fertility signals in a complex of closely related ant species supports the honest signalling hypothesis

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Abstract

Fertility signals are a crucial feature of insect societies because they largely contribute to the regulation of the reproductive division of labour. Two contrasting views have been proposed regarding fertility signals, namely as inhibiting pheromones actively suppressing worker reproduction (the queen control hypothesis), or alternatively as honest signals to which workers respond according to their best fitness interests (the honest signal hypothesis). These two hypotheses have therefore different implications for the selective pressures at work in social communication systems, one stressing the conflicting fitness benefits, and the other an agreement between cooperating units. Accordingly, the rapidity of evolution of fertility signals is predicted to differ between these two hypotheses, with a slower rate of divergence, and consequently a higher degree of conservation between close species, for the honest signalling hypothesis. Investigating the conservation of fertility signals in related species has however hardly ever been attempted. Here we compared the fertility-associated cuticular hydrocarbons in queenless reproductive hierarchies of *Neoponera apicalis*, a complex of closely related ant species. We found that putative fertility signals are highly diverse, with saturated, methyl-branched and unsaturated compounds all being linked with fertility and hierarchical status. Compared with *N. apicalis* species and *N. cooki*, *N. verenae* was characterized by reduced levels of fertility and dominance associated behaviours, which could be a consequence of a lower probability of queenlessness in this species. However, the degree of conservation of putative fertility signals was generally high, which argues in favour of the honest fertility signalling hypothesis.

Keywords: Cuticular hydrocarbons, fertility, honest signalling, *Neoponera* (formerly *Pachycondyla*) *apicalis*, social insects.

INTRODUCTION

Recognition mechanisms are a central feature of the organization of social groups. In social Hymenoptera such as ants and some bees and wasps, the occurrence of fertility signalling is of paramount importance, since these signals play a major role in the regulation of the reproductive division of labour characterizing these societies (Heinze, 2004; Monnin, 2006; Le Conte & Hefetz, 2008; Peeters & Liebig, 2009; Liebig, 2010). Workers in most species have the capacity to produce male offspring, but typically will never do so in the presence of an established reproducer (Bourke, 1988b; Ratnieks, 1988; Wenseleers et al., 2004; Ratnieks et al., 2006). Signals advertising the presence and fertility state of such a reproducer are thus thought to have a crucial influence in the regulation of worker reproduction. Cuticular hydrocarbons (CHCs) are long-chained molecules covering the insects' body, which primarily act as a waterproofing barrier, but also play a key role in chemical communication (Howard & Blomquist, 2005; Richard & Hunt, 2013). Numerous investigations have shown that CHCs are implicated in fertility signalling in ants (e.g. Monnin et al., 1998; Liebig et al., 2000; Heinze et al., 2002; Endler et al., 2004; Smith AA et al., 2009; Holman et al., 2010), bees (e.g. Ayasse et al., 1995; Hoover et al., 2003) and wasps (e.g. Sledge et al., 2001a; Dapporto et al., 2007b; Bhadra et al., 2010).

Two contrasting views have been proposed regarding fertility signals, namely as inhibiting pheromones actively suppressing worker reproduction (the queen control hypothesis), or alternatively as honest signals to which workers respond in their own interests (the honest signal hypothesis) (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009; Kocher & Grozinger, 2011). The control hypothesis sees queen-produced fertility chemicals as manipulative pheromones coercing the workers into a helper role (Hölldobler & Wilson, 1983). Evidence in favour of this hypothesis mainly comes from honey bees and fire ants where the queen bouquets are highly complex (Vargo & Hulsey, 2000; Katzav-Gozansky, 2006) and can have a direct effect on workers' physiology (Beggs et al., 2007). The honest signal hypothesis proposes on the contrary that worker reproductive self-restraint follows an informed reproductive decision ultimately favouring the workers' own inclusive fitness (Keller & Nonacs, 1993). Refraining from reproducing may indeed provide higher benefits if a related established reproducer is highly fertile. This hypothesis is supported by studies showing both the very close

relationship between fertility and CHC profiles in many species (Ayasse et al., 1995; Monnin et al., 1998; Liebig et al., 2000; Heinze et al., 2002; Dietemann et al., 2003; Cuvillier-Hot et al., 2004b; de Biseau et al., 2004; Endler et al., 2004; Hartmann et al., 2005; Smith AA et al., 2009; Holman et al., 2010; Yagound et al., 2014), and the adjustment of the workers' reproductive decisions according to the social context (Alaux et al., 2007; Malka et al., 2007; Yagound et al., 2012). Although it may be difficult to disentangle both hypotheses, it is generally predicted under the queen control hypothesis that a queen–worker arms race results in a complex and rapidly changing queen bouquet over evolutionary time. In contrast, the queen signal hypothesis argues a slower evolutionary rate of fertility-associated compounds, which consequently should be highly conserved between related species (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009; Kocher & Grozinger, 2011; van Zweden et al., 2014). To date, much of the evidence remains equivocal, because studies investigating fertility signals in a phylogenetic context are very scarce (but see Brunner et al., 2011; Holman et al., 2013a; van Zweden et al., 2014).

Here we aimed at testing the control vs honest signal hypothesis by comparing the fertility-associated CHCs between several species of the *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) *apicalis* complex (Hymenoptera: Formicidae: Ponerinae). These ants form small colonies (c. 100 individuals) with a very limited queen–worker dimorphism and a high potential for worker reproduction (Fresneau, 1994). Four closely related species are currently described in this complex (*N. apicalis*, *N. cooki*, *N. obscuricornis* and *N. verenae*; Mackay & Mackay, 2010), though at least seven more occur within the *N. apicalis* and *N. verenae* clades (Delabie et al., 2008; Ferreira et al., 2010; Yagound et al., unpublished data). These species share similar life histories (Fresneau, 1994; Wild, 2005), one difference being the facultative polygyny found exclusively in the *N. verenae* clade (Evison et al., 2012; Yagound et al., unpublished data). When hopelessly queenless, workers establish reproductive hierarchies allowing them to regulate the reproductive competition over male parentage (Oliveira & Hölldobler, 1990; Blacher et al., 2010, Yagound et al., 2014). Hierarchical and reproductive statuses are highly correlated in these hierarchies, and it has been shown in one species, *N. apicalis* morph 4, that they are also associated with the production of specific CHCs acting as putative fertility signals (Yagound et al., 2014). The set-up of such hierarchies enables to easily obtain workers with various levels of ovarian activity, and therefore

investigate their fertility-associated CHCs. The degree of conservation of putative fertility signals among these closely related species offers a potentially powerful test of the control vs honest hypothesis regarding fertility signalling in social insects.

METHODS

Ants

Colonies were collected in Brazil (Belém [PA] 01°22'44''S, 48°17'25''W; Itabuna [BA] 14°46'28"S, 39°13'20"W; Una [BA] 15°16'42"S, 39°05'06"W), French Guiana (Camp Patawa, 04°17'13"N, 52°11'12"W; Petit Saut, 05°04'16"N, 53°02'36"W), and Mexico (Volcán Tacaná [CP], 15°04'32"N, 92°12'20"W) between 2007 and 2011 (Table 1). Ants were classified in their respective species according to previous descriptions (Delabie et al., 2008; Ferreira et al., 2010; Mackay & Mackay, 2010; Yagound et al., unpublished data).

Colonies were housed in plaster nests (18 × 14 cm) connected to a foraging area of the same dimensions, where food (crickets and honey/apple mixture) was provided twice a week and water ad libitum. Each colony had a queen, more than 70 workers and brood at every developmental stage. Housing conditions were as follows: relative humidity of 60 ± 5%, temperature of 27 ± 2°C, 12:12 h light:dark cycle.

Reproductive Hierarchies

The stock colonies previously mentioned were used to form 26 experimental hopelessly queenless groups of 40 randomly chosen workers. This dequeening procedure typically induces the formation of a reproductive hierarchy manifested by ritualized agonistic behaviours (Oliveira & Hölldobler, 1990; Blacher et al., 2010; Yagound et al., 2014). All ants were individually labelled with numbered tags and dots of paint to allow the individual monitoring of their behaviour. Housing and feeding conditions were the same as above. Each experimental colony was then observed 1 h a day during a 14-day period, during which we recorded all behavioural acts linked to the establishment of the reproductive hierarchy, i.e. ritualized biting and antennal boxing (antennal strokes on

another ant's body) (Oliveira & Hölldobler, 1990; Heinze et al., 2002; Cuvillier-Hot et al., 2004b). All observations started the same day the workers were isolated from the queen, and were performed through a red plastic film to avoid disturbances that may affect the ants' behaviour. All agonistic interactions (performed and received) were then compiled in a matrix and arranged in an order minimizing the number of inconsistencies (i.e. when an individual is given a lower rank than an individual it dominates). This allowed us to reconstruct the dominance hierarchy and to assign a hierarchical rank to each individual (Blacher et al., 2010; Yagound et al., 2014). Ants at the top of the hierarchy that collectively performed more than 75% of the agonistic acts were considered high rankers. The remaining ants performing up to 95% of the agonistic acts with the exclusion of high-ranking workers were considered middle rankers, and the remaining ants at the bottom of the hierarchy low rankers.

All workers were frozen for dissection at the end of the observation period and their fertility was determined. The mean size of the six basal oocytes was taken as an ovarian index. We then determined three classes of individuals depending on their number of developed oocytes (i.e. size > 0.5 mm; Fresneau, 1994): highly fertile individuals (five to six developed oocytes), moderately fertile individuals (one to four developed oocytes) and infertile individuals (no developed oocytes). As the species of the *N. apicalis* complex differ in size (Ferreira, 2010), the ovarian index was corrected for size in order to compare it between species. Assuming an isometric relationship between ovary length and body size (measured as the mean head width), we standardized the ovarian index by dividing it by a correction factor. This factor was obtained using *N. apicalis* morph 4 (which has a medium size) as a reference, thus obtaining "*N. apicalis* morph 4 equivalent body size" for each species. The resulting corrections factors were as follows: *N. apicalis* morph 1, 1.08; *N. apicalis* morph 3, 0.95; *N. apicalis* morph 4, 1.00; *N. apicalis* morph 6, 1.19; *N. apicalis* morph 7, 1.20; *N. cooki*, 0.96; *N. verenae* morph 1, 0.87. The number of individuals analysed per colony is usually lower than 40 due to some mortality.

Table 1. Colonies used in this study.

Colony	Species	Origin	Collection date
PAP L	*N. apicalis* morph 1	Itabuna, BA, Brazil	2008
PAP M	*N. apicalis* morph 1	Una, BA, Brazil	2008
PAP 03	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 09	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 11	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 13	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 16	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 24	*N. apicalis* morph 1	Itabuna, BA, Brazil	2010
PAP 2	*N. apicalis* morph 3	Volcán Tacaná, CP, Mexico	2011
PAP 3	*N. apicalis* morph 3	Volcán Tacaná, CP, Mexico	2011
PAP D	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP E	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP F	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP G	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP Ja	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP Jb	*N. apicalis* morph 4	Petit Saut, French Guiana	2007
PAP 10	*N. apicalis* morph 6	Petit Saut, French Guiana	2011
PAP 1	*N. apicalis* morph 7	Petit Saut, French Guiana	2011
PAP 3	*N. apicalis* morph 7	Petit Saut, French Guiana	2011
PAP 7	*N. apicalis* morph 7	Petit Saut, French Guiana	2011
PVE 3	*N. verenae* morph 1	Camp Patawa, French Guiana	2007
PVE 1	*N. verenae* morph 1	Una, BA, Brazil	2008
PVE 2	*N. verenae* morph 1	Belém, PA, Brazil	2008
PVE 5	*N. verenae* morph 1	Belém, PA, Brazil	2008
PVE 23	*N. verenae* morph 1	Belém, PA, Brazil	2008
PAP C	*N. cooki*	Petit Saut, French Guiana	2007

Fertility-Associated CHCs

We then investigated in all species the existence of fertility-associated compounds by studying the link between their fertility and their CHC profiles. Extraction was performed by placing an ant (abdomen removed after dissections) in 400 µl of pentane containing 8 ng/µl of an internal standard (n-C_{17}) for 20 min. We then transferred 100 µl into a 200 µl glass insert. Following evaporation, 20 µl of pentane were added to the 200 µl glass insert. We then manually injected 2 µl of the extract into a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an HP-5MS capillary column (30 m × 25 µm × 0.25 µm) and a split–splitless injector, coupled to a 5975c mass spectrometer (Agilent Technologies) with 70 eV electron impact ionization. The carrier gas was helium at 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 1 min, then 70–180°C at 30°C/min, then 180–320°C at 5°C/min, then hold at 320°C for 5min. The areas of the peaks in the chromatogram were integrated with the MSD ChemStation software E.02.01.1177 (Agilent Technologies). Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared with known standards. Several compounds eluted in the same peak and were considered as a single variable in subsequent analyses. We obtained a total of 74 peaks for the *N. apicalis* complex (Tables S1–S7).

Statistical Analyses

Considering each species separately, we investigated the relationships between dominance, fertility and CHCs. Following hierarchy reconstruction, we calculated the K index of linearity varying from 0 (no linearity) to 1 (linear hierarchy) and tested the statistical significance of linearity with the Appleby (1983) method. We further verified if the hierarchical rank was correlated with both dominance (estimated as the proportion of agonistic acts performed) and fertility (ovarian index) with the Spearman rank correlation test.

For the chemical analyses on fertility-associated CHCs, we compared the arcsine-transformed relative quantities (Sokal & Rohlf, 2012) of all peaks using a discriminant function analysis with either fertility class (i.e. highly fertile, moderately fertile and infertile individuals) or rank class (i.e. high rankers, middle rankers and low rankers) as the categorical dependent variable to investigate their chemical divergence using Statistica 8.0 (StatSoft, Tulsa, OK, U.S.A.). We studied the relationship between fertility and relative quantities of the CHCs having the highest contribution to the discrimination of individuals according to their ovarian development using Spearman rank correlation tests. We also investigated the divergence in proportions of all CHCs between fertile (highly and moderately fertile) and infertile workers using exact permutation tests. The results of reproductive hierarchies and fertility-associated CHCs have already been reported in *N. apicalis* morph 4 (Yagound et al., 2014) but we repeat them here to ease comparison with the other species.

Unless otherwise stated, statistical analyses were performed using R-3.2.0 (R Core Team, 2012). Post-hoc corrected *P*-values following the Bonferroni–Holm method (Holm, 1979) are denoted *P'*. Statistical significance was set at $P < 0.05$.

RESULTS

Reproductive Hierarchies

There was a significantly negative correlation between dominance and hierarchical rank in most colonies across all species (Table 2). The mean number of agonistic acts performed per individual was however markedly different between colonies and between species, and was closely associated with the significance of the linearity of the hierarchy. *N. apicalis* morph 4 had the highest number of agonistic acts performed per individual and significantly near-linear hierarchies in all colonies (Yagound et al., 2014). This also tended to be the case in the other species. The linearity of the hierarchy was indeed significant in the colonies which had higher numbers of agonistic acts performed per individual (3/8 colonies in *N. apicalis* morph 1, 2/3 colonies in *N. apicalis* morph 7 and the *N. cooki* colony; Table 2). The individuals performed very few agonistic acts in colonies of *N. verenae* morph 1, none of which had a linear hierarchy. There was a significantly negative correlation between fertility and hierarchical rank in all colonies across all species, except for most colonies of *N. verenae* morph 1 (Table 2). In this species the mean fertility per individual was very low, the colony with the highest mean fertility being the only one where hierarchical rank and fertility were significantly correlated. When considering all species and controlling for colony size, we found that the number of agonistic acts performed was positively correlated with the level of fertility (Spearman rank correlation: $r_s = 0.60$, $P = 0.0017$).

Fertility Signals

Workers could clearly be discriminated by their CHC profiles according to their level of fertility and hierarchical rank in all species (Table 3). Between-group comparisons showed that highly fertile, moderately fertile and infertile individuals could be discriminated in all species (except highly and moderately fertile individuals in *N. apicalis* morph 6), with 80.7–100.0% of workers correctly assigned to their respective groups (Table 3). Despite a larger proportion of unexplained variance, high rankers, middle rankers and low rankers could also be discriminated based on their CHC profiles in all species (except high and middle rankers in *N. verenae* morph 1), with 60.4–100.0% of correct classification (Table 3).

Table 2. Reproductive hierarchy characteristics in all species.

Species	Colony	Linearity	Mean no. of dominance acts performed	Mean corrected fertility (mm)	Correlation between rank and dominance	Correlation between rank and fertility
N. apicalis morph 1	PAP L	$K = 0.46$ $P = 0.22$	11.25 ± 5.25	0.31 ± 0.06	$r_s = -0.61$ **$P = 0.0001$**	$r_s = -0.79$ **$P < 0.0001$**
	PAP M	$K = 0.17$ $P = 0.091$	20.17 ± 4.06	0.20 ± 0.08	$r_s = -0.26$ $P = 0.17$	$r_s = -0.61$ **$P = 0.0002$**
	PAP 03	$K = 0.20$ **$P = 0.023$**	29.54 ± 4.47	0.38 ± 0.09	$r_s = -0.40$ **$P = 0.019$**	$r_s = -0.87$ **$P < 0.0001$**
	PAP 09	$K = 0.20$ $P = 0.11$	14.93 ± 2.72	0.12 ± 0.06	$r_s = -0.51$ **$P = 0.0063$**	$r_s = -0.74$ **$P < 0.0001$**
	PAP 11	$K = 0.63$ **$P = 0.0011$**	31.14 ± 7.51	0.48 ± 0.09	$r_s = -0.56$ **$P = 0.0004$**	$r_s = -0.80$ **$P < 0.0001$**
	PAP 13	$K = 0.15$ $P = 0.27$	18.98 ± 2.92	0.42 ± 0.10	$r_s = -0.83$ **$P < 0.0001$**	$r_s = -0.39$ **$P = 0.026$**
	PAP 16	$K = 0.18$ $P = 0.76$	9.55 ± 2.47	0.18 ± 0.07	$r_s = -0.67$ **$P < 0.0001$**	$r_s = -0.67$ **$P < 0.0001$**
	PAP 24	$K = 0.78$ **$P < 0.0001$**	36.86 ± 7.81	0.55 ± 0.09	$r_s = -0.52$ **$P = 0.0013$**	$r_s = -0.89$ **$P < 0.0001$**
N. apicalis morph 3	PAP 2	$K = 0.07$ $P = 0.92$	12.66 ± 2.07	0.29 ± 0.08	$r_s = -0.74$ **$P = 0.0001$**	$r_s = -0.73$ **$P = 0.0001$**
	PAP 3	$K = 0.12$ $P = 0.75$	10.48 ± 5.36	0.19 ± 0.07	$r_s = -0.19$ $P = 0.32$	$r_s = -0.49$ **$P = 0.0048$**
N. apicalis morph 4	PAP D	$K = 0.92$ **$P < 0.0001$**	66.62 ± 7.92	0.37 ± 0.04	$r_s = -0.89$ **$P < 0.0001$**	$r_s = -0.61$ **$P < 0.0001$**
	PAP E	$K = 0.94$ **$P < 0.0001$**	26.82 ± 2.91	0.40 ± 0.03	$r_s = -0.69$ **$P < 0.0001$**	$r_s = -0.75$ **$P < 0.0001$**
	PAP F	$K = 0.69$ **$P < 0.0001$**	39.41 ± 13.92	0.51 ± 0.04	$r_s = -0.40$ **$P = 0.014$**	$r_s = -0.69$ **$P < 0.0001$**
	PAP G	$K = 0.45$ **$P = 0.031$**	62.46 ± 7.68	0.36 ± 0.04	$r_s = -0.80$ **$P < 0.0001$**	$r_s = -0.85$ **$P < 0.0001$**
	PAP Ja	$K = 0.85$ **$P < 0.0001$**	50.84 ± 8.46	0.33 ± 0.04	$r_s = -0.68$ **$P < 0.0001$**	$r_s = -0.75$ **$P < 0.0001$**
	PAP Jb	$K = 0.91$ **$P < 0.0001$**	69.11 ± 9.46	0.31 ± 0.04	$r_s = -0.86$ **$P < 0.0001$**	$r_s = -0.81$ **$P < 0.0001$**

Table 2. *continued*

Species	Colony	Linearity	Mean no. of dominance acts performed	Mean corrected fertility (mm)	Correlation between rank and dominance	Correlation between rank and fertility
N. apicalis morph 6	PAP 10	$K = 0.85$ $P = 0.059$	9.97 ± 5.47	0.35 ± 0.08	$r_s = -0.67$ **$P = 0.0002$**	$r_s = -0.84$ **$P < 0.0001$**
N. apicalis morph 7	PAP 1	$K = 0.71$ **$P = 0.0042$**	32.03 ± 7.51	0.49 ± 0.08	$r_s = -0.89$ **$P < 0.0001$**	$r_s = -0.84$ **$P < 0.0001$**
	PAP 3	$K = 0.81$ **$P < 0.0001$**	23.36 ± 4.82	0.77 ± 0.10	$r_s = -0.91$ **$P < 0.0001$**	$r_s = -0.77$ **$P < 0.0001$**
	PAP 7	$K = 0.41$ $P = 0.16$	3.88 ± 0.95	0.14 ± 0.05	$r_s = -0.39$ **$P = 0.030$**	$r_s = -0.74$ **$P < 0.0001$**
N. verenae morph 1	PVE 3	$K = 0.03$ $P = 0.99$	2.69 ± 0.56	0.30 ± 0.08	$r_s = -0.62$ **$P < 0.0001$**	$r_s = 0.00$ $P = 0.98$
	PVE 1	$K = 0.00$ $P = 0.99$	0.83 ± 0.21	0.30 ± 0.08	$r_s = -0.48$ **$P = 0.0031$**	$r_s = -0.07$ $P = 0.70$
	PVE 2	$K = 0.00$ $P = 0.99$	2.50 ± 0.44	0.16 ± 0.07	$r_s = -0.82$ **$P < 0.0001$**	$r_s = 0.16$ $P = 0.41$
	PVE 23	$K = 0.09$ $P = 0.79$	7.76 ± 1.22	0.10 ± 0.05	$r_s = -0.64$ **$P = 0.0002$**	$r_s = -0.40$ **$P = 0.019$**
	PVE 5	$K = 0.09$ $P = 0.95$	3.85 ± 0.91	0.25 ± 0.06	$r_s = -0.48$ **$P = 0.0040$**	$r_s = -0.07$ $P = 0.70$
N.cooki	PAP C	$K = 0.30$ **$P = 0.045$**	20.50 ± 4.08	0.55 ± 0.07	$r_s = -0.91$ **$P < 0.0001$**	$r_s = -0.72$ **$P < 0.0001$**

Significant values are highlighted in bold.

Table 3. Differences in the CHC profiles according to the fertility and hierarchical statuses in all species.

Species	Fertility			Rank		
	DA statistics	Correct classification	Between-group comparisons	DA statistics	Correct classification	Between-group comparisons
N. apicalis morph 1	Wilks's λ = 0.24 $F_{56,444}$ = 8.15 **P < 0.0001**	86.9%	all **P' < 0.0001**	Wilks's λ = 0.45 $F_{52,448}$ = 4.27 **P < 0.0001**	69.0%	all **P' < 0.0001**
N. apicalis morph 3	Wilks's λ = 0.32 $F_{18,104}$ = 4.41 **P < 0.0001**	87.3%	all **P' < 0.0060**	Wilks's λ = 0.46 $F_{18,104}$ = 2.78 **P = 0.0006**	71.4%	all **P' < 0.045**
N. apicalis morph 4	Wilks's λ = 0.13 $F_{46,396}$ = 15.58 **P < 0.0001**	80.7%	all **P' < 0.0001**	Wilks's λ = 0.30 $F_{36,406}$ = 9.45 **P < 0.0001**	77.1%	all **P' < 0.0001**
N. apicalis morph 6	Wilks's λ = 0.04 $F_{24,30}$ = 5.28 **P < 0.0001**	100.0%	all **P' < 0.0001** except HF/MF P' = 0.44	Wilks's λ = 0.08 $F_{16,38}$ = 5.84 **P < 0.0001**	100.0%	all **P' < 0.0014**
N. apicalis morph 7	Wilks's λ = 0.14 $F_{34,162}$ = 8.15 **P < 0.0001**	93.0%	all **P' < 0.0001**	Wilks's λ = 0.33 $F_{26,170}$ = 4.78 **P < 0.0001**	78.0%	all **P' < 0.0017**
N. verenae morph 1	Wilks's λ = 0.68 $F_{30,294}$ = 2.12 **P = 0.0009**	81.7%	all **P' < 0.042**	Wilks's λ = 0.64 $F_{32,292}$ = 2.22 **P = 0.0003**	60.4%	all **P' < 0.0005** except HR/MR P' = 0.27
N. cooki	Wilks's λ = 0.01 $F_{38,34}$ = 6.79 **P < 0.0001**	100.0%	all **P' < 0.0068**	Wilks's λ = 0.16 $F_{7,30}$ = 22.66 **P < 0.0001**	97.4%	n/a*

Significant values are highlighted in bold.

