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ORIGINAL ARTICLE

Tamar Katzav-Gozansky · Victoria Soroker
Fernando Ibarra · Wittko Francke · Abraham Hefetz

Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal?

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Abstract The role of Dufour's gland secretion as an egg discriminator pheromone was reevaluated by simultaneously exposing workers to two combs, one containing queen- or worker-laid eggs and the second containing treated or untreated worker-laid eggs. Treatments included extracts of Dufour's gland secretion as well as the synthetic esters that were identified in the secretion. Policing was clearly detected both in queenright and queenless colonies by the swift removal of worker, but not of queen eggs. However, neither the glandular secretion nor its synthetic ester constituents were able to protect worker-born eggs from policing. Treated worker eggs were removed significantly faster than queen eggs, and at the same rate as non-treated worker eggs. These results are not consistent with the hypothesis that the secretion serves as an egg-marking pheromone. Chemical analyses of the queen abdominal tips revealed the presence of Dufour's esters, indicating that the glandular secretion oozes out and spreads over the cuticle around the genital chamber. However, contamination while ovipositing may also explain the minute amounts of these esters that were detected on the egg surface. Dufour's gland caste-specific composition suggests that in queens it may constitute a signal that plays a role in queen-worker interactions. Attraction bioassays revealed that the queen secretion, but not that of workers, is very attractive to workers. When applied either on a glass slide or on another worker, a retinue formed around the "surrogate queen". We conclude that Dufour's gland secretion constitutes part of a complex queen signal that is the basis for the social integrity of the honeybee colony.

Keywords Dufour's gland · Worker policing · Attraction · Honeybee · Egg-marking discriminator

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T. Katzav-Gozansky (✉) · A. Hefetz
Department of Zoology,
George S. Wise Faculty of Life Sciences,
Tel Aviv University, Ramat Aviv, Tel Aviv, Israel
e-mail: katzavt@post.tau.ac.il
Tel.: +972-3-6409341, Fax: +972-3-6406991

V. Soroker
Department of Entomology, Institute of Plant Protection,
Agricultural Research Organization, The Volcani Center,
Beit Dagan, Israel

F. Ibarra · W. Francke
Institut für Organische Chemie, Universität Hamburg,
Hamburg, Germany

Introduction

Queen-worker conflict over male production is a common feature in social insects, and worker-born males are known in many species. In honeybees, however, the queen seems to have almost absolute dominance over male-egg production. In a queenright (QR) colony, only a few egg-laying workers are present (Page and Erickson 1988), and these are responsible for laying about 7% of all male eggs (Visscher 1996). However, only about 0.1% of the males in a colony are worker born (Visscher 1989). The successful domination by the queen over male production can be explained by kin selection theory. Since the queen honeybee is multiply mated, it is in the best interest of the workers to rear brothers (the queen's sons) rather than nephews (their nestmates' sons). This has resulted in the evolution of selective elimination of worker-born eggs by the workers in the colony (Woyciechowski and Lomnicki 1987), a phenomenon later called worker policing (Ratnieks 1988). Effective worker policing necessitates the development of a mechanism that enables the workers to discriminate between queen- and worker-laid eggs, e.g., an egg-discriminating pheromone. The existence of an egg-discriminatory pheromone was postulated by Ratnieks (1988), who in subsequent experiments indicated that Dufour's gland secretion serves in this role (Ratnieks 1995). Recently,the ability of anarchistic bees to mimic queen eggs, and thereby protect them from policing, was hypothesized as due to mutations in Dufour's gland that enabled the anarchistic workers to produce a queen-like secretion (Oldroyd and Ratnieks 2000). However, since normal

egg-laying workers can produce the queen-like secretion (Katzav-Gozansky et al. 1997a), an explanation other than mutations in Dufour's gland is needed to explain the ability of anarchistic workers to produce eggs that are protected from policing.

Several features of Dufour's gland in honeybees support its possible function as the source of an egg-discriminating pheromone. This abdominal gland opens into the dorsal vaginal wall (Billen 1987) and its secretion may therefore be controllably applied onto the egg before deposition. It is considerably larger in queens than in workers and contains queen-specific secretion (Katzav-Gozansky et al. 1997a). While the exudate of workers is composed of a series of odd *n*-alkanes, the glandular exudates of queens contain in addition wax-type esters, of which a mixture of tetradecyl hexadecanoate and hexadecyl tetradecanoate predominates. These esters may be regarded as a queen-specific signal that is deposited on the queen-born eggs (haploid or diploid) and protects them from policing. Other features concerning the selective expression of Dufour's gland, however, seem to negate its role as the source of egg-discrimination pheromone. Under queenless (QL) conditions, workers that develop ovaries and lay eggs also produce the main queen-specific esters (Katzav-Gozansky et al. 1997a). While ester production in QL egg-laying workers indicates that they have retained the ability to biosynthesize esters, this does not necessarily indicate that potential egg-laying workers under QR condition also do so. The production of these esters by egg-laying workers may reflect an effort to protect their eggs from policing, or as an act of competition for dominance between workers in a hopeless QL situation. In a QL honeybee colony, worker policing is expected to break down because a worker will gain maximal fitness by rearing her own brood. Therefore, unless workers are able to discriminate between their own eggs and eggs of the other workers, policing becomes maladaptive. Moreover, under hopeless QL conditions, workers that are unable to reproduce will still gain inclusive fitness by rearing their nephews. Conflict, however, may occur if bees can identify eggs of their own subfamily and favor them by eliminating all other eggs.

