Cuticular hydrocarbons correlated with reproductive status in a queenless ant

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Reproductive division of labour is regulated behaviourally in social insects lacking morphologically specialized castes. The directional nature of dominance interactions shows that recognition occurs, but little is known about its basis. In the queenless ant *Dinoponera quadriceps*, the top worker in the hierarchy ('alpha') mates and produces offspring in each colony, while other workers remain virgin. Dominant ants frequently rub one antenna of subordinates against their own cuticle, and alpha and infertile nest-mates consistently differ in their relative proportions of the cuticular hydrocarbon 9-hentriacontene (9-C_{31}). The second-ranking 'beta' occasionally lays unfertilized eggs and we show that she has less 9-C_{31} than the alpha but more than infertile workers. To investigate further the link between 9-C_{31} and ovarian activity, we experimentally removed alpha workers ($n = 11$ individuals) and used solid-phase microextraction (SPME) with gas chromatography to measure changes in 9-C_{31} on live beta workers which attained alpha status. The proportion of 9-C_{31} on the replacement alpha increased significantly after six weeks, in parallel with her gain in fecundity. We discuss whether 9-C_{31} provides honest information about egg-laying ability, enabling ants to recognize the different classes of nest-mates involved in reproductive conflicts. Such fertility cues could reliably underpin the antagonistic interactions occurring in insect societies.

Keywords: *Dinoponera quadriceps*; dominance; oogenesis; pheromones; Ponerinae; reproduction

1. INTRODUCTION

In social Hymenoptera lacking morphologically specialized queens and workers, all females in a colony are potentially capable of sexual reproduction (Wilson 1971). Most remain infertile, however, and this is behaviourally determined during adult life (Breed & Gamboa 1977; Reeve 1991; Röseler 1991). A proportion of nest-mates interact aggressively and this results in the establishment of hierarchical relationships. One or several dominant females have active ovaries, while oogenesis is suppressed in subordinates (see, for example, Röseler & Van Honk 1990). Inter-individual interactions are highly directed and thus require reliable recognition, but the basis of this recognition remains poorly understood. West-Eberhard (1977) suggested that reproductive regulation is based on both chemical communication of status and overt aggression, and this has been shown in *Polistes* and *Ropalidia* wasps (Downing & Jeanne 1985; Premnath *et al.* 1996). A similar situation may prevail in ants that have lost the queen caste (more than 100 species in the subfamily Ponerinae), because every worker is able to mate and lay fertilized eggs (Peeters 1991, 1993).

Our model species is the queenless ant *Dinoponera quadriceps*, the world's largest ant. All workers are morpho-logically equivalent in each colony (89 ± 50 workers, $n = 27$ colonies) and reproduction is regulated by a near-linear dominance hierarchy involving between five and ten workers (Monnin & Peeters 1999). Only the top-ranking worker ('alpha') can mate and reproduce; all other workers remain virgin (Monnin & Peeters 1998). The highest-ranking workers can recognize one another's status. In particular, the alpha stands in front of the second-ranking worker ('beta') for prolonged periods of time, holding out her antennae on either side of the beta's head and preventing the latter from walking away ('blocking'); alphas performed 93.8% of 386 recorded blockings. When the beta manages to free herself she often attacks recently emerged workers, which have the highest probability of replacing her (Monnin & Peeters 1999).

Another dominance behaviour of *D. quadriceps* hints that a chemical signal is also important in reproductive regulation. The alpha grasps an antenna of a hierarchy member and rubs it against her own abdomen, which is momentarily bent forward (figure 1), possibly transmitting surface cuticular chemicals to the subordinate. Gas chromatographic and mass spectroscopic analysis of the long-chain hydrocarbons (C_{23} to C_{37}) present on the cuticle of individual workers ($n = 80$ from ten colonies) differing in reproductive status has revealed an unambiguous difference between alpha and infertile workers (Monnin *et al.* 1998). The monoene 9-C_{31}:1 (9-hentriacontene) is one of the major compounds on the cuticle of alphas but is almost absent in other individuals. Similarly, in bumble-bees and

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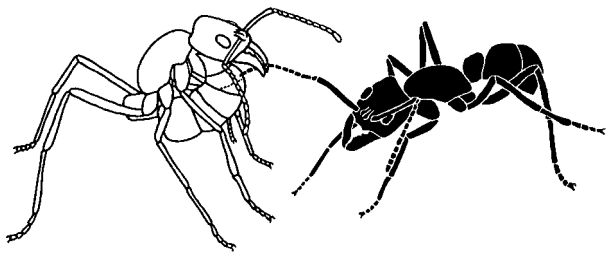†Present address: Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK.