DA, discriminant analysis; HF, highly fertile; MF, moderately fertile; HR, high rankers; MR, middle rankers.

*Only 2 classes of rank were present.

There was a large variation in the number of CHCs linked with fertility according to the species (Tables 4 and S1–S7), with for example *N. verenae* morph 1 having very few such compounds, as opposed to *N. cooki*. Here we focused on putative fertility signals, i.e. compounds whose quantities are positively correlated with the ovarian activity, but the opposite was also true for several CHCs in all species (Tables S1–S7). Most CHCs (78.6%) positively associated with fertility had an odd number of carbons. These compounds mostly had 23 (21.4%), 21 and 29 (14.3% each), and 25 and 27 (10.7% each) carbons (Table 4). The distribution of putative fertility signals was fairly even across the three main classes of CHCs (proportion of linear alkanes, methyl-branched alkanes and alkenes respectively 28.6%, 32.1% and 39.1%). Interestingly, a large number of the fertility-associated CHCs (39.3%) was present in more than one species (Table 4). Among these CHCs, 72.7% occurred in two species (*n*-C_{21}, *n*-C_{22}, *x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$, *n*-C_{23}, 11-MeC_{23}, *n*-C_{25}, 11-,13-MeC_{27} and *x*-$C_{29:1a}$ + *x,y*-$C_{29:2}$), 9.1% in three species (*n*-C_{27}), and 18.2% in five different species (*x*-$C_{25:1a}$ + *x,y*-$C_{25:2a}$ and *x*-$C_{27:1b}$ + *x,y*-$C_{27:2}$). Noteworthy was *N. apicalis* morph 3 which shared no fertility-associated CHCs with another species. All other species had at least one putative fertility signal in common with all the other species. The proportion of shared fertility-associated CHCs was modest (35.7%) in *N. cooki* whose profile was largely composed of longer-chained CHCs compared with the other species. In contrast, this proportion was high in all other cases (*N. apicalis* morph 1, 83.3%; *N. apicalis* morph 4, 66.7%; *N. apicalis* morph 6, 80.0%; *N. apicalis* morph 7, 71.4%; *N. verenae* morph 1, 100.0%; Table 4).

Table 4. Correlation between fertility and relative quantity of CHCs in all species.

Compound	*N. a* 1 *N* = 8	*N. a* 3 *N* = 2	*N. a* 4 *N* = 6	*N. a* 6 *N* = 1	*N. a* 7 *N* = 3	*N. v* 1 *N* = 5	*N. cooki* *N* = 1
x-$C_{21:1b}$		**r_s = 0.42** ***P* = 0.0008**					
x-$C_{21:1c}$					r_s = 0.31 *P* = 0.0015		
n-C_{21}	**r_s = 0.53** ***P* < 0.0001**		r_s = 0.16 *P* = 0.018				
9-,11-MeC_{21}			r_s = 0.14 *P* = 0.039				
n-C_{22}	r_s = 0.46 *P* < 0.0001			r_s = 0.37 *P* = 0.048			
x-$C_{23:1d}$	r_s = 0.13 *P* = 0.036						
x-$C_{23:1a}$ + *x,y*-$C_{23:2a}$			r_s = 0.03* *P* = 0.61		**r_s = 0.37** ***P* = 0.0002**		
x-$C_{23:1b}$ + *x,y*-$C_{23:2b}$					r_s = 0.34 *P* = 0.0005		
n-C_{23}	r_s = 0.30 *P* < 0.0001			r_s = 0.51 *P* = 0.0054			
11-MeC_{23}			r_s = 0.47 *P* < 0.0001		r_s = 0.22 *P* = 0.030		
9-MeC_{23}		r_s = 0.39 *P* = 0.0016					
n-C_{24}				r_s = 0.55 *P* = 0.0024			
x-$C_{25:1a}$ + *x,y*-$C_{25:2a}$	r_s = 0.35 *P* < 0.0001		r_s = 0.32 *P* < 0.0001	**r_s = 0.73** ***P* < 0.0001**	r_s = 0.35 *P* = 0.0005	r_s = 0.16 *P* = 0.046	
n-C_{25}					r_s = 0.35 *P* = 0.0003		r_s = 0.74 *P* < 0.0001
11-,13-MeC_{25}			**r_s = 0.79** ***P* < 0.0001**				
3-MeC_{26}							r_s = 0.75 *P* < 0.0001
x-$C_{27:1b}$ + *x,y*-$C_{27:2}$	r_s = 0.20 *P* = 0.0013		r_s = 0.50 *P* < 0.0001	r_s = 0.41 *P* = 0.027		**r_s = 0.27** ***P* = 0.0003**	r_s = 0.61 *P* = 0.0001
n-C_{27}			r_s = 0.22 *P* = 0.0011		r_s = 0.13* *P* = 0.21		r_s = 0.75 *P* < 0.0001
11-,13-MeC_{27}						r_s = 0.22 *P* = 0.0044	r_s = 0.73 *P* < 0.0001
n-C_{28}							r_s = 0.76 *P* < 0.0001
2-MeC_{28}							**r_s = 0.78** ***P* < 0.0001**
x-$C_{29:1a}$ + *x,y*-$C_{29:2}$			r_s = 0.30 *P* < 0.0001				r_s = 0.72 *P* < 0.0001
x-$C_{29:1b}$							r_s = 0.77 *P* < 0.0001
n-C_{29}							r_s = 0.53 *P* = 0.0008
11-,13-MeC_{29}							r_s = 0.64 *P* < 0.0001
10-,12-MeC_{30}							r_s = 0.59 *P* < 0.0001
x-$C_{33:1a}$							r_s = 0.62 *P* < 0.0001
x-$C_{33:1b}$							r_s = 0.40 *P* = 0.013

The highest correlation for each species is highlighted in bold. Sample sizes refer to number of colonies.

*Relative quantities in fertile > infertile individuals (see Tables S3 and S5). *N. a*, *N. apicalis*; *N. v*, *N. verenae*.

DISCUSSION

Our results offer new support to the honest fertility signalling hypothesis in social insects. This hypothesis predicts that fertility signals should be conserved between related species, because all individuals in a colony share the same fitness interests in the maintaining of the accuracy of the recognition system, which as a result is predicted to change slowly over evolutionary time. This contrasts with the directional selective pressures that would arise from a queen–worker arms race as predicted under the queen control hypothesis, thus resulting in rapidly evolving signals (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009; Kocher & Grozinger, 2011). Here we found that workers of varying fertility could be discriminated based on their CHC profiles in seven closely related species, and that there is an important degree of conservation of putative fertility signals among these species, in agreement with the honest signal hypothesis.

Except for *N. apicalis* morph 3, all species have at least one fertility-associated CHC in common with each of the other species. *N. cooki* has a very different CHC profile compared with the other species of the *N. apicalis* complex (Yagound et al., unpublished data). Despite this difference, this species shared more than one third of its fertility-associated CHCs with the other species. In the other species this proportion is comprised between two third and the totality of the fertility-associated CHCs. Most notably are the two peaks composed respectively of unsaturated C_{25} and unsaturated C_{27} that are common to five out of the seven species investigated, including *N. verenae* morph 1. These CHCs have already been reported to differ between queens and workers in this species and in *N. verenae* morph 2 (Evison et al., 2012). The conservation of CHCs in these closely-related species thus appears far from negligible, which argues in favour of the honest signalling hypothesis (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009; Kocher & Grozinger, 2011). This hypothesis is well supported in the literature (Monnin, 2006; Le Conte & Hefetz, 2008; Liebig, 2010), and is likely to be a much more common explanation in social insects than the manipulative hypothesis (Peso et al., 2014). One theoretical reason for this is that honest signals, as previously mentioned, are supposed to be evolutionarily more stable (Keller & Nonacs, 1993). However, direct evidence for this prediction has been notoriously lacking. The few recent studies investigating the congruence of fertility signals in different species all concluded in favour of the honest signal hypothesis. For example, Brunner et al. (2011) showed that, while putative

fertility signals are not fully conserved in *Temnothorax* ants, workers nevertheless respond to heterospecific queens, for which the authors concluded as a support of the honest signal hypothesis. Second, Holman et al. (2013) reported an important conservation of fertility-associated CHCs in *Lasius* ants. The same result was found among Vespine wasps (van Zweden et al., 2014), together with levels of worker reproduction following the kin-selected predictions originating from the honest signal hypothesis, i.e. where levels of reproductive workers should be affected by worker–worker relatedness and policing efficiency (Wenseleers et al., 2004). Finally, Van Oystaeyen et al. (2014) also argued in favour of a conservation of fertility signals across the social Hymenoptera, although their categorisation of CHC classes was very large.

Our results also demonstrate once more the close link between dominance and fertility in all species of the *N. apicalis* complex. However, there was a high unexpected variability in the intensity of dominance/subordination relationships and in the extent of ovary activation depending on the species. As previously described (Yagound et al., 2014), the number of agonistic acts performed during the 14 days of observation was high in *N. apicalis* morph 4, which resulted in near-linear hierarchies in all colonies. The intensity of agonistic acts was clearly reduced in the other species, particularly in *N. verenae* morph 1 where the workers hardly ever engaged in dominance/subordinate interactions. As the method of estimating hierarchy linearity heavily depends on the number of agonistic acts (Appleby, 1983; de Vries, 1995), most hierarchies failed to be statistically linear. Oliveira & Hölldobler (1991) observed one colony of *N. verenae* which had been queenless for a long time, and reported workers laying eggs together with mutual oophagy from reproductive individuals and the occurrence of males, which indicates that reproductive hierarchies also occur in this species. However, it is possible that the duration of ovarian maturation and hierarchy establishment is longer than in *N. cooki* and *N. apicalis* species. This is further indicated by the very low mean fertility level in the observed colonies of this species. This could be due to these colonies containing relatively old workers, which typically do not take part in the reproductive hierarchy (Dietemann & Peeters, 2000), but the sample size of five colonies in *N. verenae* morph 1 makes the hypothesis of artefacts in age distribution of workers unlikely. This suggests that the dynamics of hierarchy formation in *N. verenae* could actually be different from *N. cooki* and *N. apicalis* species, and that additional similarities in putative fertility

signals between *N. verenae* morph 1 and the other species could have been observed in a longer experiment.

Workers are known to adjust their reproductive decisions according to the kin structure of their colony (Ratnieks 1988; Wenseleers & Ratnieks, 2006; Moore and Liebig, 2013). *N. verenae* species have a relatively different colony genetic structure compared with *N. apicalis* species and *N. cooki*, with facultative polygyny and higher effective paternity levels (Evison et al., 2012; Yagound et al., unpublished data). The probability for a focal worker to eventually encounter queenless conditions is arguably lower in polygynous than in monogynous colonies (Bourke, 1988b; Bourke & Franks, 1995). It is therefore possible that the facultative polygynous structure in *N. verenae* could have reduced the opportunities of worker direct reproduction. This could have relaxed the selective pressures associated with worker reproduction, thus explaining the low levels of agonistic behaviours and ovary activation in this species. Exploring this hypothesis, for example by comparing the formation of hierarchies in monogynous and polygynous colonies of *N. verenae* would provide valuable insights into the factors affecting reproductive strategies in social insects.

The only exception in our results is *N. apicalis* morph 3 which has no putative fertility signal in common with another species, and a very small number of significant differences in the proportions of CHCs between workers of varying fertility. Furthermore, it clearly appears that the mean fertility in the two colonies studied in this species is lower compared with the other species. A possible explanation is thus that the individuals in these two groups did not had enough time for the ovaries to develop, and for the fertility-associated CHCs to be sufficiently differentiated between fertile and infertile individuals. Whereas this reflects actual differences between this species and the rest of the complex remains an open question. We can hypothesise the dynamics of hierarchy formation to be longer in this species, as in *N. verenae*. However, since only two colonies were observed, it is also possible that these groups contained by chance relatively older workers, resulting in a lower degree of ovarian activation in most individuals. This is also indicated by the relative low number of agonistic acts performed in these colonies.

The reduction of the intensity of agonistic acts in *N. apicalis* morphs 1, 6, and 7 and *N. cooki* compared with *N. apicalis* morph 4 is intriguing, since the levels of ovarian activity

in all these species are similar. This could suggest that agonistic behaviour plays a less important role in the formation of the reproductive hierarchies in these four species than in *N. apicalis* morph 4. However some colonies in these species are characterised by similar levels of agonistic behaviours to *N. apicalis* morph 4 (e.g. PAP 11 and PAP 24 for *N. apicalis* morph 1, and PAP 1 and PAP 3 for *N. apicalis* morph 7), and the link between fertility, dominance and hierarchical status is clear in all colonies. Therefore, it is possible that these differences partly stem from colony specificities rather than species differences. Furthermore it must be noted that only one colony was observed in *N. apicalis* morph 6 and *N. cooki*, thus making any generalisation about colony traits in these species relatively hazardous.

Our results further show that the dynamics of signal production may differ between species, with an early onset of signal production and a close association of at least one CHC and fertility after two weeks in *N. apicalis* morph 4, and apparently *N. apicalis* morph 6 and *N. cooki*. By contrast, *N. apicalis* morphs 1, 3 and 7 and particularly *N. verenae* morph 1 show lower correlations between ovarian activity and proportions of CHCs after the same duration. CHCs are incorporated into oocytes during oogenesis (Fan et al., 2002), and it is believed that a common biochemical mechanism underlies the biosynthesis of CHCs and the activation of the ovaries (Cuvillier-Hot et al., 2004b; Peeters & Liebig, 2009; Liebig, 2010). However, interspecific differences in metabolic pathways are well conceivable (Blomquist, 2010), and may explain the delay observed in some species. Given the multi-faceted role of CHCs (Howard & Blomquist, 2005; Le Conte & Hefetz, 2008), these differences could also partly stem from ecological pressures (van Wilgenburg et al., 2011b), with selection acting on a subset of CHCs affecting the production of other CHCs. For example, *N. apicalis* morphs 1 and 3 are found in different habitats (outside the Amazonian rain forest) compared with the other species of the complex (Delabie et al., 2008). Additionally, these two species do not encounter sympatric species of the complex in their habitat, and are thus not subjected to interspecific competition and risks of interbreeding with these closely-related species, which could also influence the evolution of recognition cues. Investigating the mechanisms of CHCs production in each of these species thus seems a necessary step to understand the nature of the –possibly opposing– selective forces responsible for their evolution (Smith AA et al., 2013). Indeed, despite the high similarity in fertility-associated CHCs across all species, there is a high diversity in the chemical nature of

those CHCs. As typically observed, most of these CHCs have an odd number of carbons (Monnin, 2006; Martin & Drijfhout, 2009a). Moreover, the contribution of linear alkanes, methyl-branched alkanes and alkenes is fairly even, showing that, in these species at least, all classes of CHCs have a similar potential of constituting, or contributing to, a signal of fertility. Experimentally investigating the individual role of these CHCs would now be an important, though not easy, step.

In conclusion, this study reinforces the hypothesis that fertility signals honestly reflect their bearer's reproductive activity. Reproductive division of labour is thus mostly regulated by worker reproductive decisions, with self-restriction and coercion of attempted worker reproduction being the main mechanisms responsible for the typical queen reproductive monopoly observed in insect societies (Bourke, 1988b; Ratnieks et al., 2006; Wenseleers & Ratnieks, 2006). Such reproductive decisions are ultimately affected by the genetic structure of the colony and the constraints and costs to colony-level productivity of worker reproduction (Ratnieks & Reeve, 1992; Hammond & Keller, 2004; Wenseleers et al., 2004). At a proximate level, however, they mostly depend on the detection of the established reproducer's fertility. Understanding the mechanisms responsible for the selection of particular CHCs as signals of fertility is particularly challenging, and would require a full consideration of the potential factors affecting processes as diverse as mate recognition, waterproofing balance, egg development, or nestmate recognition, from the molecular to the ecological levels.

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SUPPLEMENTARY MATERIAL

Table S1. Relative quantities of CHCs in fertile and infertile workers in *N. apicalis* morph 1.

Peak	Compound	Fertile workers (N = 89)	Infertile workers (N = 163)	P
1	*x,y*-diMeC$_{20}$	0.03 ± 0.02	0.04 ± 0.02	0.94
2	3-MeC$_{20}$	0.02 ± 0.01	0.02 ± 0.01	0.81
3	*x*-C$_{21:1a}$	0.02 ± 0.01	0.03 ± 0.01	0.55
4	*n*-C$_{21}$	0.45 ± 0.04	0.22 ± 0.01	**< 0.0001**
5	3-MeC$_{21}$	0.08 ± 0.03	0.06 ± 0.02	0.64
6	*n*-C$_{22}$	0.48 ± 0.04	0.28 ± 0.02	**< 0.0001**
7	*x,y*-diMeC$_{22}$	0.04 ± 0.02	0.08 ± 0.03	0.31
8	9-,11-MeC$_{22}$	0.03 ± 0.01	0.03 ± 0.01	0.69
9	*x*-C$_{23:1b}$ + *x,y*-C$_{23:2b}$	0.06 ± 0.01	0.13 ± 0.03	0.062
10	*x*-C$_{23:1d}$	1.74 ± 0.12	1.51 ± 0.08	0.13
11	*n*-C$_{23}$	35.28 ± 1.15	28.70 ± 0.57	**< 0.0001**
12	7-MeC$_{23}$	0.02 ± 0.01	0.01 ± 0.01	0.54
13	3-,5-MeC$_{23}$	0.16 ± 0.06	0.10 ± 0.04	0.39
14	*n*-C$_{24}$	1.04 ± 0.17	1.10 ± 0.12	0.74
15	*x,y*-diMeC$_{24}$	0.04 ± 0.02	0.05 ± 0.02	0.84
16	*x*-C$_{25:1a}$ + *x,y*-C$_{25:2a}$	1.42 ± 0.15	0.78 ± 0.07	**< 0.0001**
17	*x*-C$_{25:1b}$ + *x,y*-C$_{25:2b}$	0.49 ± 0.14	0.69 ± 0.15	0.40
18	*x*-C$_{25:1c}$	1.56 ± 0.19	2.33 ± 0.13	**0.0006**
19	*n*-C$_{25}$	13.31 ± 0.64	18.25 ± 0.45	**< 0.0001**
20	5-MeC$_{25}$	0.01 ± 0.00	0.06 ± 0.06	0.99
21	*x*-C$_{26:1a}$	0.03 ± 0.01	0.03 ± 0.01	0.76
22	*x,y*-C$_{26:2b}$	0.37 ± 0.31	0.19 ± 0.15	0.30
23	*n*-C$_{26}$	0.23 ± 0.03	0.24 ± 0.02	0.81
24	3-MeC$_{26}$	0.01 ± 0.01	0.01 ± 0.00	0.99
25	*x*-C$_{27:1b}$ + *x,y*-C$_{27:2}$	19.71 ± 1.43	15.97 ± 0.93	**0.025**
26	*x*-C$_{27:1c}$	0.22 ± 0.05	0.65 ± 0.10	**0.0002**
27	*n*-C$_{27}$	6.92 ± 0.33	6.71 ± 0.22	0.58
28	3-MeC$_{27}$	0.09 ± 0.04	0.06 ± 0.03	0.59
29	*n*-C$_{28}$	0.47 ± 0.05	0.43 ± 0.04	0.55
30	2-MeC$_{28}$	2.91 ± 0.17	2.96 ± 0.13	0.82
31	*x*-C$_{29:1a}$ + *x,y*-C$_{29:2}$	4.42 ± 0.41	4.73 ± 0.30	0.54
32	*x*-C$_{29:1b}$	0.51 ± 0.10	0.72 ± 0.11	0.19
33	*n*-C$_{29}$	2.45 ± 0.15	2.70 ± 0.08	0.093
34	11-,13-MeC$_{29}$	4.91 ± 0.48	8.73 ± 0.58	**< 0.0001**
35	*x,y*-C$_{30:2}$	0.01 ± 0.00	0.01 ± 0.00	0.84
36	3-MeC$_{30}$	0.05 ± 0.02	0.12 ± 0.02	**0.039**
37	*x*-C$_{31:1}$ + *x,y*-C$_{31:2}$	0.41 ± 0.08	1.04 ± 0.17	**0.0042**
38	*n*-C$_{31}$	0.03 ± 0.02	0.02 ± 0.01	0.38
39	*x*-C$_{33:1c}$	0.01 ± 0.00	0.04 ± 0.01	**0.015**
40	*x,y*-C$_{33:2}$	0.01 ± 0.00	0.09 ± 0.03	**0.0057**
41	11-,13-,15-MeC$_{33}$	0.01 ± 0.00	0.07 ± 0.02	**0.0027**

Significant values are highlighted in bold.

Table S2. Relative quantities of compounds in fertile and infertile workers in *N. apicalis* morph 3.

Peak	Compound	Fertile workers (*N* = 17)	Infertile workers (*N* = 46)	*P*
1	*n*-C_{20}	0.18 ± 0.01	0.17 ± 0.01	0.25
2	*x*-$C_{21:1b}$	1.78 ± 0.11	1.30 ± 0.09	**0.0033**
3	*x*-$C_{21:1c}$	0.10 ± 0.03	0.08 ± 0.01	0.42
4	*x*-$C_{21:1d}$	0.10 ± 0.02	0.07 ± 0.01	0.065
5	*n*-C_{21}	21.01 ± 0.67	20.89 ± 0.42	0.88
6	*x*-$C_{22:1a}$ + *x,y*-$C_{22:2}$	0.79 ± 0.11	0.81 ± 0.06	0.89
7	*x*-$C_{22:1b}$	0.71 ± 0.10	0.71 ± 0.07	0.98
8	*n*-C_{22}	1.51 ± 0.03	1.79 ± 0.04	**0.0002**
9	*x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$	25.14 ± 1.74	22.89 ± 1.53	0.42
10	*x*-$C_{23:1b}$ + *x,y*-$C_{23:2b}$	29.07 ± 2.08	28.13 ± 1.59	0.75
11	*x*-$C_{23:1c}$	0.04 ± 0.03	0.10 ± 0.03	0.19
12	*n*-C_{23}	14.97 ± 0.52	17.33 ± 0.39	**0.0014**
13	11-MeC_{23}	0.23 ± 0.04	0.19 ± 0.02	0.36
14	9-MeC_{23}	0.13 ± 0.02	0.07 ± 0.00	**0.0003**
15	3-,5-MeC_{23}	0.03 ± 0.01	0.02 ± 0.01	0.85
16	*n*-C_{24}	0.01 ± 0.00	0.01 ± 0.01	0.28
17	*n*-C_{25}	1.75 ± 0.20	2.33 ± 0.10	**0.0053**
18	*n*-C_{26}	0.01 ± 0.01	0.03 ± 0.01	0.41
19	*n*-C_{27}	2.08 ± 0.22	2.58 ± 0.14	**0.072**
20	*n*-C_{28}	0.01 ± 0.01	0.01 ± 0.00	0.99
21	*x*-$C_{29:1a}$ + *x,y*-$C_{29:2}$	0.01 ± 0.00	0.01 ± 0.01	0.99
22	*n*-C_{29}	0.36 ± 0.11	0.48 ± 0.08	0.47

Significant values are highlighted in bold.

Table S3. Relative quantities of compounds in fertile and infertile workers in *N. apicalis* morph 4.

