Are queen ants inhibited by their own pheromone? Regulation of productivity via negative feedback

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INTRODUCTION

The term “social physiology” describes the communication systems that facilitate group-level tasks in social organisms (Wilson and Hölldobler 1988; Seeley 1995; Johnson and Linksvayer 2010). Where physiology encompasses communication among and within the tissues of individuals, social physiology refers to information transfer among group members. In place of neurons and hormones, social physiology is analogous to physiology (Kaatz et al. 1992), and the expression of many genes (Grozinger et al. 2003) and allowing identification of fertile individuals (Adams and Balas 1999; Dietemann et al. 2003; Cuvillier-Hot et al. 2004a, 2004b; Smith et al. 2009). Queen pheromones have also been found to affect learning (Vergoz et al. 2007), physiology (Kaatz et al. 1992), and the expression of many genes (Grozinger et al. 2003) in worker honey bees (Apis mellifera). The multifaceted function of queen pheromones suggests that they comprise the sociophysiological equivalent of vertebrate sex hormones or insect juvenile hormone, which simultaneously affect diverse processes including development, immunity, and behavior (e.g., Robinson and Vargo 1997; Tillman et al. 1999; Rolff and Siva-Jothy 2002; Schroderus et al. 2010; Vinkler and Albrecht 2010).

One largely unexplored aspect of queen pheromones is their effect on queens themselves. Some social insect colonies contain multiple reproductives, which have been found to respond behaviorally and physiologically to one another’s presence, implying that queen pheromones affect queens (e.g., Vargo 1992; Cuvillier-Hot et al. 2004a; Cherón et al. 2009). For example, some colonies of Solenopsis invicta fire ants contain a single fertile queen and multiple unproductive virgin queens. Removal of the fertile queen stimulates pronounced changes in gene expression in the others, which rapidly shed their wings and activate their ovaries (Wurm et al. 2010), implying that 1 or more chemicals produced by fertile queens inhibit reproductive development. A particularly strong demonstration that queens respond to queen pheromones was provided by a recent study of Reticulitermes speratus...
termites, which showed that synthetic queen pheromone inhibits oviposition in queens (Yamamoto and Matsura 2011). Queen pheromones are also present on queen-laid eggs in a number of species (e.g., Endler et al. 2004; Holman et al. 2010b; Yamamoto and Matsura 2011), suggesting that queens are exposed to external queen pheromone sources even in colonies with a single reproductive. However, it is also possible that queens are fully or partially immune to queen pheromones, which would imply caste-specific perception or processing of chemical messages.

Here, we experimentally test whether a synthetic queen pheromone affects oogenesis and brood production in Lasius niger ant queens. The cuticular hydrocarbon 3-methylhentriacontane (hereafter 3-MeC₃₁) makes up a large proportion of the queen chemical profile in this species and has previously been shown to inhibit ovarian activation and aggression in workers and to covary with queen fertility, immunological status, and juvenile hormone titer (Holman et al. 2010a, 2010b; Holman 2012). Moreover, 3-MeC₃₁ is present on the surface of queen-laid eggs and pupae; experimental addition of extra queens or pupae to developing L. niger colonies caused a decline in queen productivity, whereas removal of pupae caused an increase (Holman et al. 2010a, 2010b). We therefore hypothesized that 3-MeC₃₁ may inhibit egg production in queens just as it does in workers. Diminished oviposition in response to brood and other queens could explain the observed effects on productivity (Holman et al. 2010a) because eggs are the sole food source of developing larvae in incipient colonies and because queens do not forage. In addition to the pheromone bioassay, we compared the hydrocarbon profiles of brood and adults and found that both queens and brood are characterized by high quantities of the queen pheromone 3-MeC₃₁ and other 3-methylalkanes relative to workers.

METHODS

Effect of synthetic 3-MeC₃₁ on queen ovarian activation and colony growth

Mated, dealate queens were collected during a mating flight on 17 July 2009 in Copenhagen, Denmark, housed immediately in individual plastic cylinders (26 × 38 mm) and kept at room temperature. Queens were given a ball of moist cotton but no food, mimicking natural “claustral” colony foundation. All queens were also provided with a plastic dummy of a similar size to a brood pile, made from a 4-mm piece of the tip of a 2-mL microcentrifuge tube, to which 10 µL of hydrocarbon solution was applied every 24 ± 0.5 h. The 3 hydrocarbon treatments were (1) pentane (HPLC grade; Sigma-Aldrich), (2) hentriacontane (n-C₃₁; Sigma-Aldrich) dissolved in pentane (0.01 µg/µL), and (3) 3-MeC₃₁ dissolved in pentane (0.01 µg/µL; synthesized as described in Holman et al. 2010b). Although n-C₃₁ has the same carbon chain length as 3-MeC₃₁ and is also abundant on queen cuticle, previous experiments using workers found no evidence that it is also a pheromone (Holman et al. 2010b). When applying hydrocarbon treatments, the dummy was carefully removed with pentane-cleaned forceps, treated with hydrocarbon solution, and replaced once the pentane solvent had evaporated. The dummy was placed away from the brood pile to avoid damaging or applying hydrocarbons to the latter, at a distance of approximately 20 mm.

