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Pre-existing differences in putative fertility signals give workers the upper hand in ant reproductive hierarchies

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In social groups, competition often gives rise to conflicts, which are regulated through a variety of mechanisms. In several social insect species, the conflict for male production that takes place between workers after queen loss, is regulated through the establishment of a reproductive hierarchy. A recent study of Neoponera apicalis showed that workers differ in their fertility levels in the presence of the queen and proposed that such idiosyncratic differences might influence access to the top of the hierarchy after queen loss. In this study, we therefore sought to characterize the influence of the initial heterogeneity in ovarian development and its chemical
and behavioural correlates on the establishment of reproductive hierarchies among orphaned workers, which can only produce males. We monitored the chemical profile before and after hierarchy establishment in four groups of orphaned workers of *N. apicalis* morph 6. The analysis of the cuticular profiles showed that tricosane (n-C_{23}) was highly correlated with ovarian development and could consequently act as a fertility signal in this ant. The relative amount of tricosane on the cuticle, both before and after the establishment of the hierarchy, was also correlated with the rank achieved within the hierarchy and with the expression of agonistic behaviours. Thus, our study experimentally shows that idiosyncratic differences in a putative fertility signal (and therefore presumably in ovarian activity) between workers in the queen’s presence reliably predict the outcome of reproductive conflict after queen loss. We propose that this signal (together with an increased agonistic motivation of the more fertile workers) could play a major role in the regulation of dominance/submission behaviours, enabling the most fertile individuals to rapidly access top ranks and monopolize reproduction, thereby maximizing the global reproductive success of all colony workers while minimizing the costs associated with the expression of agonistic behaviour.

**Key-words:** dominance behaviour, fertility signalling, idiosyncratic difference, ponerine ants, reproductive hierarchy.

Reproductive hierarchies often appear in social hymenopteran species when the queen of the colony disappears or her reproductive potential decreases. In most species, workers, although they cannot mate, maintain an ability to develop their ovaries and lay unfertilized male-destined eggs (Yagound, 2014). In these species, the establishment of reproductive hierarchies through ritualized agonistic interactions regulates the overt conflict for male production (Oliveira &
Hölldobler, 1990; Heinze et al., 1994). An individual’s rank stems from several factors that are classically described as ‘intrinsic’ and ‘extrinsic’ and which are intertwined in a network of feedback loops. Intrinsic traits refer to the state of each individual (e.g. neuroendocrine titres, reproductive status and motivation to fight, as well as potential chemical cuticular correlates) that determine its absolute fighting ability (so-called resource-holding power, or RHP, after Parker, 1974). Extrinsic factors that play a role in establishing hierarchies include the effects of past experiences (Rutte et al., 2006) and social environment whose causal role is exerted through the modification of intrinsic factors, which in turn modify future experiences. These influences are notably reflected in winner–loser effects where the outcome of an encounter (victory or defeat) induces changes in the neuroendocrine titres (Hsu et al., 2006), thus influencing individual behaviour and the outcome of future encounters (Dugatkin & Earley, 2004; Sasaki et al., 2016).

In ants, cuticular hydrocarbons (CHCs) are well known for indicating colonial affiliation, but they also convey more subtle social information about species, sex, caste, hierarchical status and reproductive status (Greene & Gordon, 2003; Liebig, 2010), thus constituting unique individual chemical profiles. This chemical signal results from quantitative or qualitative differences (or both) of endogenous and exogenous origins between one or more compounds across individuals, castes and colonies (d’Ettorre & Lenoir, 2010). The signal can therefore allow the recognition of a congener’s idiosyncratic characteristics, and many studies have demonstrated the involvement of CHCs in fertility and/or dominance signalling (Smith et al., 2009; Holman et al., 2013; Holman et al., 2016; Smith et al., 2015; Abril et al., 2018). The perception of the signal modifies the behaviour of potential partners (reviewed in Leonhardt et al., 2016). When it reflects RHP and/or fertility, the signal is thought to be honest, and workers are accordingly expected to follow their own interests in response to the signal and promote their inclusive fitness (Keller & Nonacs, 1993; Heinze & d’Ettorre, 2009). Thus, in the case of
reproductive hierarchies in a queenless colony, the most fertile worker should be selected to access the top of the hierarchy and produce males. Selection of the reproductive individuals stems from a fine balance between direct fitness costs, indirect fitness benefits and relatedness (Keller & Nonacs, 1993). Yagound et al. (2014) have shown that workers of a Neotropical ant species, *Neoponera apicalis*, can use CHCs as an index of rank in workers’ established reproductive hierarchies, the quantity of certain compounds functioning as a reliable signal of both individual ovarian development and social status.

Here, we studied the establishment of reproductive hierarchies after queen loss in workers of *N. apicalis*. In this species, workers cannot mate and therefore are unable to produce female progeny (Fresneau, 1994). While queenright workers do not usually lay eggs, they start producing males soon after being orphaned (Dietemann & Peeters, 2000). Because there is no production of new workers, which would care for the brood or adopt a new queen, males must be produced rapidly after the queen’s death or no nurses will be available. Behavioural mechanisms exploiting interindividual differences in queen presence for both reproductive physiology and chemical signalling (Yagound et al., 2015) would allow the rapid establishment of a reproductive hierarchy after queen loss and hence meet the evolutionary pressure to rapidly solve the conflict between workers over male production (Dietemann & Peeters, 2000). Namely, we propose that the most fertile workers could be more motivated to enter the reproductive race, and that fertility signalling could help resolve the ritualized agonistic encounters. Such behavioural processes would ensure the most fertile workers lead the hierarchy, thereby maximizing the inclusive fitness of the whole worker collective (Hamilton, 1964; Keller & Nonacs, 1993). To test our hypothesis, we first correlated variation in cuticular profiles and ovarian development among workers to determine which compound might be the putative fertility signal in *N. apicalis* (Liebig et al., 2000; Monnin et al., 1998; Yagound et al., 2015). We then jointly monitored the development of this compound (as a noninvasive proxy...
for ovarian development) and of ritualized agonistic behaviours by workers, from queen loss to
the stabilization of the reproductive hierarchy. We predicted that the workers most fertile in the
presence of the queen would be more active during the establishment of the hierarchy and
therefore would access the top ranks and monopolize reproduction.

