The Journal of Experimental Biology 207, 1085-1091
Published by The Company of Biologists 2004
doi:10.1242/jeb.00865

Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant *Pachycondyla inversa*

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Accepted 5 January 2004

Summary

Primitive ant societies, with their relatively simple social structure, provide an opportunity to explore the evolution of chemical communication, in particular of mechanisms underlying within-colony discrimination. In the same colony, slight differences in individual odours can be the basis for discrimination between different castes, classes of age and social status. There is some evidence from correlative studies that such inter-individual variation is associated with differences in reproductive status, but direct proof that certain chemical compounds are detected and recognized by ants is still lacking. In the ponerine ant *Pachycondyla inversa*, fertile queens and, in orphaned colonies, dominant egg-laying workers are characterized by the predominance of a branched hydrocarbon, 3,11-dimethylheptacosane (3,11-diMeC$_{27}$) on the cuticle. Using electroanntennography and gas chromatography with electroantennographic detection, we show that the antennae of *P. inversa* workers react to this key compound. 3,11-diMeC$_{27}$ is correlated with ovarian activity and, because it is detected, is likely to assume the role of a fertility signal reflecting the quality of the sender.

Key words: chemical communication, fertility signal, 3,11-dimethylheptacosane, electroantennographic detection, ant, *Pachycondyla inversa*.

Introduction

The study of mechanisms and purposes of communication is a fundamental issue of evolutionary biology. Different intentions of sender and receiver might result in an arms race where the sender benefits from cheating the receiver, who is less and less likely to react to unreliable signals (Dawkins and Krebs, 1978). Evolutionarily stable signals are supposed to be costly and to honestly reflect the characteristics of the sender (theory of 'honest signalling'; Zahavi, 1987; Grafen, 1990; reviewed in Johnstone, 1997). Reliability is therefore an important characteristic of signalling systems, even though not all signals are necessarily honest (Krebs and Dawkins, 1984; Dawkins and Guilford, 1991; Maynard Smith, 1994). Most animal societies are non-clonal and this gives rise to potential and actual conflicts over reproduction (Ratnieks and Reeve, 1992; Johnstone, 2000). Several studies have attempted to clarify the mechanisms of communication underlying the regulation of reproduction in social insects (e.g. Passera, 1984; Fletcher and Ross, 1985; Hölldobler and Bartz, 1985; Bourke, 1988). Pheromones produced by the queen appear to inhibit workers from producing their own sons from unfertilised eggs. There are several interpretations of the role of the queen's pheromone. Traditionally seen as a manipulative agent, the queen pheromone has more recently been interpreted as an honest signal of queen's fertility, thus workers benefit from refraining from reproduction (Seeley, 1979; Keller and Nonacs, 1993). Signalling of queen fertility can probably not be separated from queen recognition and is expressed as a variation in the pattern of volatile compound composition (Hölldobler and Michener, 1980).

The occurrence of pheromones that suppress the development of worker ovaries has been suggested for the queens of primitively eusocial bumble bees, such as *Bombus hypnorum* and the parasite *B. norvegicus* (Zimma et al., 2002; B. Zimma, personal observation). The nature of this putative primer pheromone has not been identified and its the source is still unclear (Bloch and Hefetz, 1999). Nevertheless, queens and workers show caste-specific volatile patterns (Ayasse et al., 1995, 1999) and dominant *Bombus hypnorum* workers, as characterized by behaviour and well developed ovaries, possess higher amounts of volatiles and special odour bouquets that may have a function as a recognition signal for fertility.

In highly eusocial honey bees, the queen mandibular pheromone (QMP) elicits several behavioural and physiological responses in workers and brood (cf. Blum, 1992; Winston and Slessor, 1998), including reproductive self-restraint. Recent work showed that additional glands might be involved in signalling the queen's presence and in the regulation of reproduction (cf. Katzav-Gozansky et al., 2002). However, due

