PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2010) **277**, 3793–3800
doi:10.1098/rspb.2010.0984
Published online 30 June 2010

Identification of an ant queen pheromone regulating worker sterility

Luke Holman[1,*], Charlotte G. Jørgensen[2,3], John Nielsen[2] and Patrizia d'Ettorre[1,†]

[1]*Centre for Social Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark*
[2]*Department of Life Sciences, Bioorganic Chemistry, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark*
[3]*Department of Medicinal Chemistry, University of Copenhagen, Jagtvej 162, 2100 Copenhagen, Denmark*

The selective forces that shape and maintain eusocial societies are an enduring puzzle in evolutionary biology. Ordinarily sterile workers can usually reproduce given the right conditions, so the factors regulating reproductive division of labour may provide insight into why eusociality has persisted over evolutionary time. Queen-produced pheromones that affect worker reproduction have been implicated in diverse taxa, including ants, termites, wasps and possibly mole rats, but to date have only been definitively identified in the honeybee. Using the black garden ant *Lasius niger*, we isolate the first sterility-regulating ant queen pheromone. The pheromone is a cuticular hydrocarbon that comprises the majority of the chemical profile of queens and their eggs, and also affects worker behaviour, by reducing aggression towards objects bearing the pheromone. We further show that the pheromone elicits a strong response in worker antennae and that its production by queens is selectively reduced following an immune challenge. These results suggest that the pheromone has a central role in colony organization and support the hypothesis that worker sterility represents altruistic self-restraint in response to an honest quality signal.

Keywords: social insect; cuticular hydrocarbon; queen signal; *Lasius niger*; handicap

1. INTRODUCTION

Worker sterility is the defining feature of eusociality, and is therefore fundamental to any explanation of its evolutionary origin and maintenance. The degree to which worker sterility is driven by cooperation or conflict (Lehmann & Keller 2006; Ratnieks *et al.* 2006; Boomsma 2009) and individual- or colony-level selection (Keller 1999; Wilson & Hölldobler 2005; Okasha 2006) remain active areas of research. At the proximate level, the genetic (Grozinger *et al.* 2003; Nelson *et al.* 2007; Schwander & Keller 2008; Alaux *et al.* 2009; Wurm *et al.* 2010) and developmental (Khila & Abouheif 2008; Roat & Landim 2008; Johnson & Linksvayer 2010) bases of reproductive division of labour have been elucidated with increasing resolution, although the systems that determine when and why individuals relinquish sterility and switch to individual reproduction are less well understood.

Queen-produced pheromones that maintain worker sterility are thought to be taxonomically widespread, as queens, their eggs and queen-derived chemicals have been shown to reduce or eliminate worker reproduction, and because queens typically produce chemicals that are absent or minimally expressed in workers (e.g. Vargo 1992; Peeters *et al.* 1999; Dietemann *et al.* 2003; Cuvillier-Hot *et al.* 2004*a*; Endler *et al.* 2004; Monnin 2006; Dengler-Crish & Catania 2007; Korb *et al.* 2009; Bhadra *et al.* 2010). However, the honeybee is the only insect in which primer pheromones (i.e. pheromones with a physiological effect) have been definitively identified (Le Conte & Hefetz 2008), meaning that it is difficult to draw general conclusions about the factors regulating sterility. Queen pheromones underpin the proximate and ultimate causes of worker sterility: in the honeybee, they cause changes in worker gene expression (Grozinger *et al.* 2003; Beggs *et al.* 2007) and physiology (Kaatz *et al.* 1992; Beggs *et al.* 2007) that mediate the transition from indirect to individual reproduction, and they have been postulated to be either a manipulation that is detrimental to workers ('queen control') or a signal to which workers are selected to respond ('queen signal'; Keller & Nonacs 1993; Heinze & d'Ettorre 2009). Queen pheromones are also interesting because they are thought to be central to the colony's 'social physiology', the superorganismal analogue of regulatory mechanisms such as hormones (Johnson & Linksvayer 2010). Elucidating the identity, *modus operandi* and fitness consequences of queen pheromones in additional taxa is therefore likely to produce new insights into social evolution.

Here, we identify a multi-functional queen pheromone from the black garden ant *Lasius niger*. In a previous study, we found that the cuticular hydrocarbon 3-methylhentriacontane (3-MeC_{31}) was strongly correlated with queen productivity, maturity and likelihood

* Author for correspondence (lholman@bio.ku.dk).
† Present address: Laboratoire d'Ethologie Expérimentale et Comparée (LEEC), University of Paris 13, 99 Avenue Jean-Baptiste Clément, 93430 Villetaneuse, France.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2010.0984 or via http://rspb.royalsocietypublishing.org.

Received 8 May 2010
Accepted 9 June 2010

of avoiding execution by workers in colonies with supernumerary queens (Holman *et al.* 2010). The results of Holman *et al.* (2010) imply that 3-MeC_{31} is a signal of queen quality, and verbal models have suggested that the queen pheromones hypothesized to regulate reproductive division of labour are likely to be honest quality signals (Keller & Nonacs 1993; Zahavi & Zahavi 1997; Heinze & d'Ettorre 2009; van Zweden 2010). We therefore developed a novel synthetic pathway for 3-MeC_{31} in order to test whether it (i) affects worker ovarian activation, (ii) influences worker behaviour and (iii) is detectable by workers. We also quantified queens' chemical profiles after an experimental immune challenge, to assess whether 3-MeC_{31} could provide information on queen condition to workers. Lastly, we found that 3-MeC_{31} is abundant on the surface of queen-laid eggs, giving insight into its function and mode of action.