**Methods**

**Ethical note**

*Neoponera apicalis* is a common ant species in central-south American tropical forests. We
obtained collection permits (No 47615) from the Chico Mendes Institute for Conservation and
Biodiversity (ICMBio/SISBIO) from the Brazilian Ministry of the Environment (MMA). Our
experimental design in the laboratory included the orphaning of four experimental groups of
workers, the labelling and behavioural observation of individual ant workers, the monitoring of
the cuticular compounds and the dissection of workers to record ovary development. Ants were
kept in artificial nests which are commonly used in ant research and in which ants do not show
abnormal or stereotypical behaviour. The whole range of expected behaviour was observed.
Ants were manipulated with soft forceps, which prevent any damage, and marked with paint,
which does not alter their behaviour in the long term. Ants were killed by freezing before
dissection. All these procedures were conducted following the institutional guidelines of animal
welfare of both Brazil and France.

**Ants**

We collected 18 colonies of *N. apicalis* in Brazil in November 2016: eight queenless and six
with fewer than 20 workers (1–56 workers, mean 22.1 workers per queenright colony, SD 17.5).
The fact that eight colonies were queenless thus suggests that queenlessness is common in this
Comparison of hierarchy establishment in the *N. apicalis* species complex showed that it occurs earlier, and agonistic behaviour is more pronounced, in monogynous species (Yagound 2014). This suggests that an increased chance of queenlessness selects for hierarchy establishment mechanisms allowing quick conflict resolution. In this study, we used four colonies: colony 1 was collected in Marituba, state of Para (1°21’18’’S, 48°20’21’’W), and colonies 8, 18 and 20 in Santa Barbara do Para (1°13’36’’S, 48°17’43’’W). Cytochrome C oxidase I sequence analysis revealed that our colonies belong to morph 6 of *N. apicalis* (Yagound et al., n.d.). *Neoponera apicalis* was divided into three morphospecies by Delabie et al. (2008) based on fine morphological differences in this complex of cryptic species. Ferreira et al. (2010) defined three additional morphs based on a set of morphological, acoustic, chemical and genetic data. Yagound (2014) added a seventh morph. Mackay and Mackay (2010) described morph 5 as *Neoponera cooki*, but the original numeration is kept in order to be consistent. Colonies were harvested in mid-October 2016 and installed in the laboratory a week later. The experiment started 2 months after their installation. During this acclimation period, workers remained with the queen. The ants were housed in plaster nests (18 x 14 cm) connected to an external environment of the same size. They were maintained at a temperature of 25 ± 2 °C, a relative humidity of 50 ± 10% and a day:night cycle of 12:12 h. Each colony was fed three times a week with an apple–honey mixture and thawed crickets (*Acheta domestica*), as well as water ad libitum.

Based on the study by Yagound et al. (2012), which showed that workers close to the queen were the first to reproduce at the onset of hierarchical competition, we assumed that the most fertile workers would stay next to the queen within the nest. We selected and individually marked 20 workers in the vicinity of the queen in each experimental colony. Each of these workers received a number label glued on the thorax and two coloured dots (Uni-ball marker). The chemical profile of ants before orphaning was extracted using SPME (see below). The
individuals were then released into the original nest. The following week, 15 of these 20
selected workers were isolated and placed in another artificial nest of the same type, to mimic
an orphaning process. We recorded agonistic interactions, that is, antennal boxing and bites, in
the nest (see Dominance Hierarchy below). Antennal boxing consists of repeated and rapid
strokes of one ant by another with the antennae. This behaviour is typical of many ant species
and is often observed during the establishment of hierarchies. In established hierarchies,
dominant individuals perform antennal boxing against subordinate individuals (Monnin &
Peeters, 1999; Blacher et al., 2010, Yagound et al., 2014). Biting occurs when the individual
uses its mandibles to grip a part of another individual’s body. In most instances, biting was
prolonged, thereby immobilizing the other individual with no apparent damage or cuts, and we
consider this behaviour ritualized biting. On the 10th day of the experiment, workers were
frozen for later extraction of their chemical profile after orphaning and measurements of their
ovarian development (Fig. 1). Of the initial 60 ants, 59 survived to this stage.

Extraction and analysis of chemical profiles

The individuals’ chemical profiles were analysed before they were orphaned and after the
establishment of the reproductive hierarchy. The initial chemical profile (before orphaning) was
obtained by solid-phase microextraction (Monnin et al., 1998). This involved rubbing an SPME
fibre (polydimethylsiloxane 100 µm) on the first segment of the abdomen for 2 min. The fibre
was then desorbed in a Varian 3900 gas chromatograph with flame ionization detection (GC-
FID). The carrier gas used was helium at 1 ml/min, with hydrogen streams at 30 ml/min and air
at 300 ml/min. The programme was as follows: the initial temperature was 70 °C for 1 min,
then it rose from 40 °C/min for 4 min to 250 °C, then increased by 1 °C/min for 8 min to 258
°C and finally increased from 40 °C/min to 320 °C and stabilized at 320 °C for 3 min. The
temperature of the injector was maintained at 280 °C and that of the FID at 340 °C. Profiles were extracted with the Varian system control software Star Chromatography workstation version 6.2 (Varian, Palo Alto, CA, U.S.A.). The compounds were identified based on their retention time (Appendix Table A1), comparing them to standard hydrocarbons already identified in a gas chromatograph coupled to a mass spectrometer (GC-MS) as well as chromatograms of Yagound (2014) for *N. apicalis* morph 6. The advantage of this method was that it was not invasive; however, it was time consuming and did not allow quantification of compounds.