to their large colony size, multiple mating of the queen and the highly complex self-organized division of labour, honey bees are probably not representative of the majority of eusocial insects. Ponerine ants stand on the other side of the range of advanced eusocial structures and give the opportunity to explore the evolution of chemical communication in such social systems. Recent studies focusing on within-colony discrimination have identified qualitative or quantitative differences in the profiles of cuticular hydrocarbons that are associated with age, castes and reproductive status. For example, in the queenless ant *Dinoponera quadriceps*, workers form linear dominance hierarchies, in which only the top-ranking individual (gamergate) reproduces. Reproductive and social status correlates with chemical differences: the top-ranking worker has a significantly higher amount of a single unsaturated cuticular hydrocarbon (9-$C_{31:1}$) than subordinates (Monnin et al., 1998; Peeters et al., 1999). Similar differences between the bouquets of reproductives and non-reproductives have been documented for several other ponerine ants (Liebig et al., 2000; Cuvillier-Hot et al., 2001, 2002) and for social wasps (Sledge et., 2001). At present, all these studies are merely correlative (but see Dietemann et al., 2003), and direct proof that certain compounds communicate fertility and are detected by the workers is still lacking. The question arises as to whether substances found to be correlated with reproductive status are indeed recognized and, if so, whether they are reliable signals reflecting the quality of the sender.

Our model system is the ponerine ant *Pachycondyla inversa*, a species with a clear morphological difference between queen and worker caste. Fertile queens and, in orphaned colonies, egg-laying workers of *P. inversa* are characterized by the predominance of 3,11-dimethylheptacosane (3,11-diMeC_{27}) on the cuticle (Heinze et al., 2002). We used chemical analyses, i.e. gas chromatography (GC) and GC with mass spectrometry (GC-MS), electroanntennography (EAG), GC with electroantennographic detection (GC-EAD) and dissection of the ovaries to clarify whether the high relative proportion of 3,11-diMeC_{27} in the cuticular bouquet of *P. inversa* mature queens and egg-laying workers is indeed detected by workers and whether it serves as an honest fertility signal.

Materials and methods

Ant collecting and housing

Colonies of *Pachycondyla inversa* Smith 1858 (cf. Lucas et al., 2002) belong to the subfamily Ponerinae. Colonies were collected in the field from knot-holes and rotten cocoa pods in an experimental cocoa plantation at Centro de Pesquisas do Cacau, CEPLAC, Itabuna, Bahia, Brazil in 2001 and 2002. In the laboratory, ants were housed in plastic boxes (19 cm × 9 cm × 9 cm) with a plaster floor, and one chamber (6 cm × 6 cm × 3 cm) serving as nest cavity. Colonies were kept under near natural conditions (27°C and 60% humidity, 12 h:12 h light:dark) and fed with diluted honey and cockroaches (*Nauphoeta cinerea*) three times per week. The plaster was regularly wetted.

Table 1. *Ovarian activity and status of fat body in individual mature queens (MQ), founding queens (FQ), virgin queens (VQ), reproductive workers (RW) and foraging workers (FW)*

Status	Total egg length (mm)	Corpora lutea	Fat bodies
Mature queen			
MQON	6	Dark orange	Orange/brown
MQ30	4.6	Yellow/orange	Yellow
MQ39	9.5	Orange	Cream/yellow
MQ18	5.8	Dark yellow	Cream/yellow
MQX1	3.4	Dark orange	Brown
MQB	7.9	Dark orange	Orange/brown
MQ71	3.5	Light yellow	White
MQ216	6.5	yellow	Yellow
MQ19	8	Dark orange	Orange/brown
MQ16	7.2	Yellow	Orange
Founding queen			
FQ4	2.7	Light yellow	White
FQ33	1.2	Light yellow	White
FQ28	3.5	Light yellow	Cream/yellow
Virgin queen			
VQ36	0	–	White
VQ30	0	–	White
VQ40	0	–	White
VQ 41	5	–	White
VQ19	0	–	White
Egg-laying worker			
RW1	6.4	Light yellow	Cream/yellow
RW2	9.3	Yellow	Yellow
RW3	6.2	Light yellow	Cream/yellow
Foraging worker			
FW4	0	–	Yellow
FW5	0	–	Yellow
FW6	0	–	Orange
FW7	0	–	Cream/yellow

Correlation between 3,11-diMeC_{27} and fertility

Mature queens from ten monogynous colonies of *P. inversa* were analysed. The cuticular profile of each queen was extracted by Solid Phase Micro Extraction (SPME), rubbing its gaster with a 7 μm polymethylsiloxane fiber (Supelco, Bellefonte, PA, USA) and analysed by gas chromatography (see Chemical analysis). For comparison, we similarly extracted cuticular hydrocarbons from three founding queens, five virgin queens and seven workers from queenless colonies (three egg-laying and four foraging workers) (Table 1). Ants were dissected the same day as the chemical analysis to assess the status of their ovaries. All developing eggs present in the ovarioles were counted and their longest length was measured under a stereomicroscope. The total egg length per individual was calculated summing the size of all developing eggs found in the ovarioles. Furthermore, we noted the coloration of the fat body, which darkens from white to brownish-orange with age, as in other ants (see Buschinger, 1968).