Peak	Compound	Fertile workers (*N* = 152)	Infertile workers (*N* = 71)	*P*
1	10-MeC_{19}	0.01 ± 0.00	0.02 ± 0.00	**< 0.0001**
2	*n*-C_{20}	0.15 ± 0.01	0.16 ± 0.01	0.12
3	*x*-$C_{21:1b}$	1.95 ± 0.44	10.19 ± 2.02	**< 0.0001**
4	*x*-$C_{21:1c}$	0.08 ± 0.00	0.08 ± 0.01	0.95
5	*x*-$C_{21:1d}$	0.46 ± 0.04	0.44 ± 0.06	0.71
6	*n*-C_{21}	15.35 ± 0.15	14.26 ± 0.43	**0.0031**
7	9-,11-MeC_{21}	0.31 ± 0.01	0.28 ± 0.07	**0.0019**
8	*x*-$C_{22:1a}$ + *x,y*-$C_{22:2}$	1.20 ± 0.04	1.35 ± 0.06	**0.030**
9	*n*-C_{22}	1.40 ± 0.02	1.43 ± 0.03	0.41
10	9-,11-MeC_{22}	0.08 ± 0.00	0.09 ± 0.01	0.27
11	*x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$	49.74 ± 0.54	40.53 ± 2.11	**< 0.0001**
12	*x*-$C_{23:1c}$	1.54 ± 0.14	1.89 ± 0.47	0.36
13	*n*-C_{23}	15.77 ± 0.21	17.80 ± 0.42	**< 0.0001**
14	11-MeC_{23}	0.44 ± 0.02	0.26 ± 0.05	**< 0.0001**
15	*x*-$C_{24:1b}$ + *x,y*-$C_{24:2}$	0.24 ± 0.01	0.25 ± 0.01	0.67
16	*n*-C_{24}	0.13 ± 0.00	0.15 ± 0.01	**0.0075**
17	*x*-$C_{25:1a}$ + *x,y*-$C_{25:2a}$	2.75 ± 0.12	1.46 ± 0.18	**< 0.0001**
18	*n*-C_{25}	1.85 ± 0.03	1.89 ± 0.07	0.53
19	11-,13-MeC_{25}	0.13 ± 0.01	0.03 ± 0.00	**< 0.0001**
20	*n*-C_{26}	0.14 ± 0.00	0.14 ± 0.01	0.56
21	*x*-$C_{27:1b}$ + *x,y*-$C_{27:2}$	0.15 ± 0.01	0.04 ± 0.00	**< 0.0001**
22	*n*-C_{27}	2.73 ± 0.07	2.60 ± 0.12	0.30
23	*n*-C_{28}	0.18 ± 0.00	0.21 ± 0.01	**0.0089**
24	2-MeC_{28}	0.63 ± 0.02	0.71 ± 0.04	0.076
25	*x*-$C_{29:1a}$ + *x,y*-$C_{29:2}$	0.11 ± 0.00	0.09 ± 0.01	**0.0011**
26	*x*-$C_{29:1b}$	0.09 ± 0.00	0.08 ± 0.00	0.43
27	*n*-C_{29}	2.22 ± 0.06	2.68 ± 0.10	**0.0001**
28	*n*-C_{31}	0.34 ± 0.02	0.60 ± 0.03	**< 0.0001**

Significant values are highlighted in bold.

Table S4. Relative quantities of compounds in fertile and infertile workers in *N. apicalis* morph 6.

Peak	Compound	Fertile workers (*N* = 15)	Infertile workers (*N* = 14)	*P*
1	*x*-$C_{21:1d}$	1.04 ± 0.16	2.43 ± 0.26	**0.0001**
2	*n*-C_{21}	1.04 ± 0.04	1.37 ± 0.08	**0.0010**
3	*x*-$C_{22:1c}$	0.15 ± 0.03	0.26 ± 0.04	**0.014**
4	*n*-C_{22}	0.75 ± 0.01	0.71 ± 0.02	0.069
5	*x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$	2.48 ± 0.21	2.46 ± 0.24	0.95
6	*x*-$C_{23:1c}$	39.03 ± 0.75	42.49 ± 0.68	**0.0024**
7	*n*-C_{23}	19.34 ± 0.80	15.30 ± 0.44	**0.0002**
8	3-,5-MeC_{23}	0.23 ± 0.05	0.37 ± 0.07	0.094
9	*x*-$C_{24:1a}$	0.38 ± 0.03	0.42 ± 0.05	0.53
10	*n*-C_{24}	0.68 ± 0.03	0.57 ± 0.05	**0.044**
11	*x*-$C_{25:1a}$ + *x,y*-$C_{25:2a}$	3.50 ± 0.23	2.04 ± 0.24	**0.0001**
12	*x*-$C_{25:1c}$	15.70 ± 0.58	16.70 ± 0.52	0.21
13	*n*-C_{25}	4.30 ± 0.38	3.62 ± 0.35	0.22
14	*x*-$C_{27:1b}$ + *x,y*-$C_{27:2}$	1.93 ± 0.21	1.28 ± 0.15	**0.018**
15	*x*-$C_{27:1c}$	1.08 ± 0.19	1.29 ± 0.12	0.39
16	*n*-C_{27}	2.52 ± 0.62	2.89 ± 0.66	0.68
17	*n*-C_{28}	4.18 ± 0.22	3.73 ± 0.47	0.40
18	2-MeC_{28}	0.37 ± 0.10	0.51 ± 0.14	0.44
19	*n*-C_{29}	1.32 ± 0.08	1.55 ± 0.16	0.21

Significant values are highlighted in bold.

Table S5. Relative quantities of compounds in fertile and infertile workers in *N. apicalis* morph 7.

Peak	Compound	Fertile workers (N = 52)	Infertile workers (N = 48)	P
1	*n*-C_{20}	0.02 ± 0.00	0.14 ± 0.05	**< 0.0001**
2	*x*-$C_{21:1b}$	0.91 ± 0.19	3.58 ± 0.31	**< 0.0001**
3	*x*-$C_{21:1c}$	0.03 ± 0.01	0.01 ± 0.01	**0.038**
4	*x*-$C_{21:1d}$	0.21 ± 0.03	0.24 ± 0.02	0.46
5	*n*-C_{21}	15.15 ± 0.31	16.52 ± 0.37	**0.0060**
6	5-MeC_{21}	0.01 ± 0.00	0.06 ± 0.02	**0.0001**
7	*x*-$C_{22:1a}$ + *x,y*-$C_{22:2}$	0.93 ± 0.09	0.91 ± 0.04	0.90
8	*n*-C_{22}	1.53 ± 0.23	1.29 ± 0.06	0.50
9	*x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$	11.33 ± 0.70	8.75 ± 0.67	**0.0097**
10	*x*-$C_{23:1b}$ + *x,y*-$C_{23:2b}$	35.20 ± 0.66	32.56 ± 0.74	**0.0089**
11	*x*-$C_{23:1c}$	1.06 ± 0.06	0.98 ± 0.06	0.36
12	*n*-C_{23}	21.46 ± 0.50	20.78 ± 0.50	0.34
13	11-MeC_{23}	0.28 ± 0.02	0.25 ± 0.02	0.17
14	9-MeC_{23}	0.04 ± 0.01	0.06 ± 0.01	0.15
15	3-,5-MeC_{23}	0.24 ± 0.03	0.18 ± 0.02	0.16
16	*n*-C_{24}	0.12 ± 0.01	0.12 ± 0.02	0.93
17	*x*-$C_{25:1a}$ + *x,y*-$C_{25:2a}$	1.17 ± 0.15	0.62 ± 0.12	**0.0053**
18	*x*-$C_{25:1b}$ + *x,y*-$C_{25:2b}$	0.81 ± 0.13	1.00 ± 0.13	0.33
19	*n*-C_{25}	2.24 ± 0.13	1.60 ± 0.09	**0.0002**
20	*n*-C_{26}	0.02 ± 0.01	0.01 ± 0.00	0.34
21	*n*-C_{27}	1.44 ± 0.11	1.13 ± 0.09	**0.033**
22	*n*-C_{28}	0.05 ± 0.01	0.09 ± 0.02	**0.039**
23	2-MeC_{28}	0.58 ± 0.07	1.10 ± 0.09	**< 0.0001**
24	*x*-$C_{29:1a}$ + *x,y*-$C_{29:2}$	0.06 ± 0.01	0.08 ± 0.01	0.37
25	*x*-$C_{29:1b}$	0.01 ± 0.00	0.03 ± 0.01	**0.0058**
26	*n*-C_{29}	1.54 ± 0.14	2.17 ± 0.16	**0.0033**
27	11-,13-MeC_{29}	0.56 ± 0.03	0.83 ± 0.04	**< 0.0001**
28	*x*-MeC_{30}	0.01 ± 0.00	0.02 ± 0.01	0.085
29	10-,12-MeC_{30}	0.21 ± 0.03	0.33 ± 0.04	**0.012**
30	3-MeC_{30}	0.58 ± 0.07	0.89 ± 0.11	**0.016**
31	*x*-$C_{31:1}$ + *x,y*-$C_{31:2}$	0.29 ± 0.03	0.47 ± 0.04	**0.0008**
32	*n*-C_{31}	0.14 ± 0.03	0.37 ± 0.04	**< 0.0001**
33	11-,13-,15-MeC_{31}	0.78 ± 0.08	1.02 ± 0.10	0.055
34	10-,12-MeC_{32}	0.17 ± 0.04	0.23 ± 0.04	0.19
35	11-,13-,15-MeC_{33}	0.58 ± 0.08	1.04 ± 0.11	**0.0010**
36	3-MeC_{33}	0.17 ± 0.04	0.28 ± 0.04	0.053

Significant values are highlighted in bold.

Table S6. Relative quantities of compounds in fertile and infertile workers in *N. verenae* morph 1.

Peak	Compound	Fertile workers (*N* = 35)	Infertile workers (*N* = 129)	*P*
1	*n*-C_{21}	6.03 ± 0.78	5.72 ± 0.37	0.71
2	3-MeC_{21}	0.03 ± 0.03	0.07 ± 0.02	0.42
3	*x*-$C_{22:1a}$ + *x,y*-$C_{22:2}$	0.10 ± 0.04	0.04 ± 0.01	0.11
4	*n*-C_{22}	1.23 ± 0.13	1.52 ± 0.08	0.070
5	9-,11-MeC_{22}	0.40 ± 0.18	0.39 ± 0.10	0.98
6	*x*-$C_{23:1a}$ + *x,y*-$C_{23:2a}$	3.59 ± 1.69	4.77 ± 0.88	0.54
7	*x*-$C_{23:1b}$ + *x,y*-$C_{23:2b}$	23.73 ± 3.68	25.54 ± 1.72	0.64
8	*x*-$C_{23:1c}$	0.73 ± 0.35	1.22 ± 0.22	0.28
9	*n*-C_{23}	22.02 ± 1.06	26.60 ± 0.74	**0.0030**
10	11-MeC_{23}	2.42 ± 0.81	1.80 ± 0.36	0.44
11	7-MeC_{23}	0.21 ± 0.18	0.01 ± 0.00	0.098
12	3-,5-MeC_{23}	0.57 ± 0.17	0.46 ± 0.09	0.58
13	*n*-C_{24}	0.62 ± 0.14	0.74 ± 0.09	0.53
14	*x,y*-diMeC_{24}	0.53 ± 0.16	0.44 ± 0.08	0.58
15	*x*-$C_{25:1a}$ + *x,y*-$C_{25:2a}$	6.44 ± 1.66	4.07 ± 0.41	**0.040**
16	*x*-$C_{25:1c}$	0.02 ± 0.02	0.03 ± 0.02	0.73
17	*n*-C_{25}	7.12 ± 0.92	6.21 ± 0.46	0.36
18	11-,13-MeC_{25}	7.47 ± 2.10	6.01 ± 1.05	0.53
19	5-MeC_{25}	1.29 ± 0.40	1.05 ± 0.20	0.59
20	*n*-C_{26}	0.37 ± 0.12	0.29 ± 0.05	0.53
21	11-MeC_{26}	0.19 ± 0.08	0.14 ± 0.04	0.55
22	*x*-$C_{27:1a}$	0.05 ± 0.05	0.03 ± 0.01	0.65
23	*x*-$C_{27:1b}$ + *x,y*-$C_{27:2}$	3.96 ± 1.29	3.72 ± 0.73	0.88
24	*n*-C_{27}	5.59 ± 0.72	5.20 ± 0.30	0.58
25	11-,13-MeC_{27}	1.83 ± 0.60	1.05 ± 0.19	0.084
26	3-MeC_{27}	1.47 ± 0.67	1.01 ± 0.24	0.43
27	*n*-C_{28}	0.21 ± 0.07	0.21 ± 0.03	0.96
28	2-MeC_{28}	0.06 ± 0.04	0.10 ± 0.03	0.64
29	*n*-C_{29}	1.98 ± 0.36	1.69 ± 0.18	0.45

Significant values are highlighted in bold.

Table S7. Relative quantities of compounds in fertile and infertile workers in *N. cooki*.

Peak	Compound	Fertile workers (N = 25)	Infertile workers (N = 13)	P
1	*n*-C_{25}	2.95 ± 0.45	0.23 ± 0.04	**< 0.0001**
2	3-MeC_{26}	2.05 ± 0.34	0.01 ± 0.00	**0.0002**
3	*x*-$C_{27:1b}$ + *x,y*-$C_{27:2}$	1.46 ± 0.41	0.23 ± 0.03	**0.017**
4	*n*-C_{27}	3.16 ± 0.35	0.42 ± 0.06	**< 0.0001**
5	11-,13-MeC_{27}	1.38 ± 0.16	0.16 ± 0.02	**< 0.0001**
6	*n*-C_{28}	0.64 ± 0.04	0.23 ± 0.03	**< 0.0001**
7	2-MeC_{28}	7.92 ± 0.54	2.14 ± 0.28	**< 0.0001**
8	*x*-$C_{29:1a}$ + *x,y*-$C_{29:2}$	2.13 ± 0.28	0.28 ± 0.03	**< 0.0001**
9	*x*-$C_{29:1b}$	3.49 ± 0.27	0.46 ± 0.05	**< 0.0001**
10	*n*-C_{29}	3.60 ± 0.22	2.57 ± 0.48	**0.032**
11	11-,13-MeC_{29}	3.70 ± 0.19	1.69 ± 0.14	**< 0.0001**
12	10-,12-MeC_{30}	1.15 ± 0.10	0.31 ± 0.13	**< 0.0001**
13	3-MeC_{30}	1.86 ± 0.17	2.57 ± 0.52	0.11
14	*x*-$C_{31:1}$ + *x,y*-$C_{31:2}$	3.57 ± 0.21	3.27 ± 0.30	0.42
15	11-,13-,15-MeC_{31}	12.46 ± 0.44	11.03 ± 0.61	0.064
16	*x*-MeC_{31}	1.45 ± 0.26	0.71 ± 0.28	0.080
17	10-,12-MeC_{32}	1.58 ± 0.13	2.10 ± 0.20	**0.030**
18	*x*-$C_{33:1a}$	1.44 ± 0.24	0.23 ± 0.08	**0.0013**
19	*x*-$C_{33:1b}$	1.34 ± 0.15	0.70 ± 0.21	**0.022**
20	11-,13-,15-MeC_{33}	9.34 ± 0.80	12.82 ± 1.18	**0.017**
21	3-MeC_{33}	9.02 ± 0.59	12.08 ± 0.68	**0.0026**
22	12-MeC_{34}	1.18 ± 0.12	2.14 ± 0.28	**0.0010**
23	*x,y*-$C_{35:2}$	1.70 ± 0.17	4.10 ± 0.39	**< 0.0001**
24	*x*-$C_{35:1}$	1.08 ± 0.21	4.46 ± 0.59	**< 0.0001**
25	11-,13-,15-MeC_{36}	18.23 ± 1.57	31.37 ± 0.63	**< 0.0001**
26	*x*-MeC_{37}	0.80 ± 0.17	1.86 ± 0.31	**0.0021**
27	*y*-MeC_{37}	1.31 ± 0.26	1.82 ± 0.52	0.34

Significant values are highlighted in bold.

CHAPITRE 5

RECONNAISSANCE COLONIALE

RÉSUMÉ

La reconnaissance coloniale est un facteur crucial dans les sociétés d'insectes car elle permet de maintenir l'intégrité du groupe en limitant l'exploitation de ses ressources par des compétiteurs ou des parasites. La réponse vis-à-vis des étrangers n'est en général pas équivalente, puisque le niveau d'agression peut être modulé selon la proximité des colonies. Cependant les mécanismes proximaux à la base de cette discrimination des voisins et des étrangers restent équivoques, notamment du fait de la grande variabilité interspécifique dans la réponse territoriale et dans les facteurs sous-jacents potentiels.

Dans cette étude nous avons comparé les processus de reconnaissance coloniale entre trois espèces proches, sympatriques et possédant des traits d'histoire de vie similaires, appartenant au complexe *Neoponera apicalis*. Les résultats montrent que les trois espèces étudiées ont toutes une reconnaissance coloniale très probablement basée sur la perception du profil d'hydrocarbures cuticulaires. De plus, *N. apicalis* morphe 7 montre une discrimination des individus hétérocoloniaux voisins et étrangers, ces derniers étant significativement plus agressés et les comportements étant de nature plus agressive à mesure que la distance spatiale séparant les nids d'origine augmente. Ce pattern de réponse comportementale est donc compatible avec un effet « cher ennemi », et nous montrons qu'il est indépendant des distances chimiques et génétiques séparant les colonies, ce qui suggère l'influence de processus d'apprentissage des visas hétérocoloniaux voisins. En revanche, *N. apicalis* morphe 4 et *N. verenae* morphe 1 n'ont pas montré de discrimination claire des individus étrangers proches et lointains.

Nous émettons l'hypothèse que cette variation interspécifique vient des différences dans les préférences de sites de nidification, affectant la fréquence des déménagements et donc la durée de contact des colonies voisines. Ces différences influenceraient au niveau proximal la possibilité de survenue des processus d'apprentissage des visas hétérocoloniaux, et au niveau ultime la compétition pour les ressources et donc les coûts et bénéfices à moduler la réponse agressive vis-à-vis des étrangers. Cette étude soutient ainsi l'hypothèse que la réponse comportementale différentielle envers les individus hétérocoloniaux familiers et non-familiers dépend de leur menace relative.

Article 6

Interspecific variation in neighbour–stranger discrimination in the *Neoponera apicalis* complex

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ABSTRACT

The ecological success of social insects lies in their ability to prevent the exploitation of colony resources by competitors or parasites. Nestmate recognition is therefore of crucial importance in maintaining the integrity of the colony. Furthermore, intercolony competitive relationships are often complex, since many species discriminate between neighbours and strangers, with reduced (the dear enemy phenomenon) or increased levels of aggression towards nearby colonies, depending on the species. However, the proximate causes underlying this neighbour–stranger discrimination remain equivocal, particularly because the interspecific diversity in life histories generates a wide variety of potential confounding factors. Here we sought to circumvent this drawback by studying three closely related sympatric ant species with very similar life histories that belong to the *Neoponera apicalis* complex. We investigated how nestmate recognition and intercolony competitive relationships were influenced by spatial, chemical and genetic distances between colonies. We found that one species, *N. apicalis* morph 7, showed a clear dear enemy phenomenon with no influence of chemical and genetic distances, suggesting the existence of a learning process. In contrast, *N. apicalis* morph 4 and *N. verenae* morph 1 failed to show any strong discrimination between close and distant non-nestmates. We propose that these differences stem from the observed interspecific variation in nesting preferences affecting both the occurrence of encounters between nearby nests and the competition for nest sites between colonies. This study further reinforces the relative threat level hypothesis as an ultimate explanation for neighbour–stranger discrimination processes.

Keywords: Aggression, dear enemy phenomenon, *Neoponera* (formerly *Pachycondyla*) *apicalis*, nestmate recognition, social insects.

INTRODUCTION

Investigating the proximate causes responsible for the maintenance of social groups' integrity is key to understanding their evolution (Bourke, 2011). The ability to distinguish group members from outsiders is one of the fundamental characteristics of all social groups. This ensures that cooperation is directed towards group members and helps to prevent the costs associated with the exploitation of group resources by competitors or parasites (Hamilton, 1964b; Sherman et al., 1997).

Discriminating group members from strangers is very common in social insects such as ants, termites and some bees and wasps (Crozier & Pamilo, 1996; d'Ettorre & Lenoir, 2010). Nestmate recognition is widely acknowledged to depend on chemical cues, namely the blend of cuticular hydrocarbons covering the insects' cuticle and acting as the main recognition cues in within- and between-colony communication processes (Howard & Blomquist, 2005; Dani, 2006; d'Ettorre & Lenoir, 2010). Chemical profiles can be influenced by a combination of heritable (Vander Meer & Morel, 1998; Lahav et al., 2001; van Zweden et al., 2010) and environmental factors (Heinze et al., 1996; Liang & Silverman, 2000; Couvillon et al., 2007), and are known to change over time (Lenoir et al., 2001a; Suarez et al., 2002). However, constant exchanges of cuticular hydrocarbons between individuals through trophallaxis (Boulay et al., 2000) and allogrooming (Soroker et al., 2003) allows the formation and permanent updating of a common chemical profile shared by all nestmates (Crozier & Dix, 1979). This colony-specific chemical profile then serves as a learned reference template which is compared with the chemical labels borne by any encountered individual (Crozier & Pamilo, 1996; d'Ettorre & Lenoir, 2010); the degree of dissimilarity between these encountered labels and the template triggers different responses towards the encountered individual, from complete acceptance to overt rejection (Reeve, 1989; Sherman et al., 1997).

This behavioural response is however not rigid and is also influenced by the costs of rejection and acceptance errors, which depend on the context of interaction (Reeve, 1989; Downs & Ratnieks, 2000; Knaden & Wehner, 2003; Thurin & Aron, 2008; Tanner & Adler, 2009). Rejecting non-nestmates can be a costly process in terms of time, energy, risk of injury, and –because recognition systems are not perfect– risk of recognition errors (Reeve, 1989; Sherman et al., 1997; Rivera-Marchand et al., 2008). In this context, adapting one's behaviour according to the familiarity of non-nestmates may be

beneficial. Decreased levels of aggression towards neighbours compared with distant strangers (i.e. the dear enemy phenomenon; Fisher, 1954) are indeed frequently observed (Jutsum et al., 1979; Heinze et al., 1996; Langen et al., 2000; Pirk et al., 2001; Dimarco et al., 2010; Tanner & Keller, 2012), and are thought to reduce the costs associated with frequent fights. However, the exact opposite, namely individuals being more aggressive towards neighbours than strangers (i.e. the nasty neighbour effect; Müller & Manser, 2007), is also common in social insects (Gordon, 1989; Dunn & Messier, 1999; Sanada-Morimura et al., 2003; Thomas et al., 2005; Newey et al., 2010). Furthermore, individuals in other cases always exhibit the same aggressive response towards non-nestmates, regardless of their spatial proximity and therefore familiarity level (Dahbi et al., 1996; Boulay et al., 2007).

The relative threat level hypothesis (Temeles, 1994) is believed to explain this diversity in the behavioural response of insect colonies towards non-nestmates (Dunn & Messier, 1999; Langen et al., 2000; Scharf et al., 2011). This hypothesis argues that neighbours and strangers may reflect different levels of threat, for example if they compete for different resources. In such cases, the behavioural response should be stronger towards the category of non-nestmates that represents the biggest threat, i.e. the greatest potential costs. However, the proximate causes responsible for this neighbour–stranger discrimination remain equivocal. For example it could be mediated by learning processes due to higher encounter rates between neighbours (Langen et al., 2000; Knaden & Wehner, 2003; Thomas et al., 2005; Dimarco et al., 2010), but environmentally and/or genetically derived recognition cues may also be important (Heinze et al., 1996; Pirk et al., 2001; Zinck et al., 2008). In fact, a plethora of potential and non-mutually exclusive factors affecting the aggressive response towards non-nestmates have been identified, including colony size (Stuart, 1991), nest density (van Wilgenburg, 2007), microhabitat variation (Heinze et al., 1996), food availability (Downs & Ratnieks, 2000), time of the year (Suarez et al., 2002; Thurin & Aron, 2008), reproductive dispersal strategy (Tanner & Keller, 2012), resource value (Sakata & Katayama, 2001), behavioural dominance (Tanner & Adler, 2009), spatial distance from the nest (Knaden & Wehner, 2003), genetic distance (Pirk et al., 2001; Zinck et al., 2008), chemical distance (Martin et al., 2012), and as previously stated, familiarity with non-nestmates (Jutsum et al., 1979; Gordon, 1989; Langen et al., 2000; Sanada-Morimura et al., 2003; Thomas et al., 2005; Dimarco et al., 2010; Newey et al., 2010). However, the interspecific

diversity in life histories combined with the methodological differences between studies generate a wide variety of potentially confounding factors rendering the comparison of specific behaviour difficult, and thus highlight the importance of controlling for their possible influence.