While producing queen-like Dufour's gland secretion may give QL workers a reproductive edge, we can not ignore a possible advantage to a QR worker in producing the secretion. If, as is assumed, the secretion serves to mark eggs, then such workers could sneak in eggs unnoticed and gain more fitness by producing sons. Should this be the case, there would be an arms race between queen and workers over reproduction. However, for such a system to evolve and be maintained, the number of QR egg-laying workers should be very low – as it is in Batesian mimics – otherwise the signaling system would break down. Studies of the biosynthesis of Dufour's gland secretion in vitro seem to lend credence to this hypothesis. These studies have shown that the glands of QR workers, after a certain delay, are also able to produce the esters (Katzav-Gozansky et al. 2000), indicating that, normally, Dufour's gland of QR workers is repressed, but also raising the possibility that at least some of the QR workers within the hive are able to alleviate this inhibition and mimic the queen's secretion. It also casts doubt on the role of Dufour's gland secretion as the egg-discrimination pheromone.

The caste specificity of Dufour's gland secretion also raises the possibility that it acts as a queen signal. Queen-specific signals in honeybees include 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), and a fecal pheromone (Page et al. 1988). 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, like the QMP, while many activities can be affected by a combination of several pheromones. One example is the inhibition of worker ovarian development, which is affected by both queen and brood pheromone (Winston 1987; Arnold et al. 1994; Mohammedi et al. 1998). Moreover, a single pheromone can act concomitantly as both a releaser and a primer pheromone. The well-studied QMP, for example, modulates a number of activities. Among these it attracts workers to the queen, and gives rise to a retinue of workers around her (Slessor et al. 1988). 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). Moreover, demandibulated queens still evoke retinue behavior (Velthuis and Van Es 1964; Velthuis 1970; Winston and Slessor 1992), suggesting that queen pheromones, other than QMP, are also important cues for worker honeybees (De Hazan et al. 1989).

The purpose of this study was to examine the role of Dufour's gland secretion as an egg-discriminating pheromone, as well as to evaluate its function as a queen signal.

Methods

Bees

All the experiments were conducted with colonies of *Apis mellifera ligustica* at the Tzrifin apiary, Israel, and in experimental hives at the I. Meier Segals Garden for Zoological Research at Tel Aviv University, between March and June 1998 and 2000.

Extract preparation and chemical analyses

Egg surface chemistry was analyzed using 500 1-day-old queen-laid eggs that were individually collected, then pooled and washed for 1 min in 5 ml dichloromethane. The extract was filtered through glass wool to remove egg particles and concentrated to 70 µl. Since preliminary analyses revealed the presence of large amounts of hydrocarbons that masked the presence of minor constituents, the egg washes were fractionated on a silica gel column (6.5 cm long and 0.6 cm wide, Macherey and Nagel 70–

230 mesh). Hydrocarbons were separated from the more polar constituents (esters included) by stepwise elution with hexane (15 ml) followed by 6% diethyl ether in hexane (15 ml). The hexane fraction contained only hydrocarbons while the second more polar fraction contained mostly esters, as evident by gas chromatography/mass spectrometry (GC/MS) analyses. To verify that the eggs removed for analysis were not contaminated with comb waxes, GC/MS analyses of wipes of the latter were also performed.

Abdominal tips of ten mated queens were wiped with an ethanol-soaked cotton ball, the pentane extract of which was concentrated and subjected to GC/MS analysis. The wipes were performed at the area of the oviposition opening of the genital chamber, using queens that had been housed for a few days with attendant workers in mailing cages. Under such conditions queens are known to accumulate a white waxy material around the genital chamber (Snodgrass 1956). The analysis aimed to identify the chemical nature of this waxy material and to verify whether it contains Dufour's gland products as was suggested by Ratnieks (1995).

Structure elucidation of volatiles was done by GC/MS. Crude extracts were concentrated in microvials (Klimetzek et al. 1989). Analyses of volatiles were carried out by combined capillary GC/MS. A Fisons GC 800 equipped with a 30-m 0.25-mm internal diameter DB5-capillary column, programmed from 60 to 300°C at a rate of 5°C/min, was linked to a Fisons MD 800 mass spectrometer, operated at 70 eV. The compounds were identified by their mass spectrometrical fragmentation patterns (Tengö et al. 1985; McLafferty and Stauffer 1989; Francke et al. 2000) and by comparison with authentic samples. Positions of double bonds were determined according to Hefetz et al. (1996). The eluting compounds were identified by their mass spectra and gas chromatographic retention times as compared to synthetic references, and previously published spectra (Katzav-Gozansky et al. 1997a). Quantification of all glandular secretions was performed by capillary GC on a 30-m SE 54 capillary column, programmed from 60–100 °C at rate of 20 °/min and then programmed to 270 °C at rate of 5 °/min.

Synthetic esters

Esters were synthesized from commercially available (Sigma) alcohols and acid chlorides (prepared from the corresponding acids) according to standard procedures: 1.1 equivalents of the alcohol were dissolved in 5 ml absolute pyridine; after addition of a catalytic amount of 4-dimethylamino pyridine, the solution was cooled in an ice bath; after addition of 1.0 equivalent of the acid chloride, the resulting suspension was stirred for 1 h at room temperature (TLC control). The reaction was quenched by addition of aqueous sodium hydrogen carbonate. The aqueous phase was extracted twice with hexane, and the hexane solution was washed with diluted hydrochloric acid, aqueous sodium hydrogen carbonate, and brine. After drying the solution with anhydrous magnesium sulfate and concentration under reduced pressure, the product was chromatographed on silica gel (Merck 60–200 mesh) using hexane/ethyl acetate 30:1 as eluent. The purity of the final products, checked by GC, proved to be 97–99%. Structural proof of the esters was obtained by nuclear magnetic resonance and MS. (Z)-9-hexadecenal and (Z)-11-hexadecenol (Sigma) served for syntheses of the corresponding carboxylic acids (Jones' reaction).