2. MATERIAL AND METHODS

(a) *Comparison of worker, queen and egg chemical profiles*

Cuticular hydrocarbons were extracted from *L. niger* queens ($n = 20$) and analysed as previously described (Holman *et al.* 2010). In short, ant cuticular hydrocarbons were extracted for 10 min in 150 µl pentane; the pentane was allowed to evaporate, and the extract was re-diluted in 60 µl pentane. We then injected 2 µl of extract into the GC-MS using an auto-sampler. Analysis of egg and worker surface hydrocarbons was the same except for the extraction and injection methods; 10 eggs or one worker were placed in a 200 µl glass insert and extracted for 3 or 10 min, respectively, in 20 µl pentane, 2 µl of which was then manually injected into the GC-MS ($n = 20$). Peak areas were analysed using multivariate statistics (using transformed data as in Holman *et al.* 2010) and univariate statistics (using proportion data and GLMs).

(b) *Synthetic cuticular hydrocarbons*

Synthesis of 3-MeC_{27} and 3-MeC_{31} is described in the electronic supplementary material. C_{29} and C_{31} were purchased from Sigma-Aldrich.

(c) *Effects of 3-MeC_{31} on worker ovarian activation and behaviour*

Lasius niger workers were collected from six wild colonies in Copenhagen, Denmark. Collected workers were divided into three equal groups, each of which was given a model queen made from the tip of a glass vial. Every 12 h for 37 days after collection, the model queen was removed, coated with 10 µl of a pentane solution of (i) 0.01 µg µl^{-1} 3-MeC_{31}, (ii) 0.01 µg µl^{-1} hentriacontane (C_{31}) or (iii) pentane only, and replaced once completely dry (blind, using a labelling code). The alkane C_{31} was chosen as a control hydrocarbon because it is also a queen-type cuticular hydrocarbon of *L. niger* (electronic supplementary material, table S1) and has the same chain length, but was previously found to be unrelated to queen productivity, maturity or survival (Holman *et al.* 2010). Highly purified HPLC-grade pentane (Sigma-Aldrich) was used throughout.

After 37 days, all colonies were frozen for dissection. Ovarian activation was scored on a scale of 1–4: (1) completely empty; (2) one or two very small eggs and/or developing nurse cell material; (3) one to three developing eggs in both ovarioles or large eggs in one ovariole; and (4) well-developed eggs in both ovarioles (blind, using a different labelling code to the behavioural observations). Production of males by workers occurs in natural colonies of *L. niger* (Fjerdingstad *et al.* 2002), although oviposition was not observed in our small laboratory colonies.

On days 3–37 of this experiment, we conducted 3 min of behavioural observations (blind to treatment) starting 10 s after the replacement of the model queen, with the aid of Ethолog v. 2.2.5 software (Ottoni 2000). We recorded aggression towards the model queen (duration of attack multiplied by number of workers attacking) and the number of aggressive worker–worker interactions. Ovary and behavioural data were analysed with quasi-Poisson GLMMs with colony (and observation day, for the behavioural observations) as a random factor, in order to account for non-normal errors, overdispersion and within-colony similarity.

(d) *Electroantennography of synthetic hydrocarbons*

We collected workers from a wild colony and used them within 6 h in electroantennography (EAG) trials (protocol adapted from d'Ettorre *et al.* 2004). The left antennal flagellum was excised ($n = 25$ workers) and mounted between two pulled glass capillaries containing insect Ringer, which bathed two Ag–AgCl electrodes. The electrode holding the proximal end of the flagellum was connected to a ground wire, while the other was connected via an amplifier to a signal acquisition interface board (IDAC; Syntech, Hilversum, The Netherlands) for signal transfer to a PC. The antenna was placed in a stream of purified, humidified air, and the amplitude of the depolarization response of the antennal neurons was recorded in millivolts (using EAG 2000 software; Syntech) following exposure to six different stimuli: a pentane control, and pentane solutions (all 0.5 µg µl^{-1}) of C_{29}, C_{31}, 3-MeC_{27} and 3-MeC_{31}, as well as a mixture containing all of these hydrocarbons. These hydrocarbons are all present on the cuticle of queen *L. niger* (electronic supplementary material, table S1), but all had a non-significant or weak (relative to 3-MeC_{31}) relationship with queen fertility, maturity and survival (Holman *et al.* 2010).

We placed 10 µl of hydrocarbon solution on a 5 × 15 mm piece of filter paper in a new Pasteur pipette heated to 70°C on a hotplate, and immediately blew a pulse of air through the pipette onto the flagellum. Before starting each run, we blew a single pulse of air onto the antenna to verify that it was responsive. The treatment order was randomized, and the experiment was conducted and analysed blind. Responses were standardized against the control for each antenna by setting the response to the pentane control as 100 and transforming the other treatments accordingly. The data were analysed using a GLMM with Gaussian errors and antenna as a random factor. There was a significant effect of order, such that the antenna tended to display a higher response to stimuli presented later in each replicate, so order was included as a covariate in the model ($t = 5.68$, $n = 25$, $p < 0.0001$).

(e) *Effect of immune challenge on production of 3-MeC_{31} by queens*

Lasius niger queens were collected during a mating flight in Copenhagen and allowed to mature and rear workers in the laboratory for 201 days. To administer an immune challenge, we starved queens for 24 h (Moret & Schmid-Hempel 2000)

