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Research article

Honeybee egg-laying workers mimic a queen signal

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Summary. In the honeybee, Dufour's gland secretion is caste specific and constitutes a component of the multi-sourced queen signal. As predicted, it is attractive to workers, which form a retinue around the scented source. Bioassays reveal the ester fraction and not the hydrocarbons to be the active constituents. This function of the esters was corroborated by assays with the synthetic queen-esters mixture, which successfully mimicked the queen's secretion. As predicted from the queen-like secretion exhibited by egg-laying workers, their glandular secretion was also attractive to nestmates, albeit to a lesser degree than that of the queen; while that of non-egg-laying workers was totally inactive.

The evolution of the multiple queen signals in honeybees can be regarded as a component in an arms race between queen and workers. We hypothesize that in response to a reduced sensitivity to a certain queen signal, queen honeybees were selected to develop an alternative signaling-source. Dufour's gland seems to be one of these sources.

Key words: Dufour's gland, esters, honeybees, *Apis mellifera*, attraction.

Introduction

Queen specific signal in the honey bee includes the Queen Mandibular Pheromone (QMP) (Barbier and Lederer, 1960; Barbier, 1986; Breed et al., 1992; Winston and Slessor 1998), a tergite pheromone (Wossler and Crewe, 1999), a fecal pheromone (Page et al., 1988), Dufour's gland pheromone (Katzav-Gozansky et al., 1997), and possibly other yet unidentified pheromones. While it is conventional to attribute a certain queen-worker interaction to specific pheromones, honeybee communication is not characterized by such simplicity. One pheromone can possess a variety of functions, while many activities can be affected by a combination of several pheromones. Moreover, a single pheromone can act concomitantly as both releaser and primer. In addition to the queen primer pheromone effect on workers, a typical queen releaser effect in workers is the formation of a retinue or royal court around the reproductive queen (Velthuis, 1985). Using this retinue behavior the full composition of the QMP was identified (Slessor et al., 1988) and the attractivity toward queens tergal glands and Dufour's glands was demonstrated (Wossler and Crewe, 1999; Katzav-Gozansky et al., 2001, respectively). Although the attractivity of the honeybee queen was studied intensively, there are many aspects that are still elusive. It became evident as early as 1954 that the mandibular glands were not the only source of pheromone production (Butler, 1954; Butler et al., 1973). Mated queens from which the mandibular glands were removed were still fully accepted by their respective colonies and successfully headed their colony for a significant period (Velthuis, 1970). This suggests that other queen pheromones produce important cues for worker honeybees, and that they can replace the QMP.

One of the caste-specific glandular sources found in the honeybee is the Dufour's gland (Katzav-Gozansky et al., 1997). While the exudates of workers are composed of a series of odd n-alkanes, the glandular exudates of queens are additionally fortified with wax-type esters. It was also found that queen Dufour's gland secretion is attractive to workers, raising the possibility that this fraction of the glandular secretion acts as a queen signal (Katzav-Gozansky et al., 2001). Worker-attraction towards the glandular secretion of virgin queens was also demonstrated by Abdalla and Cruz-Landim (2001). Both in vivo and in vitro studies have further demonstrated that ester biosynthesis in Dufour's gland is not a caste-fixed phenomenon. Queenless (QL) workers that start to develop ovaries also biosynthesize the queen-type esters. Moreover, glands from queenright (QR) nurses incubated in vitro also produce these esters, after a certain delay (Katzav-Gozansky et al., 1997). Occasionally, under QL conditions some workers attract a small but recognizable retinue, presumably by exuding some components of the queen pheromone. Such workers are designated as false queens (Crewe and Velthuis, 1980). Since Dufour's esters seem to differ significantly between QR workers and egg-laying workers,

we hypothesized that they may constitute a signal denoting these workers as false queens. Accordingly, we predicted that egg-laying workers Dufour's secretion could be attractive to other workers in a similar way to the queen secretion.

Material and methods

Bees

All the experiments were conducted with colonies of *Apis mellifera ligustica* at the Tzrifin apiary, Israel, and in experimental hives kept at the I. Meier Segals Garden for Zoological Research at Tel Aviv University, between 1998 and 2001.