For temporal constraints, the chemical profile after orphaning was obtained by a liquid phase extraction. The head and thorax of each dissected ant were soaked in 200 µl of pentane containing 4 ng/µl of compound n-C17 (representing our internal standard) in a vial tube for 5 min. The tube was then left to evaporate. After the solution was completely evaporated, the sample was analysed by GC–MS (Agilent A7890), by injecting 2 µl of the extract resuspended in 80 µl of solvent (pentane), with electron impact ionization at 70 eV. The carrier gas was helium at 1 ml/min. The same analysis programme as above was applied. The chemical profiles were integrated using the MSD ChemStation software version E.02.01.1177 (Agilent Technologies Inc., Santa Clara, CA, U.S.A.). The compounds were identified by comparing their retention time and spectra with already known compounds. The internal standard allowed us to translate peak areas to absolute quantities for the related compounds.

*<H2>Dominance hierarchy</H2>*

Antennal boxing and bites were recorded, together with the identities of the interacting ants. The loser was the ant showing submissive behaviour, that is, hunching or dodging. Twelve observation sessions were carried out (15 h total) per colony: two of 1.5 h on the first, second
and third days after being orphaned, then two of 1 h on the fourth and fifth days and finally one
of 1 h on the eighth and 10th days. During these sessions, all boxing and bites were recorded.
Observations were made under red light to avoid biasing the ants’ behaviour in the interior of
the nest (Depickère et al., 2004).

The hierarchical rank of each worker in the orphaned colonies was obtained using the
‘Glicko-rating’ method, which is a dynamic matched comparison model that calculates a score
for each individual, based on the outcome of each individual's interactions (victory or defeat;
Glickman, 1999). From this score, a ranking can be determined to deduce the hierarchy. The
Glicko-rating algorithm includes a positive constant ‘c’, which governs the size of the standard
deviation over time. This constant is defined by the user, an increased value of ‘c’ leading to a
greater average deviation per individual over time. In our study, following the guidelines of
Glickman (1999; and see So et al., 2015), we used a value of 1 for ‘c’. We checked the impact
of the ‘c’ value on our results by replicating the calculation over a range of 1–10. We obtained
similar results for the hierarchical rankings over the whole range. Glicko-rating calculations
were performed with the PlayerRatings package v1.0 (Stephenson & Sonas, 2014) in R 3.4.1
(R Development Core Team, 2017). Data were compiled in chronological order of dyadic
interactions. The same coefficient was attributed to antennal boxes and bites, so that in the
calculation of the hierarchy the two types of agonistic behaviours had the same power.

<H2>Fertility measurement</H2>

With a graduated binocular microscope, we measured the ovarian development of the ants. The
length of the three basal oocytes of the ovarioles of each ovary was measured. A fertility index
was calculated by summing the lengths of the six basal oocytes (Yagound et al., 2014). We
present this below as mean ± SD.
The distribution of the average number of agonistic interactions per hour of observation was compared between colonies to compare the dynamics of hierarchy establishment. For this, a two-sample Kolmogorov–Smirnov test was performed between each pair of the four colonies. To compensate for multiple comparisons, $P$ values were then adjusted to $P'$ values following Holm (1979).

The linearity ‘$h$’ within our four colonies was calculated between the 15 orphaned workers by the de Vries method (1995) using software R (package compete, Curley 2016).

To verify whether worker isolation led to the establishment of a reproductive hierarchy, we investigated the link between the hierarchical rank and fertility of individuals using Spearman correlations for the 59 orphaned ants dissected at the end of the experiment.

Although some intercolonial heterogeneity is expected in the proportion of each compound in the cuticular profiles, a principal coordinate analysis (PCo) and an analysis of similarity (ANOSIM) were performed to verify whether our experimental colonies (59 workers) shared a similar chemotype, due to the potential presence of cryptic morphs, differing in chemical profiles. For this, we used the PERMANOVA+ for PRIMER software (Anderson et al., 2008) using a Euclidean distance matrix calculated on square-root-transformed percentages.
Using two different methods to extract the chemical profiles was a potential source of methodological variability. To verify whether our methods were reliable, we used Spearman correlations to compare the profiles before and after orphaning using the proportions of the major compounds, namely n-C$_{21}$, C$_{23:1}$ and n-C$_{23}$, with a Bonferroni–Holm adjustment for multiple tests on the same data set (Holm, 1979). Significant correlations would indicate reliability of the two methods (even if distinct methods can generate a slight chemical distance between the profiles before and after orpharing). This calculation could be done only for three colonies (see Results for details). In addition, two individuals from colony 20 could not be included in these chemical analyses because of a technical problem when acquiring the profile before orphaning (missing data). In colony 20 there were only 14 individuals because a worker died during the experiment. We thus analysed a total of 42 workers. For these three colonies we also performed a Mantel test (package ecodist in R) between the Euclidean distance matrix of the square-root-transformed percentages of the chemical profiles before and after orphaning to evaluate global concordance between the two methods.

To identify the cuticular compound(s) potentially acting as a fertility signal in our study, we used Spearman rank correlation (on 44 workers) to assess the relationship between the fertility index measured at the end of the experiment and the final quantities of cuticular compounds. Once the putative fertility signal was identified, we also checked for correlations with the proportions before orphaning to verify pre-existing heterogeneity between the workers in the presence of the queen (42 workers). $P$ values were adjusted to account for multiple testing of the same data (Holm, 1979).