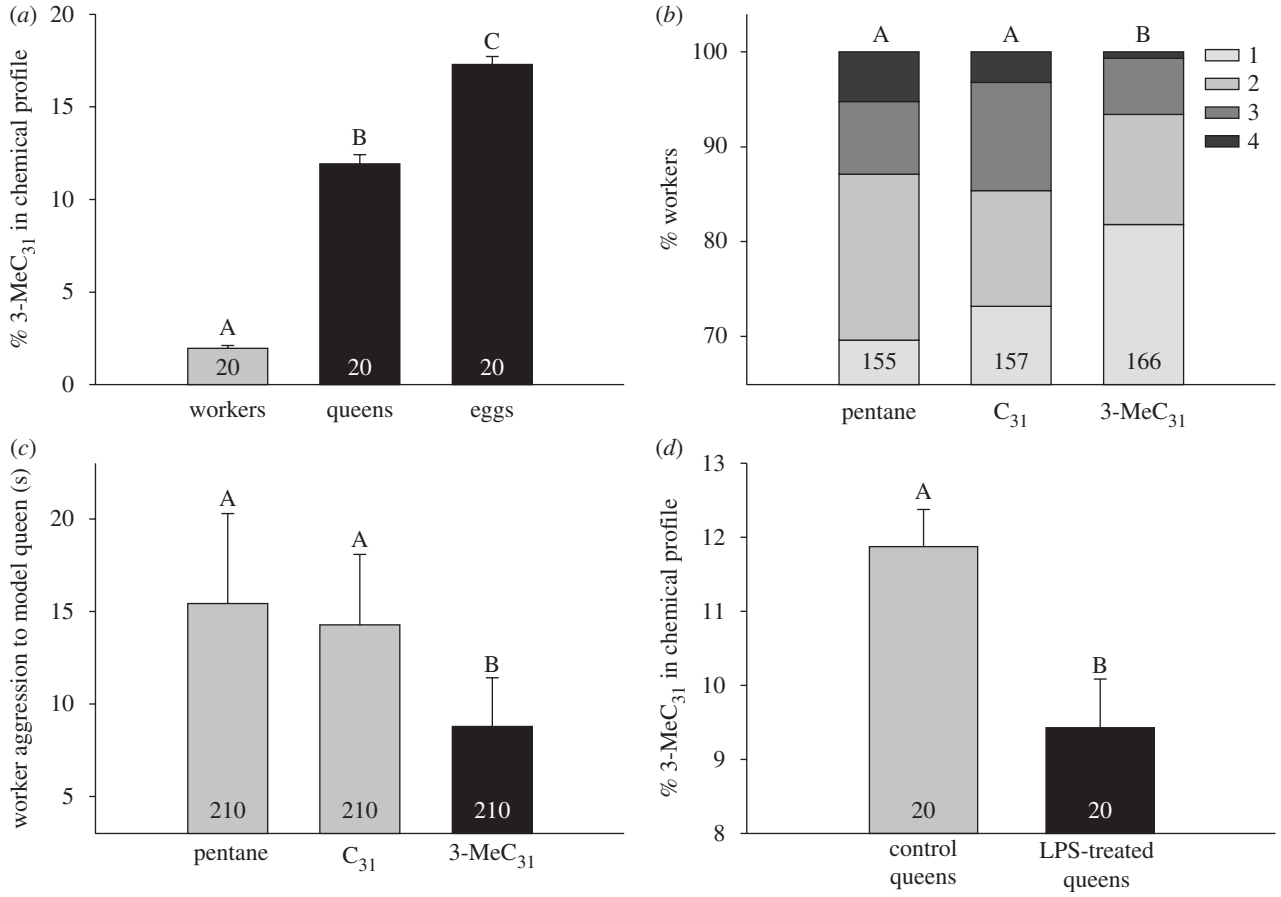

Figure 1. 3-MeC_{31} is a condition-dependent queen pheromone that affects worker physiology and behaviour in *L. niger*. (*a*) The cuticular hydrocarbon profile of queens contains a 6 times higher proportion of 3-MeC_{31} than that of a worker, while the egg profile has 9 times more; see also electronic supplementary material, table S1. (*b*) Supplementation of queenless groups of workers with synthetic 3-MeC_{31} negatively affected ovarian activation relative to controls. Bars show the frequency distributions of ovary activation on a categorical scale from 1 (no activation) to 4 (highest activation). (*c*) Glass model queens coated with 3-MeC_{31} were attacked by workers less often than controls. (*d*) A lipopolysaccharide immune challenge reduced the proportion of 3-MeC_{31} on the cuticle of queens. (*a*,*c*,*d*) Means $\pm$ 1 s.e.; shared letters indicate that two groups are not significantly different. Sample size is shown inside the bars.

and then pierced their inter-pleural membranes using a sterilized pin coated with either 2.5 $\mu g\ \mu l^{-1}$ lipopolysaccharide (Sigma-Aldrich) in sterile Ringer, or Ringer alone (blind and randomized). Queens were isolated from their colonies for 24 h then frozen for cuticular hydrocarbon analysis as described above; the peak areas were analysed blind. All statistical tests were performed in R v. 2.8.1 and validated using diagnostic plots.

3. RESULTS

(a) *3-MeC_{31} is a major component of queen and egg chemical profiles*

The cuticular hydrocarbon profiles of queens and workers were markedly different (discriminant analysis based on six principal components explaining 85% of the variance: Wilk's $\lambda = 0.03$, $F_{6,33} = 155$, $p < 0.0001$), with 3-MeC_{31} showing the strongest caste specificity (queens had a 6.1 times higher proportion than workers; figure 1*a*; electronic supplementary material, table S1) and being the most abundant single compound in the queen profile. 3-MeC_{31} was also the most abundant hydrocarbon on the surface of queen-laid eggs (figure 1*a*; electronic supplementary material, table S1).

(b) *Synthetic 3-MeC_{31} reduces worker ovarian activation and aggressive behaviour*

After 37 days of separation from the queen, worker ovarian activation was significantly lower in colony fragments that had been supplemented twice daily with synthetic 3-MeC_{31} rather than pentane solvent (figure 1*b*; GLMM: $t = 2.76$, $p = 0.006$, $n = 478$ workers) or the control hydrocarbon C_{31} ($t = 2.27$, $p = 0.024$). Ovarian activation did not differ between C_{31}- and pentane-treated workers ($t = 0.64$, $p = 0.52$). This experiment was replicated with workers from six colonies; there was no significant treatment $\times$ colony interaction term (F-test comparing models with colony fitted as a fixed factor: $F_{9,260} = 0.95$, $p = 0.47$), showing that the effect of 3-MeC_{31} was consistent across colonies (electronic supplementary material, figure S1). We therefore conclude that 3-MeC_{31} is a primer pheromone that negatively affects the activation of worker ovaries.