In this study we undertook such an approach by studying different species that belong to a complex of closely-related species to better understand the proximate factors underlying neighbour–stranger discrimination. *Neoponera* (formerly *Pachycondyla*; Schmidt & Shattuck, 2014) *apicalis* (Hymenoptera: Formicidae: Ponerinae) is a complex of partly sympatric species covering most of the Neotropics (Wild, 2005). This complex is composed of highly related species with very similar life histories (Fresneau, 1994; Wild, 2005), making this an ideal model to study the factors influencing non-nestmate discrimination. Four species have been described so far in this complex [*N. apicalis* (Latreille, 1802), *N. obscuricornis* (Emery, 1890), *N. verenae* (Forel, 1922) and *N. cooki* (Mackay & Mackay, 2010)], but recent studies have shown that *N. apicalis* and *N. verenae* in fact regroup several cryptic species (Delabie et al., 2008; Ferreira et al., 2010). These ants forage solitarily on the litter for small living or dead prey at mean distances of 20 meters but they can go as far as 40 meters. Nests density leads to an overlapping of foraging areas where intercolony encounter rates can be relatively frequent. Furthermore, strong territorial behaviour has been observed in these ants, particularly in the vicinity of the nest (Fresneau, 1985, 1994). Here we studied in three sympatric species of the *N. apicalis* complex whether non-nestmate discrimination is influenced by the nests' spatial distribution, chemical proximity and genetic distance, and further investigated if between-species differences can be found.

METHODS

Ants

Colonies of the *Neoponera apicalis* complex were collected in three localities (Petit Saut, 5°04'15.8"N, 53°02'36.3"W; Saut Sabbat, 5°23'48.45"N, 53°41'44.54"W; Montagne des Singes, 5°04'25.76"N, 52°41'55.62"W) in French Guiana in November 2011. Previous collections have revealed that several species of this complex occur in sympatry in this particular geographic area (Ferreira et al., 2010; Evison et al., 2012). Morphs were

determined by genetic analyses (Yagound et al., unpublished data) and identified according to Delabie et al. (2008) and Ferreira et al. (2010). Our population sample contained 14 colonies of *N. apicalis* morph 4, 7 colonies of *N. verenae* morph 1, and 7 colonies of a new morph that was treated as *N. apicalis* morph 7. Although the taxonomic status of these morphs is not fully resolved (Wild, 2005), we considered them based on multiple lines of evidence (Delabie et al., 2008; Ferreira et al., 2010; Yagound et al., unpublished data) as distinct species.

During collection, the locations and dimensions (estimated from the circumference of the trunk) of all nests were measured. The three species showed similar patterns of nest spatial structuring, in particular concerning their proximity between nests (Table 1). Nests of *N. apicalis* morph 7 were however often located in living trees and therefore much higher above the ground compared to the two other species which preferentially nested in rotting trunks near the ground (Table 1). *N. verenae* often nested in small rotting branches on the ground.

Table 1. Dimensions and spatial structuring of nests in all species.

Variable (mean ± SE)	*N. apicalis* morph 7	*N. apicalis* morph 4	*N. verenae* morph 1	One-way ANOVA
Nest circumference (cm)	51.8 ± 15.9	54.5 ± 8.8	10.7 ± 1.5	$F_{2,33} = 4.67$ $P = 0.087$
Nest distance above the ground (cm)	196.7 ± 59.5 (a)	25.2 ± 5.4 (b)	7.6 ± 3.9 (b)	$F_{2,33} = 16.60$ $\mathbf{P = 0.0005}$
Spatial distance between nests (m)	53.5 ± 6.6	47.4 ± 4.5	39.8 ± 6.9	$F_{2,60} = 2.30$ $P = 0.32$
Spatial distance between closest neighbours (m)	26.3 ± 5.7	31.6 ± 4.1	20.3 ± 7.3	$F_{2,31} = 1.93$ $P = 0.39$
Number of close neighbours	1.83 ± 0.49	1.14 ± 0.23	2.57 ± 0.69	$F_{2,33} = 4.45$ $P = 0.10$

Significant values are highlighted in bold. Different letters (a, b, c) denote statistical differences.

Following collection, colonies were installed in the laboratory in France and were tested straight away. Each colony contained at least a queen, 33.6 ± 5.1 (mean ± SE) workers, and brood. They were reared in plaster nests (18 × 14 cm) connected to a foraging area where ants were provided twice a week with crickets and honey/apple mixture, and water ad libitum. Housing conditions were as follows: relative humidity of 60 ± 5%, temperature of 27 ± 2°C, 12:12 h light:dark cycle. Ant collection, husbandry and experimental procedures used in this study fulfilled all the legal requirements concerning insect experimentation of France.

Aggression Tests

To test the effect of spatial distance between nests on the ants' behavioural response we conducted aggression tests for each of the three species, in which the ants originated from nests separated by different distances ranges. Five categories of distance were determined based on the mean foraging distance of these species (Fresneau, 1985, 1994), and thus reflecting their probability of encounter. Close neighbours (17.0 ± 0.9 m, range 5–20 m) were ants originating from nests sharing a large part of their foraging area and could thus have frequently encountered. Distant neighbours (32.4 ± 1.2 m, range 22–40 m) corresponded to ants originating from nests with partially overlapping foraging areas and which probably had less frequently encountered. Non-neighbours (70.2 ± 1.3 m, range 41–91 m) were ants originating from the same locality but with distinct foraging areas. Two controls were used to compare the ants' behavioural responses, namely nestmates which were individuals from the same nest, and allopatric ants (67.7 ± 20.3 km, range 38–82 km) which originated from distinct localities.

Aggression tests consisted in dyadic encounters with two ants originating from nests separated by various spatial distances. All workers were individually labelled with numbered tags glued on their thoraxes and dots of paint on their abdomens. Tests were conducted in a neutral arena (diameter = 5.3 cm) with Fluon-coated sides to prevent the ants from escaping and a filter paper as a substrate that was replaced between each test to avoid any odour residues. Ants taken from both the nest and the foraging area were gently introduced into the test arena, temporarily separated by a microscope slide for 30 s to allow them to become accustomed to the device. Following the microscope slide

removal, we video-recorded the ants' behaviours for 2 min. Using EthoLog 2.2 software (Ottoni, 2000) we then quantified the duration and occurrences of the following behaviours: antennation, transport, antennal boxing, mandible opening, biting and stinging. Antennation is an exploration behaviour corresponding to a complete absence of aggression. Transport, antennal boxing and mandible opening are ritualized behaviours representing an intermediate level of aggression, whereas biting and stinging correspond to overt aggression. Observers were blind to the origin of the ants. All tested individuals were only involved in a single test. We performed 10 encounters for each distance category, except in two occasions where 9 tests were performed due to smaller colony sizes, for a total of 148 tests.

Chemical Distance

To study the possible influence of chemical proximity on the ants' behavioural response, we analyzed the cuticular hydrocarbon profile of a sample of workers (10.3 ± 0.5) for each colony with the non-destructive method of solid-phase microextraction (SPME). A 100 μm polydimethylsiloxane fibre (Supelco, Bellefonte, PA, USA) was carefully rubbed against the abdomen of live ants for 2 min. The fibre was then desorbed in the injection port of a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an HP-5MS capillary column (30 m × 25 μm × 0.25 μm) and a split–splitless injector, coupled to a 5975c mass spectrometer (Agilent Technologies) with 70 eV electron impact ionization. The carrier gas was helium at 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 5 min, then 70–250°C at 30°C/min, then 250–260°C at 1°C/min, then 260–320°C at 20°C/min, then hold at 320°C for 5 min. Peaks areas were integrated with the MSD ChemStation software E.02.01.1177 (Agilent Technologies). Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared to known standards.

Genetic Distance

To determine the genetic distance between all pairs of colonies we genotyped 12 workers per *N. apicalis* colony (*N* = 166 and *N* = 63 for *N. apicalis* morph 4 and 7, respectively), and 24 workers per *N. verenae* colony (*N* = 164) due to its facultative polygyny (Evison et al., 2012). DNA from the head and thorax of workers preserved in ethanol was extracted in 500 µl of a 10% Chelex® 100 (Bio-Rad, Hercules, CA, USA) solution with 20 µl of proteinase K (Promega, Madison, WI, USA) at 10 mg/ml, incubated at 55°C for 40 min, then boiled at 100°C for 20 min. All samples were stored at −20°C until genotyping. Seven variable microsatellite loci were used: Pv1048, Pv1078, Pv2056, Pv2096, Pv2111, Pv4049, Pv4053 (Evison et al., 2010). Polymerase chain reactions (PCRs) were performed in a total volume of 10 µl containing 1 µl of each primer (10 µM), 5 µl of DreamTaq Master Mix (Fermentas, Vilnius, Lithuania), 2 µl of nuclease-free water and 1 µl of 1/5 diluted DNA. PCRs for loci Pv1078, Pv2096 and Pv4049 were carried out similarly, but with 1.5 µl of each primer and 1.5 µl of nuclease-free water. The DNA was amplified in a Biometra TProfessional thermocycler (Labgene Scientific, Châtel-St-Denis, Switzerland) using an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing step at 56°C, 58°C, 60°C or 62°C depending on the primer (Evison et al., 2010) for 30 s and extension step at 72°C for 1 min, then a final extension step at 72°C for 10 min. PCR products were mixed in two sets (Pv1078, Pv2056, Pv3091, Pv4053, and Pv1048, Pv2111, Pv4049) with the internal size marker LIZ500™ (Applied Biosystems, Foster City, CA, USA) and analyzed by a 3130XL Genetic Analyser (Applied Biosystems). Fragment length was then scored using Peak Scanner™ Software 1.0 (Applied Biosystems).

Statistical Analyses

The dimensions and spatial structuring of nests were compared between species using one-way ANOVAs with R-3.2.0 (R Core Team, 2012). In dyadic encounters, the duration of each behaviour performed by the two ants was summed for each test to avoid the influence of each individual on the behaviour of the other. We determined an aggression score for each behaviour according to its increasing level of aggressiveness (Errard & Hefetz, 1997): 0 for antennation, 1 for transport and antennal boxing, 2 for mandible

opening, 3 for biting and 4 for stinging. We then determined for each test an aggression index as: $(\sum^{n}_{i=1} As_i \cdot t_i)/T$, where As_i is the aggression score, t_i the duration for each act and T the total duration of interaction (Errard & Hefetz, 1997). Using R-3.2.0, we compared the aggression index as well as the duration of all behaviours between all categories of spatial distance with one-way ANOVAs, and studied the relationship between the aggression index and the spatial, chemical and genetic distance (see below) with the Spearman rank correlation test.

Chemical analyses were performed with a total of 100 peaks present in more than 50% of individuals of at least one species using Statistica 8.0 (StatSoft, Tulsa, OK, USA). We compared the arcsine-transformed relative quantities (Sokal & Rohlf, 2012) of all peaks using a factor analysis followed by a discriminant function analysis with colonies as the categorical dependent variable to investigate their chemical divergence. A cluster analysis was then performed to compute the Euclidean distances with Ward's method between all individuals. The mean pairwise Euclidean distance was then used as a measure of the chemical distance between each pair of colony.

We determined the linkage disequilibrium between all pairs of loci for each species to verify the independence of the loci with GENEPOP on the Web (Raymond & Rousset, 1995; Rousset, 2008). To investigate the amount of genetic variation at each locus we calculated for each species the number of alleles (A), allelic richness (A_r; El Mousadik & Petit, 1996), unbiased expected heterozygosity (H_e), and the inbreeding coefficient (F_{IT}; Weir & Cockerham, 1984) as a measure of the mating structure of each population using FSTAT 2.9.3 (Goudet, 2001). The average relatedness within colonies (r; Queller & Goodnight, 1989) was then determined with GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). The fixation index (F_{ST}; Weir & Cockerham, 1984) between all pairs of colonies was calculated as a measure of their genetic distance with FSTAT 2.9.3.

Correlations between the chemical distance (mean pairwise Euclidean distance), the (ln-transformed) spatial distance and the genetic distance (pairwise F_{ST}) were performed using partial Mantel tests (Mantel, 1967) with 20 000 permutations as implemented in FSTAT 2.9.3. Statistical significance was set at $P < 0.05$. Post-hoc corrected P-values following the Bonferroni–Holm method (Holm, 1979) are denoted P'.

RESULTS

Aggression Tests

The aggression index varied significantly according to the spatial distance for all three species (one-way ANOVAs: *N. apicalis* morph 7, $F_{4,49} = 53.64$, $P < 0.001$; *N. apicalis* morph 4, $F_{4,49} = 10.87$, $P < 0.001$; *N. verenae*, $F_{4,50} = 14.16$, $P < 0.001$; Figure 1). The aggression index was always significantly lower for nestmates encounters than for all other non-nestmates encounters (post-hoc tests: *N. apicalis* morph 7, all $P' < 0.002$; *N. apicalis* morph 4, all $P' < 0.028$; *N. verenae*, all $P' < 0.003$; Figure 1). This indicates that ants of all species are able to behaviourally discriminate group-members from strangers. This is further corroborated by the longer duration of antennations during non-nestmates encounters compared with nestmates encounters (*N. apicalis* morph 7, $F_{4,49} = 9.41$, $P < 0.001$, all $P' < 0.002$; *N. apicalis* morph 4, $F_{4,49} = 2.92$, $P = 0.028$, all $P' < 0.015$; *N. verenae*, $F_{4,50} = 2.49$, $P = 0.043$, all $P' < 0.017$ except for the comparison with close neighbours $P' = 0.055$; Supplementary Figures S1a, S2a and S3a).

In addition to these similar characteristics, the three species studied here showed different patterns of aggressive responses towards strangers. *N. apicalis* morph 7 presented the most structured behavioural response, with a clear effect of spatial distance on the level of aggression. The aggression index was indeed positively correlated with spatial distance (Figure 1 and Table 2), with close neighbours encounters showing a reduced level of aggression compared to all other non-nestmates encounters (all $P' < 0.014$; Figure 1). When looking at each behaviour specifically, we found an even clearer pattern of behavioural response depending on the spatial distance separating nests (Supplementary Figure S1). Control tests between nestmates triggered as expected the shortest durations of most aggressive behaviours (transport, $F_{4,49} = 2.92$, $P < 0.001$, all $P' < 0.010$ except for the comparison with close neighbours $P' = 0.075$; mandible opening, $F_{4,49} = 12.09$, $P < 0.001$, all $P' < 0.010$; biting, $F_{4,49} = 5.77$, $P < 0.001$, all $P' < 0.028$; stinging, $F_{4,49} = 9.69$, $P < 0.001$, all $P' < 0.041$; Supplementary Figure S1). Encounters between close neighbours were characterized by a longer duration of antennal boxing (all $P' < 0.003$; Supplementary Figure S1b), whereas encounters between distant neighbours involved longer durations of more aggressive behaviours such as mandible opening (all $P' < 0.016$; Supplementary Figure S1d), and there was a trend for biting (all $P' < 0.086$; Supplementary Figure S1e).

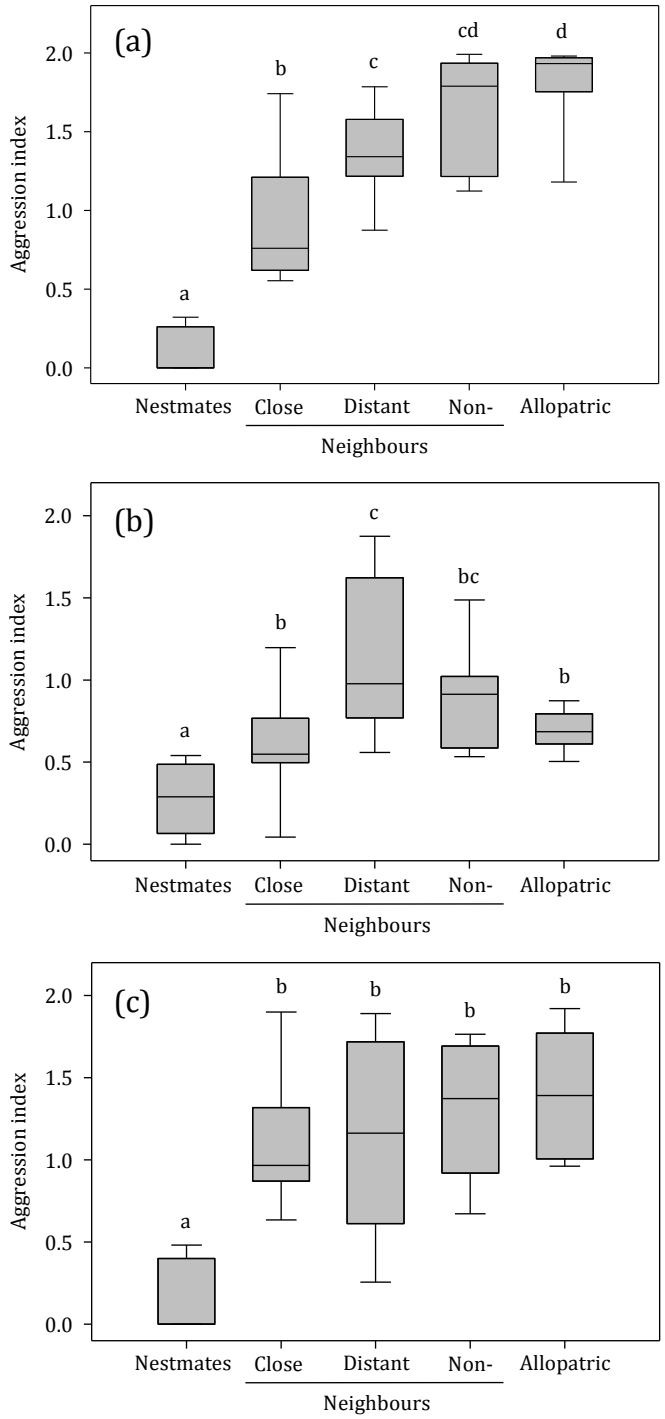

Figure 1. Aggression index in dyadic encounters between ants originating from nests separated by various categories of spatial distance. (a) *N. apicalis* morph 7. (b) *N. apicalis* morph 4. (c) *N. verenae* morph 1. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of encounters are $N = 10$ for each box plot, except for allopatric encounters in (a) and distant neighbours encounters in (b) where $N = 9$. Different letters denote statistical differences.

Dyadic encounters between non-neighbours were finally characterized by the highest level of aggression, with a similar duration of stinging (i.e. the behaviour with the highest aggression score most likely to cause fatal wounds) compared with encounters between allopatric ants (P' = 0.22), and a longer duration of stinging compared with encounters between distant neighbours (P' = 0.041) and there was a strong trend for close neighbours (P' = 0.063; Supplementary Figure S1f). The duration of transport appeared not to be affected by the spatial distance between nests (all P' > 0.29; Supplementary Figure S1c). The overall pattern appeared clearly when looking at the proportions of all behaviours in each category of distance, with mostly antennation behaviours in nestmates encounters, a large part of ritualized behaviours at close spatial distances ranges, and an increasing proportion of overt aggression as the spatial distance increases (Figure 2a). Ritualized behaviours accounted for 82.9% and 60.3% of the aggressive behaviours in close and distant neighbours encounters, respectively. By contrast, overt aggressions were responsible respectively for 61.3% and 81.9% of the aggressive behaviours in non-neighbours and allopatric encounters (Figure 2a).

Table 2. Relationship (Spearman rank correlation) between the aggression index and the spatial (ln-transformed), genetic (F_{ST}) and chemical (Euclidean) distances in all species.

Variable	*N. apicalis* morph 7	*N. apicalis* morph 4	*N. verenae* morph 1
Spatial distance	r_s = 0.71 **P < 0.0001**	r_s = 0.08 P = 0.63	r_s = 0.34 **P = 0.034**
Genetic distance	r_s = 0.01 P = 0.97	r_s = −0.29 P = 0.08	r_s = 0.02 P = 0.90
Chemical distance	r_s = 0.28 P = 0.08	r_s = 0.06 P = 0.73	r_s = 0.22 P = 0.17

Significant values are highlighted in bold.

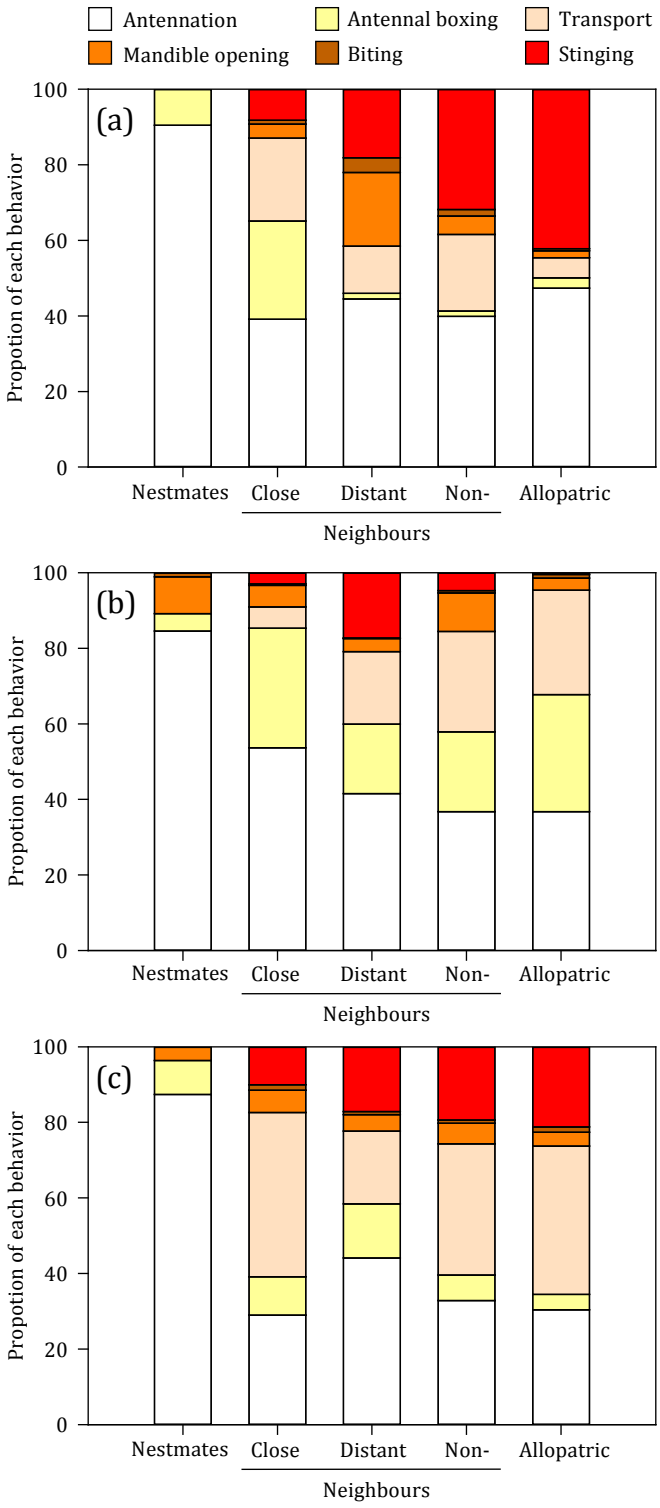

Figure 2. Proportion of all behaviours (duration) during dyadic encounters between ants originating from nests separated by various categories of spatial distance. (a) *N. apicalis* morph 7. (b) *N. apicalis* morph 4. (c) *N. verenae* morph 1. Sample sizes of encounters are $N = 10$ for each bar, except for allopatric encounters in (a) and distant neighbours encounters in (b) where $N = 9$.

N. apicalis morph 4 showed a much less structured pattern of behavioural response depending on the spatial distance. The aggression index was uncorrelated with spatial distance (Figure 1 and Table 2). There was an increased level of aggression for the encounters between distant neighbours compared with both close neighbours and allopatric encounters (both $P' < 0.018$), and there was a similar trend for the encounters between non-neighbours compared with both close neighbours and allopatric encounters (both $P' < 0.086$). The aggression indices of encounters involving close neighbours and allopatric ants were similar ($P' = 0.45$), as were the aggression indices of encounters between distant neighbours and between non-neighbours ($P' = 0.22$; Figure 1). Control tests between nestmates were again characterized by shorter durations of most aggressive behaviours (antennal boxing, $F_{4,49} = 3.36$, $P = 0.015$, all $P' < 0.003$; mandible opening, $F_{4,49} = 3.36$, $P = 0.020$, all $P' < 0.027$ except for the comparison with close neighbours $P' = 0.080$; stinging, $F_{4,49} = 3.34$, $P = 0.021$, all $P' < 0.047$ except for the comparison with close neighbours $P' = 0.16$ and allopatric ants $P' = 0.078$; Supplementary Figure S2). Whereas the duration of most behaviours investigated did not significantly differ between all categories of non-nestmates tests (Supplementary Figure S2), the duration of stinging was significantly longer during encounters between distant neighbours than during both close neighbours and allopatric encounters ($F_{4,49} = 3.34$, $P = 0.021$, both $P' < 0.048$; Supplementary Figure S2f) but was similar during non-neighbours encounters ($P' = 0.30$). This relatively unstructured pattern is also seen in the proportions of all behaviours, apart from nestmates encounters characterized by antennation behaviours (Figure 2b). Overt aggressions always represented the minor part (< 10%) of aggressive behaviours in all encounters, but their proportion reached 32.5% in distant neighbours encounters (Figure 2b).