Since the ester constituents of Dufour's gland secretion comprised the queen-specific compounds, their activity was tested using synthetic material. The ester blend was prepared according to the relative proportion of the esters in the queens' total glandular constituents. All esters present in the gland (Table 1) were used, except tetradecyl (Z)-9-tetradecenoate that was found as a trace in the gland. Hydrocarbons generally common to queen and worker secretions were not used in the mixture preparation.

Table 1 Chemical composition of queen Dufour's gland secretion, egg surface, and abdomen wipes of *Apis mellifera* (*ND* not detected, + present in the extract). Numbers refer to the corresponding peaks in Fig. 1

Esters		Dufour's gland	Egg coating	Abdomen wipes
1	Isopropyl tetradecanoate	ND	+	+
2	Isopropyl hexadecanoate[a]	ND	+	ND
3	(1-Methyl)hexyl hexadecanoate	ND	+	ND
4	Decyl hexadecanoate	ND	+	ND
5	Tetradecyl dodecanoate	+	ND	+
6	Tetradecyl (Z)-9-tetradecenoate	+	ND	+
7	Tetradecyl tetradecanoate	+	+	+
8	Dodecyl hexadecanoate	ND	+	ND
9 10	Tetradecyl (Z)-9-hexadecenoate + Tetradecyl (Z)-11-hexadecenoate[a]	+	+	+
11 12	Tetradecyl hexadecanoate + Hexadecyl tetradecanoate[b]	+	+	+
13	Tetradecyl (Z)-9-octadecenoate	+	ND	ND
14	(Z)-9-hexadecenyl hexadecanoate	+	ND	ND
15	Hexadecyl hexadecanoate	+	+	ND
16	Octadecyl tetradecanoate	+	ND	ND
17	(Z)-9-hexadecenyl (Z)-9-octadecenoate	+	ND	+
18	Octadecyl (Z)-9-hexadecenoate	ND	+	+
19	Hexadecyl (Z)-9-octadecenoate	ND	+	ND
20	(Z)-9-octadecenyl (Z)9-hexadecenoate	ND	ND	+
21	Octadecyl hexadecanoate	+	ND	+
22	Hexadecyl octadecanoate	ND	+	ND
23	Octadecyl (Z)-9-octadecenoate	ND	ND	+
24	Octadecyl octadecanoate	ND	ND	+
25	Eicosyl hexadecanoate	ND	ND	+
26	Eicosyl octadecanoate	ND	+	ND
27	Tetracosyl hexadecanoate	ND	+	ND
28	Decyl (Z)-9-octadecenoate	ND	+	ND

[a] Trace amounts only [b] Relative proportions ca 1:5

Table 2 Comb types simultaneously inserted into a QR discriminator colony to assess worker policing

First comb	Second comb
Queen-laid haploid eggs	Worker-laid haploid eggs
Worker-laid haploid eggs sprayed with ethanol	Queen-laid diploid eggs sprayed with ethanol
Worker-laid haploid eggs sprayed with Dufour's gland secretion	Queen-laid diploid eggs sprayed with ethanol
Worker-laid haploid eggs sprayed with Dufour's gland secretion	Worker-laid haploid eggs sprayed with ethanol
Worker-laid haploid eggs sprayed with synthetic ester blend	Queen-laid diploid eggs sprayed with ethanol
Worker-laid haploid eggs sprayed with synthetic ester blend	Worker-laid haploid eggs sprayed with ethanol

Egg-policing bioassay:

The effect of Dufour's gland secretion on egg discrimination by workers was tested in a policing bioassay. The present assay was different from a previously published assay in which the tested eggs were removed from their cell and temporarily positioned in rows on glass slides for 0.5–2 h prior to treatment. Following treatment both queen and worker eggs were transferred to a single comb of drone cells (Ratnieks 1995). In our modified assay, we avoided possible dehydration of eggs and damage during transfer by treating the eggs in situ using 1- to 2-day-old eggs that were laid directly in the tested comb. This increased the survival rate of the queen-laid eggs from 0–40% in Ratnieks setup to 75–100% in this setup. QR workers were simultaneously exposed to two combs as listed in Table 2, and the extent of egg removal was the parameter selected for assessing policing.

Preparation of queen- and worker-born eggs

Queens were induced to lay haploid or diploid eggs by inserting combs containing drone or worker cells into the hive.

Worker-born eggs were obtained from QL groups of about 2,000 workers created from a strong populated colony (Katzav-Gozansky et al. 1997a). QL colonies were established without any brood, thus no queen rearing occurred. Egg-laying workers and eggs were observed about 1 week after the QL colonies had been established.

Setting of discrimination colonies

QR discriminator colonies constituted strongly populated colonies, housed in two Langstroth hive bodies, unless otherwise stated. Only alien colonies (with respect to the egg source) were used as discriminator colonies in order to eliminate any bias due to nestmate recognition. Judging from the high retention of queen-laid eggs, we conclude that introducing an alien comb into the discriminator colony did not have much effect on egg survival. During the bioassay, the queen was confined to the lower level of the hive by a queen excluder to prevent egg deposition in the upper hive body (the test area). The test combs were inserted into the upper hive body for 24 h, between two brood combs.

