Queen-signal modulation of worker pheromonal composition in honeybees

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Worker sterility in honeybees is neither absolute nor irreversible. Whether under queen or worker control, it is likely to be mediated by pheromones. Queen-specific pheromones are not exclusive to queens; workers with activated ovaries also produce them. The association between ovarian activation and queen-like pheromone occurrence suggests the latter as providing a reliable signal of reproductive ability. In this study we investigated the effect of queen pheromones on ovary development and occurrence of queen-like esters in workers’ Dufour’s gland. Workers separated from the queenright compartment by a double mesh behaved like queenless workers, activating their ovaries and expressing a queen-like Dufour’s gland secretion, confirming that the pheromones regulating both systems are non-volatile. Workers with developed ovaries produced significantly more secretion than sterile workers, which we attribute primarily to increased ester production. Workers separated from the queenright compartment by a single mesh displayed a delayed ovarian development, which we attribute to interrupted transfer of the non-volatile pheromone between compartments. We suggest that worker expression of queen-like characters reflects a queen–worker arms race; and that Dufour’s gland secretion may provide a reliable signal for ovarian activation. The associative nature between ovary development and Dufour’s gland ester production remains elusive.

Keywords: Dufour’s gland; Apis mellifera; pheromones; worker reproduction

1. INTRODUCTION
Reproductive skew, a process whereby one or several individuals in a colony reproduce while most individuals are sterile and help the reproduc tives to rear their offspring, is a fundamental phenomenon characterizing eusocial insects. This reproductive division of labour is often accompanied by some morphological and physiological differences. Nonetheless, in many social insects there is an ongoing conflict between queen and workers over the parentage of males. In species with relatively small colonies, the conflict may be accompanied by aggression, whereas in species with more populous colonies, e.g. the honeybee Apis mellifera, the conflict is subtler and without apparent aggression. In A. mellifera the queen apparently wins the conflict because only very few worker-born males develop to maturity (Page & Erickson 1988; Visscher 1998). A key question about the mechanism of reproductive skew is whether the queen actively inhibits worker reproduction (queen control), or whether workers have power over their own reproduction and behave in ways that optimize their inclusive fitness (worker control) (Keller & Nonacs 1992). Whatever the mechanism, it is likely to involve queen pheromones. Under the ‘queen-control hypothesis’ it is assumed that the queen pheromone actively inhibits both ovarian development and oviposition in workers. By contrast, under the ‘worker-control hypothesis’ the queen pheromone acts as an honest signal advertising the queen’s presence and/or her quality. Supercedure of less fecund queens is known in honeybees (Breed et al. 1985; Winston 1987, p.197), supporting the ‘worker-control hypothesis’.

Honeybee queens are endowed with caste-specific pheromones, including the mandibular glands (Slessor et al. 1988), Dufour’s gland (Katzav-Gozansky et al. 1997), tergal glands (Wossler & Crewe 1999a) and queen faeces (Page et al. 1988). Of these, the queen mandibular pheromone (QMP) was long considered as a primer pheromone affecting worker reproduction (Butler 1959), although later studies challenged this finding (Willis et al. 1990). However, a recent study reconfirmed the inhibitory effect of QMP on ovarian development (Hoover et al. 2003). From all of these studies it is clear that QMP is not as effective as a living queen. Moreover, another queen-specific pheromone produced by the tergal glands and inhibiting worker reproduction in small worker groups was recently discovered in A. m. capensis (Wossler & Crewe 1999b). Although it has not been tested in full-scale colonies, this suggests, along with the incomplete effect of QMP, that the queen may possess additional pheromones that act in concert to regulate worker physiology and behaviour. It also suggests that the queen advertises her presence in additional ways to pheromone emission.