To estimate the amount of 3,11-diMeC$_{27}$ on the cuticle of the ants, we additionally extracted four mature queens from monogynous colonies and eight workers from queenless colonies (three egg-laying and five foraging workers) in 300 µl of pentane for 10 min. After evaporation of solvent, residues were redissolved in 50 µl of pentane containing an internal standard (n-C$_{18}$) and 2 µl of this solution was analysed by GC (see Chemical analysis).

Chemical analysis

The SPME fibre or the solvent extract was injected into a gas chromatograph (Agilent Technologies 6890N, Waldbronn, Germany) with a flame ionisation detector, equipped with a capillary column (Rtx-5; 30 m × 0.25 mm × 0.50 µm, Restek, Bellefonte, PA, USA). The injector was a *split-splitless* type, the carrier gas Helium (flow rate 1 ml min^{-1}), and the temperature was raised from 70°C to 180°C at 20° min^{-1} and from 180°C to 280°C at 4°C min^{-1}, then held at 280°C for 15 min. Compounds were identified from their mass spectra, which were produced by electron ionisation mass spectrometry using a Hewlett Packard (Palo Alto, CA, USA) 5890A gas chromatograph coupled to an HP 5917A mass selective detector (70 eV electron impact ionisation).

Synthesis of 3,11-diMeC$_{27}$

Commercially available 6-bromo-1-hexanol (Aldrich, Stanheim, Germany) was protected as the tetrahydropyranyl derivative and transformed into the Wittig salt by using triphenylphosphane. The Wittig reaction with 2-methylbutanal (Aldrich), catalytic hydrogenation of the reaction product, deprotection and Swern oxidation furnished 8-methyldecanal. In a parallel approach, commercial heptadecanol (Aldrich) was converted to the bromide and, subsequently, to the Wittig salt. This was methylated at the α-position by using butyl lithium and methyl iodide. The resulting Wittig salt was reacted with 8-methyldecanal (see above) to yield 3,11-dimethyl-10-heptacosene. The latter was hydrogenated to yield the target 3,11-dimethylheptacosane. In our hands, the two-step method, i.e. alkylating the Wittig salt of a primary bromide in α-position, gave much higher yields than a secondary bromide in a one-step approach (Fig. 1).

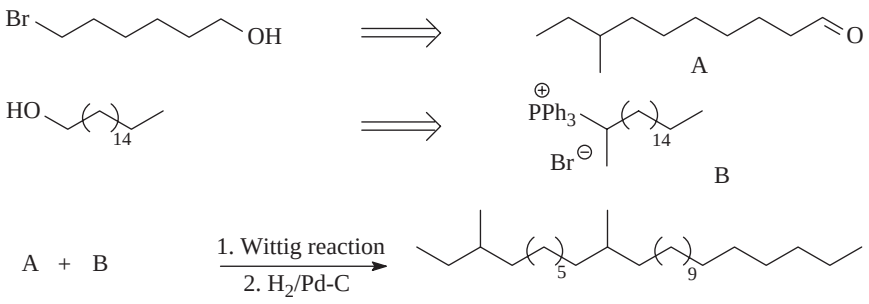

Fig. 1. Pathway for the synthesis of 3,11-dimethylheptacosane. PPh_3, triphenyl phosphonium group; Pd-C, catalyst consisting of palladium on charcoal.

Detection of 3,11-diMeC$_{27}$ by worker antennae

Gas chromatography with electroantennographic detection (GC-EAD)

The GC-EAD analyses were performed using a HP 6890 gas chromatograph (Hewlett-Packard) equipped with a DB5-MS column (30 m × 0.32 mm i.d., 0.25 µm film; J&W Scientific, Folsom, Ca, USA). The temperature was raised from 50°C to 310°C at a rate of 10°C min^{-1}, using helium as the carrier gas. A GC effluent splitter (split ratio 1:1) was used and the outlet was added to a purified and humidified airstream, directed over the excised antenna of *P. inversa* workers. The tip of the excised antenna was cut off and the antenna mounted between two glass electrodes filled with insect Ringer solution. The electrode holding the base was connected to a grounded Ag–AgCl wire, the electrode at the antenna's tip being connected *via* an interface box to a signal acquisition interface board (IDAC; Syntech, Hilversum, The Netherlands) for signal transfer to a PC. The responses of the flame ionization detector (FID) and the EAD signals were recorded simultaneously. The GC-EAD analyses were performed first with cuticular surface extracts of *P. inversa* queens and then with the synthetic compound 3,11-diMeC$_{27}$. To detect 'physiologically active' compounds and to discriminate electrophysiological responses from noise we performed 40 GC-EAD runs for each type of sample.