Last, we investigated the presence of a correlation between the putative fertility signal and the observed behaviour using Spearman correlation. The deviation from the mean quantity of fertility-related compound(s) (within each colony) was correlated with the different behaviours expressed by each individual (42 workers). Using a mean deviation index allowed
us to buffer the effect of intercolonial variation in the quantity of compound.

### Results

#### Setting up reproductive hierarchies

Despite some variability among colonies in the intensity of agonistic displays (Fig. 2), the dynamics of agonistic behaviour did not differ significantly between them. No two by two comparison between colonies was significant (Kolmogorov–Smirnov test with Bonferroni–Holm correction: colony 1–8: $D = 0.571$, $P' = 0.203$; colony 1–18: $D = 0.571$, $P' = 0.203$; colony 1–20: $D = 0.571$, $P' = 0.212$; colony 8–18: $D = 0.571$, $P' = 0.203$; colony 8–20: $D = 0.571$, $P' = 0.203$; colony 18–20: $D = 0.429$, $P' = 0.575$). Agonistic interactions rose rapidly within the first 2 days of being orphaned and then returned to basal level.

#### Establishment of hierarchies

Hierarchy established in colonies 8, 18 and 20 had linearities of $h' = 0.52$ ($P = 0.001$), $h' = 0.66$ ($P < 0.001$) and $h' = 0.65$ ($P < 0.001$), respectively. Hierarchy in colony 1 did not show a significant linearity ($h' = 0.19$, $P = 0.48$).

#### Rank and ovarian development

All but 10 of our 59 workers had activated ovaries. The average ovarian development, measured at the end of the experiment, was highest for colony 18 (4.16 ± 2.59 mm), followed by colony 8 (3.72 ± 3.10 mm), colony 1 (3.42 ± 2.25 mm) and finally colony 20 (2.97 ± 2.06 mm). Within
each colony the fertility index of workers was significantly correlated with their hierarchical rank from the first day of being orphaned (Table 1).

**Chemical profiles, fertility and behaviours**

The chemical profiles were grouped by colony in the PCo and with the ANOSIM, suggesting the existence of a characteristic colonial signature (Appendix Fig. A1). Considering the average chemical distance calculated between colonies, colony 18 was very different from the others (0.75 on average with colony 18, against 0.20 between the other three colonies; Appendix Table A2).

The cuticular profile of each ant was composed of 28–30 peaks and included several series of n-alkanes, branched mono and dimethyl-alkanes and alkenes, with carbon atom numbers ranging from 19 to 33. The majority of compounds were linear alkanes and alkenes. Consistent with the chemical distance results, colonies 1, 8 and 20 displayed a qualitatively distinct chemotype from colony 18 (Appendix Fig. A2). Colony 18 was thus excluded from correlation analysis with the chemical profiles. The chemical profiles of the workers from colony 18 were heterogeneous, some appearing separated and others represented among the other colonies’ profiles (Appendix Fig. A1). As several morphs of *N. apicalis* occur in the collection area, this unusual result could thus be a consequence of a chance hybridization between two morphs (i.e. a male from another morph), which cannot be detected using nuclear DNA. Although interesting, we have no additional means to explain this discrepant chemotype.

**Reliability between the two chemical extraction methods**
The proportions of the three major compounds (n-C_{21}, C_{23:1} and n-C_{23}) in the SPME samples analysed by GC–FID before orphaning were significantly correlated with those analysed by GC–MS after orphaning (Table 2). The Mantel test between the two chemical profile matrices before and after orphaning were significantly correlated ($P < 0.001$) with a Mantel value of 0.70, which, considering the potential variation due to fertility and environmental changes between the two analyses, is sufficient to validate the use of the two methods. Initial and final n-C_{23} proportions were also correlated (Spearman correlation: $r_s = 0.75$, $P < 0.001$).

**<H3>Correlation between chemical profiles and fertility**

The amount of the alkane tricosane (n-C_{23}) after orphaning was significantly correlated with the fertility index for each individual (Spearman correlation: $r_s = 0.63$, $N = 44$, $P < 0.001$; Fig. 3a), as was the proportion of n-C_{23} in the chemical profile before orphaning (Spearman correlation: $r_s = 0.69$, $N = 42$, $P < 0.001$; Fig. 3b). This compound was the component of the chemical profile that best correlated with ovarian development. Correlation values of the other compounds are presented in Appendix Table A3.

**<H3>Fertility and behaviour**

We focused on n-C_{23} which was the best correlated compound with fertility. Both the number of fights won by an ant and the number of interactions it was involved in were significantly correlated with the quantity of n-C_{23} after orphaning (Table 3). The same results were obtained when considering only the first 2 days of interaction. Last, behaviours at the beginning of the experiment (first 2 days) were highly correlated with all behaviours observed during the whole
10 days of the experiment (Table 4), showing that the hierarchy was established during the first 48 h after queen loss.

### Discussion

Our results confirmed the rapidity of hierarchy establishment over a period of 48 h after queen loss. The number of agonistic behaviours decreased drastically after this period, which is typical of a stabilized hierarchy. The cuticular profiles analysis showed tricosane to be highly correlated with ovarian development, therefore putatively acting as a fertility signal. Interestingly, the relative amounts of tricosane on the cuticle both before and after the establishment of the hierarchy were also correlated with (1) the rank achieved within the hierarchy and (2) the frequency of the agonistic behaviours displayed.