Worker policing was also tested in QL colonies to assess whether it is context dependent, i.e., do workers react to the queen pheromone independently of her presence, or does policing break down under a hopeless QL situation? Furthermore, since QL egg-laying workers possess the main queen like-esters, this may provide another indication as to whether or not the secretion is used for marking the eggs. To test egg discrimination by QL workers, we used QL discriminator hives of about 2,000 bees that were established as previously described. To avoid biased results due to the deposition of new eggs by egg-laying workers and consequently interfering with the scoring of policing, the experiments were conducted before the workers commenced egg laying (during the 2–6 days after the QL discriminator colonies were created). Egg laying in these colonies was inspected daily. In these experiments, the tested egg-combs were inserted between two honey-combs.

Bioassay and treatments

The policing bioassay was based on simultaneously introducing two egg-combs side by side. Worker policing was evaluated by counting the number of eggs in each of the combs in a constant sample area (15.5×10 cm) at time 0 and 24 h. To test the effect of Dufour's gland secretion or its synthetic ester constituents on egg policing, ethanolic extracts (test solutions) or pure ethanol (control) were utilized (Table 2). In addition, we tested isopropyl tetradecanoate as an alternative egg-marking pheromone, since it was identified as a component on both the egg surface and abdominal tip. One queen equivalent (Qeq) in 2 ml ethanol of the test solutions or 2 ml of ethanol was applied to one side of each comb using an aerosol sprayer. In a preliminary experiment, ethanol was found to have no effect on egg survival or brood development. Chemical analysis of the eggs after the experiment revealed the presence of the applied esters on the egg surface. Since each comb contained approximately 1,000 eggs, and honeybee queens lay between 1,000–1,500 eggs daily during the most active period (Gary 1992), applying the combs with 1 Qeq/day simulated the use of one Dufour's content a day by the queen. For comparison, Ratnieks (1995) in his experiments used one queen Dufour's gland to treat up to 150 eggs (10 Qeq/day).

Worker policing in QR colonies was evaluated by examining the sets of combs described in Table 2. Worker policing in QL colonies was carried out using queen-laid diploid eggs versus worker-laid haploid eggs that were both sprayed with ethanol.

Egg removal as a function of number of eggs per cell

The pattern of egg laying by workers differs from that of the queen (Page and Erickson 1988). Workers lay multiple eggs per cell, while the queen lays one egg per cell. It was therefore necessary to test whether the number of eggs per cell is important in discrimination between worker- and queen-laid eggs resulting in worker policing. Counts were conducted using the same discriminatory colonies at time 0 and after 3 h.

Attraction bioassay

Two types of attraction bioassays were performed: one using a treated glass slide as a queen substitute, while in the second assay, live workers treated with the test solution were used. The tests were performed in plastic Petri dishes (15×2 cm) containing two side slits for insertion of the tested material. Bees (20 for the slide bioassay or 10 for the live-bee bioassay) from the brood area of a QR colony were used in each assay shortly after they had been collected (modified from Kaminski et al. 1990). In the glass slide bioassay, queen's Dufour's or mandibular gland extracts in ethanol were applied to one slide and ethanol (control) to the second slide. After allowing the solvent to evaporate, both slides (treatment and control) were simultaneously inserted into the arena through the side slits. The number of workers contacting each slide was recorded at 30-s intervals during a 5-min test (total of ten replicates). For each replicate, the sum of contacts for treatment versus control over 5 min was used as a measure of attraction and tested for significance using the Wilcoxon signed-rank test. To empha-

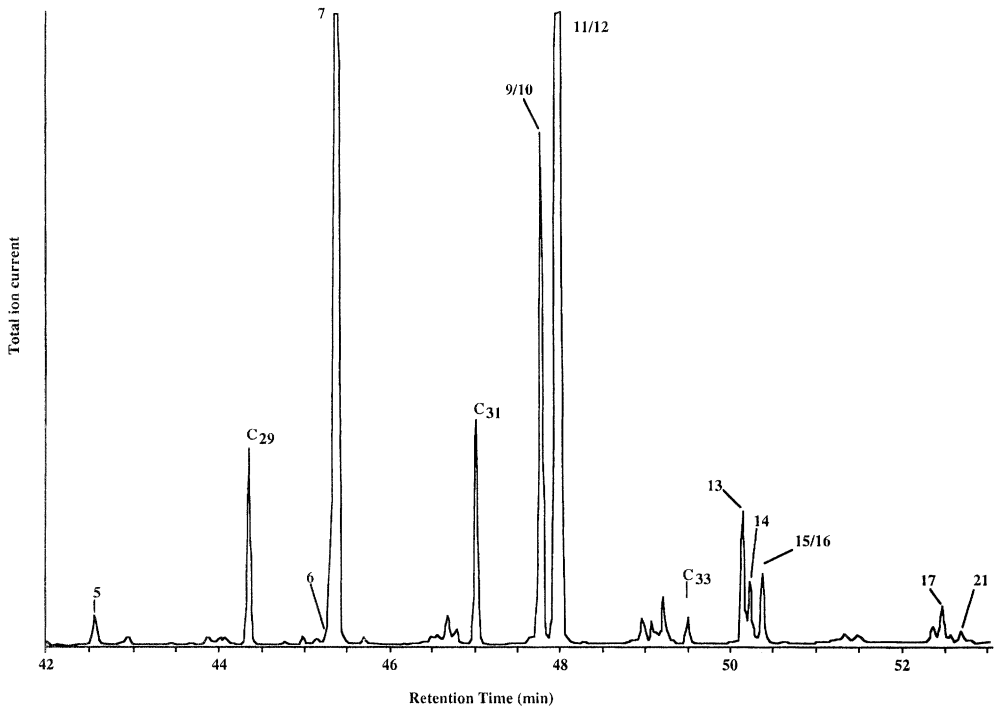

Fig. 1 Section of the gas chromatogram showing the wax-ester region of queens' Dufour's gland contents; *numbers* refer to compounds listed in Table 1. C_{29}, C_{31}, and C_{33} represent nonacosane, hentricontane, and tritriacontane, respectively. They are accompanied by small amounts of earlier-eluting alkenes

size the level of preference of the treatment versus control, the results are presented as the percentage of assays.