All of the queen-specific pheromones identified so far appear to be non-volatiles, judging from their respective chemistries. Studies with QMP have confirmed this and also revealed the mechanism for its dispersion throughout the hive, mostly by allogrooming (Naumann et al. 1992). Although not explicitly tested, it is assumed that the other non-volatile queen signals are dispersed in the hive in a
similar manner. Another interesting feature of queen pheromones is their plasticity in workers. So far, for the mandibular and Dufour’s glands, it has been demonstrated that queenless (QL) egg-laying workers produce the major queen-characteristic compounds (Crewe & Velthuis 1980; Plettner et al. 1996; Katzav-Gozansky et al. 2000). The concomitant occurrence of ovarian development and royal pheromone expression in workers suggests not only that these two processes are associated, but most likely that the regulation of both is correlated. The proposed regulatory system for queen pheromone expression is that signals emanating from the queen may in turn inhibit the expression of these very same chemicals in workers. The aim of this study was to examine whether the queen emits chemical signal(s) that simultaneously inhibit both worker ovarian development and queen-like Dufour’s gland ester production.

2. MATERIALS AND METHODS
(a) Volatility and transmission of the queen pheromones
All the experiments were conducted with colonies of A. mellifera ligustica at the I. Meier Segals Garden for Zoological Research at Tel Aviv University, between March and September 2001.

The queen effect on ovarian development and Dufour’s gland expression in workers was studied in four-comb mini-hives (22 cm × 27 cm × 24 cm) each of which was separated into two compartments by either a single-mesh (SM) or double-mesh (DM) screen (8 mm mesh size). Separation by a single mesh allowed limited contact between the bees from each compartment, presumably enabling transfer of non-volatile as well as volatile pheromones. In the DM hives, the distance between the meshes was 1.5 cm, preventing any between-compartment physical contact, but allowing volatile substances to pass through. Each compartment contained ca. 1500 workers, a comb with empty cells and a comb with honey and pollen. Bees in the QL compartment were allowed to forage freely. Thus, in the SM hives, food could flow from the QL to the queenright (QR) compartment through trophallaxis, but not in the DM hives. Therefore, the QR compartment of the latter was supplemented with candy (a mixture of honey and sugar powder) and pollen throughout the experiment. Similarly partitioned hives that housed completely sterile situations as these eggs are usually policed (T. Katzav-Gozansky, personal observation). Bee sampling started once egg laying was observed in the QL compartments (Kruskal–Wallis: H_{2\times 15} = 6.19; p = 0.045). In the QL control colonies, eggs appeared significantly earlier than in the QL compartment of the DM colonies (8.6 ± 0.4 versus 13.0 ± 2.7 days (mean ± s.e.m.); Mann–Whitney U-test: n = 5, U = 2, p = 0.028). The occurrence of eggs in the SM colonies was intermediate (11.8 ± 2.3), not significantly different from either the QL control or DM colonies (U = 4, p = 0.08 and U = 8.5, p = 0.4, respectively). Figure 1 depicts the time course of ovarian development in bees from the three different treatments starting on day 1 after eggs were observed in the QL control colonies (day 10 of the experiment). Both the time after colony establishment and the level of queen isolation had a significant effect on ovarian development (GLM, univariate test of significance: F_{1,66} = 6.8, p = 0.01 and F_{2,66} = 5.8, p = 0.005, respectively). Ovarian development in workers from the DM and control QL hives was already as high as 55% on day 10 and did not increase significantly thereafter (ordering test: \(\tau = 0.12, n = 25, p = 0.4\) and \(\tau = 0.2, n = 25, p = 0.16\), respectively). In the SM hives, however, the initial proportion of workers with developed ovaries on day 10 was lower than that of other treatments (25%; ANOVA: \(F_{2,11} = 4.04, p = 0.04\), followed by an LSD test, \(p = 0.03\)), but it progressively increased with time, reaching the levels of the control QL hives by day 16 of the experiment (ordering test: \(\tau = 0.42, n = 20, p = 0.01\)). Comparing the percentages of workers with developed ovaries, taking into

(b) Chemical analysis of Dufour’s gland composition
Dufour’s gland content in bees with developed ovaries is greater than that of bees with undeveloped ovaries (Katzav-Gozansky et al. 1997). Therefore, Dufour’s glands from bees having ovaries at stage 3 were extracted individually, whereas glands from bees having ovaries at stage 1 or 2 were extracted in pairs to obtain sufficient quantities for proper analysis. In the analyses the amount was calculated as per gland. All extracts were prepared in 50 \(\mu\)l of dichloromethane containing 100 ng of eicosane as an internal standard. Samples were stored at −20°C until analysis. Glandular components were quantified by gas chromatography (Varian CP 3800) equipped with DB-1 fused silica, column-temperature-programmed from 150–300°C at 5°C min\(^{-1}\).