Electroantennography (EAG)

The antennae of *P. inversa* workers (*N*=16) were prepared as described above for GC-EAD trials. Antennal response was expressed as total depolarisation of the olfactory neurons in mV. Activity was amplified, recorded and analysed using software from Syntech Company. The testing solutions were applied on pieces of filter paper (Schleicher & Schuell, Dassel, Germany) introduced into Pasteur pipettes heated to approximately 50°C. The odour was applied by blowing a pulse of carbon-filtered humidified air (250 ml min^{-1}) generated by a mechanical stimulus air controller (Syntech Company) through the Pasteur pipette into a tube carrying a continuous stream of carbon-filtered humidified air over the antennal preparation. A recovery period of 30 s was allowed between each stimulus. We tested each antenna in the following order: air (filter paper without odour), solvent (pentane), pentane solutions of synthetic 3,11-diMeC27 (10 ng, 100 ng and 1000 ng) and 10 µl of *P. inversa* cuticular extract (one ant extracted in 300 µl of pentane for 10 min). Odour cartridges were replaced after the use of each antenna. For statistical analysis, the amplitude of the response to the six different samples was measured for each antenna. Data were normalized calculating the proportion (%) of each reaction over the total amplitude and analysed by Friedman repeated-measures analysis of variance (RM-ANOVA) and Wilcoxon test.

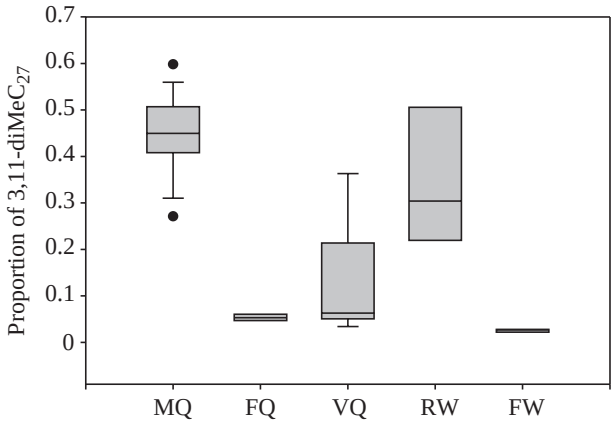

Fig. 2. Differences in the cuticular level of 3,11-diMeC27 among mature queens (MQ), founding queens (FQ), virgin queens (VQ), reproductive workers (RW) and foraging workers (FW). Box plots show median, 10th, 25th, 75th, 90th, percentiles. Datapoints that lie outside the 10th and 90th percentiles are shown as dots.

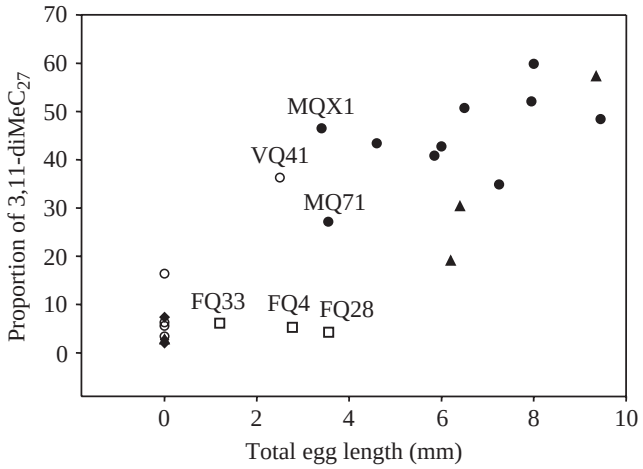

Fig. 3. Scattergram of relative amount of 3,11-diMeC$_{27}$ on the cuticle and total egg length (indicating ovarian activity; see Materials and methods) of mature queens (filled circles), founding queens (open squares), virgin queens (open circles), reproductive workers (triangles) and foraging workers (diamonds).