The number of each brood type was scored on days 10, 20, and 28 after the mating flight in order to assess the rate of colony growth in the 3 treatments. Brood were scored as 1 of 5 classifications, namely eggs, small (instar 1), medium (instar 2–3) and large (instar 4) larvae, and pupae. Brood were counted using a binocular microscope without disturbing the colony. After the experiment, queens were freeze-killed and dissected in order to quantify the degree of ovarian activation; this was accomplished by counting the number of fully developed eggs (those possessing a fully developed chorion) present in the ovaries. Four queens did not survive until day 28 and were excluded, giving a final sample size of 59 colonies (pentane = 19, n-C₃₁ = 20, and 3-MeC₃₁ = 20). We also quantified body size as the distance between queens’ eyes (measured from digital photographs taken at ×40 magnification) in order to control for the possible effect of body size on fecundity. Queens were randomly allocated to treatments, and the hydrocarbon application, dissections, brood counts, and head measurements were performed blind to treatment.

Statistical analyses were performed in R 2.15.0 (www.r-project.org). The composition of the brood pile represents multivariate data, so the effects of hydrocarbon treatment and time on the brood pile were analyzed by first reducing the dimensionality of the brood count data set using nonmetric multidimensional scaling (NMDS) implemented with the “vegan” package for R. Prior to NMDS, we computed the Bray–Curtis distance matrix of the brood pile data. The NMDS scores were then used as response variables in linear mixed models with queen ID as a random factor (to account for the 3 repeated measures per colony) and treatment, time, queen head size, and the treatment × time interaction as fixed factors. We also performed a similar analysis with the more widely used principal component analysis, which gave qualitatively identical results (not shown), but we here present the NMDS results as this method is more robust for use with count data. Treatment levels were compared using contrasts, and time of sampling (i.e., 10, 20, and 28 days postmating) was treated as a continuous variable. The day-28 data were also examined with univariate analyses, namely generalized linear models (GLM) with Poisson errors, or quasi-Poisson errors if the data were overdispersed. The aim of the univariate analyses was to clarify which brood types varied most between treatment groups and thereby aid interpretation of the multivariate analysis.

Chemical analysis of L. niger larvae

Larvae were obtained from 1-year-old colonies of L. niger reared in the lab in Paris. Cuticular hydrocarbons were extracted from n = 9 samples, each containing 10 larvae placed in 20 µL of pentane for 5 min in a 200-µL glass insert. We preferentially selected final instar larvae, but younger larvae were used when 10 of these were not available. Each larval sample came from a different colony. Two microliters of the extract were then manually injected into an Agilent 7890A gas chromatograph (capillary column: Agilent HP-5MS, 30 m × 25 µm × 0.25 µm; split-splitless injector; carrying helium gas at 1 mL/min) coupled to an Agilent 5975c mass spectrometer and analyzed with the following temperature program: 70–270 °C at 30 °C/min, then 270–300 °C at 1 °C/min, then hold at 300 °C for 1 min (all settings as in Holman et al. 2010a, 2010b; Holman 2012). Hydrocarbons were identified by visual inspection of the mass spectra and retention times of each peak and comparing these with known standards and previous studies of L. niger. The larval hydrocarbon data were then compared with the chemical profiles of workers, queens, and pupae collected in previous studies (Holman et al. 2010a, 2010b) using NMDS followed by discriminant analysis.