(c) Statistical analysis
Statistical analyses were performed using STATISTICA for Windows; v. 6.0, Statsoft, Inc. The Kruskal–Wallis non-parametric test was used to compare the onset of egg-laying behaviour and the change in ester production, followed by a Mann–Whitney U-test. The percentage of bees with developed ovaries in each colony and amount of glandular secretion (response variables) were fitted by means of general linear models (GLM) to test the effect of treatment and time after colony establishment (categorical variables). Pairwise comparisons were conducted by the Bonferroni post hoc test. Worker ovarian development 10 days after colony establishment was compared using ANOVA followed by a least significant difference (LSD) test. The effect of time on ovary development in each treatment and ester: hydrocarbon ratio was tested using the ordering test (Sokal & Rohlf 1995).

3. RESULTS
(a) Workers’ ovarian development and egg laying
The treatment had significant effect on the onset of worker-egg appearance in the QL compartments (Kruskal–Wallis: \(H_{2,15} = 6.19; p = 0.045\)). In the QL control colonies, eggs appeared significantly earlier than in the QL compartment of the DM colonies (8.6 ± 0.4 versus 13.0 ± 2.7 days (mean ± s.e.m.); Mann–Whitney U-test: n = 5, U = 2, p = 0.028). The occurrence of eggs in the SM colonies was intermediate (11.8 ± 2.3), not significantly different from either the QL control or DM colonies (U = 4, p = 0.08 and U = 8.5, p = 0.4, respectively).

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account all the bees sampled throughout the experiment, did not reveal any significant difference between control QL and DM colonies (developed ovaries: 57.1% and 57.7%, respectively; GLM, univariate test of significance followed by Bonferroni post hoc test: \( p = 1.0 \)). In the SM colonies, by contrast, the proportion of bees with developed ovaries was significantly lower than that of QL or DM workers (41.8%; GLM, Bonferroni post hoc test: \( p = 0.01 \) and \( p = 0.009 \), respectively).

(b) Dufour’s gland composition
Concomitant with ovarian development in workers, there was an increase in Dufour’s gland secretory amount (all workers analysed irrespective of treatment; figure 2). To assess which of the classes of compounds—esters or hydrocarbons—contributed to secretory augmentation, we calculated the ester to hydrocarbon ratios for each ovarian developmental stage. There was a significant increase in ester:hydrocarbon ratio in the glandular secretion with ovarian development (ordering test: \( z = 0.57, n = 338, p < 0.001; r^2 = 0.998 \)), indicating that the increase in secretory amounts can be attributed to a great increase in ester amounts. To evaluate the effect of queen-signal dispersion on Dufour’s gland secretion plasticity in workers, we comparatively analysed the secretion of workers with developed ovaries (stage 3) from each treatment (QL control, SM and DM hives). The treatment did not affect the total amount of secretion found in Dufour’s glands (figure 3; numbers above columns denote total amounts ± s.e.m.; Kruskal–Wallis test: \( H_{2,117} = 2.1, p = 0.4 \)), but significantly affected the amount of esters found in the glands of these bees (figure 3; Kruskal–Wallis test: \( H_{2,117} = 7.7, p = 0.02 \)). Surprisingly, stage 3 bees from the QL control hives had significantly less esters than those from the SM or the DM bees (Mann–Whitney \( U \)-test: \( n = 40, U = 483, p = 0.01 \) and \( n = 40, U = 579, p = 0.02 \), respectively). However, there was no difference in the amount of esters between the SM and DM bees (\( n = 41, U = 690, p = 0.62 \)).