The results for *N. verenae* finally showed an even simpler pattern where only nestmates and non-nestmates were behaviourally discriminated. Although there was a positive correlation between spatial distance and the aggression index (Table 2), there was no significant difference in the aggression index between all non-nestmates encounters (all $P' > 0.21$; Figure 1). As with the two *N. apicalis* species, control tests between nestmates were characterized by shorter durations of most aggressive behaviours (antennal boxing, $F_{4,49} = 2.79$, $P = 0.039$, all $P' < 0.030$ except for the comparison with non-neighbours $P' = 0.068$; transport, $F_{4,49} = 4.47$, $P = 0.005$, all $P' < 0.015$; mandible opening, $F_{4,49} = 2.77$, $P = 0.045$, all $P' < 0.045$; biting, $F_{4,49} = 2.31$, $P = 0.048$, all $P' < 0.008$;

Supplementary Figure S3). There was however no significant difference in the duration of all aggressive behaviours between all non-nestmates encounters (Supplementary Figure S3). The encounters between nestmates were again mostly characterized by antennation behaviours, whereas the proportions of all behaviours were similar in all other categories of spatial distance, although the part of overt aggressions among aggressive behaviours tended to increase with spatial distance, from 19.4% in close neighbours encounters to 40.4% in allopatric encounters (Figure 2c).

Cuticular Hydrocarbons

There were clear chemical differences between the cuticular hydrocarbon profiles of all colonies for all three species (Wilks's $\lambda < 0.0001$, $F_{504,3281} = 289.23$, $P < 0.0001$), with 95.14% (235/247) of workers correctly assigned to their colony by the discriminant function analysis.

There was no significant correlation between pairwise Euclidean distance and spatial distance for all species (Mantel tests: *N. apicalis* morph 7, $r = 0.24$, $P = 0.29$; *N. apicalis* morph 4, $r = -0.09$, $P = 0.52$; *N. verenae*, $r = 0.31$, $P = 0.17$). Likewise, no correlation was found between the aggression index and the chemical distance in all species (Table 2).

Genetic Analyses

We detected no signs of linkage disequilibrium between all pairs of loci. Measures of genetic variability showed a relatively higher genetic diversity in *N. apicalis* morph 4 and *N. verenae* than in *N. apicalis* morph 7, but all species exhibited high levels of heterozygosity (Supplementary Table S1). The inbreeding coefficient was not significantly different from zero in each case (*t*-test: *N. apicalis* morph 7, $F_{IT} = -0.07$, $t = -0.67$, $P = 0.57$; *N. apicalis* morph 4, $F_{IT} = 0.19$, $t = 1.49$, $P = 0.25$; *N. verenae*, $F_{IT} = 0.05$, $t = 0.26$, $P = 0.81$). Average intra-nest relatedness was similar to the expected $r = 0.75$ under monogyny and monandry for *N. apicalis* morphs 7 and 4 (respectively $r = 0.72 \pm 0.04$, *t*-test: $t = -0.68$, $P = 0.42$; and $r = 0.73 \pm 0.02$, $t = -1.44$, $P = 0.16$) and was significantly lower for *N. verenae* ($r = 0.61 \pm 0.06$, $t = -2.32$, $P = 0.015$) indicating moderate levels of polygyny. Average inter-nest relatedness indicated that colonies were not related (*N.*

apicalis morph 7, r = −0.09 ± 0.05; *N. apicalis* morph 4, r = −0.06 ± 0.03; *N. verenae*, r = −0.11 ± 0.03). Moreover, pairwise F_{ST} values were high in all species (*N. apicalis* morph 7, 0.35 ± 0.03; *N. apicalis* morph 4, 0.38 ± 0.02; *N. verenae*, 0.31 ± 0.01), indicating a high genetic divergence between colonies (Wright, 1978).

There was no pattern of isolation by distance in all species (Mantel tests: *N. apicalis* morph 7, r = −0.16, P = 0.48; *N. apicalis* morph 4, r = −0.13, P = 0.35; *N. verenae*, r = −0.18, P = 0.44). Genetic and chemical distances were positively correlated in *N. apicalis* morph 4 (r = 0.58, P = 0.0001) but there was no significant correlation between these two variables in *N. apicalis* morph 7 and *N. verenae* (respectively r = 0.11, P = 0.53 and r = −0.12, P = 0.82). Finally, no correlation was found between the aggression index and the genetic distance in all species (Table 2).

DISCUSSION

This study reveals that three closely-related and sympatric species belonging to the *Neoponera apicalis* complex all show clear nestmate recognition abilities, but nevertheless exhibit strong differences in their behavioural response underlying the discrimination of non-nestmates.

N. apicalis morph 7 showed a marked neighbour–stranger discrimination, with reduced levels of aggression at near spatial distance ranges. Beyond this close link, ants in this species also showed a well structured pattern of behavioural response depending on the spatial proximity between nests. Indeed, dyadic encounters between close nests differed both quantitatively and qualitatively from encounters between more distant colonies, with a shift from ritualized behaviours –antennal boxing and mandible opening towards close and distant neighbours, respectively– to overt aggressions –stinging towards non-neighbours, be they sympatric or allopatric. Overall, this pattern of behavioural response is therefore compatible with a dear enemy phenomenon, as already found in a number of species from different ant subfamilies (Heinze et al., 1996; Langen et al., 2000; Pirk et al., 2001; Dimarco et al., 2010; Tanner & Keller, 2012).

The results for the other two species however failed to provide strong evidence in favour of a neighbour–stranger discrimination. There was no obvious influence of

spatial distance between nests on the aggressive response towards strangers in *N. apicalis* morph 4. The only visible effect consisted in an increased level of overt aggression during the encounters between distant neighbours, and to a lesser extent between non-neighbours. However, the aggression level during allopatric encounters reached the same level as during the encounters between close neighbours. Recognition errors are unlikely to explain this result given the marked differences in the chemical profiles between allopatric colonies, and no obvious pattern of encounters or nest emigration provides a simple mechanism underlying this response. Additional studies, possibly with a longitudinal monitoring of the responses, therefore seem necessary to confirm the existence of a specific effect of spatial distance in this species, and to exclude possible artefacts caused by the context of encounter in our tests (Tanner & Adler, 2009). Meanwhile, although *N. verenae* exhibited a low positive correlation between aggression and spatial distance, no neighbour–stranger discrimination could be found in this species, as also reported in other cases (Dahbi et al., 1996; Boulay et al., 2007). Also noteworthy is the general important variability in the behavioural responses that could partly stem from inter-individual differences of the tested ants in terms of physiology and experience. However such an effect probably occurs in all species, and we are therefore confident that it cannot alone account for the observed interspecific differences.

The dear enemy phenomenon can potentially be explained by a variety of non-mutually exclusive factors, such as a greater proximity of recognition cues between nearby colonies (Martin et al., 2012), deriving either from microhabitat variations (Heinze et al., 1996), common diet (Liang and Silverman, 2000), or genetic proximity (van Zweden et al., 2010). This last factor could furthermore functionally explain a reduced level of aggression towards neighbours if nearby colonies are related, in which case the balance between costs and benefits to inclusive fitness may prevent a strong rejection and even favour affiliative behaviours towards neighbours (Zinck et al., 2008). Here, even though all colonies showed distinct cuticular hydrocarbon profiles, the chemical proximity between neighbouring colonies was not higher than the one between distant nests. There was no pattern of isolation by distance, at least at these fine scales, in all species. Furthermore, the level of aggression was also uncorrelated with both genetic and chemical distances in each case. Therefore, the dear enemy phenomenon observed in *N.*

apicalis morph 7 cannot be explained by a similarity in recognition cues, be they environmentally-derived or heritable, between nearby colonies.

Alternatively, this neighbour–stranger discrimination could be mediated by a learning process. Social insects are able to learn new chemical recognition cues during adult life (van Wilgenburg et al., 2011a), which can modify their recognition template (Errard & Hefetz, 1997; Orivel et al., 1997). Foragers may thus learn the specific chemical profiles of neighbouring colonies following repeated encounters during their foraging trips (Langen et al., 2000; Knaden & Wehner, 2003; Sanada-Morimura et al., 2003; Dimarco et al., 2010), and then adjust their behaviour accordingly. The nature of this learning phenomenon remains unknown, but it can theoretically consist either in a simple habituation process (Langen et al., 2000; Dimarco et al., 2010; Tanner & Keller, 2012), or in the formation of a new –neighbour-specific– template (Knaden & Wehner, 2003; Newey et al., 2010). Our results suggest that the specificity of the behavioural responses towards the different categories of neighbours does not simply result from the formation of a broader template encompassing nestmates and non-nestmates recognition cues (Helanterä et al., 2007), but instead that multiple templates may be learned from particular non-nestmates. This hypothesis is compatible with the fine discrimination abilities of *N. apicalis* ants (Yagound et al., 2014), and could explain the qualitative differences in aggressive behaviour between close, distant and non-neighbours. Another open question is whether and how the neighbours' labels are transmitted between nestmates, since only a fraction of the colony members will actually be in direct contact with particular non-nestmates. Contrasting elements of evidence exist (Brown & Gordon, 1997; Sanada-Morimura et al., 2003; Gill et al., 2012), possibly because of interspecific variations in learning dynamics, thus warranting further investigation. Interestingly, the individual path fidelity and regional specialization that persists for long periods in the field in *N. apicalis* (Fresneau, 1985) might be sufficient for individual foragers to increase their familiarity towards specific foreign colonies with increased chances of encounters due to shared foraging areas. Intracolonial information transfer may thus be less necessary, but this should be confirmed by individual-level studies.

As previously stated, the great interspecific diversity in the behavioural response towards non-nestmates can mostly be explained because of context and life history

differences favouring one strategy over the others. For example, the nasty neighbour effect appears to be favoured in species with large colonies able to control stable territories or perform mass recruitment (Gordon, 1989; Dunn & Messier, 1999; Newey et al., 2010). By contrast, *N. apicalis* morph 7 has small colonies without an established territory and where workers forage solitarily for randomly distributed and unpredictable resources (Fresneau, 1994). Therefore monopolizing food resources is impossible, possibly favouring a dear enemy phenomenon (Heinze et al., 1996; Langen et al., 2000; Tanner & Keller, 2012). However, the most striking result of this study is that *N. apicalis* morph 4 and *N. verenae* also share all these characteristics but do not exhibit any neighbour–stranger discrimination. The relative threat level hypothesis proposes that a higher level of aggression should be directed towards the category of non-nestmates representing the biggest threat (Temeles, 1994). According to this hypothesis, strangers thus represent a bigger threat than neighbours in *N. apicalis* morph 7, whereas their level of threat is equivalent in the other two species. Here we propose that this hypothesis is likely to explain our results, and that it is mediated by the interspecific variation in nesting preferences influencing the competition for nest sites between colonies.

Nest relocation following colony growth, predation event or nest deterioration is common in ants (McGlynn, 2012) and this is particularly true in the tropical habitat of the *N. apicalis* species complex (Fresneau, 1994; Pezon et al., 2005). However, our results show that, even if all species had very similar patterns of nest spatial structuring, they exhibited different nesting preferences. *N. apicalis* morph 7 indeed showed an arboreal nesting preference, whereas the other species commonly nested in rotting wood near or on the ground. Consequently, the temporal stability of nest sites is very likely to be much more important in *N. apicalis* morph 7, which implies that these ants must have a relatively low frequency of nest relocation. In this situation, familiar neighbours are likely to compete for food only, and therefore they represent a relatively low threat level since the competition for food is unlikely to trigger costly competitive relationships between colonies. It may then be beneficial to reduce the costs of frequent fights with close neighbours. By contrast, unfamiliar strangers may represent scouts from emigrating colonies looking for a new nest (Hölldobler & Wilson, 1990). These individuals thus represent a greater level of threat, particularly since suitable nesting sites in living trees are likely to be rare compared with cavities in rotting trunks. This is

further suggested by the fact that *N. apicalis* morph 7 is more aggressive towards distant non-nestmates than the other two species. Overall, this neighbour–stranger discrimination is thus likely to ultimately result from the relative threat level they represent.

In contrast, the more ephemeral nature of nest sites in *N. apicalis* morph 4 and *N. verenae* suggests higher frequencies of nest relocation. All non-nestmates may then be potential competitors for both food and nests, and thus represent similar threat levels. Discriminating neighbours from strangers may therefore bring no obvious benefits in these species, thus explaining the high proportions of ritualized behaviours towards all categories of non-nestmates, contrary to *N. apicalis* morph 7. Furthermore, the rates of encounters between neighbouring colonies might simply not have been high enough for any familiarization to occur. Whereas quick neighbour-stranger discrimination has been shown in some cases (Sanada-Morimura et al., 2003), other studies suggest that this process may take a relatively long time (Newey et al., 2010), possibly because of the necessary duration for the information to be integrated at the colony level. Investigating the dynamics of aggressive response between newly neighbouring colonies as a function of their history of encounters would provide a valuable direct test for these hypotheses.

In conclusion, our study reveals important differences in intercolony competitive relationships between closely-related sympatric species with very similar life histories. Variation in nesting preference appears to be a critical factor influencing the average encountering rate between close colonies, which in turn affects the balance between costs and benefits of discriminating neighbours from strangers, and possibly constrains its establishment. A more thorough investigation of population densities, relocation frequencies and effective encounter rates in the wild should now be conducted to fully confirm these findings. These results indicate that the proximate factors underlying neighbour–stranger discrimination may be even more diverse than previously thought. This further stresses the importance of learning in non-nestmates discrimination processes and reinforces the relative threat level hypothesis as an ultimate explanation for the dear enemy phenomenon.

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SUPPLEMENTARY MATERIAL

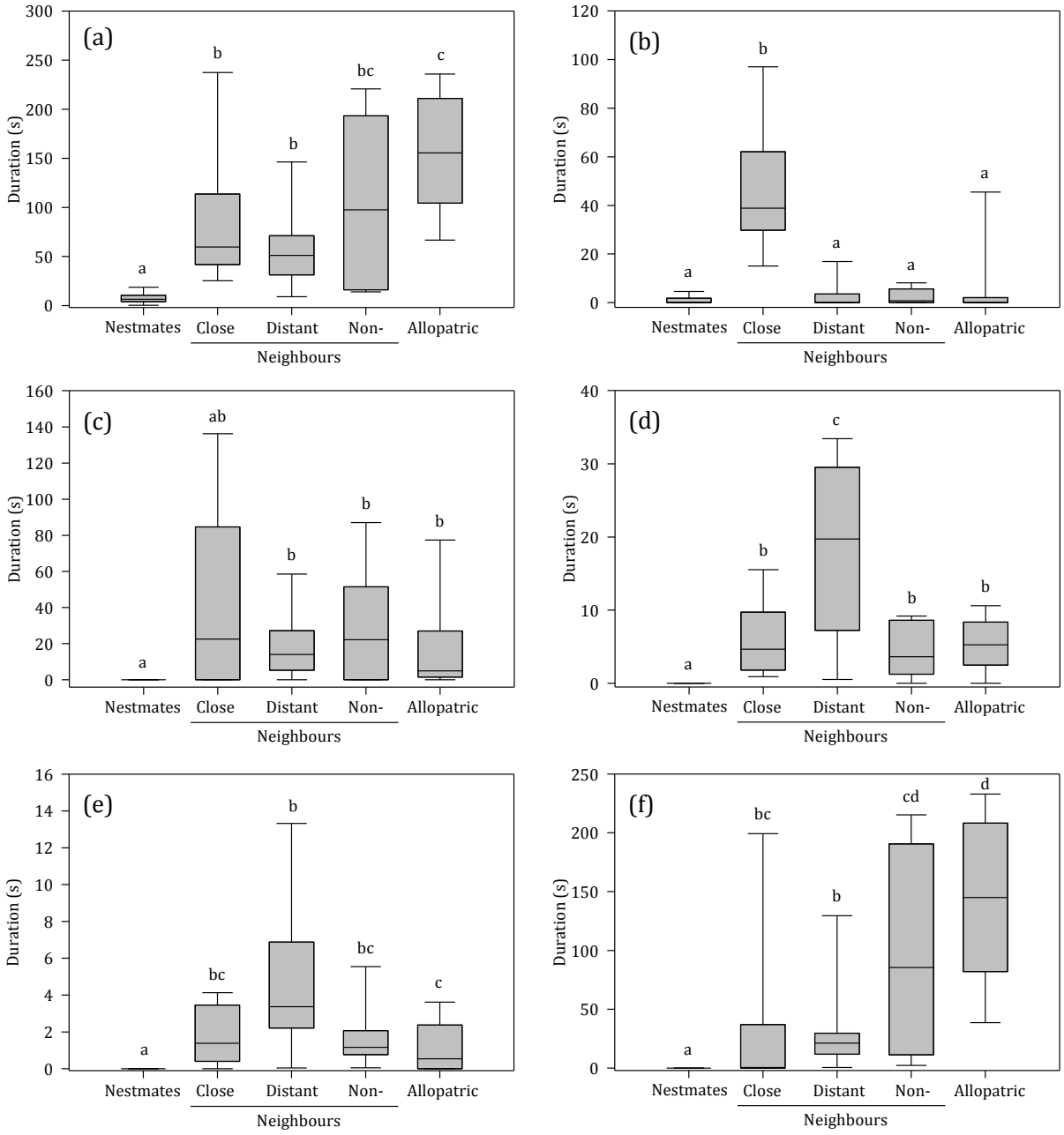

Figure S1. Duration (s) of each behaviour in dyadic encounters between ants originating from nests separated by various categories of spatial distance in *N. apicalis* morph 7. (a) Antennation. (b) Antennal boxing. (c) Transport. (d) Mandible opening. (e) Biting. (f) Stinging. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of encounters are $N = 10$ for each box plot, except for allopatric encounters where $N = 9$. Different letters denote statistical differences.

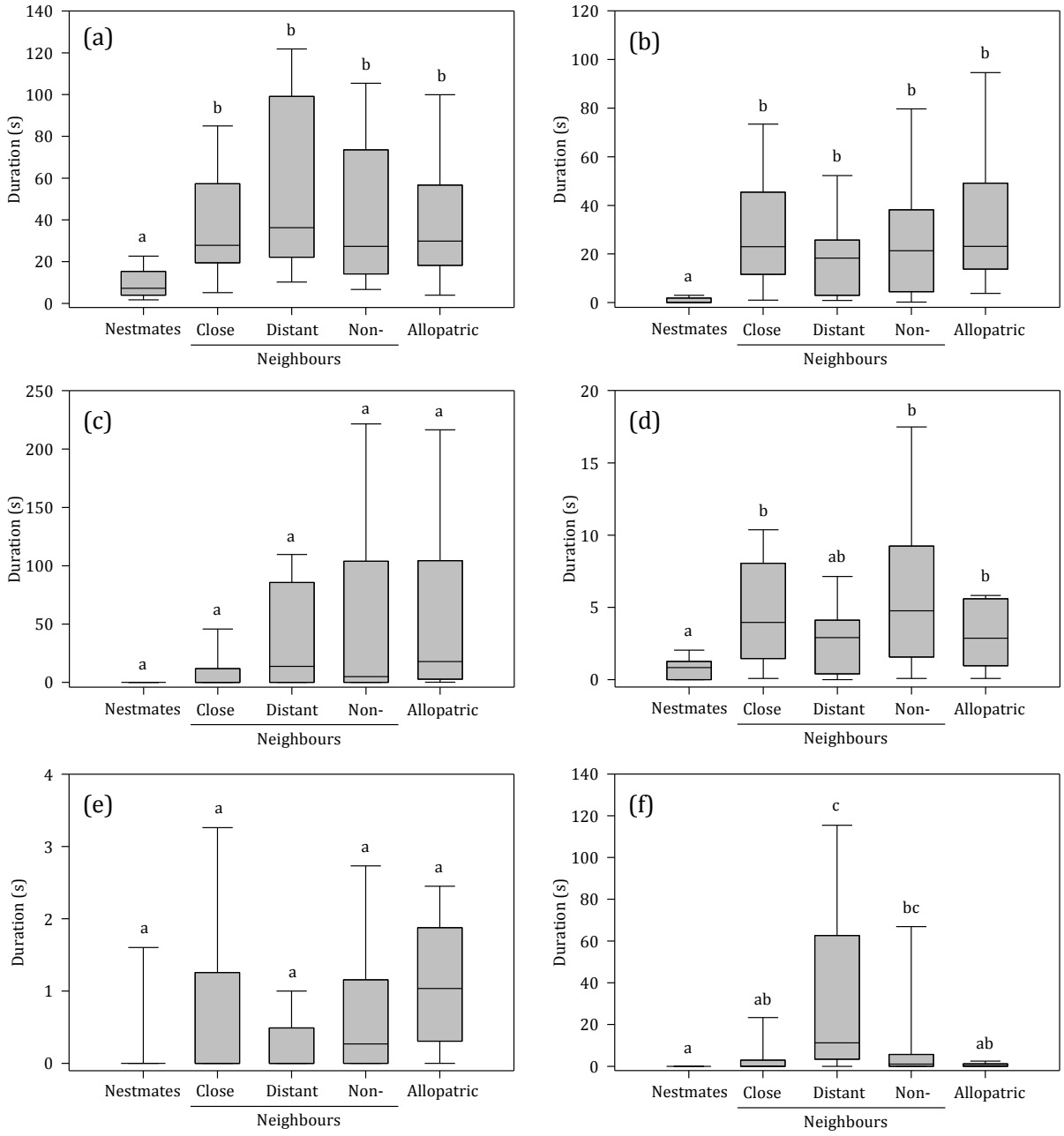

Figure S2. Duration (s) of each behaviour in dyadic encounters between ants originating from nests separated by various categories of spatial distance in *N. apicalis* morph 4. (a) Antennation. (b) Antennal boxing. (c) Transport. (d) Mandible opening. (e) Biting. (f) Stinging. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of encounters are $N = 10$ for each box plot, except for distant neighbours encounters where $N = 9$. Different letters denote statistical differences.