Figure 1. Abdomen rubbing between nestmates in
D. quadriceps. A dominant worker (shown here in white) rubs
the tip of the antenna of a subordinate (shown in black)
against the intersegmental membranes, between her fifth and
sixth as well as her sixth and seventh abdominal segments.
There is no immediate reaction by the subordinate. (Each ant
is 3 cm long.)

Polistes wasps, breeders and sterile helpers have different
cuticular hydrocarbon profiles (Ayasse *et al.* 1995;
Bonavita-Cougourdan *et al.* 1991). We hypothesize that
some of the hydrocarbons on the cuticle have a communi-
catory function during the behavioural regulation of
reproduction.

In *D. quadriceps*, the beta worker can lay unfertilized
eggs infrequently (Monnin & Peeters 1997), and this
gives an opportunity to study the cuticular profile of
individuals with an intermediate level of ovarian activity.
Furthermore, the beta worker is most likely to replace an
ageing alpha (Monnin & Peeters 1999), and her ovarian
activity will then increase to levels typical of established
alphas. The recently developed technique of solid-phase
microextraction (SPME) (Arthur & Pawliszyn 1990)
permits the removal of chemicals from the cuticle of
insects without any effect on their survival, and this
allows the study of temporal changes in the hydrocarbon
profile of live individuals. Such a non-destructive tech-
nique is essential to show that the chemical difference
between breeders and helpers is directly related to
ovarian activity. *D. quadriceps* is especially well-suited to
this because the chemical difference between the alpha
egg-layer and her infertile nest-mates is expressed by a
single compound: the alpha yields approximately seven
times more 9-C_{31} on her cuticle than infertile workers
(Monnin *et al.* 1998). Using SPME and gas chromato-
graphy, we first determined the relative proportion of
9-C_{31} for beta individuals in the presence of an alpha.
Thereafter, we documented the change in 9-C_{31} when
the beta assumed alpha status after the experimental
removal of the established alpha. Information about
fertility could be a reliable basis for the antagonistic
interactions that determine the reproductive division of
labour in insect societies, including policing behaviour
by sterile individuals (Ratnieks 1988).

2. MATERIAL AND METHODS

All workers from eight colonies collected in Brazil (along the
road between Sambaiba and Tobias Barreto, Bahia state) in
January 1996 were individually marked. Antagonistic inter-
actions were recorded daily to assess the ranks of workers in the
dominance hierarchy, and these were dissected at the end of the
observations to determine their ovarian activity (Monnin &
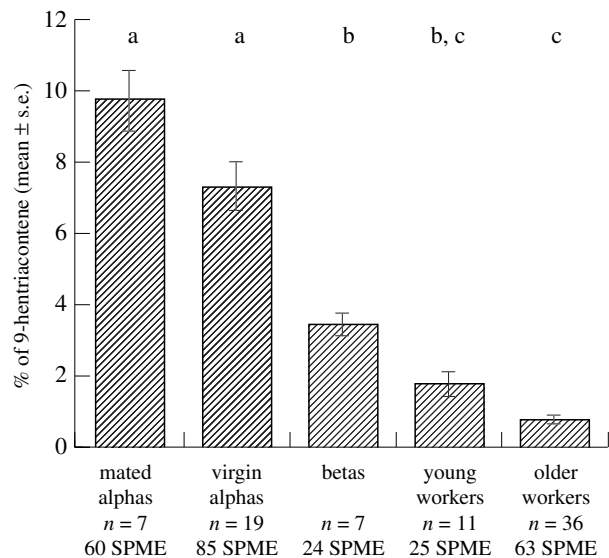Peeters 1997). When a new alpha becomes established, she can