Workers frequently attacked the glass model queens to which we applied the hydrocarbon solutions. However, models coated with 3-MeC_{31} were attacked significantly less than those treated with C_{31} (GLMM: $t = 2.27$, $p = 0.001$) or pentane ($t = 5.83$, $p = 0.03$). The duration of attack did not differ between the pentane and C_{31}

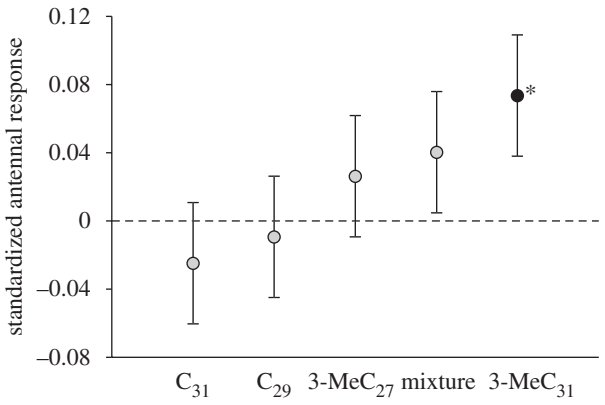

Figure 2. The queen pheromone 3-MeC_{31} elicits a strong response in worker antennae. Response of worker antennae ($n = 25$) to the five treatment solutions, relative to the pentane control. The plots show the estimated effect ± 1 s.e. from contrasts of a mixed model with antenna as a random factor and treatment order as a covariate; a value of zero indicates an antennal response equal to that of the control (pentane only) stimulus. Only 3-MeC_{31} produced a significantly higher (starred; $p < 0.05$) electrophysiological response than the control.

treatments ($t = 1.16$, $p = 0.25$) (figure 1*c*; $n = 630$ observations). We also recorded mild aggression among workers in the form of body-jerking threat displays, although the number of aggressive acts did not differ between treatment groups ($p > 0.1$; $n = 630$). There was therefore no evidence that the onset of worker ovarian activation was accompanied by increased worker–worker aggression as in some other social insects (e.g. Cuvillier-Hot *et al.* 2004*a*; Korb *et al.* 2009).

(c) *3-MeC_{31} elicits a strong electrophysiological response in worker antennae*

In EAG trials, 3-MeC_{31} induced a stronger response in excised worker antennae than the pentane control (figure 2; mixed-effects model with antenna as a random factor: $t = 2.04$, $n = 25$, $p = 0.043$), demonstrating that the queen pheromone is detected by worker antennae. None of the other synthetic hydrocarbons tested (C_{29}, C_{31} and 3-MeC_{27}) induced a significantly greater antennal response than the control (all $p > 0.46$). The response to 3-MeC_{31} was significantly higher than that to C_{29} ($t = 2.33$, $p = 0.048$) and C_{31} ($t = 2.75$, $p = 0.020$), and non-significantly higher than that to 3-MeC_{27} ($t = 1.31$, $p = 0.22$) and the mixture of all four hydrocarbons ($t = 0.92$, $p = 0.35$).

(d) *Immune challenge reduces the amount of 3-MeC_{31} present on queen cuticle*

The cuticular hydrocarbon profiles of queens subjected to a lipopolysaccharide immune challenge were significantly different from those of controls (discriminant analysis based on eight principal components explaining 86% of the variation; Wilk's $\lambda = 0.60$, $\chi^2_8 = 16.9$, $p = 0.031$), primarily because the mean proportion of the chemical profile composed of 3-MeC_{31} was 21 per cent lower in challenged queens (figure 1*d*; ANOVA: $t_{38} = 2.83$, $p = 0.007$). The proportions of all 29 of the other hydrocarbon peaks did not significantly change following immune challenge (all $p_{38} > 0.06$).

4. DISCUSSION

These experiments demonstrate that 3-MeC_{31}: (i) is a primer pheromone that negatively affects worker ovarian activation; (ii) is also a releaser pheromone (i.e. one that affects behaviour) that is perceptible to workers and influences aggressive behaviour; (iii) elicits a comparatively strong response in worker antennae, implying the presence of many olfactory receptor neurons sensitive to 3-MeC_{31}, consistent with its function as a pheromone; and (iv) displays condition-dependent expression. We also found that 3-MeC_{31} is the major component of the chemical profile of queen-laid eggs.

To our knowledge, 3-MeC_{31} is the first insect primer pheromone to be definitively identified outside of the honeybee (Le Conte & Hefetz 2008), and the first cuticular hydrocarbon demonstrated to affect conspecific reproductive physiology in any species. Primer pheromone activity of queen cuticular hydrocarbons is nevertheless likely to be common throughout the social insects. Differences in the cuticular hydrocarbon profiles of queens and workers have been reported in many species of ants, bees, wasps and termites (e.g. Peeters *et al.* 1999; Dietemann *et al.* 2003; Monnin 2006; Sramkova *et al.* 2008; Liebig *et al.* 2009; Peeters & Liebig 2009). Moreover, in *Camponotus floridanus* ants, workers in queenless colonies do not reproduce while queen-laid eggs are present (Endler *et al.* 2004); the eggs are coated with a hydrocarbon mixture similar to the cuticle of queens, consistent with regulation of worker sterility by one or more hydrocarbons. Similarly, queen corpses reduce the reproductive output of live queens in *Solenopsis invicta* ants (Vargo 1992), and in the wasps *Polistes gallicus* and *Ropalidia marginata* queens apparently prevent subordinate reproduction with chemicals from an abdominal gland (Dapporto *et al.* 2007) and Dufour's gland (Bhadra *et al.* 2010), respectively. Interestingly, *R. marginata* queens had more 3-MeC_{31} in Dufour's gland than did workers, suggesting that this compound may act as a primer pheromone in distantly related species. Cuticular hydrocarbons have also been shown to regulate reproduction indirectly through their role in 'worker policing': illegitimate reproductives are identified by their cuticular hydrocarbons and aggressed by their nest-mates (e.g. Peeters & Liebig 2009; Smith *et al.* 2009).