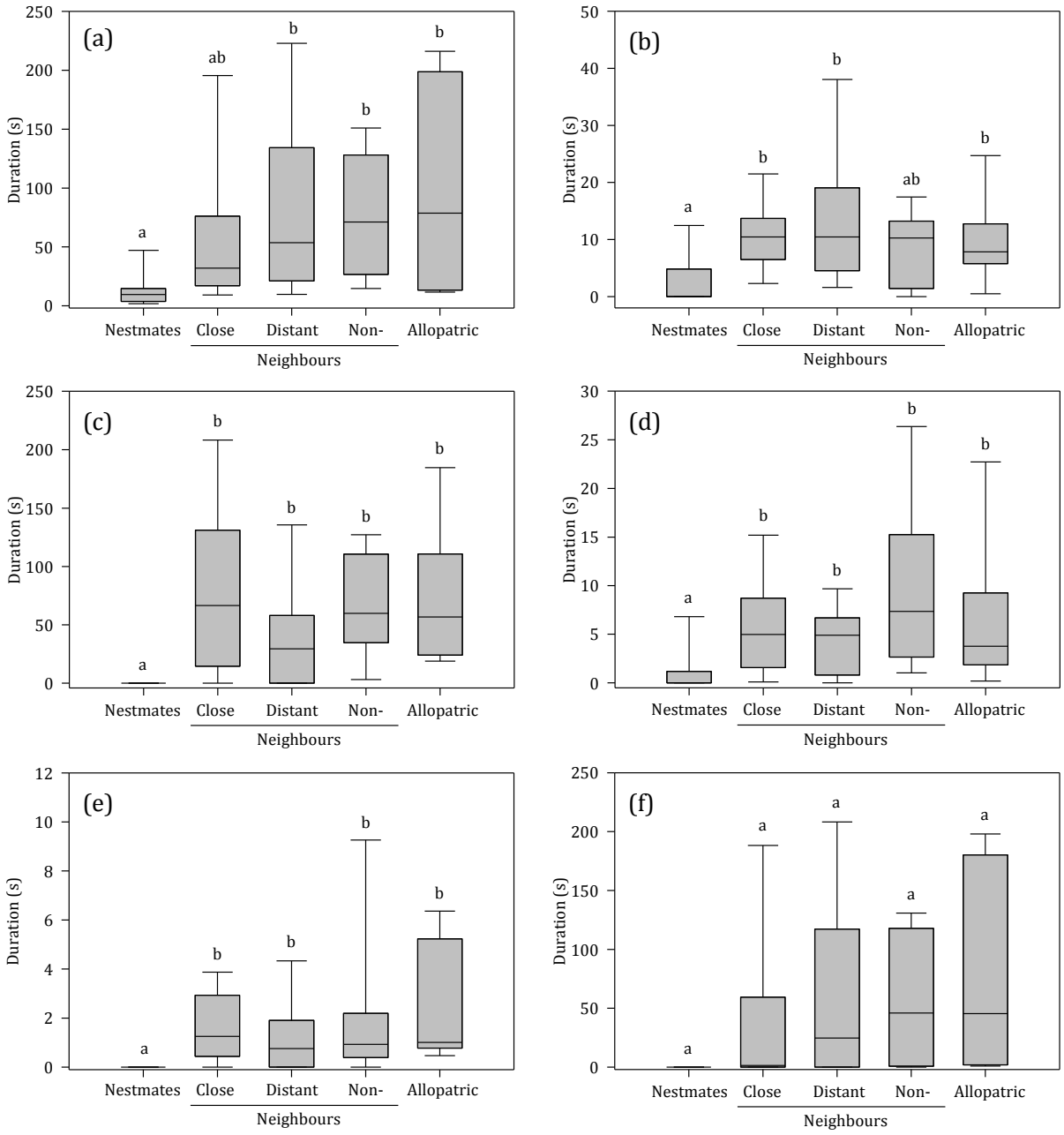

Figure S3. Duration (s) of each behaviour in dyadic encounters between ants originating from nests separated by various categories of spatial distance in *N. verenae* morph 1. (a) Antennation. (b) Antennal boxing. (c) Transport. (d) Mandible opening. (e) Biting. (f) Stinging. Box plots represent 10th, 25th, 50th (median), 75th and 90th percentiles. Sample sizes of encounters are $N = 10$ for each box plot. Different letters denote statistical differences.

Table S1. Genetic variation at seven microsatellite loci in all species.

Locus	*N. apicalis* morph 7			*N. apicalis* morph 4			*N. verenae* morph 1		
	A	A_r	H_e	A	A_r	H_e	A	A_r	H_e
Pv1048	6	2.89	0.82	16	7.20	0.88	12	10.19	0.93
Pv1078	3	2.02	0.62	2	1.90	0.26	1	1.00	0.00
Pv2056	5	2.62	0.69	10	5.50	0.79	8	7.57	0.85
Pv2111	2	1.83	0.47	14	7.15	0.90	11	8.64	0.89
Pv3091	4	1.94	0.52	10	5.41	0.79	2	1.69	0.05
Pv4049	3	2.03	0.54	4	2.41	0.31	3	3.00	0.60
Pv4053	3	1.91	0.41	8	5.33	0.79	3	2.03	0.09
Mean ± SE	3.71 ± 0.52	2.18 ± 0.15	0.58 ± 0.05	9.14 ± 1.90	4.99 ± 0.79	0.67 ± 0.10	5.71 ± 1.71	4.87 ± 1.43	0.49 ± 0.16

A, number of alleles; A_r, allelic richness; H_e, unbiased expected heterozygosity.

DISCUSSION GÉNÉRALE

Les travaux présentés dans cette thèse apportent de nombreuses informations affinant notre compréhension des mécanismes permettant de maintenir la coopération dans les groupes sociaux face aux possibilités d'exploitation internes et externes. Ils révèlent de plus l'intérêt d'utiliser une approche comparative et intégrative pour appréhender cette problématique centrale de l'étude des comportements sociaux. Cette dernière partie a pour but d'établir la synthèse des résultats obtenus, mais également de discuter de diverses perspectives ouvertes par ce travail.

I. Evolution du complexe *Neoponera apicalis*

Le chapitre 1 (Article 1) avait pour objectif de clarifier le statut taxonomique du complexe *N. apicalis*. L'identification de ces espèces cryptiques constitue un défi taxonomique pouvant bénéficier de l'utilisation combinée de divers caractères dans une approche intégrative (Bickford et al., 2007 ; Seifert, 2009). En effet, si des caractères morphologiques, tels la forme du pétiole, le nombre d'antennomères jaunes ou la pilosité, ont été utilisés pour définir certains morphes de *N. apicalis* et *N. verenae* (Delabie et al., 2008), l'ampleur de la variation intra-morphe et le chevauchement inter-morphe de ces caractères utilisés seuls les rendent peu fiables pour définir les espèces de ce complexe (F. Prada & F. Fernández, communication personnelle), au moins en ce qui concerne les ouvrières. La morphologie de l'organe stridulatoire est en revanche un caractère pertinent pour délimiter ces espèces (Ferreira et al., 2010), mais son observation précise n'est directement accessible qu'à l'aide de la microscopie électronique.

Notre approche intégrative mêlant taxonomie chimique et phylogénie moléculaire montre une forte divergence chimique et génétique aussi bien entre les groupes qu'au sein des groupes étudiés. Elle confirme la monophylie de tous les morphes identifiés à l'aide de caractères morphologiques, cytogénétiques et bioacoustiques (Delabie et al., 2008 ; Ferreira et al., 2010) et identifie un nouvel haplotype au sein de *N. verenae* (morphe 3) et de *N. apicalis* (morphe 7). Les relations phylogénétiques entre ces groupes ne sont cependant que partiellement résolues avec la phylogénie actuelle (Figure 1), ce qui implique la nécessité d'inclure l'étude de nouveaux gènes, notamment nucléaires. Nos résultats montrent une forte divergence génétique entre les différentes

espèces et morphes de ce complexe, notamment pour les morphes en sympatrie. Associés aux différences morphologiques, moléculaires, comportementales, cytogénétiques et écologiques déjà rapportées (Delabie et al., 2008 ; Ferreira, 2010 ; Ferreira et al., 2010 ; Evison et al., 2012 ; Articles 5 et 6), ces résultats soutiennent fortement la présence de 11 espèces valides dans le complexe *N. apicalis* : *N. apicalis* morphes 1, 2, 3, 4, 6 et 7, *N. cooki*, *N. obscuricornis* et *N. verenae* morphes 1, 2 et 3.

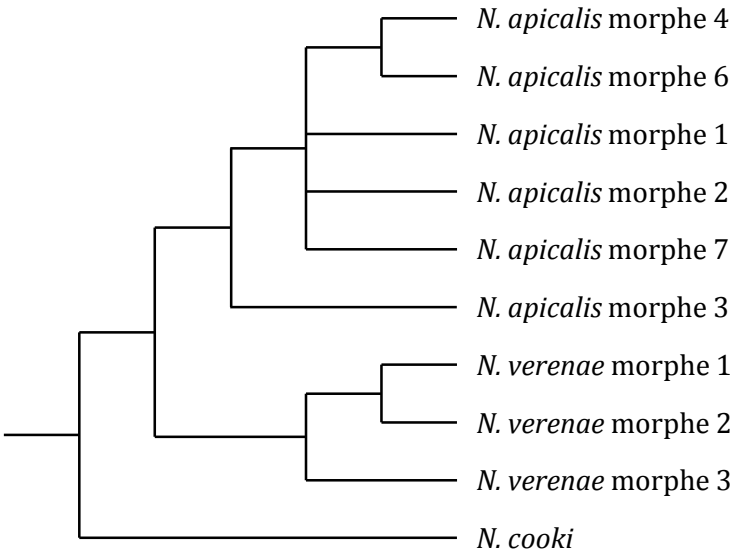

Figure 1. Arbre consensus des relations phylogénétiques bien soutenues au sein du complexe *N. apicalis*. La position de *N. obscuricornis* n'est pas connue.

Dans une étude récente, Touchard et al. (2014) ont identifié deux morphes (1 et 2) de *N. apicalis* en Guyane Française à l'aide des peptides de la glande à venin et du gène mitochondrial COI. Cela montre l'intérêt de ce nouveau caractère pour contribuer à délimiter ces espèces cryptiques. Cependant, leur identification ne correspond pas à la classification actuelle des morphes de ce complexe (Delabie et al., 2008 ; Ferreira et al., 2010), et ces auteurs n'ont pas déposé leurs séquences dans une banque de données publique. Etant donné le nombre de morphes de *N. apicalis* présents en sympatrie en Guyane Française, il est impossible pour l'instant de faire correspondre leurs identifications avec les nôtres, et ainsi tirer parti de ce nouvel outil taxonomique. Ce cas est donc révélateur de la nécessité de décrire et nommer ces différentes espèces pour

stabiliser leur statut taxonomique comme cela a été fait pour *N. cooki* (Mackay & Mackay, 2010).

Nos résultats peuvent servir de base pour étudier la biogéographie du complexe *N. apicalis* et émettre des hypothèses quant à son histoire évolutive. La région Néotropicale est connue pour abriter la plus forte diversité de Formicidae (Wilson & Hölldobler, 2005 ; Fisher, 2010), aussi bien en tant que réservoir d'espèces que zone d'apparition de nouvelles espèces (Moreau & Bell, 2013). Les phylogénies moléculaires datent l'apparition du genre *Neoponera* il y a 16–19 Ma (Schmidt, 2013). Il a été proposé que le bassin amazonien soit la zone d'origine de l'ancêtre du complexe *N. apicalis* (Delabie et al., 2008), en raison notamment des haut niveaux de sympatrie que l'on y trouve. Cette hypothèse est corroborée par nos résultats montrant le plus fort taux de sympatrie en Guyane Française, et par la présence de l'espèce basale *N. cooki* inféodée à cette région (Mackay & Mackay, 2010). Des processus de divergence sans doute anciens et complexes ont alors suivi (Gavrilets, 2003 ; Delabie et al., 2008) mettant possiblement en jeu à la fois des processus de spéciations sympatrique et allopatrique (Fitzpatrick et al., 2008), et entrainant une diversification au sein du groupe *N. verenae* et surtout *N. apicalis*. Par exemple, la position basale au sein du groupe *N. apicalis* du clade *N. apicalis* morphe 3 inféodée à l'Amérique Centrale et donc en allopatrie avec les autres espèces peut correspondre à une spéciation sympatrique suivie d'une colonisation vers le nord après la fermeture de l'isthme de Panama il y a environ 3 Ma (Kirby et al., 2008). On peut noter que les deux autres taxons présents en allopatrie, *N. apicalis* morphe 1 et *N. verenae* morphe 2, occupent également un habitat différent (forêt atlantique) (Delabie et al., 2008). La plupart des espèces du complexe demeurent en revanche en sympatrie. Leur morphologie très similaire est alors sans doute le résultat d'une sélection stabilisatrice permettant à ces espèces d'échapper à leurs prédateurs potentiels en conservant un morphotype associé à leur piqure douloureuse (Lucas et al., 2002). Cette hypothèse est également suggérée par les cas de mimétisme batésien mettant en jeu la fourmi *Gigantiops destructor* (Formicinae) (Wheeler, 1922) mais également plusieurs espèces d'araignées telles *Castianeira memnonia* et *C. tenuiformis* mimant à la fois la morphologie et le comportement de *N. apicalis* (Reiskind, 1977 ; McIver & Stonedahl, 1993).

Cette étude permet donc de clarifier le statut taxonomique du complexe *N. apicalis* et confirme l'existence d'une diversité bien supérieure à celle actuellement décrite au sein

de ce complexe. Elle pose les jalons de l'étude de la biogéographie de ces fourmis, mais révèle en même temps la nécessité de compléter l'échantillonnage dans leur aire de répartition extrêmement large, afin de définir précisément la distribution des différentes espèces du complexe et pouvoir ensuite recréer leur histoire évolutive.

Nous démontrons par ailleurs dans cette étude que les morphes 1, 3, 4, 6 et 7 de *N. apicalis*, *N. cooki* et *N. verenae* morphe 1 possèdent tous des profils d'HCC qui leur sont spécifiques (Article 1). De plus, seuls quelques HCC possédant des proportions distinctes dans chacune de ces espèces sont suffisants pour les discriminer de manière non ambigüe. Les trois classes principales d'HCC (alcanes linéaires, branchés et alcènes) sont toutes susceptibles de constituer des caractères taxonomiques fiables, mais les HCC insaturés ou possédant des groupements méthyl sont ceux qui présentent la plus forte dissimilitude entre les espèces étudiées, et ont ainsi le plus fort potentiel à servir d'outils taxonomiques. Ce pattern a été observé de nombreuses fois chez les fourmis (Dahbi et al., 2008 ; Martin et al., 2008a ; Martin & Drijfhout, 2009a ; Drescher et al., 2010 ; Guillem et al., 2012), et il est vraisemblablement la conséquence du plus grand potentiel de communication de ces HCC lié à leur conformation spatiale (Châline et al., 2005b ; Monnin, 2006 ; Blomquist, 2010). Au sein de chaque morphe, ces HCC semblent aussi coder l'information relative à l'appartenance coloniale comme typiquement chez les insectes sociaux (Howard & Blomquist, 2005 ; d'Ettorre & Lenoir, 2010). De plus, nos résultats montrent également l'existence de chémotypes dans certains morphes étudiés, dont certains sont probablement liés à des différences géographiques (Evison et al., 2012), mais dont d'autres sont l'indication possible d'évènements d'hybridation entre lignées partiellement divergentes (Seifert, 2009 ; Ferreira, 2010), et reflétant des variations dans les HCC liées aux lignées maternelles (Helanterä et al., 2013) ou plus probablement paternelles (Nehring et al., 2011).

Ces résultats montrent donc que des profils différents d'HCC peuvent coexister au sein d'une même colonie, confirmant le peu d'échanges d'HCC entre membres de la même colonie chez ces espèces (Soroker et al., 1998, 2003) et suggérant leur fort déterminisme génétique (Vander Meer & Morel, 1998 ; van Zweden et al., 2010). Ils suggèrent aussi que la formation du visa colonial n'implique qu'une partie des HCC similaire entre les chémotypes, ce qui semble être généralement le cas chez les fourmis (Greene & Gordon, 2007 ; van Wilgenburg et al., 2010 ; Smith AA et al., 2013). Il a par exemple été montré

chez *Formica exsecta* que seuls les *Z*9-alcènes interviennent dans la reconnaissance coloniale (Martin et al., 2008b). Maintenant que les profils d'HCC propres à chaque colonie et chémotype sont disponibles, il devient possible d'effectuer des tests où certaines parties du profil chimique sont modifiées et de tester ainsi la perception de ces indices de reconnaissance et la réponse comportementale qu'ils induisent.

Cette étude confirme donc l'utilité des HCC en tant qu'outil taxonomique, notamment pour différencier les espèces cryptiques (Bagnères & Wicker-Thomas, 2010 ; Kather & Martin, 2012). Ces outils paraissent particulièrement pertinents dans les études se focalisant sur le comportement du fait des possibilités d'extractions non-invasives des HCC via la micro-extraction en phase solide (e.g. Monnin et al., 1998), contrairement aux caractères moléculaires nécessitant de sacrifier tout ou partie des individus.

II. Régulation des conflits liés à la reproduction

1. Organisation coloniale

Un des aspects centraux de cette thèse a été l'étude des facteurs responsables de la régulation des conflits associés à la reproduction dans les colonies du complexe d'espèces *Neoponera apicalis*. Nous nous sommes particulièrement intéressés aux mécanismes proximaux intervenant dans le conflit lié la production des mâles. Dans le chapitre II (Article 2), nous avons déterminé la structure génétique des colonies de plusieurs espèces du complexe. Les résultats confirment tout d'abord pour les morphes de *N. apicalis*, et probablement *N. cooki*, le statut monogame (au moins d'un point de vue fonctionnel) classiquement attribué aux ponérines, bien qu'exceptionnellement mesuré (Peeters, 1993). Ce trait représente très probablement l'état ancestral de l'organisation coloniale chez les insectes sociaux (Hughes et al., 2008a), ce qui renforce l'intérêt d'utiliser ce modèle pour étudier les questions relatives à l'évolution de la socialité. Cette structure monogyne–monoandre révèle par ailleurs l'existence d'un conflit potentiel pour la production des mâles tel que prédit par la théorie de l'*inclusive fitness*, qui découle des différences d'optimums reproductifs entre la reine et les ouvrières, celles-ci étant plus apparentées à leurs fils et neveux qu'à leurs frères (Trivers & Hare, 1976 ; Ratnieks & Reeve, 1992 ; Wenseleers et al., 2004). De façon intéressante, et nous y reviendrons, ces résultats montrent aussi des différences entre les morphes de *N.*

verenae facultativement polygynes et les autres espèces du complexe. Cette polygynie pourrait avoir en partie relâché les pressions associées à la reproduction des ouvrières, du fait d'une probabilité moindre d'accéder à la reproduction directe (Bourke & Franks, 1995).

2. Rôle des processus de reconnaissance

Après avoir estimé l'existence de conflits potentiels, nous avons étudié en détail les conditions dans lesquelles ces conflits s'expriment dans les colonies, en nous focalisant sur une espèce en particulier, *N. apicalis* morphe 4. Ainsi, dans le chapitre III (Articles 3 et 4) nous avons examiné les mécanismes proximaux responsables du partage de la reproduction. Nous avons pour cela étudié la manifestation la plus claire de ce conflit reproducteur, à savoir l'établissement par les ouvrières d'une hiérarchie reproductive lorsque la reine devient sénescente et perd son potentiel reproducteur ou disparait (Heinze et al., 1994). Nous avons montré que les interactions agonistiques ritualisées font émerger une hiérarchie linéaire ou quasi linéaire où les statuts hiérarchiques et reproducteurs sont très fortement corrélés, et que les processus de reconnaissance jouent un rôle capital dans la formation et la stabilisation de la structure hiérarchique (Article 3). Nos résultats révèlent que tous les individus peuvent discriminer le statut (haut ou bas rang) des autres membres de la colonie, et que ces capacités sont particulièrement fines pour les individus de haut rang qui peuvent discriminer individuellement, d'un point de vue fonctionnel, les autres hauts rangs, alors que les bas rangs n'ont pas montré de telles capacités. A l'inverse, ni les hauts rangs ni les bas rangs n'ont discriminé comportementalement deux individus de bas rang.

Ces résultats ont plusieurs implications. Ils montrent tout d'abord que la discrimination de statut est un mécanisme particulièrement pertinent pour expliquer la mise en place et le maintien des hiérarchies reproductives. Ceci est notamment dû à son niveau de précision, qui ici dépasse ce qui est couramment admis à son sujet. En effet, la possibilité de discriminer le statut de deux individus séparés par un rang d'écart n'est en général pas considérée comme possible du fait du manque de fiabilité dans les indices de reconnaissance (Liebig, 2010). En outre, de telles capacités de discrimination de deux individus ont déjà été mises en évidence, mais via le prisme de la reconnaissance

individuelle (d'Ettorre & Heinze, 2005 ; Dreier et al., 2007). L'intérêt de nos résultats est donc de montrer que la discrimination de statut est bien plus précise que ce qui a été admis jusqu'à présent. Par conséquent, nous pensons qu'il est crucial de reconsidérer la discrimination de statut comme une sérieuse alternative à la reconnaissance individuelle. Cette distinction est importante car si ces deux processus cognitifs peuvent aboutir au même résultat d'un point de vue fonctionnel (discriminer deux individus), ils divergent en revanche grandement dans leur niveau de complexité cognitive (Wiley, 2013). La reconnaissance individuelle dépend de processus de mémorisation des traits propres à chaque individu, y compris leur caractère dominant ou subordonné, processus qui demandent une dépense énergétique non négligeable (Dukas, 2008 ; Burns et al., 2011). Ceci est particulièrement vrai dans une hiérarchie qui, comme dans notre cas, comprend une douzaine d'individus. En outre les études démontrant l'existence de reconnaissance individuelle se basent sur la reconnaissance d'un individu (Tibbetts, 2002 ; d'Ettorre & Heinze, 2005 ; Dreier et al., 2007 ; Sheehan & Tibbetts, 2008), mais la capacité à reconnaitre individuellement au moins deux individus (et jusqu'à plus de dix dans notre cas) n'a jamais été démontrée à notre connaissance. Certains auteurs ont d'ailleurs sur ce point argumenté que la reconnaissance d'au moins deux individus était nécessaire pour démontrer l'existence de reconnaissance individuelle (Gheusi et al., 1994 ; Thom & Hurst, 2004). Prouver l'existence de reconnaissance individuelle selon ces critères (sans parler de la reconnaissance dans des contextes différents) parait donc très difficile. La discrimination de statut, en revanche, est un mécanisme cognitif bien moins coûteux car il ne repose sur aucun processus de mémorisation des traits individuels, et il fonctionne tout aussi bien quel que soit le degré de familiarité entre les individus (Hemelrijk, 2000). Sa simplicité et son efficacité en font un mécanisme potentiellement très répandu dans la stabilisation des hiérarchies sociales, et plus généralement dans la structuration des groupes sociaux. L'établissement de relations privilégiées et durables entre des sous-groupes d'individus semble être un phénomène fréquent dans les sociétés d'insectes (Jeanson, 2012). Les capacités fines de discrimination de statut que nous venons de mettre à jour sont ainsi un mécanisme à même d'expliquer l'existence de tels réseaux d'interactions.

La deuxième implication de ces résultats concerne les différences de capacités de discrimination observées entre les individus de hauts rangs et les individus de bas rangs. Ces différences pourraient être dues à une simple différence de motivation. Les

individus de bas rangs n'étant pas impliqués dans la hiérarchie reproductive, nous l'avons vu, ceux-ci n'ont en effet aucun bénéfice *a priori* à discriminer deux hauts rangs. Dès lors que leur statut de haut rang est reconnu, une réponse d'évitement similaire dans les deux cas peut être suffisante. Cependant, ces résultats pourraient aussi indiquer de réelles différences de capacités cognitives entre hauts rangs et bas rangs. Ces différences pourraient être influencées par des différences dans l'activité du système neuroendocrinien (Bloch et al., 2000b ; Cuvillier-Hot & Lenoir, 2006 ; Kamhi & Traniello, 2013), les patterns d'expression génique (Toth et al., 2014), voire le développement de certaines structures cérébrales (Ehmer et al., 2001 ; Molina & O'Donnell, 2007 ; Smith et al., 2010). L'existence de variations dans les niveaux d'amines biogènes, comme l'octopamine et la dopamine, est une hypothèse particulièrement intéressante, en raison de leur implication dans les processus cognitifs liés à l'apprentissage et à la mémoire (Farooqui, 2007 ; Verlinden et al., 2010), et de leur forte association avec le statut hiérarchique (Bloch et al., 2000b ; Cuvillier-Hot & Lenoir, 2006) et reproducteur (Dombroski et al., 2003 ; Sasaki et al., 2007 ; Penick et al., 2014). Tester cette hypothèse n'a malheureusement pas été possible dans cette étude, mais apparait clairement être l'une des perspectives importantes de cette thèse. Montrer l'existence de différences dans les niveaux d'amines biogènes serait en effet un argument important en faveur de l'existence de différences réelles dans les capacités cognitives selon le statut hiérarchique des individus. Ceci ouvre la voie à de nombreuses questions quant aux causes et conséquences de ces différences. On peut effectivement se demander si ces variations sont une conséquence d'un état physiologique général associé au statut hiérarchique et reproducteur des individus, et sont modifiées progressivement lors de l'établissement des relations de dominance/subordination. D'un autre côté, les observations d'interactions clairement dirigées dès les premières heures de mise en place de la hiérarchie posent la question de l'influence de ces amines biogènes non seulement pour développer un comportement de dominance et une fertilité caractéristique d'un haut rang (Kamhi & Traniello, 2013), mais également comme pré requis pour avoir des capacités cognitives suffisamment fines et ainsi discriminer individuellement le statut des autres individus de haut rang directement en compétition.