Figure 1. Time-dependent changes in ovarian development (percentage of bees with developed ovaries in each colony; mean ± s.e.m.) in control QL colonies (diamonds) and the QL half of colonies with SM (triangles) or DM (squares). Each point represents an average of five replicates (colonies) from which bees were sampled (a total of 345 bees dissected per treatment).

Figure 2. Total secretionary amounts (grey bars) and hydrocarbon to ester ratios (solid line with squares) in Dufour’s gland (nanograms per gland) of QL workers expressing different ovarian development (mean ± s.e.m.). Different letters denote statistical differences at \( p < 0.005 \) according to a Kruskal–Wallis test, followed by a Mann–Whitney \( U \)-test.

4. DISCUSSION
Two of the most pronounced differences between queen and worker honeybees—reproduction and pheromone composition—are not fixed phenomena, but rather show plasticity. Workers that are removed from the queen influence initiate reproductive activity (Butler et al. 1970; Hoover et al. 2003) and also express a queen-like pheromone composition (Crewe & Velthuis 1980; Plettner et al. 1993; Katzav-Gozansky et al. 1997). The co-occurrence of ovarian development and queen-like pheromone expression in workers suggests a link between these two traits. For example, the queen-like pheromones may constitute a reliable signal denoting ovarian development in workers that express it. Whether this linkage is also physiological, that is, ovarian development invariably results in queen-like pheromone composition, is not clear. In the present study we investigated in greater detail the degree of association between ovarian development and queen pheromone expression in workers. By controlling the flow of information about the presence of the queen, we attempted to assess whether the pheromones affecting ovary activation are also those that affect worker pheromone composition; or, if different, whether they are transmitted in a similar manner.

The basic premise of the experiment was that continuous detection of the queen’s presence is adaptive both to the queen and workers, and that in populous colonies the queen presence is likely to be conveyed by pheromones. Studies with QMP have shown that the queen produces copious amounts of this pheromone and that workers actively disperse it throughout the colony (Naumann et al. 1992, 1993), consistent with the adaptive value of broadcasting the queen presence. In this study we controlled information flow, i.e. the passage of queen pheromones, by dividing experimental hives with either an SM or a DM. We hypothesized that volatile pheromones could readily pass between the QR and the QL compartments, in both the SM and DM hives; whereas non-volatile pheromones could pass between compartments in the SM hives, albeit at reduced rates, but could not pass at all in the DM hives.

The percentage of bees in the DM hives that developed ovaries was not different from the QL control hives and had reached maximum already by day 14. This indicates that
the queen signal that regulates worker reproduction is indeed non-volatile. The dynamics in ovarian development in these hives is in agreement with previous reports which showed that the degree of ovary development levels off in the second week after dequeening (Miller & Ratnieks 2001). The results obtained from the SM hive are more complex. Although ovarian activation in workers in the QL compartment of these hives eventually occurred, it was delayed compared with the QL control or the DM hives. We postulate that the rate at which the pheromone was transferred between compartments was rather inefficient, resulting in a gradual decline in pheromone titre in the QL compartment. Accordingly, ovarian activation in workers started once the pheromone titre fell below the detection threshold. The fact that the rate at which the pheromone was transferred between compartments was rather inefficient, resulting in a gradual decline in pheromone titre in the QL compartment, suggests that workers may differ in their threshold sensitivity to the pheromone (Pankiw et al. 1995, 2000). We assume that once the pheromone was transferred to the QL compartment it was effectively distributed among workers, but that workers with low sensitivity to the pheromone initiated ovarian development earlier than their sensitive nestmates. Successful worker reproduction depends not only on ovarian development but also upon decline in egg policing. The fact that consistent egg laying by workers occurred in the QL control hives significantly earlier may indicate that the egg policing broke down in the QL control hives earlier than in the DM hive. Although this also occurred earlier than in the SM hive, this difference was not significant. We do not know whether this delay was a result of more extensive egg policing in the treated hives, but if so, it suggests that the queen signal that directs worker policing is more volatile, because the DM hives were clearly affected. Why the full effect of this postulated pheromone was not expressed in the SM hives remains elusive.