In honeybees, queen pheromones are detected via an odorant binding protein in workers' antennae (Wanner *et al.* 2007), leading to reductions in juvenile hormone titre (Kaatz *et al.* 1992), dopamine production (Beggs *et al.* 2007) and dopamine receptor gene expression (Beggs *et al.* 2007) that cause workers to remain sterile. Available data suggest that the physiological mechanisms by which 3-MeC_{31} affects worker reproduction in *L. niger* are probably very similar. Firstly, our results demonstrate that workers perceive 3-MeC_{31} via their antennae. A recent study of *L. niger* found a gene (Ln385_5) with worker-biased expression that encodes a homologue of the pheromone-binding protein ASP1 (Graff *et al.* 2007). This protein is found in the antennal olfactory sensillae of worker and drone honeybees, where it binds to queen pheromone (Danty *et al.* 1999), so it is possible that Ln385_5 is also involved in the perception of queen pheromones. Secondly, juvenile hormone also regulates ovarian activation in *L. niger*

(Sommer & Hölldobler 1995), and in *S. invicta* loss of the dominant queen affects the expression of genes that regulate juvenile hormone levels in subordinate queens, causing them to activate their ovaries (Wurm *et al.* 2010).

Our immune challenge experiment indicates that 3-MeC_{31} provides information about a queen's immune status or overall condition. This result implies that production of 3-MeC_{31} is physiologically costly relative to other cuticular hydrocarbons (assuming that pheromone production is always beneficial to queens), and therefore supports the prediction that queen pheromones should only be evolutionary stable when they honestly signal a queen's reproductive potential (Keller & Nonacs 1993), because costly traits act as handicaps that constrain dishonest signalling (Johnstone & Grafen 1993; Zahavi & Zahavi 1997; Heinze & d'Ettorre 2009; van Zweden 2010). Several other lines of evidence suggest that putative social insect queen pheromones are handicaps. Queen-specific cuticular hydrocarbons are typically methylated alkanes or alkenes, which are thought to confer inferior protection against desiccation compared with the hypothetically ancestral compounds, linear alkanes (Monnin 2006; Le Conte & Hefetz 2008). Also, reproductive development in insects is correlated with hormone titres (Heinze & Schrempf 2008) as well as surface chemicals; hormones influence condition and survival, for example through effects on immune function (Rolff & Siva-Jothy 2002) and anti-oxidant activity (Heinze & Schrempf 2008). Therefore, the costs of pheromone synthesis might arise from the physiologically expensive hormone levels required for their production (as suggested for sexual signals; Folstad & Karter 1992), although further biochemical data are required to test this hypothesis. First steps in this direction have been achieved by studies supplementing queens with juvenile hormone analogues, which suppress reproduction; hormone treatment was associated with a reduction in reproductive-like chemicals in queenless ants (Cuvillier-Hot *et al.* 2004*b*), but not in honeybees (Malka *et al.* 2009). Cuticular hydrocarbons associated with reproductive activity can also attract aggression in certain contexts (e.g. when expressed by individuals with relatively low reproductive potential), and thereby incur costs (Peeters & Liebig 2009; Smith *et al.* 2009).

An alternative to the handicap hypothesis of honest queen pheromones is based on the 'index' concept (*sensu* Maynard Smith & Harper 2003); pheromone production might be inextricably linked to reproductive physiology (e.g. by shared dependence on common biosynthetic pathways), so that dishonest signalling is impossible (Heinze & d'Ettorre 2009; Smith *et al.* 2009; van Zweden 2010). Our immune challenge data are also consistent with this hypothesis; immune activation might have depressed queens' reproduction, which in turn lowered pheromone production. Future studies will need to investigate the costs, genetics and biochemistry of pheromone production in order to distinguish between these hypotheses.

As well as being abundant on the cuticle of queen *L. niger*, 3-MeC_{31} is the most plentiful hydrocarbon on the surface of queen-laid eggs, and is also found on cocoons (Holman *et al.* 2010). Being present on brood may increase the frequency with which workers encounter 3-MeC_{31}, which has very low volatility; ant brood has been shown to inhibit sexual production (Edwards 1987) and worker oviposition (Endler *et al.* 2004), suggesting that eggs are a means of distributing queen pheromones. Many *L. niger* cuticular hydrocarbons, including 3-MeC_{31}, are also present on the nest soil (Lenoir *et al.* 2009), which could be another mechanism of dispensing the signal. Another function of brood-borne 3-MeC_{31} may be the regulation of queen productivity via negative feedback. *Lasius niger* queen productivity was lower in the presence of brood and other queens (Holman *et al.* 2010), so it is possible that 3-MeC_{31} affects the reproductive state of queens as well as workers (although the dose–response curve of the two castes would probably be different). The presence of the queen pheromone on eggs may also contribute to ensuring that the pheromone is an honest signal of fertility, because fertile queens will produce more eggs and thereby expose workers to greater quantities of pheromone. Lastly, 3-MeC_{31} might serve as an egg-marking signal used by workers to decide which eggs to rear (e.g. Endler *et al.* 2006; van Zweden *et al.* 2009).