RESULTS

Effect of synthetic 3-MeC₃₁ on queen ovary activation

Queens that received 3-MeC₃₁ had significantly fewer fully developed eggs in their ovaries at the end of the experiment than queens treated with pentane (Figure 1; GLM...
Comparison of the brood pile of queens treated with different hydrocarbon solutions. (A) T

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382

from those treated with 3-MeC31 (\( z_{53} = 2.46, P = 0.014 \)). The \( n\)-C31-treated queens were intermediate and did not differ significantly from those treated with 3-MeC31 (\( z_{53} = 2.38, P = 0.38 \)) or pentane (\( z_{55} = 1.55, P = 0.12 \)). We also tested if this result still held if the number of eggs present in the colony at the time of dissection (a measure of queens’ oviposition rate over the last ca. 10 days) was included as a covariate; as expected, this predictor had a significant negative effect, showing that queens that had recently laid many eggs had fewer eggs in their ovaries (\( z_{52} = 2.28, P = 0.03 \)). As in the previous analysis, 3-MeC31-treated queens had fewer eggs in their ovaries than pentane-treated queens (\( z_{52} = 2.00, P = 0.046 \), and the \( n\)-C31-treated queens were intermediate and did not differ significantly from those treated with 3-MeC31 (\( z_{52} = 0.74, P = 0.46 \)) or pentane (\( z_{52} = 1.25, P = 0.22 \)). This suggests that the negative effect of 3-MeC31 on the number of eggs in queens’ ovaries resulted from an inhibition of fecundity rather than stimulation that caused queens to oviposit more and deplete their egg reserves.

Effect of synthetic 3-MeC31 on colony growth

A scree plot of stress versus number of dimensions indicated that reducing the brood count data to 2 NMDS scores provided the best fit. The ordination converged on a solution with a stress level of 0.09, suggesting a good fit. NMDS score 1 was negatively affected by the number of larvae and positively affected by the number of eggs and cocoons, whereas score 2 increased with the number of larvae (Table S1). NMDS score 1 significantly increased with time since mating (\( F_{1,108} = 78, P < 0.0001 \)) and was affected by hydrocarbon treatment (\( F_{2,55} = 5.63, P = 0.006 \)) (Figure 2A). There was no significant effect of queen head size (though there was a trend for positive effect: \( F_{1,55} = 3.95, P = 0.053 \)), and there was no significant interaction between treatment and time (\( F_{2,108} = 0.80, P = 0.46 \)). The queens treated with 3-MeC31 had significantly higher values of NMDS score 1 than those treated with \( n\)-C31 (contrast: \( b_0 = 2.44, P = 0.018 \)) or pentane (\( b_0 = 2.0, P = 0.047 \)), and there was no difference between the pentane and \( n\)-C31 groups (\( b_0 = 0.55, P = 0.58 \)). These results suggest that addition of the queen pheromone 3-MeC31 to the colony significantly affected the composition of the brood pile. The loadings on each brood type on NMDS score 1 (Table S1) suggest that 3-MeC31 reduced the number of larvae produced or elevated the production of eggs or cocoons, or a combination of these.

NMDS score 2 also changed significantly with time since mating (\( F_{1,10} = 65, P < 0.0001 \)). However, treatment (\( F_{2,55} = 0.51, P = 0.60 \)) and head size (\( F_{1,55} = 0.66, P = 0.42 \)) had no significant effect on this score.

We also investigated the effect of treatment on the number of each brood type present in the colony on day 28 (Figure 2B) using univariate analyses in order to identify the brood types that differed between treatment groups.

![Figure 1](https://beheco.oxfordjournals.org/)

Queens treated with 3-MeC31 had significantly fewer eggs in their ovaries than those that received pentane, whereas \( n\)-C31-treated queens were intermediate. Bars show the mean + SE, and bars sharing a letter are not significantly different.

![Figure 2](https://beheco.oxfordjournals.org/)

Composition of the brood pile of queens treated with different hydrocarbon solutions. (A) Treatment with 3-MeC31 significantly affected the composition of the brood pile relative to \( n\)-C31 or pentane. The boxplots show the distribution of NMDS score 1 on days 10, 20, and 28 after colony foundation. (B) Mean + SE number of brood items on day-28 postmating. The number of eggs has been divided by 10 for ease of comparison. Bars sharing a letter are not significantly different; NS indicates that treatment did not significantly affect the numbers of that brood type.

Downloaded from http://beheco.oxfordjournals.org/ at The Australian National University on February 10, 2013
The number of pupae present at the end of the experiment was significantly decreased by 3-MeC31 treatment relative to pentane (Poisson GLM: $z_{55} = 1.99, P = 0.046$), though the decrease was not significant relative to $n$-C31 ($z_{55} = 1.38, P = 0.17$); head size was a significant predictor of the number of pupae produced ($z_{55} = 1.99, P = 0.050$) and was therefore used as a covariate. The numbers of the other brood types (eggs and the 3 classes of larvae) were not significantly affected by treatment (all $P > 0.17$) although egg number was positively related to head size ($z_{55} = 2.19, P = 0.03$). Together with the results of the multivariate analysis, the data suggest that addition of 3-MeC31 had subtle but detectable effects on the composition of the brood pile and may have reduced the number of cocoons produced by queens relative to the pentane control.