3. Indices de reconnaissance

Nous avons recherché les indices de reconnaissance sous-jacents à cette discrimination de statut (Article 3). Nous avons alors pu montrer que les individus de statuts hiérarchiques et reproducteurs différents ont des profils d'HCC spécifiques permettant clairement leur discrimination. Un HCC en particulier, le 13-méthylpentacosane (13-MeC_{25}), dont les proportions sont fortement corrélées à l'activité ovarienne, et consécutivement au rang hiérarchique, des individus, semble constituer, ou au moins contribuer à, un signal de fertilité (Monnin, 2006 ; Liebig, 2010). De plus, la relation exponentielle qui lie les quantités de cet HCC au statut des individus renforce cette hypothèse, en même temps qu'elle fournit un mécanisme probable aux résultats observés dans les tests de discrimination. Si le 13-MeC_{25} est effectivement l'indice de reconnaissance utilisé dans les processus de discrimination de statut, alors la trop grande similarité dans les proportions de ce composé entre les individus de bas rang pourrait imposer une contrainte forte empêchant leur discrimination, *a contrario* des individus de haut rang. Ce résultat confirme par ailleurs l'hypothèse de l'absence de reconnaissance individuelle du fait d'une absence d'indices de reconnaissance. En effet, si reconnaissance individuelle il y a, on peut s'attendre par définition à ce que les indices permettant cette reconnaissance ne varient pas selon le statut des individus (d'Ettorre & Heinze, 2005). Une tâche consistant à reconnaitre individuellement deux bas rangs ou deux hauts rangs est similaire d'un point de vue cognitif, alors qu'une tâche cognitive consistant à discriminer le statut de deux hauts rangs est nécessairement plus simple du fait de cette relation exponentielle que s'il faut discriminer le statut de deux bas rangs. Le fait que les individus de haut rang discriminent individuellement deux hauts rangs mais pas deux bas rangs suggère ainsi l'absence de reconnaissance individuelle.

Les bioessais réalisés corroborent en outre l'implication du 13-MeC_{25} dans la perception du statut des individus (Article 4). Nos résultats montrent en effet que les individus de haut rang (impliqués dans la compétition reproductive) diminuent leur réponse agonistique envers des individus dont les proportions de cet HCC ont été expérimentalement augmentées, ce qui correspond à la réponse attendue si ces individus sont perçus comme affichant un plus haut statut hiérarchique. Le test contrôle réalisé en traitant les individus avec du *n*-C_{20} a cependant entrainé des effets similaires au traitement impliquant le 13-MeC_{25}, même si leur amplitude est moindre. Ce résultat

inattendu atténue donc l'interprétation que l'on peut faire du rôle actif du 13-MeC_{25}, bien que les résultats suggèrent néanmoins que les individus perçoivent et réagissent de manière spécifique à cet HCC. La confirmation de ces résultats par des expériences complémentaires parait donc nécessaire. La réponse des individus dans ce genre d'expériences est très fortement influencée par le contexte même du test (Howard, 1993 ; Peeters et al., 1999 ; Buczkowski & Silverman, 2005). Il est possible que des réintroductions d'individus manipulés dans des groupes de plusieurs individus (e.g. Smith et al., 2012) soient nécessaires pour induire un contexte plus « signifiant » et entrainant des réponses non ambigües.

4. Régulation du partage de la reproduction

Nos résultats montrent par ailleurs que le 13-MeC_{25} est fortement associé au statut reproducteur quelle que soit la caste et le niveau de développement ovarien (Article 4), et ils suggèrent que ce composé pourrait réguler le partage de la reproduction durant toutes les étapes de la vie de la colonie. Tant que la reine est suffisamment fertile tous les individus ont intérêt à maintenir son monopole reproducteur (Keller & Nonacs, 1993 ; voir Introduction). Les deux principaux mécanismes pouvant intervenir sont alors l'auto-restriction reproductive des ouvrières et la coercition sous forme de *policing* exercé par les ouvrières vis-à-vis des ouvrières qui développent leurs ovaires et/ou de leurs œufs (Ratnieks & Reeve, 1992 ; Bourke & Franks, 1995 ; Ratnieks et al., 2006). De tels comportements de *policing* ne sont pas connus chez *N. apicalis* et ils ne sont pas non plus prédits par la structure d'apparentement de la colonie (Wenseleers et al., 2004 ; Wenseleers & Ratnieks, 2006), mais ils pourraient survenir comme moyen de régulation si la reproduction des ouvrières entraine des coûts importants sur la productivité coloniale (Ratnieks & Reeve, 1992 ; Gobin et al., 2003 ; Hammond & Keller, 2004). Une autre possibilité, prédite cette-fois par la théorie de l'*inclusive fitness*, serait un *policing* réalisé par la reine elle-même et contribuant ainsi à maintenir son monopole reproducteur (Monnin & Peeters, 1997 ; Kikuta & Tsuji, 1999 ; Wenseleers et al., 2005 ; Smith et al., 2011). Cependant il n'a jamais été reporté d'actes coercitifs exercés par la reine chez ces espèces (Fresneau & Dupuy, 1988 ; Oliveira & Hölldobler, 1990 ; Fresneau, 1994 ; Dietemann & Peeters, 2000) et nous n'en avons jamais observé non plus. En l'état actuel de nos connaissances, il semble plus probable que l'auto-restriction

reproductive soit le principal facteur responsable de l'absence de reproduction des ouvrières en présence d'une reine fertile, bien qu'en tout état de cause, les décisions reproductives des ouvrières soient basées sur les mêmes informations, c'est-à-dire la surveillance des signaux de fertilité émis par la reine (et éventuellement les ouvrières).

Ces mécanismes de régulation permettent donc de maintenir le monopole reproducteur de la reine, mais on peut se demander si le biais reproducteur est total ou si les ouvrières peuvent néanmoins se reproduire dans une certaine mesure en présence de la reine. En effet, la structure d'apparentement des colonies (Article 2) et l'absence supposée de *policing* prédisent un conflit ouvert en présence d'une reine fertile où 16% des ouvrières se reproduisent (Wenseleers et al., 2004). Des pontes d'ouvrières en présence de reine ont déjà été observées chez *N. apicalis* (Oliveira & Hölldobler, 1990 ; Fresneau, 1994). Dietemann & Peeters (2000) ont montré que ces œufs correspondent majoritairement à des œufs trophiques offerts pour la plupart à la reine, mais que dans certaines colonies les ouvrières pondent des œufs reproducteurs en présence de la reine. Ces résultats peuvent être mis en relation avec les données démographiques obtenues chez *N. apicalis* morphe 3 (Fresneau, 1994) mais qui sont très vraisemblablement applicables aux autres espèces du complexe de par la similitude de leurs traits d'histoire de vie (Delabie et al., 2008). Bien que les sexués soient produits tout au long de l'année dans cet habitat tropical, il existe un pic de production des mâles en fin de saison sèche (fin avril au Mexique), qui suit le pic de production des gynes d'environ 1,5 mois. Cela signifie que les œufs mâles sont majoritairement produits lorsque les larves femelles terminent leur développement (la durée de développement est d'environ 75 jours pour les mâles et 80 jours pour les gynes, dont 35 jours au stade nymphe [Fresneau, 1994]). Il est donc possible que certaines ouvrières, notamment celles qui pondent des œufs trophiques et possèdent des ovaires au moins partiellement actifs, participent à la ponte des mâles à ce moment-là, en détectant par exemple la présence de larves de gynes ou les facteurs abiotiques induisant leur développement (Hölldobler & Wilson, 1990). La durée de maturation probablement plus longue pour les gynes (Fresneau, 1994) implique que les mâles produits légèrement plus tard émergent au moment où la majorité des gynes sont réceptives, maximisant ainsi leurs opportunités de reproduction et donc augmentant l'*inclusive fitness* des ouvrières. Déterminer par génotypage la parenté des mâles dans les colonies récoltées sur le terrain, notamment à cette période de l'année, permettrait de vérifier simplement cette

hypothèse. Cela suggère donc que le monopole reproducteur de la reine ne serait pas absolu, et que les opportunités de reproduction des ouvrières pourraient être plus nombreuses que ce qui était pensé jusqu'à présent. Le faible différentiel de fécondité entre la reine et les ouvrières (Fresneau, 1994 ; Dietemann & Peeters, 2000), les coûts probablement faibles sur la productivité coloniale et les bénéfices indirects associés à la production des fils des ouvrières dans cette structure génétique monogame pourraient alors avoir favorisé l'existence de ces rares évènements de reproduction directe par les ouvrières en présence d'une reine fertile.

5. Mise en place des hiérarchies reproductives

Si la possibilité de reproduction occasionnelle des ouvrières en présence de la reine doit être envisagée, leur principale opportunité de reproduction survient lorsque la colonie se retrouve « irrémédiablement sans reine ». Une telle situation ne se produit qu'une seule fois chez *N. apicalis*, étant donné que l'unique reine n'est jamais remplacée quand celle-ci disparait (Peeters, 1993 ; Fresneau, 1994). En ce sens *N. verenae* peut faire figure d'exception, étant donné sa polygynie facultative qui peut diminuer les opportunités de reproduction directe de la part des ouvrières (voir Article 5). On peut noter que le début du conflit ouvert entre les ouvrières n'est pas prédit à la mort de la reine, mais dès lors que sa fécondité (ou son potentiel reproducteur) décline en deçà d'un seuil correspondant au potentiel reproducteur du collectif des ouvrières une fois la régulation du partage de la reproduction effectuée (Monnin & Ratnieks, 2001). Si l'on raisonne en termes d'*inclusive fitness*, il est clair que passé un certain seuil, la reine elle-même a intérêt à favoriser la reproduction des ouvrières au détriment de sa reproduction directe (Bourke, 1994). Ce changement de comportement des ouvrières passe ainsi très probablement par un suivi des signaux de fertilité émis par la reine, notamment les proportions de 13-MeC_{25}. Ce changement de signal semble être détecté via des contacts directs, et il est en effet fréquent d'observer que les ouvrières dont les ovaires sont déjà au moins partiellement actifs ont une proximité supérieure avec la reine chez plusieurs espèces dont *N. apicalis* (Fresneau, 1984 ; van Doorn & Heringa, 1986 ; Dietemann & Peeters, 2000 ; Yagound et al., 2012), ce qui peut leur permettre de surveiller en permanence ses productions d'HCC et ainsi détecter le moment le plus opportun pour débuter la mise en place de la hiérarchie reproductive. Nos observations montrent que

les ouvrières peuvent détecter et réagir à l'absence de la reine très rapidement, typiquement en moins de deux heures, et parfois en seulement 20 minutes. Ceci suggère que la seule présence de couvain produit par la reine (et potentiellement porteurs des mêmes HC [Monnin & Peeters, 1997 ; d'Ettorre et al., 2004 ; Endler et al., 2004 ; Smith et al., 2008 ; Bonckaert et al., 2012]) n'est pas suffisante pour observer une absence de reproduction des ouvrières, contrairement à ce qui a été montré chez *Camponotus floridanus*, espèce qui diffère grandement dans son organisation sociale, avec des colonies bien plus populeuses et polydomiques (Endler et al., 2004).

Une fois détecté le changement dans les signaux de fertilité de la reine débute alors la mise en place de la hiérarchie à proprement parler. Nous l'avons vu (Introduction, IV. 2), les facteurs intervenant dans l'établissement et le maintien des hiérarchies sociales sont extrêmement divers (Dugatkin & Earley, 2004 ; Hsu et al., 2006). Nous pouvons néanmoins en dresser le mécanisme probable chez *N. apicalis*, en insistant comme le montrent nos résultats sur l'importance du signalement du statut reproducteur et des mécanismes de reconnaissance associés. Les différences interindividuelles présentes au début du processus de formation de la hiérarchie ont vraisemblablement une importance capitale dans la détermination des rangs hiérarchiques, et parmi ces différences le niveau de développement ovarien est sans doute le facteur le plus important. Nos résultats suggèrent que les individus présentant des ovaires déjà partiellement actifs ont en effet un avantage très clair lors de la mise en place de la structure hiérarchique (Article 4). Ces individus correspondent probablement aux ouvrières pondant des œufs trophiques (et occasionnellement des œufs reproducteurs) en présence de la reine. Leur état physiologique est alors fortement influencé par leur âge car les individus trop âgés perdent tout potentiel reproducteur (Dietemann & Peeters, 2000). En plus des différences intrinsèques probables, notamment dans l'agressivité (Rutte et al., 2006), ces individus possèdent ainsi des niveaux d'hormones gonadotropes (e.g. hormone juvénile ; Robinson & Vargo, 1997 ; Hartfelder, 2000), ainsi que d'amines biogènes (e.g. octopamine et dopamine ; Kamhi & Traniello, 2013) associés à cette fertilité et qui influencent leur comportement de dominance. Enfin ils possèdent nous l'avons vu (Article 4) un profil chimique déjà caractéristique de leur statut reproducteur. Ces individus ont donc toutes les chances d'être dominants dès leurs premières interactions agonistiques, du fait de leur plus grande motivation et/ou aptitude (Hurd, 2006) influencée par leur état physiologique d'une part, et par le

signalement de leur statut reproducteur reconnu par les individus avec qui ils interagissent d'autre part. Cette reconnaissance de statut, même basée sur des différences très faibles d'HCC, parait en effet très probable étant donné les capacités fines de discrimination démontrées par ces ouvrières (Article 3). En outre, la nature hautement ritualisée des comportements agonistiques émis dans ces interactions de dominance/subordination (Oliveira & Hölldobler, 1990) indique assez clairement que l'issue de la rencontre ne se décide pas par l'aptitude des individus à dominer physiquement leur adversaire, mais bien par la perception d'indices de reconnaissance associés au statut reproducteur des individus. De plus l'individu dominé adopte la majorité du temps une posture de soumission avant même qu'il y ait contact direct entre les deux fourmis. Les quelques cas de lutte réelle entre deux individus doivent ainsi correspondre à des rencontres entre ouvrières présentant des niveaux très semblables de signaux de fertilité empêchant une discrimination non ambigüe de leur statut respectif. Ceci renforce donc l'importance du signalement du statut et des mécanismes de reconnaissance associés dans la détermination des relations de dominance/subordination entre les individus.

Commence alors une boucle auto-entretenue (Figure 2) dans laquelle les comportements de dominance émis et de subordination reçus vont modifier les niveaux d'amines biogènes cérébrales (Bloch et al., 2000b ; Cuvillier-Hot & Lenoir, 2006), l'activité des corps allates et possiblement d'autres structures productrices d'hormones gonadotropes (Tibbetts & Izzo, 2009 ; Smith AR et al., 2013). Ces paramètres vont alors influencer d'une part l'ovogenèse et donc renforcer le développement de la fertilité (Tibbetts & Izzo, 2009 ; Penick et al., 2014), et d'autre part la biosynthèse des HC au niveau des œnocytes associés notamment aux corps gras (Martins & Ramalho-Ortigão, 2012). Certains HC spécifiques, et particulièrement le 13-MeC_{25}, sont ensuite transportés dans l'hémolymphe via des lipophorines et acheminés à la fois sur la cuticule où ils renforcent le signal de fertilité, et dans les ovaires où ils sont incorporés dans les oocytes en croissance (Fan et al., 2002). La très forte association entre l'activité des ovaires et la biosynthèse des HCC semble ainsi expliquée par le fait que ces deux processus dépendent directement du même mécanisme biochimique (Cuvillier-Hot et al., 2004b ; Peeters & Liebig, 2009 ; Liebig, 2010). Les lipophorines peuvent de plus transporter les hormones gonadotropes (Okot-Kotber & Prestwich, 1991), ce qui peut modifier leur conformation et donc la nature des HC transportés vers la cuticule (Sevala

et al., 2000), et ainsi participer à la spécificité du lien entre HCC associés à la fertilité et activité ovarienne. L'interdépendance de la dominance, de la fertilité et des signaux chimiques contribue alors au renforcement du rang hiérarchique, et il est probable que par cette boucle auto-entretenue, les individus présentant une pré-fertilité lors de la mise en place de la hiérarchie atteignent les rangs hiérarchiques les plus élevés. A ces phénomènes peuvent se rajouter les processus d'auto-organisation où la dominance lors des interactions précédentes augmente la probabilité de dominer dans les interactions futures (Dugatkin & Earley, 2004 ; Hsu et al., 2006), là encore en modifiant sans doute l'état physiologique et plus particulièrement les niveaux d'amines biogènes. Ce mécanisme potentiel est bien entendu ensuite modulé pour chaque individu en fonction de ses caractéristiques intrinsèques, de l'histoire de ses interactions agonistiques et sans doute d'une part de hasard, pour aboutir à des niveaux variables d'activation ovarienne et de signal de fertilité reflétant le statut hiérarchique de chaque ouvrière. Une fois les rangs définis, la structure hiérarchique linéaire émergente (Article 3) peut ensuite être stabilisée selon les mêmes processus de discrimination de statut basés sur les signaux de fertilité. La diminution très nette des actes agonistiques caractéristique de cette seconde phase coïncide alors avec le début de la ponte des individus de haut rang (Oliveira & Hölldobler, 1990 ; Blacher et al., 2010). D'une manière plus générale, ces résultats tendent à remettre en cause l'idée selon laquelle la production des signaux de fertilité commence après la détermination des rangs hiérarchiques et le début de l'ovogenèse (Peeters et al., 1999 ; Liebig et al., 2000 ; Cuvillier-Hot et al., 2004b ; Hartmann et al., 2005 ; Monnin, 2006 ; Peeters & Liebig, 2009), puisqu'ils suggèrent qu'au contraire ces signaux sont présents dès le départ et jouent un rôle décisif dans la détermination des rangs hiérarchiques.

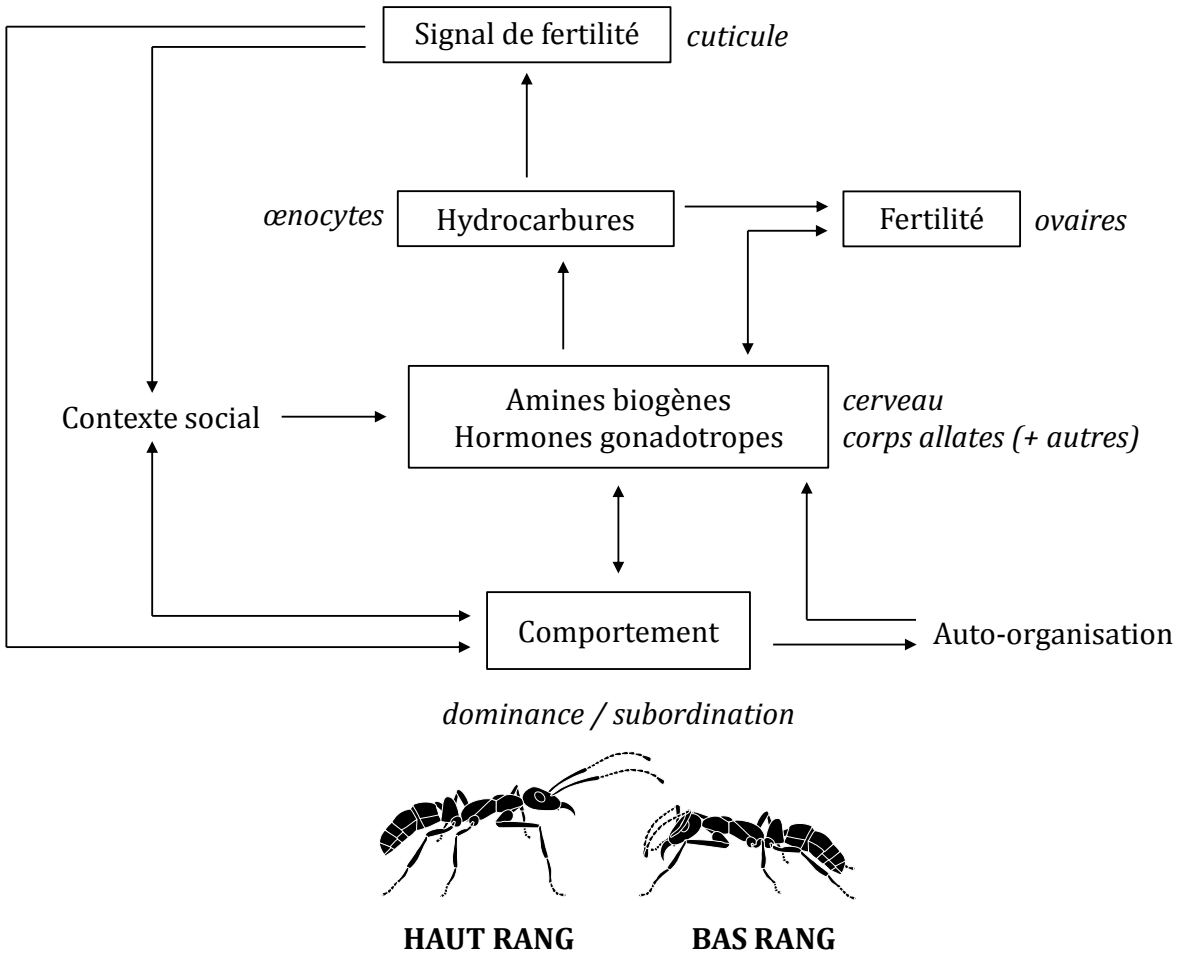

Figure 2. Mécanismes proximaux intervenant dans la détermination des rangs hiérarchiques et leurs influences mutuelles.

En résumé, on voit donc le rôle capital des signaux de fertilité dans la détermination des relations de dominance/subordination entre les individus, donc dans l'émergence puis la stabilisation de la hiérarchie reproductive. Oliveira & Hölldobler (1990) ont reporté dans une colonie l'existence d'une hiérarchie en présence de la reine. Nos résultats et l'hypothèse qui en découle suggèrent qu'au contraire la hiérarchie n'est formée qu'à la sénescence de la reine. En effet, cette sénescence étant un évènement imprévisible, la hiérarchie ainsi formée devrait être maintenue pendant de très longues périodes, et serait remise en cause perpétuellement à l'émergence des nouvelles ouvrières (Monnin & Peeters, 1999 ; Cuvillier-Hot et al., 2001). Il est donc probable que dans ces conditions les bénéfices associés à la formation de la hiérarchie ne compensent pas ses coûts (Monnin & Ratnieks, 2001), et contraignent ainsi l'expression du conflit à la perception

du déclin du signal de fertilité émis par la reine. De plus, la rapidité de mise en place de la hiérarchie reproductive ne rend pas *a priori* nécessaire son expression précoce.

Si ce mécanisme de discrimination de statut est si efficace et présent dès la mise en place de la hiérarchie, on peut se demander pourquoi les actes agonistiques, nécessairement coûteux malgré leur ritualisation (Gobin et al., 2003), sont maintenus dans les colonies. Une hypothèse est que ces agressions sont nécessaires pour un développement rapide de la fertilité (Lamba et al., 2007 ; Shukla et al., 2014), probablement via leurs effets sur les amines biogènes et les hormones gonadotropes comme expliqué précédemment. Ce système pourrait avoir été sélectionné du fait des pressions importantes liées à la courte fenêtre temporelle disponible pour la reproduction des ouvrières une fois la colonie irrémédiablement sans reine (Fresneau, 1994 ; Dietemann & Peeters, 2000 ; voir Article 4). Ces contraintes sont aussi susceptibles d'avoir favorisé la pré-fertilité des ouvrières en présence de la reine, permettant d'après le mécanisme proposé une régulation rapide du conflit lié à la production des mâles, et ainsi une augmentation de l'*inclusive fitness* pour tous les membres de la colonie avant que celle-ci ne périclite totalement.

Pour finir sur ce point, on notera que l'hypothèse présentée ci-dessus fait la synthèse d'études réalisées sur des espèces différentes. Aussi, bien que les mécanismes présentés soient sans doute communs à ces différents taxons, il reste nécessaire de les démontrer dans leur ensemble chez *N. apicalis*. La caractérisation des processus biochimiques gouvernant les comportements de dominance, la biosynthèse des HCC et l'activité des ovaires est donc une perspective clé pour les études futures. L'identification des patterns d'expression génique sous-jacents semble de ce point de vue une piste de recherche intéressante pour lever le voile sur cet aspect essentiel de l'organisation des sociétés d'insectes (Monnin, 2006).