Together with previous work, our results show how condition-dependent queen pheromones could act as parsimonious 'master signals' at the centre of the colony's social physiology that quantitatively modulate multiple colony-level traits. If pheromone production declines with the condition of the queen (e.g. when the queen becomes old or ill), worker behaviour associated with the absence of the queen may be initiated before the queen dies. Where the same pheromones are present on brood (Endler *et al.* 2004; van Zweden *et al.* 2009), declining brood number may similarly contribute to these worker responses (Edwards 1987; Endler *et al.* 2004), as well as allowing queens to tune their reproductive rate to the current number of brood as mentioned above. If the queen pheromone also affects worker aggression (as implicated here and in several other ants; Vander Meer & Alonso 2002; Peeters & Liebig 2009; Smith *et al.* 2009; Moore & Liebig 2010; Wurm *et al.* 2010), the pheromone could also be used by the colony to decide who should reproduce. In *L. niger*, colonies are often co-founded by multiple queens, but only one queen survives after the first workers eclose (Sommer & Hölldobler 1995). A queen's likelihood of being spared execution by workers is correlated with the amount of 3-MeC_{31} on her cuticle, implying that workers use this chemical to selectively kill the least fertile queens (Holman *et al.* 2010). Queen-like hydrocarbons also facilitate identification and punishment of reproductive workers in the ant *Aphaenogaster cockerelli* (Smith *et al.* 2009) and are thought to signal reproductive rank in queenless ants with dominance hierarchies (Peeters & Liebig 2009). A queen-derived cue also modulates worker aggression and consequently adoption of new queens in *S. invicta* (Vander Meer & Alonso 2002), and chemicals from the queen's sting gland prevent subordinate queens from shedding their wings and becoming reproductive (Vargo 1997). In the honeybee, production of most queen pheromone components is lower in 'drone-producing' and virgin queens relative to fully fertile queens (Strauss *et al.* 2008), resulting in a reduced behavioural response by workers (Kocher *et al.* 2009), and pheromone quantity or quality deteriorates in old

queens (Butler 1957). In bumblebees, developing colonies reach a 'competition point' at which workers begin to reproduce, the timing of which is thought to depend on changes in queen-produced pheromone(s) (Alaux *et al*. 2007).

In the honeybee, a single queen-produced chemical (9-keto-2(E)-decenoic acid) induces near-complete worker sterility in bioassays (Kaatz *et al*. 1992); several other honeybee pheromones are known, but to our knowledge, there is little evidence that they directly affect worker sterility (although they may have an indirect effect via their positive chemotactic effect on workers; e.g. Hoover *et al*. 2003). Similarly, in termites, silencing one gene in the colony's queen induces worker behaviour characteristic of recently de-queened colonies (Korb *et al*. 2009). Furthermore, the chemical profiles of reproductives frequently only differ from sterile workers by a single compound, or a single family of hydrocarbons (reviewed in Monnin 2006). In the present study, we showed that 3-MeC_{31}, but not C_{31}, affects worker sterility, even though both compounds are characteristic of queens; 3-MeC_{31} was also active in isolation. Based on these data, we suggest that worker sterility might often be regulated by single- rather than multi-component pheromones, and that at present there is insufficient evidence to rule out either of these hypotheses in any species. Single-component queen pheromones imply a mutualistic model of the origin of eusociality characterized by minimal parent–offspring conflict (Boomsma 2009), because in a high-conflict scenario, an evolutionary arms race over reproductive rights is predicted (Keller & Nonacs 1993). This arms race is expected to be characterized by the evolution of resistance to the queen pheromone in subordinates, followed by elaboration of the pheromone (e.g. by adding more component chemicals) and restoration of its manipulative effects (Le Conte & Hefetz 2008; Heinze & d'Ettorre 2009). Under the low-conflict model, workers might instead co-opt a single, arbitrary chemical that honestly indicates the presence of a healthy reproductive as a regulatory mechanism for their self-imposed sterility. Identification of queen primer pheromones in other taxa may reveal universal evolutionary trends and produce unexpected advances in our understanding of the origin and maintenance of eusociality.

We are grateful to all members of the Center for Social Evolution, Copenhagen, for a stimulating work environment, and to J. J. Boomsma for comments on the manuscript. This work was supported by the Marie Curie Excellence grant CODICES (MEXT-CT-2004-014202) assigned to P.d'E. and by a Marie Curie Intra-European Fellowship to L.H. (no. 235403; CHEMDOC).

REFERENCES

Alaux, C., Boutot, M., Jaisson, P. & Hefetz, A. 2007 Reproductive plasticity in bumblebee workers (*Bombus terrestris*)—reversion from fertility to sterility under queen influence. *Behav. Ecol. Sociobiol.* **62**, 213–222. (doi:10.1007/s00265-007-0455-6)

Alaux, C. *et al.* 2009 Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc. Natl Acad. Sci. USA* **106**, 15 400–15 405. (doi:10.1073/pnas.0907043106)

Beggs, K. T., Glendining, K. A., Marechal, N. M., Vergoz, V., Nakamura, I., Slessor, K. N. & Mercer, A. R. 2007 Queen pheromone modulates brain dopamine function in worker honey bees. *Proc. Natl Acad. Sci. USA* **104**, 2460–2464. (doi:10.1073/pnas.0608224104)

Bhadra, A., Mitra, A., Deshpande, S., Chandrasekhar, K., Naik, D., Hefetz, A. & Gadagkar, R. 2010 Regulation of reproduction in the primitively eusocial wasp *Ropalidia marginata*: on the trail of the queen pheromone. *J. Chem. Ecol.* **36**, 424–431. (doi:10.1007/s10886-010-9770-x)

Boomsma, J. J. 2009 Lifetime monogamy and the evolution of eusociality. *Phil. Trans. R. Soc. B* **364**, 3191–3207. (doi:10.1098/rstb.2009.0101)

Butler, C. 1957 The process of queen supersedure in colonies of honeybees (*Apis mellifera* Linn.). *Insect. Soc.* **4**, 211–223. (doi:10.1007/BF02222154)

Cuvillier-Hot, V., Lenoir, A., Crewe, R., Malosse, C. & Peeters, C. 2004*a* Fertility signalling and reproductive skew in queenless ants. *Anim. Behav.* **68**, 1209–1219.

Cuvillier-Hot, V., Lenoir, A. & Peeters, C. 2004*b* Reproductive monopoly enforced by sterile police workers in a queenless ant. *Behav. Ecol.* **15**, 970–975. (doi:10.1093/beheco/arh072)

Danty, E. *et al.* 1999 Cloning and expression of a queen pheromone-binding protein in the honeybee: an olfactory-specific, developmentally regulated protein. *J. Neurosci.* **19**, 7468–7475.