Extract preparation

Dufour's glands were dissected under double distilled water and extracted in dichloromethane for component separation or in ethanol for bioassay. Foragers were selected as donors of worker glands since among QR workers they tend to have higher quantities of glandular secretion (Katzav-Gozansky et al., 1997). In order to separate between esters and hydrocarbons the glandular extracts were fractionated on a Florisil column (6.5 cm long and 0.6 cm wide; Merck, 0.150–0.250 mm). The Florisil was prewashed with ethyl acetate to remove all possible contaminants. Hydrocarbons were separated from the more polar constituents (esters included) by stepwise elution with pentane (6 ml) followed by ethyl acetate (6 ml). The pentane fraction contained only hydrocarbons while the second, more polar fraction contained mostly esters, as confirmed by GC/MS analyses (only few contaminants were observed).

Attraction bioassay

All attraction tests were performed in plastic Petri dishes (15 × 2 cm). Seven-day old bees, the age at which worker attraction to QMP is the greatest (Kaminski et al., 1990), were collected from the brood area of a QR colony. For each assay 10 freshly collected bees served as the responding bees (Katzav-Gozansky et al., 2001), one bee was treated with 2 µl of experimental mixture in ethanol, and a second bee was treated with ethanol alone (control). The treated bees were color marked on the thorax while the extracts were applied on the abdomen. Bees were treated with either queen or worker total Dufour's gland extract, its separated ester and hydrocarbon fractions, or a synthetic ester mixture. The doses used in both assays were calculated as queen equivalents (Qeq), considering that 1 Qeq equals 20 µg of all glandular constituents (Katzav-Gozansky et al., 1997). Both bees, extract and solvent treated, were introduced simultaneously to the arena. The number of workers contacting each bee was recorded at 30 sec intervals during a 5 min test (total of 10 times). For each replicate the sum of contacts for treatment vs. control over 5 min. was used as a measure of attraction. In order to emphasize the level of preference of the treatment vs. control the results are presented as the percentage of assays in which there was preference towards the treated bee.

Synthetic esters

Esters were synthesized and kindly supplied by Prof. Wittko Francke of the Department of Chemistry at Hamburg University, Germany, as described in Katzav-Gozansky et al. (2001). The blend of the esters was prepared according to the relative proportion of the esters in the queen total glandular constituents. The proportions were calculated as average of newly mated and one-year old mated queens. All esters present in the gland were used (Katzav-Gozansky et al., 1997), except tetradecyl (Z)-9-tetradecenoate that was found as a trace in the gland. The composition the blend of esters was: Tetradecyl dodecanoate (1%), Tetradecyl tetradecanoate (20%), Tetradecyl-(Z)-9-hexadecenoate (24%), Tetradecyl hexadecanoate (5.3%), Hexadecyl tetradecanoate (21.1%), Hexadecenyl hexadecanoate (6.1%), Tetradecyl-(Z)-9-octadecenoate (7%), Tetradecyl-(Z)-9-hexadecenoate (6.5%), Hexadecyl hexadecanoate (7.1%), Octadecyl hexadecanoate (1.9%). Hydrocarbons, being generally common to queen and worker secretions, were not added to the synthetic mixture.

Statistical analysis

Statistical analyses were performed using Statistica for Windows; version 6.0, Statsoft, Inc. Wilcoxon Signed Rank test was used to compare the attraction of workers to the glandular secretion and the control. Statistical significance was accepted at $p = 0.05$. Data are presented as means ± SE.

Results

Worker attraction to Dufour's gland secretion from the various bee groups tested is depicted in Figure 1. Queens' glandular secretion was highly attractive to the tested workers, which formed a retinue around the signal source in concentrations as low as 1/8 queen-equivalents. In contrast, the secretion of QR workers (foragers) was unattractive. While on average 40 ± 6.2 contacts were directed towards a bee treated with 1/8 Qeq of queen Dufour's gland secretion, only 8 ± 1.6 were directed towards the ethanol treated bee (Wilcoxon Signed Rank test, $P = 0.01$, $T = 0.0$). This can be compared to the treatment with 1/8 Qeq worker Dufour's gland secretion, which elicited only 8 ± 1.2 contacts with the treated bee, not significantly different from the ethanol control (9 ± 1.0, Wilcoxon Signed Rank test, $P = 0.8$, $T = 6.5$). The reaction of worker bees to Dufour's gland secretion from egg-laying workers was intermediate between that of queens and that of non-laying workers. The mean contacts directed towards bees treated with 1/8 Qeq of egg laying worker glandular secretion was 10 ± 1.6 as compared to 6 ± 0.8 towards bees treated with