In the second type of bioassay, live workers were used. Presenting a secretion on a bee versus an inanimate object such as a glass slide provides a more natural situation. In addition, since egg-laying workers possess a queen-like Dufour's secretion, it was interesting to investigate how other workers react to a worker-borne queen signal. In these assays, 2 µl of either queen or worker Dufour's gland extracts were applied on two individually marked nurse bees. The bees were color marked on the thorax while the extracts were applied on the abdomen. Both bees, extract and solvent treated, were introduced simultaneously to the arena and the number of attracted workers was recorded as described above. The doses used in both assays were calculated as queen equivalents, with 1 Qeq equaling 20 µg of all glandular constituents. One queen equivalent of mandibular gland secretion was considered as the extract of the paired queen glands.

Statistical analysis:

Statistical analyses were performed using Statistica for Windows, version 5.5 (Statsoft). To test whether egg removal in each pair of simultaneously inserted combs differed, a Wilcoxon signed-rank test was used. The same statistical analysis was also used to compare the attraction of workers to the glandular secretion and the control. The effect of number of eggs in each cell was tested using a Kruskal-Wallis test. Statistical significance was accepted at P=0.05. Data are presented as means±SE.

Results

Egg coating and abdominal tip extractions

Comparative analyses of Dufour's gland extract (Fig. 1), a wipe of queen abdominal tips, and a solvent wash of diploid eggs laid by the queen revealed that both abdominal tips and egg washes contained the esters characteristic of the queen's Dufour's gland (Table 1). In the abdominal wipes, uneven-numbered *n*-alkanes ranging from C_{23} to C_{31} that are characteristic of cuticular lipids (Arnold et al. 1996) were also identified. Dufour's gland secretion contained small amounts of high-boiling alkenes. The abdominal tip wipes also contained a series of even-numbered aliphatic primary alcohols ranging from tetradecanol to eicosanol, and fatty acids including nonanoic, tetradecanoic, hexadecanoic, and octadecanoic acid. No traces of mandibular gland components were found in the analysis of the abdominal tip wipes. Egg coating contained enormous amounts of odd-numbered hydrocarbons (similar to those identified in abdominal tip wipes and several lower-boiling ones), but when fractionated by column chromatography, the major Dufour's gland esters as well as long-chain aldehydes (C_{18}–C_{22}) of unknown origin could be detected in the more polar fraction. One of the esters found in the egg coating wash was isopropyl tetradecanoate, which was also present in the abdominal wipes but was completely absent in Dufour's gland secretion. The most abundant wax-type ester present in the egg extracts was decyl (Z)-9-octadecenoate which was absent in both Dufour's gland extracts and abdominal wipes. Comb wax analysis revealed that none of its characteristic waxes were found in the egg extracts.

Egg-policing bioassay

First, we verified that workers could discriminate between haploid queen-laid and worker-laid eggs, by simultaneously introducing both comb types into a QR colony for 24 h. The bees largely retained the haploid queen-laid eggs (68%) whereas most of worker-laid eggs

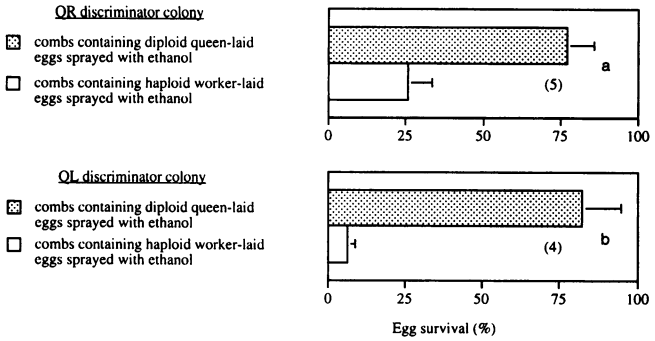

Fig. 2a,b Worker policing in treated combs (value in *parentheses* number of replicates)

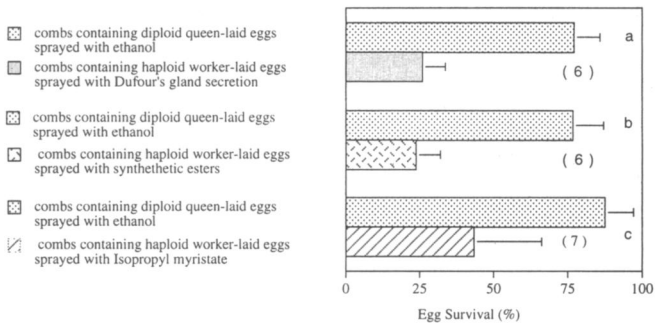

Fig. 3a–c Egg survival after 24 h in combs containing queen-laid eggs (ethanol treated) and combs containing worker-laid eggs treated with various extracts simultaneously introduced into QR colonies (value in *parentheses* number of replicates)