**Chemical profiles of eggs, larvae, workers, and queens**

*L. niger* larvae were found to possess qualitatively similar cuticular hydrocarbons to eggs and adults, namely a blend of $n$-alkanes, methylalkanes, dimethylalkanes, trimethylalkanes, and alkenes with carbon chain lengths of 25–33 (Figure S1; Table S2). The pheromone 3-MeC31 and other 3-methylalkanes were abundant in the larval chemical profile, as in queens, eggs, and pupae. Larvae also possessed some short-chain hydrocarbons (e.g., $n$-C25 and 3-MeC25; Table S2) that are not present in detectable quantities on queens or workers.

We also performed a discriminant analysis on the hydrocarbon data (expressed as proportions) for which replicates were available (i.e. the larval data from the present study plus the egg, worker, and queen data from Holman et al. 2010b). After reducing a Euclidean distance matrix of the data set to 3 variables (again, the number of variables was selected using a scree plot of stress vs. number of dimensions) using NMDS (stress: 0.02, suggesting a very good fit), we found that the cuticular hydrocarbons of each group were highly distinct (MANOVA on NMDS scores: Wilk’s $\lambda = 0.005$, $F_{9,153} = 134, P < 0.0001$) and were able to correctly classify 100% of samples as eggs, larvae, workers, or queens (jackknife leave-one-out cross-validation), suggesting that each life stage has distinct cuticular hydrocarbons (Figure S3). Larvae and queens were the most similar groups, and brood were more chemically similar to queens than to workers (Figure 3; for Euclidean distances between life stages, see Table S3).

**DISCUSSION**

Addition of the queen pheromone 3-MeC31 to incipient colonies affected the composition of the brood pile and appeared to slow the rate of colony growth: queens had slightly fewer cocoons at the end of the experiment. A possible explanation for this result is that 3-MeC31 reduced the fertility of queens (just as it does in workers; Holman et al. 2010b), delaying colony development. This interpretation is supported by the ovary dissections, which revealed that 3-MeC31-treated queens had fewer eggs in their ovaries than those treated with pentane. 3-MeC31 could also have slowed colony development by depressing parental care behaviors in queens; such a behavioral effect cannot be ruled out by this experiment, but the ovary dissections suggest that at least part of the decline in productivity resulted from a physiological effect of 3-MeC31 on ovarian activity. These results imply that brood-borne queen pheromones allow queens to regulate their productivity via negative feedback, and therefore that ants and termites have independently evolved similar social physiology (though using very different chemical messages) (Yamamoto and Matsuura 2011).

The observed effect sizes were relatively small: 3-MeC31 treatment significantly reduced the number of pupae produced by around 11%, and the number of eggs in queens’ ovaries by 26% relative to the pentane control. In a previous study of *L. niger*, addition of extra pupae to 2-queen colonies reduced the production of workers and pupae
by an average of 10%; for 3-queen colonies the reduction was 25%, whereas in 1-queen colonies no reduction was observed (Holman et al. 2010a). Differences in the experimental design mean that it is not possible to accurately ascertain whether 3-MeC31 alone has an inhibitory effect equal to that of whole pupae, but the effect appears to be of the same order of magnitude.

The cuticular hydrocarbon profile of *L. niger* larvae was found to contain large amounts of 3-methylalkanes, particularly 3-MeC29 and 3-MeC31, relative to workers. Interestingly, larval cuticular hydrocarbon extracts contained the short-chain hydrocarbons n-C25 and 3-MeC25, which are present in negligible quantities on workers and queens (Holman et al. 2010b). This result suggests that larvae synthesize some or all of the hydrocarbons present on their body surface rather than acquiring them from contact with adults, and that the expression or activity of the enzymes involved in hydrocarbon synthesis are somewhat different in larvae. For example, larvae may have fewer or less-active elongases, the enzymes that sequentially add units to the carbon chain (Blomquist and Bagnères 2010; Bonasio et al. 2010), than adults. Although it has not been experimentally tested whether 3-methylalkanes other than 3-MeC31 are queen pheromones in *L. niger*, this is plausible; experiments have suggested that *Linepithema humile* ants treat hydrocarbons of different chain lengths synonymously provided that the methyl branch is in the same position (van Wilgenburg et al. 2010).