Figure 2. Percentages of 9-hentriacontene present on the
cuticle of various functional categories of workers. Each
individual was measured by SPME over several weeks
(5.1 ± 0.7 measures per individual, mean ± s.e.), and the
mean values obtained were averaged for each category.
Tenure of alpha status was always superior to 14 days;
'young' workers are less than one month old. Categories not
significantly different (ANOVA: Tukey HSD for unequal
sample sizes) are denoted with identical letters.

mate only when foreign males visit the nests, but she begins to
lay eggs before then (Monnin & Peeters 1998). Counts of devel-
oping and mature oocytes revealed no difference between virgin
alphas and mated alphas (when mated, egg-laying workers are
called 'gamergates' in queenless ants), especially since the rate of
oviposition is low (one egg per day). Gamergates do not have
significantly different proportions of 9-C_{31} relative to virgin
alphas, but it tends to be higher (Monnin *et al.* 1998; figure 2).

The relative proportion of 9-C_{31} in betas ($n = 7$ workers, from
five colonies) was determined with a novel application of SPME.
A fused-silica fibre (Supelco^{TM}) coated with 7 μm polydimethyl-
siloxane was rubbed directly against the intersegmental
membranes of the abdomen (where the antenna of subordinate
workers is rubbed; figure 1) for 2 min, during which time
organic compounds are adsorbed directly into the stationary
phase. The fibre was then inserted into the injector port
(260 °C) of a gas chromatograph (HP 5890). The temperature
of the non-polar fused-silica capillary column (HP-5) was
programmed from 260 °C (isothermal for 15 min) to 300 °C at
5 °C min^{-1}. Integration of peak areas was done with HP Chem-
Station. Fourteen major peaks representing 39 long-chain
hydrocarbons identified by mass spectrometry, including 9-C_{31},
were selected for multivariate analysis (see Monnin *et al.* 1998).
Betas were sampled 3 ± 1 times (mean ± s.e., range 1–7) over a
period of 13 ± 4 days (mean ± s.e., range 0–31), and averages of
the relative proportions of 9-C_{31} were compared with those of
alpha and infertile workers determined previously (Monnin *et
al.* 1998) by using an ANOVA.

We removed the alpha ($n = 11$, in eight natural colonies or
experimental groups) and used SPME to measure the subse-
quent changes in the hydrocarbon profile of the high-ranking
worker that acceded to alpha status. The replacement alphas
were measured 7 ± 1 times (mean ± s.e., range 3–15) over a
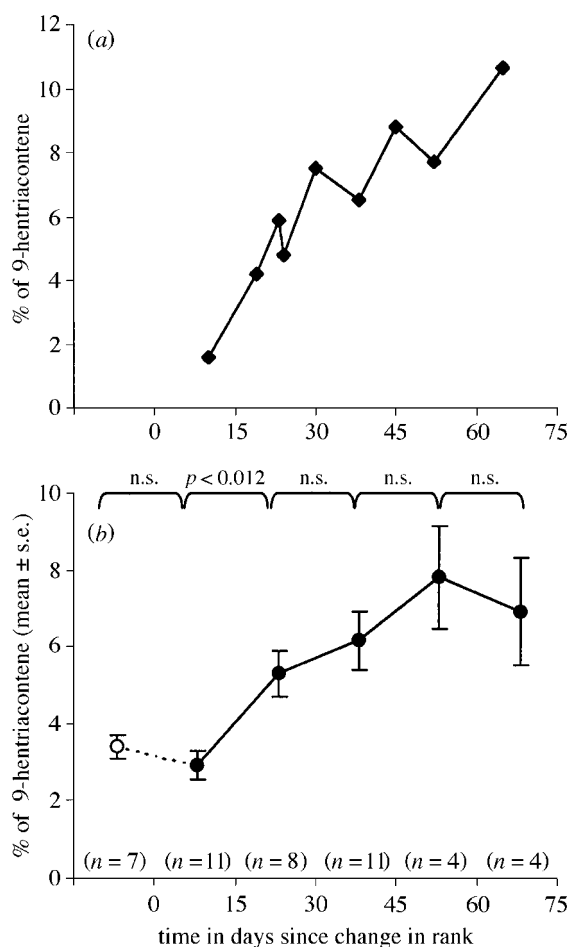period of 54 ± 13 days (mean ± s.e., range 19–147), with the first