These results constitute the first experimental evidence that differences in ovarian activity (estimated by an indirect method) between workers in the presence of the queen accurately predict the outcome of the reproductive hierarchy, which is a consequence of a tournament between workers. They also fully support our hypothesis that physiological differences between workers are mirrored in modulated motivations to fight (Stevenson et al., 2000). The outcome of the dominance/submission could then be facilitated with chemical signalling (Yagound et al., 2015).

Fertility signals have been identified in other morphs of *N. apicalis* (Yagound, 2014), but not in morph 6. In three colonies (the fourth having a different chemotype) tricosane was the cuticular compound best correlated with fertility. Thus, tricosane is the most probable fertility signal in these colonies. We used tricosane as a proxy for the fertility signal, but we are aware that it may also be part of a mixture of compounds used for fertility recognition. This does not, however, change the conclusions of our study. In his comparative study of fertility signalling in the *N. apicalis* complex, Yagound (2014) found that an alkene was best correlated
with fertility in morph 6 \( (r_s = 0.75, P < 10^{-4}) \). However, Yagound could analyse only one colony and he also found that tricosane was correlated with ovarian development \( (r_s = 0.51, P < 0.01) \). This finding and the larger sample that we analysed legitimize the interpretation of tricosane as a putative signal of fertility for *N. apicalis* morph 6. Other compounds were also correlated with fertility in our sample but tricosane appeared to be the best correlated compound with both fertility and behavioural parameters (the expression of agonistic behaviours and the social ranks achieved) during the establishment of the hierarchy. This consistency between physiological and behavioural data fulfils the condition for tricosane to be an index of fertility. The correlation between the putative fertility signal and the hierarchical ranks also strengthens the idea of it being an honest signal (Heinze & d’Ettorre, 2009). A reliable index of fertility allows adequate reproductive decision making, depending on individual interests in terms of inclusive fitness (Yagound, 2014). This signal would allow workers to identify the best potential reproducer within the colony.

The initial heterogeneity between ants in queenright colonies could be amplified during subsequent agonistic interactions. Idiosyncratic variations could initially reflect the differences in workers’ ages affecting their physiological and hormonal states, and hence their ovarian activation (Yagound et al., 2015). Workers with an already partially active ovarian system would have a clear advantage during the establishment of hierarchies. Lamba et al. (2007) hypothesized that in other eusocial insects (wasps) fighting could be used not to exclude the other females from dominance, but rather to speed up the development of the ovarian system of the future reproductive (via an action on biogenic amines) and so facilitate the monopolization of colony reproduction. Aggressive behaviours also lead to a decrease in juvenile hormone titre (usually positively correlated with fertility) in subordinate individuals (Tibbetts et al., 2018). Physical contact between workers in the ant *Diacamma* has also been shown to affect dopamine secretion in the worker’s brain and to regulate reproduction inside
the nest (Shimoji et al., 2017). Agonistic interactions in *Neoponera* may thus impact ovarian development through similar neuroendocrine changes.

Hierarchical status discrimination based on the putative fertility signal can generate a linear hierarchy. Fertility signalling would be involved in both the establishment (Yagound et al., 2015) and the maintenance (Heinze et al., 2002) of the reproductive hierarchy. Agonistic interactions acting on the physiological and hormonal secretions would reinforce the pre-existing differences in fertility between individuals, and this would accelerate cooperation within the nest. Subordinates would maximize their fitness by quickly resolving conflicts by reducing their ability to reproduce (Tibbetts et al., 2018).

Interestingly, our results showed a strong correlation between fertility and the number of fights an individual is involved in (whatever the outcome). This result suggests two mutually nonexclusive hypotheses. First, tricosane could be correlated with both fertility and motivation to fight and/or involvement in the colony’s hierarchy. Biogenic amines such as octopamine or dopamine could possibly be involved in this process. Indeed, biogenic amines mediate changes in dominance behaviour linked with fertility in the ant *Harpegnathos saltator* (Penick et al., 2014). Moreover, it has been demonstrated in the cricket *Gryllus bimaculatus* that these bioamines are necessary to trigger aggressive behaviour (Stevenson et al., 2000). Second, tricosane could act as a fertility signal and thus attract aggression from competitors attempting to gain dominance. Such behaviour where workers attack congeners that display fertility signals has been shown, for example, in the context of worker policing in social insects (ants: Hartmann et al., 2005; Monnin & Peeters, 1999; Smith et al., 2009; bees: Visscher and Dukas, 1995; wasps: Wenseleers et al., 2005). This mechanism could thus ensure the fertility signal has similar functions in the contexts of worker policing and establishment of the reproductive hierarchy, namely regulating reproduction at the level of the worker collective.
The fact that tricosane was also correlated with the percentage of fights won favours the first explanation. Attacked individuals in the case of worker policing are indeed more likely to be defeated (and their reproductive activity suppressed; Monnin & Peeters, 1999), while highly motivated animals could have an advantage in a tournament system. Tricosane could thus both reflect ovarian development and be correlated with a network of neuroendocrine activity that ensures fighting motivation and, maybe more generally, the ability to mobilize resources (RHP; Parker, 1974). One mechanism ensuring the honesty of the fertility signal (and its role in the reproductive hierarchy) could be the strong links between the neuroendocrine networks involved in the regulation of reproduction, agonistic behaviour and fighting abilities. A second mechanism could be that individuals ‘motivated’ to fight, but lacking the skills required to occupy the top of the hierarchy, would be defeated by others workers. This mechanism would be in line with theories proposing that the costs (both physiological and social) of maintaining a signal ensure its honesty (Zahavi, 1975; Heinze & d’Ettorre, 2009).