*L. niger* queens were previously found to produce fewer brood if they were sharing a colony with other queens and/or if brood was experimentally added (Holman et al. 2010a). The present results suggest that the 3-MeC31 present on queens and brood caused some or all of the observed reductions in productivity. Restraining reproduction in response to the presence of other queens and/or a large brood pile may be adaptive because there is likely a trade-off between current reproductive effort and future survival and reproduction (Sears 1992), and the marginal benefits of producing additional brood presumably decline with the number already present in the colony. Moreover, unrelated *L. niger* queens often co-found new colonies, but the first clutch of workers kill surplus queens until only 1 remains; queens also sometimes fight one another directly (Sommer and Hölldobler 1995; Holman et al. 2010a). The decreased reproductive output of cohabiting queens may therefore serve to conserve energy for the fighting stage.

The inhibitory effect of 3-MeC31 on queen fecundity might also be adaptive in established, single-queen colonies containing a large worker force. Queens could adjust their reproductive output based on the frequency with which they encounter brood-borne hydrocarbons. The value to queens of adjusting their fecundity based on external cues, as opposed to relying solely on internal factors (e.g., their own endocrine or nutritional state), may be that the former provides better information on the composition of the colony. For example, queens with a large worker force may be exposed to brood-borne pheromones less often than queens in incipient colonies because *L. niger* workers transport much of the older brood away from the queen to separate brood chambers (Urbani 1991). A buildup of brood around the queen may indicate that there are insufficient workers to process the existing ones, signifying that the queen should reduce its oviposition rate. Moreover, queens may be unable to assess the number and developmental state of brood in the colony without relying on external cues such as brood-borne 3-MeC31 because the queen’s physiological state might often be a poor predictor of the quantity of brood present in the colony. For example, queen fecundity declines greatly during winter, yet colonies may contain many overwintering larvae.

Recently, a study of *R. speratus* termites found that the reproductive output of queens is reduced by addition of synthetic queen pheromone, which is naturally found on both queens and their eggs (Yamamoto and Matsuura 2011). Similarly, addition of queen-laid eggs to queenless groups of *Camponotus floridanus* ant workers inhibited worker reproduction; the eggs are coated with queen-like hydrocarbons (which include 3-methylalkanes, as in *Lasius*), suggesting that egg-borne pheromones inhibited worker reproduction (Endler et al. 2004). However, workers that received larvae and pupae, but not eggs, were not reproducitively inhibited, suggesting that the worker response may be specific to eggs rather than brood in general (Endler et al. 2004). Accordingly, the hydrocarbons that characterize queens and eggs are not major components of the larval chemical profile (Moore D, personal communication), suggesting that *C. floridanus* larvae do not produce pheromones that inhibit oviposition. Honey bee larvae produce a volatile pheromone that inhibits ovary activation in workers (Maisonasse et al. 2009), but to our knowledge, its effects on queens are unstudied. Together with the present work, these studies suggest that inhibition of queen fecundity by pheromones present on other queens and brood may be a widespread phenomenon among social insects and imply that this sociophysiological regulatory mechanism has evolved multiple times.

An alternative, or complementary, regulatory system for queen fecundity has been proposed based on data from the fire ant *S. invicta*. In that species, queens are apparently stimulated to oviposit by anal secretions produced by larvae that are about to pupate (Tschinkel 1988, 1995). This queen response could allow queens to match their oviposition rate to the number of available workers because pupating larvae will soon become adult workers capable of tending brood. This mechanism is stimulatory, rather than inhibitory as in the present study, and may allow queens to increase oviposition rate as the colony grows in size (an example of positive feedback; Wilson and Hölldobler 1988). Queens might base their reproductive rate on cues to both the number of brood and the availability of nurse workers, combining negative and positive feedback in order to improve regulation of colony growth rate.

In summary, our results provide evidence for a novel function of queen pheromones in the social physiology of ant colonies. Brood are coated with queen-like hydrocarbons including 3-MeC31, and larvae appear to synthesize their own hydrocarbons. Queen productivity and egg load were significantly affected by 3-MeC31 treatment, though these effects were subtle, suggesting that queens are less sensitive to the sterility-inducing effect of queen pheromone than are workers. Together with previous data showing that queen productivity is inhibited by exposure to brood (Holman et al. 2010a), these results suggest that egg production may be self-limiting because productive queens are exposed to more brood-borne pheromones (as in termites), and that the brood pheromone may be the same as the sterility-inducing pheromone produced by the queen. This regulatory mechanism mirrors endocrine negative feedback loops inside individual organisms.

**SUPPLEMENTARY MATERIAL**

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

**FUNDING**

The work was supported by a Marie Curie Intra-European Fellowship to L.H. (#235403; CHEMDOC) and by the Danish
National Research Foundation (Copenhagen Centre for Social Evolution).

We are grateful to Dani Moore for discussing unpublished data, and to P. García-Gonzalez and 2 anonymous reviewers for constructive comments on the manuscript.

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