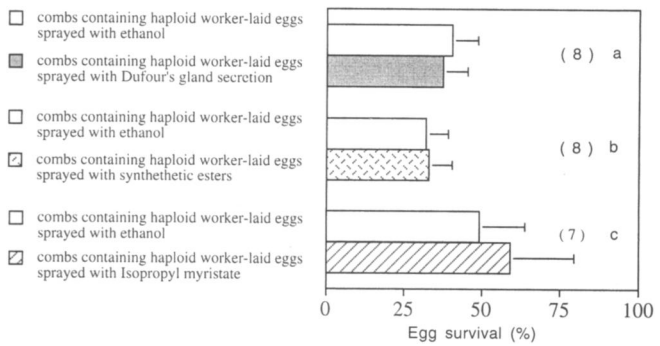

Fig. 4a–c Egg survival after 24 h when two combs containing worker-laid eggs had been introduced to a QR hive, one of which was sprayed with ethanol and the other with the tested compounds (value in *parentheses* number of replicates)

were eliminated (only 13.5% survived). Likewise, the survival of queen-laid diploid eggs in a QR discriminatory colony was very high: about 80% of the eggs were conserved after 24 h. Similarly, Oldroyd and Ratnieks (2000) also found no significant difference in policing rate between haploid or diploid queen-laid eggs. Thus, we used diploid queen-laid eggs for all subsequent experiments.

Figure 2 presents egg removal in combs containing queen-laid eggs compared to combs containing worker-laid eggs. Both comb types were ethanol treated and simultaneously introduced into QR or QL colonies. In a QR discriminatory colony, 69% of queen-laid eggs were conserved after 24 h as compared to 31% survival of worker-laid eggs (Wilcoxon signed-rank test T=0, n=5, P=0.043; Fig. 2a). Worker policing behavior in QL hives seemed as effective. Queen-laid eggs were mostly retained whereas worker-laid eggs were mostly eliminated (82% vs 6% queen- and worker-eggs were retained after 24 h, respectively; Wilcoxon signed-rank test T=0, n=4, P=0.068; Fig. 2b).

Figures 3 and 4 present the effect of Dufour's gland secretion as well as synthetic Dufour's esters on egg discrimination, i.e., egg policing by workers in QR colonies. Figure 3 compares egg survival in combs containing queen-laid eggs (ethanol treated) and combs containing worker-laid eggs treated with various extracts simultaneously introduced into QR colonies. Spraying the comb of worker-laid eggs with Dufour's gland secretion did not protect them from worker policing. Only 25% of the worker eggs survived compared to 77% of the queen eggs (Wilcoxon signed-rank test T=1, n=6, P=0.046; Fig. 3a). Worker-laid eggs from combs sprayed with synthetic esters and inserted into the discriminator colony along with queen-laid eggs (Fig. 3b) were eliminated at similar rates (20%). Although treating the eggs with isopropyl tetradecanoate improved survival to 43%, it was not significantly different from that of ethanol-treated eggs (43% vs 31%; Mann-Whitney U-test U=10, n=12, P=0.22).

The second set of experiments aimed to assess worker policing when two combs containing worker-laid eggs were introduced to a QR hive, one of which was sprayed with ethanol and the other with the tested extract (Fig. 4). Treating one comb with Dufour's gland secretion did not significantly alter the rate of egg removal compared to the ethanol-treated comb (37% and 41%, respectively; Wilcoxon signed-rank test T=14, n=8, P=0.58; Fig. 4a). Likewise, spraying one of the combs with synthetic esters did not reduce egg removal from that comb (32% vs 33% survival in the ester and ethanol treatments, respectively, after 24 h; Wilcoxon signed-rank test T=18, n=8, P=1.0; Fig. 4b). Spraying one of the combs with isopropyl tetradecanoate enhanced the survival rate of the eggs, but this difference was not statistically significant (59% vs 49% for the ester and ethanol treatments, respectively; Wilcoxon signed-rank test T=4, n=7, P=0.09; Fig. 4c).

The pattern of egg laying by workers differs from that of the queen. At the onset of worker egg laying, eggs laid by workers are placed erect in the bottoms of cells like queen eggs, but as more eggs are deposited, they are poorly placed in the bottom of the cells with multiple eggs laid per cell (Page and Erickson 1988). We therefore tested the hypothesis that egg-number per cell provides a cue for recognition of worker-laid eggs. The extent of worker-laid egg removal after 3 h was calculated

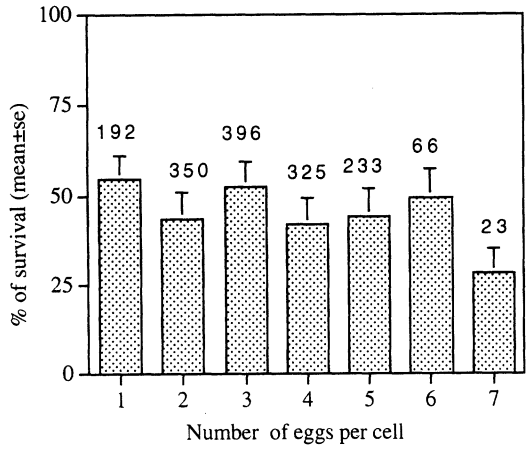

Fig. 5 Worker egg removal by QR workers as a function of the original number of eggs deposited per cell after 3 h. The *numbers* above the columns represent the total number of cells counted in four combs

in relation to their original number per cell (t=0). As can be seen in Fig. 5, egg removal was independent of the original number of eggs in the cell (Kruskal-Wallis H=1.996, n=27, P=0.92). Removal of eggs from cells that originally contained seven eggs was higher, but not significantly so (Mann-Whitney U-test U=21, n=27, P=0.247)