The loss of the queen probably lifts an inhibition for already fertile individuals which very quickly start competing to reproduce. The highest motivation for fighting of these individuals probably drives the expression of ritualized agonistic encounters within the colony. The impact of social experience and especially winner–loser effects would then help amplify the pre-existing differences at the physiological (Oliveira et al. 2009), cognitive and behavioural (Hsu & Wolf, 2000; Rutte et al., 2006) levels. The social system would then develop from the queenright state, where all workers refrain from reproducing, to the establishment of the reproductive hierarchy based on self-organized processes. After a short period of social perturbation with intense fighting behaviour, the social system stabilizes again with the selection of a new reproductive individual and the disappearance of agonistic interactions.

To our knowledge, this is the first study to monitor the development of the chemical profile from the queenright state to the stabilization of a reproductive hierarchy by orphaned ant
workers. Our study supports the hypothesis that the pre-existing fertility differences between individuals in the queenright condition predict the destiny of workers in the reproductive hierarchy. The most fertile workers reach the high ranks and produce males. The selective pressures are strong after queen loss, with a short time window for producing the last batch of reproductive ants (Dietemann & Peeters, 2000). In response to these strong ecological constraints, ants have developed a recognition system based on cuticular hydrocarbons related to ovarian development and acting as a fertility signal (Yagound et al., 2015). This fertility signal, already perceptible in the presence of the queen, makes it possible for workers to evaluate the interindividual differences and, subsequently, agonistic interactions help to establish and stabilize the reproductive hierarchy (especially with winner–loser effects; Chase et al., 2002). All these mechanisms allow a quick resolution of the overt conflict and ensure division of reproductive and ergonomic tasks inside orphaned colonies to allow the production of males.

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References


Figure legends

Figure 1. Timeline of the experiment. The queen was removed from each colony 7 days after individuals were marked and the experiment ran for 10 days. ‘Initial profile’ corresponds to the workers’ chemical profile in the presence of the queen (determined by flame ionization detection, GC–FID); ‘final profile’ corresponds to the chemical profile at the end of the experiment (determined by gas chromatography–mass spectrometry, GC–MS). Agonistic interactions (antennal boxing and bite) were used to calculate the hierarchical rank of the 15 orphaned individuals per colony.

Figure 2. Number of agonistic interactions per hour of observation as a function of observation day during the 10 days after the queen was removed.

Figure 3. Variation in the fertility index (summed lengths of the six basal oocytes in the ovary, mm) as a function of (a) the amount of tricosane in the ants’ chemical profile after queen removal and (b) the proportion of tricosane in the profile before queen removal.

Figure A1. PCO (principal coordinate analysis) of the chemical profiles of the four colonies (based on the Bray–Curtis similarity matrix calculated with the square-root-transformed proportions). N = 15 individuals in each colony.

Figure A2. Representative examples of the chemical profiles from (a) colony 1 (chemotype A) and (b) colony 18 (chemotype B).

Figure A3. Plot of the data used in the Spearman correlations presented in Table 3. Correlations are shown between the recorded behaviours and the mean deviation of tricosane per colony.
before (left, $n$-C$_{23}$ initial) and after queen removal (right, $n$-C$_{23}$ final). (a) Hierarchical rank on
the 10th day (the end of the experiment), (b) the number of fights won for days 1 and 2, (c) the
total number of fights from day 1 to day 10, (d) the percentage of fights won for days 1 and 2,
(e) the total percentage of fights won from day 1 to day 10, (f) the number of fights individuals
were involved in for days 1 and 2 and (g) the total number of fights individuals were involved
in from day 1 to day 10.
Table 1. Spearman correlations between an individual ant’s fertility index and hierarchical rank on the first, second and 10th (final) day of the experiment

<table>
<thead>
<tr>
<th>Day</th>
<th>$r_s$</th>
<th>$P^*$</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 rank</td>
<td>-0.3</td>
<td>0.03</td>
<td>59</td>
</tr>
<tr>
<td>Day 2 rank</td>
<td>-0.39</td>
<td>0.006</td>
<td>59</td>
</tr>
<tr>
<td>Day 10 rank</td>
<td>-0.44</td>
<td>0.002</td>
<td>59</td>
</tr>
</tbody>
</table>

To compensate for multiple comparisons, $P$ values were adjusted to $P^*$ values following Holm (1979).
Table 2. Spearman correlations between the proportions of the three main compounds within individual ants’ chemical profiles before and after queen removal.

<table>
<thead>
<tr>
<th>Cuticular hydrocarbons</th>
<th>$r_s$</th>
<th>$P'$</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-C$_{21}$</td>
<td>0.48</td>
<td>0.001</td>
<td>42</td>
</tr>
<tr>
<td>C$_{23:1}$</td>
<td>0.34</td>
<td>0.03</td>
<td>42</td>
</tr>
<tr>
<td>n-C$_{23}$</td>
<td>0.74</td>
<td>2.1e-8</td>
<td>42</td>
</tr>
</tbody>
</table>

To compensate for multiple comparisons, $P$ values were adjusted to $P'$ values following Holm (1979).
Table 3. Spearman correlations between the behaviours expressed after (final n-C\text{23}) and before (initial n-C\text{23}) queen removal and the mean deviation in amount of n-C\text{23} between individuals of the same colony.

To compensate for multiple comparisons, P values were then adjusted to P’ values following Holm (1979).