Dapporto, L., Santini, A., Dani, F. R. & Turillazzi, S. 2007 Workers of a *Polistes* paper wasp detect the presence of their queen by chemical cues. *Chem. Sens.* **32**, 795–802. (doi:10.1093/chemse/bjm047)

Dengler-Crish, C. M. & Catania, K. C. 2007 Phenotypic plasticity in female naked mole-rats after removal from reproductive suppression. *J. Exp. Biol.* **210**, 4351–4358. (doi:10.1242/jeb.009399)

d'Ettorre, P., Heinze, J., Schulz, C., Francke, W. & Ayasse, M. 2004 Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant *Pachycondyla inversa*. *J. Exp. Biol.* **207**, 1085–1091. (doi:10.1242/jeb.00865)

Dietemann, V., Peeters, C., Liebig, J., Thivet, V. & Hölldobler, B. 2003 Cuticular hydrocarbons mediate discrimination of repoductives and nonreproductives in the ant *Myrmecia gulosa*. *Proc. Natl Acad. Sci. USA* **100**, 10 341–10 346. (doi:10.1073/pnas.1834281100)

Edwards, J. P. 1987 Caste regulation in the pharaoh's ant *Monomorium pharaonis*: the influence of queens on the production of new sexual forms. *Physiol. Entomol.* **12**, 31–39. (doi:10.1111/j.1365-3032.1987.tb00721.x)

Endler, A., Liebig, J., Schmitt, T., Parker, J. E., Jones, G. R., Schreier, P. & Holldobler, B. 2004 Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. *Proc. Natl Acad. Sci. USA* **101**, 2945–2950. (doi:10.1073/pnas.0308447101)

Endler, A., Liebig, J. & Hölldobler, B. 2006 Queen fertility, egg marking and colony size in the ant *Camponotus floridanus*. *Behav. Ecol. Sociobiol.* **59**, 1–10.

Fjerdingstad, E. J., Gertsch, P. J. & Keller, L. 2002 Why do some social insect queens mate with several males? Testing the sex-ratio manipulation hypothesis in *Lasius niger*. *Evolution* **56**, 553–562.

Folstad, I. & Karter, A. J. 1992 Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**, 603–622. (doi:10.1086/285346)

Graff, J., Jemielity, S., Parker, J. D., Parker, K. M. & Keller, L. 2007 Differential gene expression between adult queens and workers in the ant *Lasius niger*. *Mol. Ecol.* **16**, 675–683. (doi:10.1111/j.1365-294X.2007.03162.x)

Grozinger, C. M., Sharabash, N. M., Whitfield, C. W. & Robinson, G. E. 2003 Pheromone-mediated gene expression in the honey bee brain. *Proc. Natl Acad. Sci. USA* **100**, 14 519–14 525. (doi:10.1073/pnas.2335884100)

Heinze, J. & d'Ettorre, P. 2009 Honest and dishonest communication in social Hymenoptera. *J. Exp. Biol.* **212**, 1775–1779. (doi:10.1242/jeb.015008)

Heinze, J. & Schrempf, A. 2008 Aging and reproduction in social insects—a mini-review. *Gerontology* **54**, 160–167. (doi:10.1159/000122472)

Holman, L., Dreier, S. & d'Ettorre, P. 2010 Selfish strategies and honest signalling: reproductive conflicts in ant queen associations. *Proc. R. Soc. B* **277**, 2007–2015. (doi:10.1098/rspb.2009.2311)

Hoover, S. E. R., Keeling, C. I., Winston, M. L. & Slessor, K. N. 2003 The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* **90**, 477–480. (doi:10.1007/s00114-003-0462-z)

Johnson, B. R. & Linksvayer, T. A. 2010 Deconstructing the superorganism: social physiology, groundplans, and sociogenomics. *Q. Rev. Biol.* **85**, 57–79.

Johnstone, R. A. & Grafen, A. 1993 Dishonesty and the handicap principle. *Anim. Behav.* **46**, 759–764. (doi:10.1006/anbe.1993.1253)

Kaatz, H.-H., Hildebrandt, H. & Engels, W. 1992 Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **162**, 588–592.

Keller, L. (ed.) 1999 *Levels of selection in evolution*. Princeton, NJ: Princeton University Press.

Keller, L. & Nonacs, P. 1993 The role of queen pheromones in social insects: queen control or queen signal? *Anim. Behav.* **45**, 787–794. (doi:10.1006/anbe.1993.1092)

Khila, A. & Abouheif, E. 2008 Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. *Proc. Natl Acad. Sci. USA* **105**, 17 884–17 889. (doi:10.1073/pnas.0807351105)

Kocher, S. D., Richard, F.-J., Tarpy, D. R. & Grozinger, C. M. 2009 Queen reproductive state modulates pheromone production and queen–worker interactions in honeybees. *Behav. Ecol.* **20**, 1007–1014. (doi:10.1093/beheco/arp090)

Korb, J., Weil, T., Hoffmann, K., Foster, K. R. & Rehli, M. 2009 A gene necessary for reproductive suppression in termites. *Science* **324**, 758. (doi:10.1126/science.1170660)

Le Conte, Y. & Hefetz, A. 2008 Primer pheromones in social Hymenoptera. *Annu. Rev. Entomol.* **53**, 523–542.

Lehmann, L. & Keller, L. 2006 The evolution of cooperation and altruism—a general framework and a classification of models. *J. Evol. Biol.* **19**, 1365–1376. (doi:10.1111/j.1420-9101.2006.01119.x)

Lenoir, A., Depickere, S., Devers, S., Christides, J. P. & Detrain, C. 2009 Hydrocarbons in the ant *Lasius niger*: from the cuticle to the nest and home range marking. *J. Chem. Ecol.* **35**, 913–921. (doi:10.1007/s10886-009-9669-6)

Liebig, J., Eliyahu, D. & Brent, C. S. 2009 Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. *Behav. Ecol. Sociobiol.* **63**, 1799–1807. (doi:10.1007/s00265-009-0807-5)

Malka, O., Katzav-Gozansky, T. & Hefetz, A. 2009 Uncoupling fertility from fertility-associated pheromones in worker honeybees (*Apis mellifera*). *J. Insect Physiol.* **55**, 205–209. (doi:10.1016/j.jinsphys.2008.11.002)

Maynard Smith, J. & Harper, D. 2003 *Animal signals*. Oxford, UK: Oxford University Press.