Chemical analysis of Dufour’s gland secretion confirmed that under QL conditions the queen-characteristic esters are produced in workers. Grouping the bees according to their ovarian development shows clearly that the total secretory amount increases with increased ovarian development. Workers with developed ovaries at stage 3 possessed as much as 541 ± 50 ng, a greater than twofold increase in secretory amount compared with sterile workers. This increase can be attributed to a greater increase in ester production, as revealed by the linear correlation between ovarian development and ester/hydrocarbon ratio. In workers with mature ovaries at stage 3, the proportion of esters reached 27%. Assuming that production of queen-characteristic secretion (esters) provides a reliable and quantitative indicator of the degree of ovarian development, it becomes clear why egg-laying workers never reach the queen level: the total amount of secretion in queens is 20 ± 4.1 µg, of which 65% are the esters (Katzav-Gozansky et al. 1997). Worker ovaries contain at most two dozen ovarioles, compared with the several hundred present in queens. If workers are sensitive to the amount of Dufour’s pheromone in the queens and this in turn reflects the number of developed ovarioles, this may constitute the mechanism for evaluating queen quality. An alternative explanation is that the synthesis ability of worker glands has declined during the evolution of worker sterility.

The regulatory mechanism underlying Dufour’s gland chemical plasticity in workers is another unsolved problem. The association between ovarian development and glandular expression may also suggest a physiological link, although direct evidence for this is still lacking. The results of the above experiment suggest, to the contrary, that these processes have different dynamics and therefore may be physiologically uncoupled. Comparing the ester amounts in the secretion in bees with stage 3 ovaries showed that the treatment had a significant effect. Bees from the QL hives had a significantly lower amount than those from the SM or DM hives. This suggests that the bees in the QL compartment of the SM and DM hives still perceived a different social environment (the presence of the interrupted queen signal?) and reacted by boosting further the queen-characteristic esters. The interrupted queen signal under our experimental conditions possibly mimics a weakening signal that may occur in normal hives, informing the workers about queen fecundity. Worker bees will benefit most if they begin ovipositing within the short time window between queen weakening and her death. The fact that these differences become apparent only in bees with developed ovaries, suggests that although the two processes, ovarian development and Dufour’s ester production, may be physiologically uncoupled, they are probably under a mutual regulatory system; that is they respond similarly to the changes in the hive social environment.

The ability of egg-laying workers to mimic the queen pheromone is probably part of an ongoing queen–worker conflict, which is exhibited as a pheromonal arms race. Theoretically, selection should favour queens having measures that control worker signal expression so as to maintain the queen’s reproductive dominance, and workers that resist this control. Why should workers maintain this pheromone plasticity in view of the successful queen control by worker policing (Ratnieks & Visscher 1989; but see Pirk et al. 2004) for critics on worker egg policing in honeybees)? Possessing a queen-like Dufour’s composition may also be adaptive under the hopeless QL situation,

Figure 3. Ester amounts (nanograms per gland; mean ± S.E.M.) in bees with developed ovaries (stage 3). The numbers above the bar denote the total amount of secretion. The data are pooled for all bees per treatment (n = 118 samples from 15 colonies). Different letters denote statistical differences at p < 0.05 according to a Kruskal–Wallis test, followed by a Mann–Whitney U-test.
when workers race for male production. There is a small time window between queen disappearance and social breakdown when male-rearing is possible (Page & Erickson 1988). Becoming a ‘false queen’ by possessing the queen characteristic esters (Katzav-Gozansky et al. 2003) may give a head start to these workers and enhance their chance of successfully rearing males. Such a worker–worker pheromone contest was demonstrated also for the mandibular gland (Moritz et al. 2004) as well as for Dufour’s gland (R. Dor, T. Katzav-Gozansky and A. Hefetz, unpublished data). It is therefore not inconceivable that queen-pheromone plasticity in workers may result in the formation of ‘worker false queens’ under the QR condition, which reflects an escalation of the arms race. Queens, therefore, at least under the queen-control hypothesis, are predicted to evolve new pheromonal means for controlling workers, and workers are predicted to respond accordingly. This arms race may explain why queens possess multiple pheromone bouquets that are produced by different exocrine glands.

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