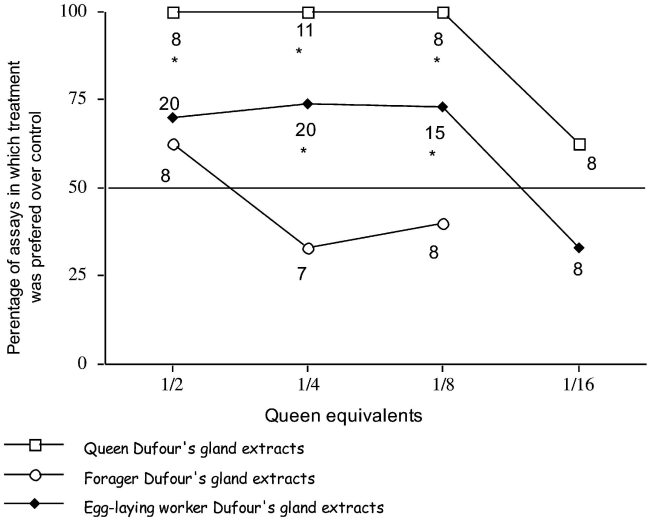

Figure 1. Attraction of worker bee treated with queen or worker Dufour's gland secretion at different concentration. Data are presented as preference, expressed as the percentage of tests in which the glandular secretion was preferred over the control. Statistical analysis was performed using Wilcoxon Signed rank test (n- number of replicates)

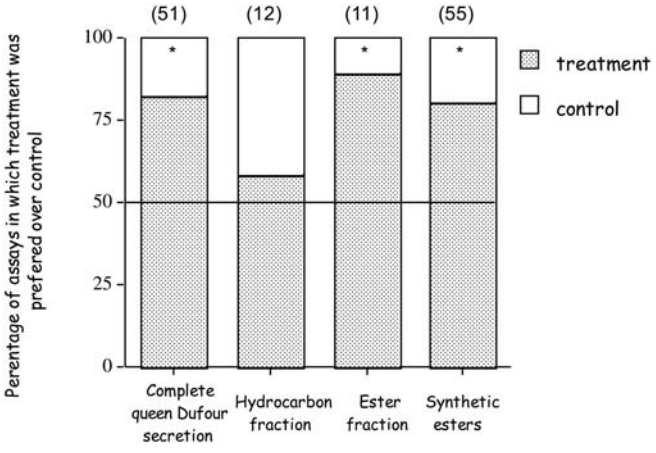

Figure 2. Attraction of worker honeybees to a worker bee treated with queen, fractionated Dufour's gland secretion or its synthetic esters using 1/4–1/2 Qeq. Data are presented as preference, expressed as the percentage of tests in which the glandular secretion was preferred over the control. Statistical analysis was performed using Wilcoxon Signed rank test (n- number of replicates)

solvent, Wilcoxon Signed Rank test, $P = 0.04$, $T = 24$). Since the amount of secretion found in the glands of egg-laying workers is about 1/7–1/8 Qeq, attraction of the workers to such doses is within the physiological range.

The two major classes of compounds present in the glandular secretion, hydrocarbons and esters were separated by column chromatography, and each fraction was tested for retinue-eliciting activity. Figure 2 demonstrates the preference of treatment vs. control using 1/2–1/4 Qeq of the hydrocarbons and ester fractions. The esters, whether from natural or synthetic source were as active as the queen complete Dufour's gland secretion (mean of contacts for the ester fraction vs control, 10 ± 1.4 vs. 6 ± 0.8, $P = 0.01$, $T = 3$; Synthetic ester fraction 10 ± 0.7 vs. 6 ± 0.5, $P > 0.0001$, $T = 212$; Queen's complete secretion 19 ± 1.8 vs. 8.1 ± 0.7, $P > 0.0001$, $T = 106$; Wilcoxon Signed Rank test). The hydrocarbon fraction, on the other hand, did not induce any response towards the treated bee (mean of contacts for hydrocarbon fraction 9 ± 1.2 vs. the control 6 ± 0.7, Wilcoxon Signed Rank test, $P = 0.16$, $T = 21$).

Differences between the preference to the queen glandular secretion in Figure 1 and 2 are probably a result of variation between experiments, hives, extracts and seasonal effects since the attraction experiments were conducted at two different periods.