<table>
<thead>
<tr>
<th></th>
<th>( r_S ) (final n-C\text{23})</th>
<th>( r_S ) (initial n-C\text{23})</th>
<th>( P' )</th>
<th>( P' )</th>
<th>( N )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final hierarchical rank</td>
<td>-0.52</td>
<td>0.002</td>
<td>-0.48</td>
<td>0.003</td>
<td>42</td>
</tr>
<tr>
<td>No. of fights won during days 1 and 2</td>
<td>0.46</td>
<td>0.008</td>
<td>0.53</td>
<td>0.002</td>
<td>42</td>
</tr>
<tr>
<td>Total no. of fights won</td>
<td>0.53</td>
<td>0.001</td>
<td>0.59</td>
<td>2.5e-4</td>
<td>42</td>
</tr>
<tr>
<td>Percentage of fights won during days 1 and 2</td>
<td>0.37</td>
<td>0.01</td>
<td>0.43</td>
<td>0.008</td>
<td>42</td>
</tr>
<tr>
<td>Total percentage of fights won</td>
<td>0.44</td>
<td>0.008</td>
<td>0.44</td>
<td>0.008</td>
<td>42</td>
</tr>
<tr>
<td>Fight number during days 1 and 2</td>
<td>0.45</td>
<td>0.008</td>
<td>0.51</td>
<td>0.002</td>
<td>42</td>
</tr>
<tr>
<td>Fight number total</td>
<td>0.5</td>
<td>0.002</td>
<td>0.59</td>
<td>2.2e-4</td>
<td>42</td>
</tr>
</tbody>
</table>

Holm (1979). ‘Final’ and ‘total’ correspond to the behaviours expressed from day 1 to day 10 of being orphaned. ‘Fight number’ corresponds to the number of fights an individual was involved in. Data dispersions are presented in Fig. A3.
Table 4. Spearman correlations between behaviours during combined days 1 and 2 and from day 1 to day 10 of the experiment

<table>
<thead>
<tr>
<th></th>
<th>$r_S$</th>
<th>$P$</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hierarchical rank</td>
<td>0.86</td>
<td>&lt;2.2e-16</td>
<td>59</td>
</tr>
<tr>
<td>No. of fights won</td>
<td>0.96</td>
<td>&lt;2.2e-16</td>
<td>59</td>
</tr>
<tr>
<td>Percentage of fights won</td>
<td>0.90</td>
<td>&lt;2.2e-16</td>
<td>59</td>
</tr>
<tr>
<td>No. of fights</td>
<td>0.95</td>
<td>&lt;2.2e-16</td>
<td>59</td>
</tr>
</tbody>
</table>
Table A1. Identification of cuticular hydrocarbons (CHCs) on *N. apicalis* morph 6 for a moderately fertile individual (corresponding to chemotype A in Fig. A2)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time</th>
<th>Relative abundance</th>
<th>Characteristic fragments</th>
<th>CHC ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.876</td>
<td>-</td>
<td>-</td>
<td>n-C_{17} (internal standard)</td>
</tr>
<tr>
<td>2</td>
<td>6.532</td>
<td>0.05</td>
<td>268</td>
<td>n-C_{19}</td>
</tr>
<tr>
<td>3</td>
<td>6.915</td>
<td>0.08</td>
<td>282</td>
<td>n-C_{20}</td>
</tr>
<tr>
<td>4</td>
<td>7.265</td>
<td>0.7</td>
<td>294</td>
<td>C_{21:1}</td>
</tr>
<tr>
<td>5</td>
<td>7.399</td>
<td>14.85</td>
<td>296</td>
<td>n-C_{21}</td>
</tr>
<tr>
<td>6</td>
<td>7.563</td>
<td>0.06</td>
<td>140 196 295</td>
<td>9-MeC_{21}</td>
</tr>
<tr>
<td>7</td>
<td>7.694</td>
<td>0.07</td>
<td>70 267 295</td>
<td>4-MeC_{21}</td>
</tr>
<tr>
<td>8</td>
<td>7.785</td>
<td>1.41</td>
<td>308</td>
<td>C_{22:1}</td>
</tr>
<tr>
<td>9</td>
<td>7.906</td>
<td>0.97</td>
<td>310</td>
<td>n-C_{22}</td>
</tr>
<tr>
<td>10</td>
<td>8.139</td>
<td>0.07</td>
<td>169 182 309</td>
<td>11-MeC_{22}</td>
</tr>
<tr>
<td>11</td>
<td>8.523</td>
<td>62.42</td>
<td>322</td>
<td>C_{23:2} + C_{23:1}</td>
</tr>
<tr>
<td>12</td>
<td>8.633</td>
<td>11.13</td>
<td>324</td>
<td>n-C_{23}</td>
</tr>
<tr>
<td>13</td>
<td>8.847</td>
<td>0.19</td>
<td>168 196 323</td>
<td>11-MeC_{23}</td>
</tr>
<tr>
<td>14</td>
<td>8.962</td>
<td>0.16</td>
<td>85 252 281 323</td>
<td>5-MeC_{23}</td>
</tr>
<tr>
<td>15</td>
<td>9.167</td>
<td>0.09</td>
<td>336</td>
<td>C_{24:1}</td>
</tr>
<tr>
<td>16</td>
<td>9.371</td>
<td>0.07</td>
<td>338</td>
<td>n-C_{24}</td>
</tr>
<tr>
<td>17</td>
<td>10.135</td>
<td>0.99</td>
<td>350</td>
<td>C_{25:1}</td>
</tr>
<tr>
<td>18</td>
<td>10.392</td>
<td>1.4</td>
<td>352</td>
<td>n-C_{25}</td>
</tr>
<tr>
<td>19</td>
<td>11.606</td>
<td>0.14</td>
<td>366</td>
<td>n-C_{26}</td>
</tr>
<tr>
<td>20</td>
<td>12.756</td>
<td>0.04</td>
<td>378</td>
<td>C_{27:1}</td>
</tr>
<tr>
<td>21</td>
<td>13.168</td>
<td>2.45</td>
<td>380</td>
<td>n-C_{27}</td>
</tr>
<tr>
<td>22</td>
<td>14.374</td>
<td>0.12</td>
<td>394</td>
<td>n-C_{28}</td>
</tr>
<tr>
<td>23</td>
<td>14.824</td>
<td>0.33</td>
<td>365 393</td>
<td>2-MeC_{28}</td>
</tr>
<tr>
<td>24</td>
<td>14.918</td>
<td>0.06</td>
<td>406</td>
<td>C_{29:1}</td>
</tr>
<tr>
<td>25</td>
<td>15.046</td>
<td>0.83</td>
<td>408</td>
<td>n-C_{29}</td>
</tr>
<tr>
<td>26</td>
<td>15.225</td>
<td>0.24</td>
<td>168 196 252 281 407</td>
<td>11-16-diMeC_{28}</td>
</tr>
<tr>
<td>27</td>
<td>15.961</td>
<td>0.2</td>
<td>393 421</td>
<td>2-MeC_{30}</td>
</tr>
<tr>
<td>28</td>
<td>16.065</td>
<td>0.04</td>
<td>434</td>
<td>C_{31:1}</td>
</tr>
<tr>
<td>29</td>
<td>16.174</td>
<td>0.09</td>
<td>436</td>
<td>n-C_{31}</td>
</tr>
<tr>
<td>30</td>
<td>16.352</td>
<td>0.32</td>
<td>168 196 224 252 281 309 435</td>
<td>11-13-15-MeC_{31}</td>
</tr>
<tr>
<td>31</td>
<td>17.634</td>
<td>0.44</td>
<td>168 308 337 463</td>
<td>11-MeC_{33}</td>
</tr>
</tbody>
</table>
Table A2. Analysis of similarity between the chemical profiles of the four colonies