Monnin, T. 2006 Chemical recognition of reproductive status in social insects. *Ann. Zool. Fenn.* **43**, 531–549.

Moore, D. & Liebig, J. 2010 Mixed messages: fertility signaling interferes with nestmate recognition in the monogynous ant *Camponotus floridanus*. *Behav. Ecol. Sociobiol.* **64**, 1011–1018. (doi:10.1007/s00265-010-0916-1)

Moret, Y. & Schmid-Hempel, P. 2000 Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**, 1166–1168. (doi:10.1126/science.290.5494.1166)

Nelson, C. M., Ihle, K. E., Fondrk, M. K., Page, R. E. & Amdam, G. V. 2007 The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol.* **5**, 673–677.

Okasha, S. 2006 *Evolution and the levels of selection*. Oxford, UK: Oxford University Press.

Ottoni, E. B. 2000 EthoLog 2.2: A tool for the transcription and timing of behavior observation sessions. *Behavior Research Methods Instruments & Computers* **32**, 446–449.

Peeters, C. & Liebig, J. 2009 Fertility signaling as a general mechanism of regulating reproductive division of labor in ants. In *Organization of insect societies: from genome to socio-complexity* (eds J. Gadau & J. Fewell), Cambridge, MA: Harvard University Press.

Peeters, C., Monnin, T. & Malosse, C. 1999 Cuticular hydrocarbons correlated with reproductive status in a queenless ant. *Proc. R. Soc. Lond. B* **266**, 1323–1327. (doi:10.1098/rspb.1999.0782)

Ratnieks, F. L. W., Foster, K. R. & Wenseleers, T. 2006 Conflict resolution in insect societies. *Annu. Rev. Entomol.* **51**, 581–608. (doi:10.1146/annurev.ento.51.110104.151003)

Roat, T. C. & Landim, C. D. 2008 Temporal and morphological differences in post-embryonic differentiation of the mushroom bodies in the brain of workers, queens, and drones of *Apis mellifera* (Hymenoptera, Apidae). *Micron* **39**, 1171–1178. (doi:10.1016/j.micron.2008.05.004)

Rolff, J. & Siva-Jothy, M. T. 2002 Copulation corrupts immunity: a mechanism for a cost of mating in insects. *Proc. Natl Acad. Sci. USA* **99**, 9916–9918. (doi:10.1073/pnas.152271999)

Schwander, T. & Keller, L. 2008 Genetic compatibility affects queen and worker caste determination. *Science* **322**, 552. (doi:10.1126/science.1162590)

Smith, A. A., Hölldobler, B. & Liebig, J. 2009 Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. *Curr. Biol.* **19**, 78–81. (doi:10.1016/j.cub.2008.11.059)

Sommer, K. & Hölldobler, B. 1995 Colony founding by queen association and determinants of reduction in queen number in the ant *Lasius niger*. *Anim. Behav.* **50**, 287–294. (doi:10.1006/anbe.1995.0244)

Sramkova, A., Schulz, C., Twele, R., Francke, W. & Ayasse, M. 2008 Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). *Naturwissenschaften* **95**, 515–522. (doi:10.1007/s00114-008-0353-4)

Strauss, K., Scharpenberg, H., Crewe, R. M., Glahn, F., Foth, H. & Moritz, R. F. A. 2008 The role of the queen mandibular gland pheromone in honeybees (*Apis mellifera*): honest signal or suppressive agent? *Behav. Ecol. Sociobiol.* **62**, 1523–1531. (doi:10.1007/s00265-008-0581-9)

Vander Meer, R. K. & Alonso, L. E. 2002 Queen primer pheromone affects conspecific fire ant (*Solenopsis invicta*) aggression. *Behav. Ecol. Sociobiol.* **51**, 122–130.

van Zweden, J. S. 2010 The evolution of honest queen pheromones in insect societies. *Commun. Integr. Biol.* **3**, 50–52. (doi:10.4161/cib.3.1.9655)

van Zweden, J. S., Heinze, J., Boomsma, J. J. & d'Ettorre, P. 2009 Ant queen egg-marking signals: matching deceptive

laboratory simplicity with natural complexity. *PLoS ONE* **4**, e4718. (doi:10.1371/journal.pone.0004718)

Vargo, E. L. 1992 Mutual pheromonal inhibition among queens in polygyne colonies of the fire ant *Solenopsis invicta*. *Behav. Ecol. Sociobiol.* **31**, 205–210.

Vargo, E. L. 1997 Poison gland of queen fire ants (*Solenopsis invicta*) is the source of a primer pheromone. *Naturwissenschaften* **84**, 507–510. (doi:10.1007/s001140050435)

Wanner, K. W., Nichols, A. S., Walden, K. K. O., Brockmann, A., Luetje, C. W. & Robertson, H. M. 2007 A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proc. Natl Acad. Sci. USA* **104**, 14 383–14 388. (doi:10.1073/pnas.0705459104)

Wilson, E. O. & Hölldobler, B. 2005 Eusociality: origin and consequences. *Proc. Natl Acad. Sci. USA* **102**, 13 367–13 371. (doi:10.1073/pnas.0505858102)

Wurm, Y., Wang, J. & Keller, L. 2010 Changes in reproductive roles are associated with changes in gene expression in fire ant queens. *Mol. Ecol.* **19**, 1200–1211. (doi:10.1111/j.1365-294X.2010.04561.x)

Zahavi, A. & Zahavi, A. 1997 *The handicap principle: a missing piece of Darwin's puzzle*. New York, NY: Oxford University Press.