Discussion

Queen fecundity is a fundamental parameter affecting colony fitness, and the replacement of a less fecund queen was reported for several species, the honeybee included. Accordingly, a queen must be selected according to her fecundity advertised either by marking her eggs with a queen-specific signal or by emitting a signal that reliably indicates fecundity. Evidence for marking eggs was provided for the fire ant *Solenopsis invicta* (Vander Meer and Morel, 1995) and the ponerine ant *Dinoponera quadriceps* (Monnin and Peeters, 1997). We suggest that Dufour's gland secretion may serve as such a fecundity signal in the honeybee. Although the physiological mechanism is still elusive, there is a strict link between ovarian development and the occurrence of the queen specific esters in the gland. Queen fecundity signal is predicted to be of low volatility and attractive to workers, two conditions met by Dufour's secretion. The present experiments demonstrate that the secretion constitutes a component in the array of pheromones that contribute to the formation of a retinue around the honeybee queen (i.e. mandibular glands; tergal glands). Our finding that egg-laying worker secretion, or the queen specific esters, can also elicit retinue by mimicking the queen signals are consistent with this hypothesis.

In honeybee colonies, under a hopeless queenless situation a race among the workers over male production will arise. The right time for egg laying is crucial for successful reproduction: premature egg layers suffer disproportionate agonistic behavior while late egg layers miss reproductive opportunities due to a breakdown of the social organization necessary for successful brood rearing (Page and Erickson, 1988). The presence of queen-like esters, possibly in synergism with other glandular secretions, may provide an edge for the egg-laying workers in becoming pseudoqueens and in regulating dominance patterns in honeybee colonies. Moreover, under these conditions, an association of nestmates that have lost the reproductive race among the egg-laying workers, and ultimately any chance of raising their brood, becomes adaptive, since late-breeders have little chance of reproducing successfully. Again, the presence of the queen-like esters in the egg-laying workers provides a reliable signal of which bee is likely to win the race.

Under queenright conditions where worker policing is adaptive, on the other hand, these esters may act as kairomones that help the nest members to identify the potential egg-layers and aggress them. Visscher and Dukas (1995) have shown that, indeed, workers are able to detect certain characteristics in nestmates, most likely olfactory cues that are correlated with ovarian development, and selectively attack them. The fact that the queen-like esters in Dufour's gland can be synthesized *in vitro* even in glands that have been removed from queenright workers, and the seemingly obligatory link between ovarian development and the occurrence of these esters in the gland, suggests that they can reliably disclose potential egg-laying workers. Preliminary studies in our laboratory revealed that bees treated with Dufour's gland secretion tend to be more aggressed and suffer higher mortality than bees treated with solvent. This finding, if substantiated, is consistent with a reliable fecundity signal that bears cost if misused.

Does the production of a reliable fecundity signal by the queen ensure that she will monopolize reproduction in the colony? It seems that in some cases the arms race between the queen and workers is still evident. In the Cape honey bee (*A. m. capensis)* reproductive workers apparently evade both queen pheromonal control and worker policing (Moritz et al., 2001). They develop ovaries and a queen-like pheromonal signal themselves (mandibular gland (Crewe and Velthuis, 1980;

Hepburn and Crewe, 1990) as well as Dufour's gland (Sole et al., in press). It was further observed that worker distribution in the colony is variable and based on the location of the queen. There are bees that are attracted to her, but there are also workers that are repelled (Moritz et al., 2001). In *A. mellifera* (of European origin) there are mutant colonies termed "anarchistic", in which worker reproduction is successful in the presence of the queen (Oldroyd et al., 1994). However, it is still unknown whether this phenomenon is accompanied by mimicking queen pheromonal signals. Even in normal colonies there is evidence for some worker reproduction under QR conditions. Using electrophoretic markers it was shown that up to 7% of the haploid eggs in normal colonies are worker derived (Visscher, 1996). How these workers evade the queen control and/or worker policing is still enigmatic. All these findings raise the possibility that while multiple insemination may have resulted in queen reproductive dominance, it did not stop the ongoing arms race between queen and workers. We suggest that Dufour's gland secretion mimicry reflects this ongoing arms race.

It is now evident that in honeybees there are multiple sources of queen signals. While it is possible that each of these secretions serves a certain role in queen-worker interaction, it is the whole complement that signals the queen's quality, as a possible result of the queen-worker arms race. It is known that honeybee colonies differ in their sensitivity to QMP (there are high and low-responding colonies to the retinue effect of the QMP; Pankiw et al., 1994). This reduced sensitivity may provide an opportunity for nest members to start reproducing without being attacked by their nestmates, since their pheromonal changes will not be sensed. This expressed escalation in the arms race may have selected for queens to produce additional queen signals, thus bypassing the insensitivity to the existing queen signal. Once evolved, this queen signal may have been used later for regulating more specific queen-worker interactions.

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