Results

Correlation between 3,11-diMeC$_{27}$ and fertility

Dissections confirmed that all mature and young founding queens were inseminated. Mature queens had dark-yellow/orange *corpora lutea*, indicating that they had been laying eggs (median total egg length=6.25 mm; range 3.4–9.45 mm). They also had dark-yellow fat bodies, suggesting they were not very young queens. Queen 71 appeared to be a young queen, not fully fertile (light yellow *corpora lutea* and white fat bodies). Queen X1 had very dark *corpora lutea* and a brownish fat body, indicating that she was an old reproductive queen with few developing eggs. Founding queens were young and produced few eggs (median total egg length=2.7 mm; range 1.2–3.55 mm). All but one (VQ41, total egg length=2.5) virgin queens had undeveloped ovaries and white fat bodies. Reproductive workers had developed ovaries (median total egg length=6.4 mm; range 6.2–9.35 mm). Ovaries were undeveloped in all foraging workers (Table 1).

The identification of the cuticular chemical profile of *P. inversa* is published elsewhere (Heinze et al., 2002). In the present study we focus attention on the variation of a particular unsaturated hydrocarbon: 3,11-diMeC$_{27}$ (Fig. 2). Mature queens contained the highest relative amount of 3,11-diMeC$_{27}$ on the cuticle (median 44.9%; range 27.1–59.8%). Founding queens had a considerably lower relative amount of the compound (median 5.3%; range 4.5–6.3%), as well as virgin queens with undeveloped ovaries (median 5.91%; range 3.3–16.3%). Fertile workers had high levels of 3,11-diMeC$_{27}$ on the cuticle (median 30.3%; range 19–57.2%), while foraging workers showed the lowest proportion of the compound (median 2.76%; range 2–7.4%). There is a strong positive correlation between the total egg length (sum of the length of eggs in the ovarioles) and the proportion of 3,11-diMeC$_{27}$ on an individual's cuticle (Spearman Rank correlation coefficient, Rs=0.82, P<0.001, Fig. 3).

Solvent extractions with internal standard allowed us to quantify the amount of 3,11-diMeC$_{27}$ on the ant cuticle. *P. inversa* queens are significantly larger than workers [thorax length of queens: 4.78±0.11 mm (mean ± S.D., N=8); of worker: 3.95±0.16 mm (N=10)]. Mature queens had a higher quantity of 3,11-diMeC$_{27}$ (median 5.15 μg; range 4.6–7.11 μg), while egg-laying workers had considerable less (median 0.69 μg; range 0.61–1.32 μg). Foraging workers showed a very low quantity of the compound (median 0.05 μg; range 0.02–0.09 μg).

Detection of 3,11-diMeC$_{27}$ by workers' antennae

Gas chromatography with electroantennographic detection (GC-EAD)

The antennae of *P. inversa* workers reacted positively to the cuticular extract of *P. inversa* queens. We received a strong response for 3,11-diMeC$_{27}$ (Fig. 4), indicating that this is a biological active compound that is detected by the workers' antennae. A similar reaction was elicited using synthetic 3,11-diMeC$_{27}$. There were 21 GC-EAD runs with a good baseline. We received a strong response for 3,11-diMeC27 in 11 runs and a weaker response in the other runs, indicating a good repeatability of the GC-EAD.

Five further compounds (linear and methyl-branched alkanes and two unidentified substances) showed an EAD signal in several GC-EAD runs. However, since they can be found in small amounts only, they did not produce strong and clearly visible EAD signals. GC-EAD and EAG runs with synthetic compounds in higher concentrations have to be performed to clearly reveal if those compounds are active.

Electroantennography (EAG)

The antenna of *P. inversa* workers reacted to the EAG

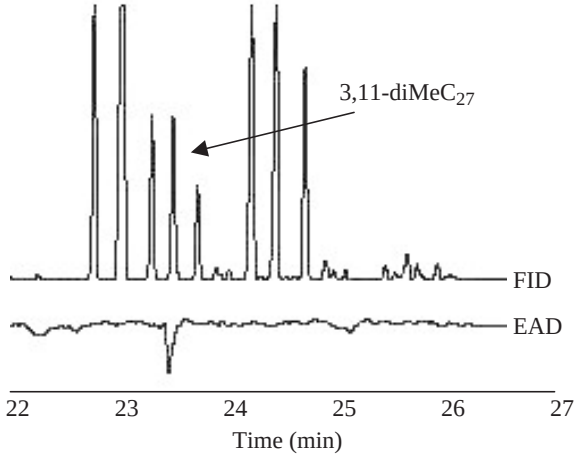

Fig. 4. Gas chromatograph recording with electroantennographic detection (GC-EAD) of the cuticular extract of a *P. inversa* queen using an antenna of a worker of *P. inversa* (for the identification of the cuticular chemical profile of *P. inversa*, see Heinze et al., 2002). FID, flame ionization detector.