<table>
<thead>
<tr>
<th>Groups</th>
<th>$R$</th>
<th>$P$</th>
<th>Permutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 8</td>
<td>0.148</td>
<td>0.01</td>
<td>9999</td>
</tr>
<tr>
<td>1, 18</td>
<td>0.758</td>
<td>0.0001</td>
<td>9999</td>
</tr>
<tr>
<td>1, 20</td>
<td>0.208</td>
<td>0.0002</td>
<td>9999</td>
</tr>
<tr>
<td>8, 18</td>
<td>0.771</td>
<td>0.0001</td>
<td>9999</td>
</tr>
<tr>
<td>8, 20</td>
<td>0.281</td>
<td>0.0001</td>
<td>9999</td>
</tr>
<tr>
<td>18, 20</td>
<td>0.747</td>
<td>0.0001</td>
<td>9999</td>
</tr>
</tbody>
</table>

The global test of the analysis of similarity gives a global $R$ of 0.549 ($P = 0.0001$, number of permutations = 9999). Pairwise test results are given in the table.
Table A3. Spearman correlations (with Bonferroni–Holm adjustment for multiple comparisons) between the fertility index and the compounds present in the chemical profile after orphaning of colonies 1, 8 and 20 (*N* = 44)

<table>
<thead>
<tr>
<th>Cuticular hydrocarbons</th>
<th>( r_s )</th>
<th>( p' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-C(_{19})</td>
<td>-0.51</td>
<td>0.009</td>
</tr>
<tr>
<td>n-C(_{20})</td>
<td>-0.15</td>
<td>1</td>
</tr>
<tr>
<td>C(_{21:1})</td>
<td>-0.23</td>
<td>1</td>
</tr>
<tr>
<td>n-C(_{21})</td>
<td>0.18</td>
<td>1</td>
</tr>
<tr>
<td>9-MeC(_{21})</td>
<td>-0.28</td>
<td>1</td>
</tr>
<tr>
<td>4-MeC(_{21})</td>
<td><strong>0.44</strong></td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td>C(_{22:1})</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>n-C(_{22})</td>
<td>0.48</td>
<td>0.023</td>
</tr>
<tr>
<td>11-MeC(_{22})</td>
<td>0.41</td>
<td>0.14</td>
</tr>
<tr>
<td>C(<em>{23:2} + C</em>{23:1})</td>
<td>0.27</td>
<td>1</td>
</tr>
<tr>
<td>n-C(_{23})</td>
<td><strong>0.63</strong></td>
<td><strong>1.5e-4</strong></td>
</tr>
<tr>
<td>11-MeC(_{23})</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>5-MeC(_{23})</td>
<td><strong>0.61</strong></td>
<td><strong>2.9e-4</strong></td>
</tr>
<tr>
<td>C(_{24:1})</td>
<td>0.34</td>
<td>0.42</td>
</tr>
<tr>
<td>n-C(_{24})</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>C(_{25:1})</td>
<td><strong>0.45</strong></td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td>n-C(_{25})</td>
<td><strong>0.48</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>n-C(_{26})</td>
<td>0.27</td>
<td>1</td>
</tr>
<tr>
<td>C(_{27:1})</td>
<td>-0.01</td>
<td>1</td>
</tr>
<tr>
<td>n-C(_{27})</td>
<td>0.41</td>
<td>0.13</td>
</tr>
<tr>
<td>n-C(_{28})</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>2-MeC(_{28})</td>
<td>0.08</td>
<td>1</td>
</tr>
<tr>
<td>C(_{29:1})</td>
<td>-0.22</td>
<td>1</td>
</tr>
<tr>
<td>n-C(_{29})</td>
<td>0.24</td>
<td>1</td>
</tr>
<tr>
<td>11-16-diMeC(_{28})</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td>2-MeC(_{30})</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>C(_{31:1})</td>
<td>-0.11</td>
<td>1</td>
</tr>
</tbody>
</table>
For colony 18, only the compound n-C_{27} was correlated with fertility \((r_S = -0.5, P' = 0.057, N = 15)\). To compensate for multiple comparisons, \(P\) values were adjusted to \(P'\) values following Holm (1979). Significant values are highlighted in bold.