Attraction bioassay

Attraction of workers to queen Dufour's gland secretion applied to a glass slide is presented in Table 3. For comparison, worker attraction toward queen mandibular gland secretion, previously reported to induce retinue behavior in worker honeybees (Kaminski et al. 1990), is also shown. Queen mandibular gland secretion was attractive to workers at all concentrations tested. Dufour's gland secretion was significantly attractive at concentration of 1/3 and 1/4 Qeq, but not at the lower or higher concentration tested. In a second set of experiments, we tested the retinue response of workers toward a nestmate applied with various doses of queen or worker Dufour's gland secretion (Table 4). In each trial, two workers, one treated with glandular secretion and the second with ethanol (solvent control) were introduced into the arena. Workers treated with queen Dufour's gland secretion were significantly more attractive than their solvent-applied paired workers at concentrations of 1/2, 1/4, 1/6 and 1/8 Qeq, but not at the lower concentrations of 1/16 or 1/32 Qeq. At the attractive doses of Dufour's gland secretion, workers were seen to form a court around the treated nestmate. No attraction was observed to any concentration of workers' Dufour's gland secretion. In one case (1/6 Qeq of workers' secretion), the secretion may have repelled the workers, since the preference was significant in the opposite direction.

Table 3 Attraction of worker honeybees to a glass slide to which queen mandibular or Dufour's gland secretion had been applied at different concentrations. Data are presented both as the mean values of contact with the treatment and control slide and as preference, expressed as the percentage of tests in which the glandular secretion was preferred over the control. Statistical analysis was performed using the Wilcoxon signed-rank test (n number of replicates)

Gland concentration	Queen mandibular gland extract					Queen Dufour's gland extract				
	Mean of contacts (treatment vs control) ± SE	T	P	n	Preference (%)	Mean of contacts (treatment vs control) ± SE	T	P	n	Preference (%)
1/2 Qeq	23.8±2.6 vs 14.1±2.2	5.5	0.025	10	80.0	13.0±1.4 vs 9.2±1.4	15.5	0.065	12	83.3
1/3 Qeq	26.4±3.4 vs 13.5±1.8	2	0.006	12	91.6	19.3±5.2 vs 8.2±1.2	11.5	0.031	12	83.3
1/4 Qeq	33.1±3.5 vs 12.9±2.3	0	0.011	8	100.0	16.8±3.0 vs 11.3±1.3	6	0.009	8	75.0
1/6 Qeq	26.3±2.8 vs 10.8±1.6	6	0.008	18	83.3	11.6±1.7 vs 8.6±1.2	49	0.110	18	61.0

Table 4 Attraction of worker honeybee to a worker bee applied with queen or worker Dufour's gland secretion at different concentrations. Data are presented both as the mean values of contact with the treatment and control bee and as preference, expressed as the percentage of tests in which the glandular secretion was preferred over the control. Statistical analysis was performed using a Wilcoxon signed-rank test (n number of replicates

Gland concentration	Queen's Dufour's gland secretion					Forager's Dufour's gland secretion				
	Mean of contacts (treatment vs control) ± SE	T	P	n	Preference (%)	Mean of contacts (treatment vs control) ± SE	T	P	n	Preference (%)
1/2 Qeq	39.8±4.1 vs 8.5±2.1	0	0.011	8	100.0	10.1±2.8 vs 7.6±1.8	11.5	0.36	8	62.5
1/4 Qeq	15.0±1.3 vs 8.0±0.7	145.5	<0.001	47	88.3	7.7±0.8 vs 9.5±1.9	8	0.600	7	50.0
1/6 Qeq	10.0±1.0 vs 5.0±0.9	1.5	0.020	8	87.5	6.8±1.1 vs 13.5±3.3	4	0.049	8	12.5
1/8 Qeq	39.6±6.2 vs 7.6±1.6	0	0.011	10	100.0	7.7±1.4 vs 8.9±1.1	6.5	0.786	8	37.5
1/16 Qeq	21.3±5.4 vs 13.1±2.1	7.5	0.270	8	62.5					
1/32 Qeq	17.9±2.8 vs 15.3±2.8	23	0.640	10	60.0					

Discussion

Queen-worker conflict in honeybees seems to have been resolved by the queen's multiple mating and the evolution of worker policing. Thus, from kin selection theory, it follows that worker sterility is driven by the mutual interest of workers to rear brothers rather than nephews (Woyciechowski and Lomnicki 1987; Ratnieks 1988). Using electrophoretic markers, 7% of the haploid eggs in normal colonies were found to be worker derived, but only a few worker-derived males develop (Visscher 1996). Indeed, worker sterility is not absolute, and under QL conditions, at least some of the workers lay viable male eggs that produce males. This suggests that there are still signs of queen-worker conflict even in this highly evolved social insect. Worker policing necessitates the evolution of an egg-discrimination mechanism, i.e., an egg-marking pheromone. Dufour's gland secretion of the queen has been suggested as the source of such a pheromone that is applied onto the egg during oviposition (Ratnieks 1995). Two predictions stem from this hypothesis: Dufour's gland secretion can be detected on the egg surface, and application of the queen secretion on worker-laid eggs will protect them from policing.