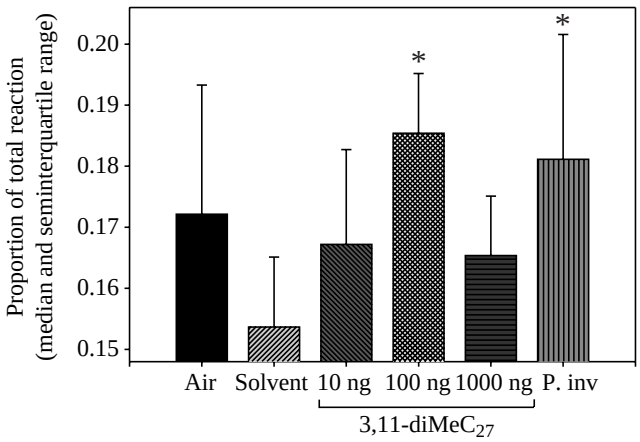

Fig. 5. Reaction of the workers' antennae to different odour samples (expressed as proportions of the total reaction, N=16 for each odour). Air, filter paper without odour; solvent, pentane, 10 ng, 100 ng and 1000 ng, pentane solutions of synthetic 3,11-diMeC$_{27}$, P. inv, cuticular extract of *P. inversa*. The response is significantly different (Friedman RM-ANOVA: Fr=16.42; P=0.006. *Significant differences (P<0.05), Wilcoxon test).

stimulation with the six different samples. Friedman analysis of variance indicates that the differences among the treatment groups are statistically significant (Friedman ANOVA, Fr=16.42; P=0.006, Fig. 5). Pair-wise comparison showed that only the solution containing 100 ng of 3,11-diMeC$_{27}$ and the *P. inversa* cuticular extract elicited a response significantly larger than the other treatments (Fig. 5). This indicates that there is probably a threshold for the amount of 3,11-diMeC$_{27}$ to be detected, since the solution containing 10 ng of the compound did not elicit a reaction different from that of the solvent or the air alone. On the other hand, a highly concentrated solution (with 1000 ng) of 3,11-diMeC$_{27}$ did not produce a stronger response, presumably because of habituation of the receptors.

Discussion

This study provides the first direct evidence that a chemical compound in the cuticular blend of an ant can be detected by its antennae. In GC-EAD trials, the antennae of *Pachycondyla inversa* workers reacted strongly to 3,11-diMeC$_{27}$, both when tested with natural queen's extracts and with the synthetic compound. Electroantennography confirmed that workers' antennae respond to 3,11-diMeC$_{27}$ and showed that this response varies with the concentration, suggesting a possible threshold level for 3,11-diMeC$_{27}$ detection. This branched hydrocarbon is characteristic of the cuticular blend of mature, fertile queens, where it can represent over 50% of the total hydrocarbon composition, but reaches very low levels on the cuticle of young founding and virgin queens. Egg-laying workers from queenless colonies possess a high proportion of 3,11-diMeC$_{27}$ (see also Heinze et al., 2002), but the absolute amount does not reach that of mature queens, perhaps due to the smaller body size of workers. We showed that the proportion of 3,11-diMeC$_{27}$ on the ant cuticle is positively correlated with ovary development and egg production. Our results are in line with recent correlative studies showing that ovarian activity affects cuticular compounds in other ponerine ants (Monnin et al., 1998; Peeters et al., 1999; Liebig et al., 2000; Cuvillier-Hot et al., 2001, 2002), in bumble bees (Ayasse et al., 1995) and social wasps (Sledge et al., 2001). This supports the hypothesis that 3,11-diMeC$_{27}$ is a 'pheromonal queen signal' (*sensu* Keller and Nonacs, 1993), from which workers can deduce reliable information about the productivity of the queen or a worker. Workers are probably also capable of quantitatively distinguishing between fertile, inseminated queens and fertile, uninseminated workers, as the latter have much smaller total amount of this substance on their body surface.