Figure 3. Temporal change in the percentage of 9-hentriacontene present on the cuticle of beta workers that acceded to the alpha rank. (*a*) Example of worker G4/25; (*b*) eleven individuals (filled circles) were repeatedly measured over several weeks. Decreasing sample sizes in consecutive data points reflect mortality and experimental removal. Values for alphas are compared between two consecutive periods of time (Wilcoxon matched-pairs test). Independent values for betas (open circle) are added for comparison (Mann–Whitney *U*-test).

measure done 7 ± 1 days after the removal of the alpha (mean $\pm$ s.e., range 1–14). To analyse the changes in 9-C_{31} over time, the relative proportion of this chemical was averaged for each worker over periods of two weeks, and these values were compared between two consecutive periods of time with a Wilcoxon matched-pairs test.

3. RESULTS

Betas had half as much 9-C_{31} as virgin alphas, and four times as much as infertile workers aged more than one month (figure 2; $p < 0.001$ and $p < 0.05$, respectively). Betas did not differ significantly from young infertile workers of less than one month old, but multivariate analysis previously indicated that the latter have a distinct cuticular hydrocarbon profile (Monnin *et al*. 1998). The intermediate level of 9-C_{31} found in betas is meaningful: dissections indicated that alphas ($n = 13$) had 2.9 ± 0.4 mature oocytes compared with 0.8 ± 0.3 mature oocytes in betas ($n = 11$) ($p < 0.001$, Mann–Whitney *U*-test), and

betas laid only 18 out of 129 eggs recorded in 15 colonies (Monnin & Peeters 1997).

When the alpha was experimentally removed, she was replaced by a beta or by a gamma ($n = 9$ and 2, respectively). (A gamma worker is third ranking in the hierarchy.) Repeated SPME measures of the replacement alpha over several weeks showed a clear increase in her level of 9-C_{31} (figure 3). This increase was significant between the second and fourth weeks following the removal of the alpha ($p < 0.05$, Wilcoxon matched-pairs test). 9-C_{31} continued to increase over the following weeks, and after six to eight weeks attained levels similar to those of established virgin reproductives (see figure 2).

4. DISCUSSION

In the queenless ant *D. quadriceps*, dominance ranks are reflected in clear differences in ovarian activity between alpha, beta and other subordinate workers. Our chemical analyses of cuticular hydrocarbons showed a compelling correlation between the relative proportion of 9-C_{31} and egg-laying behaviour (figure 2). This pattern was confirmed by the increase in 9-C_{31} on the high-ranking worker who assumed alpha status after the experimental removal of the established alpha (figure 3). It takes six to eight weeks for the replacement alpha to exhibit a relative proportion of 9-C_{31} similar to that of an established alpha (figure 3), and this may correspond to the delay needed for the ovaries to become fully developed. This first study in live insects of the temporal changes of a cuticular molecule has been made possible by the non-destructive technique of SPME.

Our data strongly suggest a relationship between ovarian activity and production of a long-chain hydrocarbon. Such a relationship exists in some solitary insects (such as Diptera), where the onset of vitellogenesis parallels the synthesis of various cuticular hydrocarbons that function as sex pheromones (see, for example, Dillwith *et al*. 1983). This synthesis is correlated with changes in hormonal titres in the hemolymph (Trabalon *et al*. 1990). Similarly, in both *Polistes dominulus* and *Bombus hypnorum*, pentane rinses of the cuticle revealed that the alpha egg layer differed in the relative proportions of several long-chain hydrocarbons relative to sterile subordinates (Bonavita-Cougourdan *et al*. 1991; Ayasse *et al*. 1995). Thus, several insects investigated exhibit a basic relationship between ovarian physiology (including its endocrine mediators) and cuticular hydrocarbons.