6. Potentiel reproducteur des ouvrières

Le système décrit chez *N. apicalis* montre l'importance du potentiel reproducteur des ouvrières, supposé représenter un état ancestral dans l'évolution des sociétés d'insectes, et on peut alors s'interroger sur les mécanismes responsables de la diminution généralement observée de ce potentiel reproducteur des ouvrières chez les espèces hautement eusociales (Ratnieks, 1988 ; Bourke, 1999, 2011 ; Wenseleers et al., 2004). La

comparaison des modalités d'établissement des hiérarchies reproductives entre espèces différentes peut apporter un éclairage intéressant à ces questions. Nous montrons ainsi que la dynamique de formation des hiérarchies est différente chez *N. verenae* par rapport aux autres espèces du complexe (Article 5). En effet, il apparait qu'à durée égale d'isolement de la reine, le développement ovarien moyen des individus est clairement réduit par rapport aux autres espèces, ceci étant probablement lié à la très faible intensité d'interactions agonistiques observées dans ces colonies. L'apparentement est plus faible dans les colonies de *N. verenae* de par leur polygynie facultative et leur plus haut niveau de paternité effective (Evison et al., 2012 ; Article 2). La probabilité pour une ouvrière d'accéder à la reproduction directe (autrement dit de se retrouver dans une situation irrémédiablement sans reine) est ainsi théoriquement réduite chez cette espèce (Bourke, 1988b ; Bourke & Franks, 1995). Les pressions liées au conflit pour la production des mâles sont ainsi réduites et on peut émettre l'hypothèse que ces facteurs ont entrainé une diminution du potentiel reproducteur des ouvrières chez *N. verenae* par rapport aux espèces *N. apicalis* et *N. cooki*. En effet, la reproduction des ouvrières est susceptible d'entrainer des coûts sur la productivité coloniale du fait du compromis opposant la reproduction au travail ergonomique (Wenseleers et al., 2004 ; voir Introduction III. 2). Les bénéfices plus faibles associés à la reproduction des ouvrières sont ainsi moins susceptibles de compenser les coûts qui y sont associés. La résultante serait ainsi une dynamique plus lente de formation des hiérarchies reproductives chez cette espèce, qui sont néanmoins maintenues (Oliveira & Hölldobler, 1991) du fait des gains en *fitness* directe (même réduits) qu'elles procurent. Cette dynamique d'établissement des hiérarchies contraste donc fortement avec celle observée chez *N. apicalis* où les bénéfices plus forts liés à la production des mâles ont pu sélectionner une mise en place plus rapide de la hiérarchie face aux contraintes exercées par la courte fenêtre temporelle disponible pour la reproduction directe des ouvrières (Dietemann & Peeters, 2000 ; Article 4).

Cette hypothèse ouvre alors des perspectives très intéressantes pour des études comparatives entre *N. verenae* et *N. apicalis* afin appréhender les questions liées à l'évolution sociale, et particulièrement aux mécanismes proximaux intervenant dans la régulation du partage de la reproduction. Elle suggère en effet que *N. verenae*, bien que très proche phylogénétiquement et présentant des traits d'histoire de vie similaires (Wild, 2005 ; Delabie et al., 2008), pourrait représenter un état légèrement dérivé dans

son organisation sociale par rapport à *N. apicalis*. Ceci est en effet corroboré par sa structure génétique facultativement polygyne et polyandre (Evison et al., 2012 ; Article 2), sa structure coloniale facultativement polydomique (N. Châline, communication personnelle), sa possible reproduction par fission (Delabie et al., 2008), et donc son potentiel reproducteur des ouvrières réduit, qui sont tous supposés être des traits dérivés dans l'évolution sociale (Bourke, 1999, 2011 ; Hughes et al., 2008a ; Cronin et al., 2013). Le complexe *N. apicalis* parait ainsi être un modèle de choix pour comprendre les déterminants comportementaux, écologiques et génétiques responsables de la divergence entre les individus reproducteurs et stériles au cours de l'évolution de la socialité (Maynard Smith & Szathmáry, 1995 ; Queller & Strassmann, 1998 ; Bourke, 2011).

III. Evolution des indices de reconnaissance

1. Signalement de la fertilité

Les études comparatives revêtent une importance capitale dans notre compréhension des processus évolutifs, et une partie de cette thèse s'est ainsi focalisée sur l'évolution des indices de reconnaissance au sein du complexe *N. apicalis*. Nous avons dans le chapitre IV (Article 5) comparé les HCC associés à la fertilité chez plusieurs espèces du complexe. Les résultats montrent que les signaux putatifs de fertilité sont fortement conservés chez ces espèces proches, malgré leur nature chimique diverse (les composés en question pouvant être linéaires, insaturés, ou présentant des groupements méthyl). Ces résultats confirment les études réalisées sur d'autres groupes d'espèces (Brunner et al., 2011 ; Holman et al., 2013a ; van Zweden et al., 2014), et ils soutiennent l'hypothèse du signalement honnête de la fertilité (voir Introduction V. 2) qui prédit une vitesse lente d'évolution et en conséquence un important degré de conservation de ces signaux entre espèces proches (Keller & Nonacs, 1993 ; Heinze & d'Ettorre, 2009 ; Kocher & Grozinger, 2011).

Cette hypothèse s'accorde avec les résultats précédemment exposés au chapitre III qui montrent que la prise de décision reproductive des individus semble dépendre principalement de leurs intérêts en termes d'*inclusive fitness*, une caractéristique rendue possible par la présence d'indices de reconnaissance fiables signalant la fertilité des

individus, et qui est probablement très générale au sein des insectes sociaux (Monnin, 2006 ; Le Conte & Hefetz, 2008 ; Peeters & Liebig, 2009 ; Liebig, 2010). Le ou les HCC associés à la fertilité semblent donc jouer le rôle de signaux, qui dans la communication animale sont classiquement considérés comme honnêtes, c'est-à-dire véhiculant, la plupart du temps, une information fiable et bénéfique pour le receveur (Maynard Smith & Harper, 1995 ; Searcy & Nowicki, 2005). La problématique encore non totalement résolue est de comprendre quels peuvent être les mécanismes qui permettent de maintenir l'honnêteté du signal, autrement dit l'association entre l'intensité du signal (ici la proportion d'un ou de plusieurs HCC dans le profil chimique) et le trait (le niveau de fertilité) qu'il reflète (Rendall et al., 2009 ; Ruxton & Schaefer, 2011 ; Számadó, 2011). La théorie la plus largement invoquée pour expliquer le maintien de l'honnêteté est le principe du handicap (Zahavi, 1975). Cette théorie propose que la balance coût/bénéfice d'un signal est plus faible pour les individus émettant des signaux plus forts, parce que ces individus sont soit les plus à même d'assumer les coûts plus importants associés à un signal fort, soit sont ceux qui bénéficient le plus de la réponse du receveur (Maynard Smith & Harper, 1995). L'idée est donc que les signaux pour être honnêtes doivent être associés à des coûts supérieurs au coût minimum nécessaire pour produire et émettre le signal (Zahavi, 1975 ; Grafen, 1990 ; Guilford & Dawkins, 1995).

Nous avons montré que les individus hautement fertiles, moyennement fertiles et non-fertiles divergent dans leurs quantités relatives d'HCC, mais la quantité absolue d'HCC dans l'ensemble du profil est la même (Articles 3 et 4). Il parait vraisemblable que les coûts de production de ces HCC soient donc similaires entre individus fertiles et non-fertiles (Blomquist, 2010 ; Wyatt, 2014), et qu'ils soient ainsi peu à même d'imposer de fortes pressions sur l'honnêteté du système de signalisation. L'existence de coûts liés à la détection des tricheurs (hypothèse du contrôle social ; Rowher, 1975 ; Számadó, 2011), semble en revanche corroborée chez plusieurs espèces qui montrent que les individus dont le signal est expérimentalement manipulé sont agressés par les autres membres du groupe (Tibbetts & Dale, 2004 ; Tibbetts & Izzo, 2010 ; Smith AA et al., 2009 ; Smith et al., 2012). Il convient néanmoins ici de faire une distinction dans le sens du mot tricheur employé dans ces études. Selon Tibbetts et Dale (2004) et Tibbetts et Izzo (2010), les tricheurs chez la guêpe *Polistes dominula* correspondent à des individus montrant une incongruence entre un signal (reflétant le RHP) et un trait associé (la dominance). Les individus dont le signal est manipulé, de sorte qu'il ne reflète pas leur statut réel, sont la

cible d'agressions régulant les relations de dominance/subordination entre les agresseurs et les agressés. Selon Smith A. A. et al. (2009, 2012), les tricheurs chez les fourmis *Aphaenogaster cockerelli* et *Odontomachus brunneus* correspondent à des individus affichant un phénotype reproducteur (via des HCC, respectivement le pentacosane et le *Z*9-nonacosadiene, associés au statut reproducteur) en présence d'un reproducteur déjà établi, autrement dit des individus à même d'exploiter les ressources du groupe pour leur propre profit (Bourke & Franks, 1995 ; Ratnieks et al., 2006 ; Bourke, 2011). Les individus dont le signal est manipulé sont alors la cible d'agressions régulant le partage de la reproduction. Ce qui est perçu ici n'est donc pas la dissociation entre le niveau du signal et le trait associé, mais la présence d'un comportement reproducteur égoïste dans un contexte social où la restriction reproductive est favorisée. Cette distinction est importante pour envisager les mécanismes à l'œuvre dans le maintien de l'honnêteté du signal, puisque cela suggère que la pression sociale n'exerce pas une influence majeure sur l'association entre certains HCC et l'activité ovarienne, mais qu'elle influence par contre très fortement le contexte dans lequel les individus vont afficher un phénotype reproducteur. Il apparait donc que les coûts associés à la production et/ou au maintien du signal de fertilité ne sont probablement pas les mécanismes principaux garantissant son honnêteté. En outre, si les actes agonistiques très nombreux effectués par les individus de haut rang sont très probablement associés à des coûts, rien ne dit que le coût lié à l'évitement des individus dominants pour les individus de plus bas rang est moindre (Senar et al., 2000). Cette conclusion vient en parallèle des études récentes minimisant l'emphase de la théorie du handicap comme mécanisme général expliquant l'honnêteté dans la communication animale (Getty, 2006 ; Számadó, 2011).

En revanche, le déterminisme commun de l'activité des ovaires et de la biosynthèse des HCC, sous l'égide probable des hormones gonadotropes et des amines biogènes est un mécanisme très général permettant d'expliquer l'honnêteté du signal de fertilité (Keller & Nonacs, 1993 ; Cuvillier-Hot et al., 2004b ; Heinze & d'Ettorre, 2009; Peeters & Liebig, 2009 ; Smith AA et al., 2009 ; Liebig, 2010 ; Article 4). En effet, ce mécanisme entraine une contrainte physiologique très forte sur l'association entre la production des HCC et l'ovogenèse, et il a de plus a été démontré qu'il pouvait exister une contrainte génétique dans cette association (Holman et al., 2013b ; Hoffmann et al., 2014). De tels signaux « infalsifiables » sont classiquement nommés des index (Maynard Smith & Harper,

1995). Ces contraintes garantissent ainsi l'honnêteté du système de signalisation sans la nécessité de coûts liés à la production ou au maintien du signal, mais cela ne signifie pas pour autant que les coûts n'interviennent pas. En effet, si estimer la contribution relative des coûts et contraintes dans le maintien de l'honnêteté des signaux est particulièrement difficile, ils ne doivent pas pour autant être considérés comme deux processus opposés (Holman, 2012).

2. Evolution des hydrocarbures cuticulaires

De manière plus générale, nos résultats ouvrent des perspectives intéressantes liées à l'évolution des indices de reconnaissance au sein du complexe *N. apicalis*, à la fois dans les processus de reconnaissance du partenaire et de reconnaissance coloniale. Deux modes d'évolution des HCC peuvent survenir (Symonds & Elgar, 2008 ; van Wilgenburg et al., 2011b). En premier lieu, un mode d'évolution « progressive » suppose que de légères variations dans le profil d'HCC sont accumulées au cours du temps en même temps que les espèces divergent les unes des autres, ce qui résulte en une plus grande similitude des HCC entre espèces proches (Roelofs et al., 1982). Alternativement selon un mode d'évolution « brusque », des changements plus radicaux interviennent lors des processus de spéciation, résultant dans une divergence plus importante des HCC entre espèces proches (Baker, 2002). Par conséquent, il est prédit une forte correspondance entre la distance phylogénétique et la similarité chimique selon le mode d'évolution progressive, alors que la correspondance devrait être beaucoup moins forte dans l'hypothèse alternative (Baker, 2002 ; Symonds & Elgar, 2004, 2008).

Nos résultats montrent que la similarité chimique entre les espèces du complexe ne reflète pas leurs relations phylogénétiques (Article 1), ce qui suggère un mode d'évolution brusque des HCC. Ce mode d'évolution est notamment prédit dans les cas où il y a un bénéfice associé au fait de posséder des indices de reconnaissance uniques, comme par exemple lorsque des espèces fortement apparentées vivent en sympatrie (Symonds & Elgar, 2004, 2008). On peut donc supposer que la forte proximité phylogénétique des espèces du complexe *N. apicalis* et leur haut niveau de sympatrie ont favorisé la différentiation claire de leurs HCC, d'autant plus du fait des pressions de sélection stabilisatrices affectant leur morphologie. Ces molécules sont en effet les

principaux indices de reconnaissance utilisés dans les processus de communication intra et intercoloniale (Howard & Blomquist, 2005). La forte dissimilarité entre les profils de ces espèces proches et possédant des traits d'histoire de vie très similaires (Wild, 2005 ; Delabie et al., 2008) est donc sans doute un facteur clé dans leur spéciation et leur maintien en sympatrie. On peut noter de façon intéressante que s'il y a une forte dissemblance dans le profil total d'HCC entre ces espèces, les HCC associés à la fertilité sont eux fortement conservés (Article 5). Cette distinction peut indiquer des différences de vitesse d'évolution dans les HCC en lien avec leur fonction. Il a en effet été montré que les HCC pouvaient avoir différentes héritabilités (van Zweden et al., 2010) et on peut supposer que les HCC associés à la fertilité soient davantage conservés à cause des contraintes liées à leur mécanisme de production associé à l'ovogenèse (Fan et al., 2002), comme nous l'avons vu précédemment. Ainsi des populations différentes chez *Odontomachus brunneus* possèdent des chémotypes divergents et des différences importantes d'HCC liés à la reconnaissance coloniale, alors que les HCC intervenant dans le signalement de la fertilité sont conservés entre ces populations (Smith AA et al., 2013). Plus généralement, nos résultats confirment ainsi que le profil d'HCC peut servir à véhiculer simultanément plusieurs types d'informations dans des contextes différents (e.g. reconnaissance coloniale et reconnaissance de la fertilité) (Denis et al., 2006 ; Le Conte & Hefetz, 2008).

Il convient néanmoins de garder à l'esprit que ces résultats sont directement dépendants de la phylogénie de ce complexe d'espèces, et qu'ils sont ainsi susceptibles de changer partiellement en même temps que les relations phylogénétiques entre certaines de ces espèces sont plus clairement définies.

IV. Relations intra et interspécifiques

Enfin, le dernier aspect étudié dans cette thèse concerne les mécanismes permettant de maintenir l'intégrité du groupe en limitant l'exploitation de ses ressources par des compétiteurs ou des parasites. Dans le chapitre 5 (Article 6), nous nous sommes ainsi intéressés aux processus sous-jacents à la reconnaissance coloniale et à la discrimination des voisins et étrangers au sein du complexe *N. apicalis*. Nos résultats montrent que les trois espèces sympatriques étudiées (*N. apicalis* morphes 4 et 7 et *N.*

verenae morphe 1) présentent toutes une reconnaissance coloniale claire très probablement basée sur la perception du profil d'HCC. De plus, *N. apicalis* morphe 7 montre une discrimination des individus hétérocoloniaux voisins et étrangers compatible avec un effet « cher ennemi » (Fisher, 1954 ; Heinze et al., 1996 ; Pirk et al., 2001 ; Tanner & Keller, 2012), et probablement dépendant de processus d'apprentissage des visas hétérocoloniaux voisins (Langen et al., 2000 ; Knaden & Wehner, 2003 ; Sanada-Morimura et al., 2003 ; Thomas et al., 2005 ; Dimarco et al., 2010). En revanche, ni *N. apicalis* morphe 4 ni *N. verenae* morphe 1 ne montrent de discrimination claire des individus étrangers en fonction de la distance spatiale séparant leurs colonies d'origine. Nos observations ont de plus montré des préférences de sites de nidification différentes entre ces espèces, qui entrainent des différences dans les fréquences de déménagement (Fresneau, 1994 ; Pezon et al., 2005) et peuvent conséquemment moduler la compétition pour les sites de nidification (Delabie et al., 1997), et donc la réponse territoriale vis-à-vis des colonies étrangères.

Cette étude clarifie donc les mécanismes proximaux responsables de la variation interspécifique très importante dans la réponse agressive face aux voisins et aux étrangers (Gordon, 1989 ; Dahbi et al., 1996 ; Heinze et al., 1996 ; Langen et al., 2000 ; Sanada-Morimura et al., 2003 ; Boulay et al., 2007 ; Newey et al., 2010 ; Tanner & Keller, 2012). Elle montre en effet que des espèces proches phylogénétiquement, ayant des traits d'histoire de vie très similaires et vivant dans le même habitat en sympatrie peuvent néanmoins varier, d'un point de vue fonctionnel, dans leur réponse comportementale vis-à-vis des individus hétérocoloniaux originaires de colonies proches ou éloignées. Nos résultats permettent d'envisager un mécanisme simple permettant d'expliquer cette variation interspécifique et basé sur des différences de sites de nidification affectant la durée de cohabitation des colonies voisines et modulant au niveau proximal les possibilités de mise en place de processus d'apprentissage des visas hétérocoloniaux, et au niveau ultime la menace relative que ces colonies voisines représentent par rapport aux colonies éloignées (Temeles, 1994).

La perspective la plus claire émanant de cette étude consiste à tester directement l'hypothèse proposée. Il parait ainsi particulièrement intéressant d'examiner les modalités de mise en place de cet apprentissage des visas hétérocoloniaux. L'approvisionnement en solitaire avec régionalisation des aires de recherche (Fresneau,

1985) suggère que chaque fourrageuse va avoir tendance à rencontrer les mêmes individus lors de ses sorties à l'extérieur. Ceci peut donc favoriser l'apprentissage de visas hétérocoloniaux particuliers lors de la répétition des rencontres. La dynamique de formation et la durée de rétention de cet apprentissage pourraient ainsi être testées en observant la réponse comportementale des fourrageuses face à des individus hétérocoloniaux après un nombre variable de rencontres. Il serait aussi intéressant d'observer si les autres membres de la colonie peuvent moduler leur réponse vis-à-vis des voisins sans avoir eu de contacts directs avec eux. Cette hypothèse parait *a priori* peu vraisemblable car elle supposerait une rétention des indices de reconnaissance des individus hétérocoloniaux sur les fourrageuses « expérimentées », mais des résultats allant dans ce sens ont déjà été reportés (Gill et al., 2012). Le deuxième aspect à tester est l'effet des fréquences de déménagement sur les occurrences de rencontres entre colonies voisines, et la mise en place de réponses particulières entre ces colonies. On peut alors se demander si ces paramètres peuvent contribuer à structurer les populations dans l'espace, aussi bien au niveau intraspécifique qu'interspécifique. En effet, ces espèces vivant en sympatrie, la compétition pour les sites de nidification se joue également au niveau interspécifique (Delabie et al., 1997). Les communautés de fourmis sont typiquement structurées par des hiérarchies où certaines espèces sont dominantes par rapport à d'autres (Savolainen & Vepsäläinen, 1988 ; Blüthgen & Fiedler 2004), et il est possible que les préférences de sites de nidification soient le reflet de telles relations de dominance. Il faut également envisager la possibilité d'interactions privilégiées entre colonies d'espèces différentes (Delsinne et al., 2007 ; Tanner & Adler, 2009). L'étude des relations territoriales devient alors particulièrement intéressante pour appréhender la coexistence de ces espèces en sympatrie (Parr & Gibb, 2010), ainsi que les pressions intervenant dans les processus de divergence entre ces espèces, notamment dans les indices de reconnaissance à la base de ces interactions.

V. Conclusion

L'évolution de la socialité représente l'une des transitions majeures de l'évolution. Elle entraine des niveaux de coopération sans précédent, mais introduit en même temps de nouveaux conflits résultant de son plus haut niveau d'organisation. Les travaux réalisés au cours de cette thèse soulignent l'importance des mécanismes de reconnaissance dans le maintien de la coopération dans les groupes sociaux face à leur exploitation par des éléments à la fois externes et internes au groupe. Nous montrons que des mécanismes simples peuvent néanmoins être la base de prises de décision plastiques résultant en des réponses adaptatives dans des contextes aussi variés que la régulation intracoloniale du partage de la reproduction ou la compétition intercoloniale pour les sites de nidification. Ces prises de décisions résultent la plupart du temps d'indices de reconnaissance dont la fiabilité dépend d'une balance entre coûts, bénéfices et contraintes.

Cette étude révèle par ailleurs l'intérêt d'une approche comparative et intégrative pour appréhender ces processus vitaux dans le maintien de l'organisation sociale. Elle réaffirme de plus la pertinence de la théorie de l'*inclusive fitness* comme grille de lecture de l'évolution de la socialité. Les traits d'histoire de vie, la position phylogénétique et les connaissances accumulées sur le complexe d'espèces *Neoponera apicalis* en font un modèle pertinent pour étudier le fonctionnement et l'évolution des groupes sociaux. Si l'étude des causes proximales a été privilégiée dans cette thèse, on s'est cependant également attaché, dans une tradition éthologique, à envisager les différentes questions sous les angles complémentaires de l'ontogenèse, de la fonction adaptative et de la phylogenèse, afin d'obtenir une description plus complète des phénomènes étudiés. Les nombreuses perspectives émanant de ce travail ouvrent la voie à des recherches prometteuses permettant d'affiner encore davantage notre compréhension de l'évolution sociale.

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Résumé :
La coopération et les conflits sont les deux facettes de l'évolution sociale. L'objectif principal de cette thèse a été d'étudier chez les fourmis du complexe d'espèces *Neoponera apicalis* les mécanismes de régulation permettant de maintenir la coopération dans les groupes sociaux face aux risques d'exploitation internes et externes au groupe, à travers une approche comparative et intégrative. Nous montrons que la structure génétique des colonies entraine des conflits d'intérêts liés à la reproduction, notamment en ce qui concerne la production des mâles. L'étude de la régulation du partage de la reproduction révèle que les décisions reproductives des individus sont principalement basées sur la détection de signaux associés à la fertilité grâce à des capacités fines de discrimination de statut. Ces informations permettent aux ouvrières d'ajuster leur comportement reproducteur selon le contexte social et en fonction de leurs intérêts en termes d'*inclusive fitness*. En effet, alors qu'une auto-restriction reproductive des ouvrières est observée en présence d'une reine fertile, un conflit ouvert se déclare quand celle-ci disparait, régulé par la mise en place d'une hiérarchie reproductive linéaire dans laquelle les ouvrières de haut rang accèdent à la reproduction. Le signalement du statut reproducteur parait jouer un rôle capital dans la régulation des interactions de dominance/subordination, et donc dans la détermination des rangs hiérarchiques. Les signaux associés à la fertilité sont par ailleurs fortement conservés entre les différentes espèces de ce complexe, ce qui souligne leur honnêteté et donc leur stabilité évolutive. Nous montrons enfin l'existence d'une reconnaissance coloniale chez ces espèces, basée sur les mêmes indices de reconnaissance, et permettant de moduler la réponse territoriale selon le niveau de familiarité des colonies étrangères. Cette étude démontre donc l'importance des mécanismes de reconnaissance dans la régulation de la vie sociale.

Conflicts, cooperation and recognition systems in ants of the *Neoponera apicalis* species complex

Abstract:
Social evolution implies both cooperation and conflicts. The main objective of this thesis was to study the regulatory mechanisms allowing to maintain cooperation in social groups against exploitation from within and outside. We choose a comparative and integrative approach using ants of the *Neoponera apicalis* species complex. We show that the colony genetic structure gives rise to reproductive conflicts, particularly over male production. The study of the regulation of the partitioning of reproduction reveals that the individuals' reproductive decisions are mainly based on the detection of fertility-associated signals through fine-scale status discrimination abilities. This information allows the workers to adjust their reproductive behaviour according to the social context and following their inclusive fitness interests. Whereas worker reproductive self-restraint is observed with a fertile queen, an overt conflict arises in queenless conditions, which is regulated through the formation of a linear reproductive hierarchy where high-ranking workers reproduce. Reproductive status signalling seems to play a crucial role in the regulation of the dominance/subordination relationships, and thus in the determination of hierarchical ranks. Furthermore, fertility-associated signals are highly conserved among the species of the complex, which highlights their honesty and thus their evolutionary stability. We finally show that the nestmate recognition processes in these species are based on the same recognition cues and allow to modulate the territorial response depending on the familiarity with non-nestmates. This study demonstrates the importance of recognition mechanisms in the regulation of social life.

Discipline : Ethologie

Mots clés : *Neoponera* (*Pachycondyla*) *apicalis*, conflit reproducteur, insectes sociaux, reconnaissance, hiérarchie reproductive, dominance, signal honnête, espèces cryptiques

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