Workers need to assess the queen's fertility, since they benefit from refraining from reproduction and helping the queen (increasing their own inclusive fitness) only if it is mated and highly productive (Bourke, 1988). The reliability of cuticular levels of 3,11-diMeC$_{27}$ as a signal of fertility is based on its physiological connection with ovarian development. When there is a direct link between a signal and some underlying aspect of the signaller's condition, lying may be physically impossible. Physical constraint is the simplest mechanism for the maintenance of honestly, and its effectiveness was shown, for example, in birds displaying ornamental carotenoid colorations (e.g. Johnstone, 1997). A *P. inversa* queen or worker, with undeveloped ovaries, seems to simply be unable to produce high amount of 3,11-diMeC$_{27}$: virgin queens and foraging workers had undeveloped ovaries and very low levels of 3,11-diMeC$_{27}$. The only virgin queen producing eggs had a proportionally high level of the cuticular compound, confirming that this is not connected with the mating status. This suggests that workers might refrain from

reproducing in the presence of an unmated queen to whose offspring they would be less closely related than to their own offspring. Nothing is known about hierarchy formation and rank acquisition in colonies containing virgin queens and workers only. Virgin queens are possibly better egg-layers and also more capable of behavioural dominance than workers. Further studies are needed to investigate this point.

Social insects provide a suitable model system to investigate whether signals are honest (Zahavi, 1987; Grafen, 1990) or manipulative (Krebs and Dawkins, 1984). It has been argued that manipulative 'pheromonal queen control' has never been demonstrated in social insects and is difficult to explain evolutionarily (Keller and Nonacs, 1993). Nonetheless, in the case of *P. inversa*, the action of 3,11-diMeC_{27} as a physiological inhibitor of ovarian development (pheromonal control over reproduction) cannot be completely excluded. Intriguingly, in our study, the cuticular level of 3,11-diMeC_{27} in founding queens stays quite low, despite their capability of laying eggs. During the founding stage, queens do not need to communicate their fertility since the worker caste is absent. Moreover, in one old queen (QX1), egg-production appeared to have decreased, while the 3,11-diMeC_{27} level remained quite high. The fact that 3,11-diMeC_{27} is the cuticular compound producing the strongest reaction of workers' antennae, supports the persuasive hypothesis that it is a clear informative signal. The positive correlation with egg production, and its occurrence in egg-laying workers, suggests that it is an honest signal of fertility. Further studies are needed to clarify whether there is a possibility for manipulation in this signalling system. As pointed out by Keller and Nonacs (1993), worker reproduction should decrease with increased number of queens in a colony (polygyny). More queens would produce more pheromone and exhibit a greater control over worker reproduction. Alternatively, if the pheromone is only an informative signal, the number of queens should not affect worker reproduction. *P. inversa* is facultatively polygynous, thus suitable for testing this hypothesis. Occasional worker reproduction is likely to occur in queenright colonies of *P. inversa* since there is evidence for worker policing by egg-eating (cf. Ratnieks, 1988). Workers of *P. inversa* are able to discriminate between worker-laid eggs and queen-laid eggs. In a queenright discriminator colony, workers eliminate eggs laid by non-nestmate workers but care for eggs laid by non-nestmate queens (P. D'Ettorre, J. Heinze and F. L. W. Ratnieks, personal observation). Worker-laid eggs and queen-laid eggs have a different chemical signature, which may allow workers to discriminate between them. Queen-laid eggs possess a significantly higher amount of 3,11-diMeC_{27} on the surface than worker-laid eggs, probably due to a contamination from the cuticle to the egg-surface (cf. similar findings in *Dinoponera quadriceps*; Monnin and Peeters, 1997*)*. This is likely to be an example of the widespread pheromonal parsimony, with 3,11-diMeC_{27} serving as fertility signal and protecting queen-laid eggs from policing.

The authors were supported by the 'INSECTS' network of the Universities of Copenhagen, Firenze, Keele, Lausanne, Oulu, Regensburg, Sheffield and the ETH Zurich, financed by the European Commission *via* the Research Training Network established under the Improving Human Potential Programme. We are grateful to Jacques Delabie for his help in the field and to Andreas Böttcher, Gunda Dutzler, Raphaelo Fraberger, Tina Wanke and Bernhard Zimma for their assistance in the laboratory. David Morgan made helpful comments on earlier versions of the manuscript. Research was permitted by the Brazilian Minister of Science and Technology (RMX 004/02).

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