In *D. quadriceps*, high levels of 9-C_{31} were characteristic of different alphas from unrelated colonies. We suggest that this cue yields information about individual fecundity. It is necessary to test empirically whether 9-C_{31} induces a specific behavioural change in ants that are exposed to it, but such response is highly context dependent and influenced by the motivation of tested workers, thus complicating the design of a realistic bioassay. The frequent behaviour of abdomen rubbing (figure 1) is evidence for the involvement of hydrocarbons with low volatility (such as 9-C_{31}) in inter-individual interactions; direct antennal contact may enable enhanced perception of long-chain molecules.

Several authors have invoked chemical signalling of ovarian activity, but there has seldom been direct

evidence for it. Wilson (1971, p. 277) explicitly stated that wasps and bees 'with the greatest ovarian development recognize each other as rivals and display open hostility to each other', while Ratnieks (1988, p. 277) discussed that in orphaned honeybees, 'ovarian development can be detected by other workers, perhaps via subtle morphological or chemical changes'. Visscher & Dukas (1995) demonstrated such recognition in *Apis* and hypothesized that 'concomitants of ovarian development' with a communicative function exist. Such a fertility cue would exhibit 'anonymous' properties, i.e. it identifies the emitter as a member of a class but does not distinguish it from other instances of the same class (Hölldobler & Carlin 1987). A fertility cue fulfils the conditions for honesty (Keller & Nonacs 1993) because it indicates the level of oogenesis of individuals.

In *D. quadriceps*, the beta competes aggressively with younger workers over the replacement of the gamergate. High levels of $9\text{-}C_{31}$ were not a prerequisite for acceding to the alpha rank, because the replacement alpha became behaviourally distinct (i.e. performed frequent abdomen rubbing and 'blocking' directed at the new beta) within a few hours of replacing the gamergate (Monnin & Peeters 1999), that is before any modification in cuticular hydrocarbons. In established colonies, all workers are daughters of the singly mated gamergate, and subordinate workers gain equal fitness whichever high ranker becomes the alpha. The ability to win physical contests is generally a reliable correlate of the quality of participants (West-Eberhard 1979; Grafen 1990), and dominance interactions alone determine the identity of the replacement alpha.

Once the alpha is mated, however, it is in the interest of nest-mates to prevent the beta from replacing her, even though the beta may be more vigorous than the gamergate. If a full-sister of high rank replaces the gamergate and mates with a foreign male, the majority of nest-mates lose inclusive fitness (Monnin & Peeters 1999). Thus, the beta and other colony members have conflicting interests over the production of future offspring, and the latter should favour the retention of the established egg layer (which is likely to be their mother) so long as her fecundity remains constant. Chemical information about oogenesis seems appropriate to regulate this situation, and it is in the interest of all colony members to keep this information honest. Importantly, the emission of cuticular hydrocarbons cannot be controlled, in contrast to the secretions of an exocrine gland with a reservoir. The alpha benefits from signalling her reproductive capacity. Any worker having the beta rank does not benefit from revealing her intermediate egg-laying ability, because she is always recognized by the alpha, which directs most of her interactions towards the beta (Monnin & Peeters 1999). However, the beta cannot cheat by producing a counterfeit signal to prevent detection of her increase in ovarian activity, because if she succeeds in acceding to the alpha rank, she will in turn benefit from advertising her newly gained ability. Experimental short-term removals of the alpha ($n = 6$) indicate that the beta becomes the victim of intense aggression from the alpha when the latter is re-introduced, which then leads to her immobilization by subordinates (Monnin & Peeters 1999).

Fertility cues could reliably underpin most antagonistic interactions that determine the reproductive division of labour in insect societies, especially policing behaviour by infertile individuals (Ratnieks 1988). Two independent components (aggressive ability and information about fertility) seem essential to ensure an evolutionarily stable regulation of sterility. Reproductive differentiation based on behavioural interactions presumably corresponds to the ancestral condition in social Hymenoptera, but queens influence the egg-laying activity of workers by olfactory means in many species (Passera 1980; Fletcher & Ross 1985; Bourke & Franks 1995; Seeley 1985; Vargo 1998). Our data on fertility cues suggest an origin for the evolution of this pheromonal signalling.

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