Among social insects there is some evidence for active egg marking that permits egg discrimination. In many monogynous QL ants, the alpha worker is often able to destroy subordinates' eggs. In *Diacamma* sp., the single gamergate eats most of the haploid eggs occasionally laid by virgin workers (Nakata and Tsuji 1996; Kikuta and Tsuji 1999). In *Dinoponera quadriceps*, oophagy seems to be regulated by an identified chemical signal (9-hentriacontane) present on the cuticle of alpha workers and on their eggs (Monnin and Peeters 1997). In some polistine wasps, alpha wasps selectively destroy the eggs laid by subordinates during the initial stage of colony foundation (Reeve 1991). In *Polistes fuscatus* and the honeybee, Dufour's gland secretion seems to be used for that recognition (Downing 1991; Ratnieks 1995).

Our chemical analyses of honeybee eggs revealed an abundance of hydrocarbons that mask almost entirely any minor constituents that may have been present. However, removing the hydrocarbon fraction by column chromatography revealed that the major esters of queen Dufour's gland constitute part of the egg surface chemistry, accompanied by acids and aldehydes of unknown origin, and relatively large amounts of isopropyl tetradecanoate. Dufour's esters and isopropyl tetradecanoate were also detected in wipes of the abdominal tips. It should be noted that although the esters comprise about 60–70% of queen Dufour's gland secretion (about 12 μg esters per queen; Katzav-Gozansky et al. 1997a), only minute amounts of esters were detected on the eggs. This raises the possibility that the esters detected on the egg surface result from passive contamination by the queen Dufour's gland secretion during oviposition rather than active marking of the eggs by the queen. The presence of Dufour's esters in the queen abdominal tips supports this suggestion and indicates that the secretion oozes out of the gland and spreads over the cuticular area of the abdominal tip.

Our present experiments showed that worker policing occurs under both QR and QL conditions. When workers are exposed simultaneously to queen- and worker-born eggs, they rapidly selectively eliminate the workers' eggs. The fact that policing is also effective under QL conditions indicates that even QL workers are still sensitive to the differences between queen- and worker-born eggs. Moreover, workers react to the putative queen signal present on the egg irrespective of the context of perception. Theoretically, in the absence of the queen, it is adaptive for workers to retain both queen- and worker-born eggs; this, however, does not happen. Under a hopeless QL situation and in the absence of a reference of queen-laid eggs, policing probably breaks down and workers do not devour their sisters' eggs. However, since preferentially rearing drones according to relatedness (sons > sons of super-sisters > sons of half-sisters) confers higher inclusive fitness, discrimination with respect to kinship is predicted (Robinson et al. 1990). However, this would necessitate a more refined egg-discrimination signal, evidence for which is lacking at the moment. Egg discrimination is probably mediated by chemical signals and not because workers lay several eggs per cell and in a disorderly manner, since the original number of worker-laid eggs per cell did not affect the policing rate. However, the source of this chemical signal does not seem to be Dufour's gland secretion. Neither the queen glandular secretion nor its synthetic ester constituents protected worker-laid eggs from oophagy. In the first set of experiments, we tested whether worker eggs artificially marked with Dufour's gland secretion or with queen-specific esters would acquire the protection of naturally marked eggs (i.e., queen-derived eggs). This was not the case: worker eggs were swiftly removed. In the second set of experiments, we tried to "upgrade" worker eggs to a "queen egg status" with the above chemicals in a comparison with regular, untreated, worker-laid eggs. The "upgraded" worker eggs were not treated differently by the test bees, and were removed as fast. Application of isopropyl tetradecanoate, a compound that was detected on the egg surface and on the queen's abdominal tips, reduced egg removal, but not to a statistically significant degree. These results contradict previously published observations that suggested that the queen Dufour's gland is the source of a discriminator pheromone that protects eggs from policing (Ratnieks 1995). One of the possibilities for the contradictory results may reside in the experimental design. In Ratnieks' (1995) experiments only a single comb was inserted into the hive, into which both the treated and control eggs were transferred. In the present experiments, the two types of eggs were naturally deposited on each of the two combs that were inserted into the discriminator hive. The quantity of Dufour's gland extract applied on the eggs was ten times less than in Ratnieks' design, which may have also contributed to the different results. Applying queen attractant (as found in this study) to the egg at

high dose may have interfered with egg recognition. Our experiments did not exclude the possibility that egg discrimination occurs through signals found on the comb. The comb can serve as a medium for the transmission of cues (Breed and Stiller 1992). Combs with queen-laid eggs possibly absorbed a queen signal like a footprint pheromone (Lensky and Slabetzki 1981), thus rendering them automatically more attractive. Theoretically, this would seem unlikely to interfere with egg discrimination, since in a normal hive, workers may insert their egg in a comb already containing queen-born eggs. Therefore, spraying the combs with the tested solution, in our judgment, did not disrupt the recognition of any signal deposited on the egg. In any case, these putative signals do not seem to originate from Dufour's gland secretion. The eggs used in this study were of random age, which may have introduced a small bias into the experiment, since older eggs become more acceptable (Ratnieks 1993). However, egg age seems not to have been a critical factor since worker eggs were swiftly eliminated irrespective of their age.

Supporting the suggestion that the egg discrimination pheromone does not originate from Dufour's gland is the similarity of Dufour's gland secretion between egg-laying workers and queens (Katzav-Gozansky et al. 1997a). If Dufour's gland secretion were the discrimination cue, it would be reasonable to assume that egg-laying workers that possess the queen-like secretion would use it to protect their own eggs even under a hopeless QL situation. In this case, since worker-born eggs were used in the assay, policing would not have been detected. The fact that under QL conditions, workers still performed policing effectively demonstrates that having the queen Dufour's gland components did not help the egg-laying workers to camouflage their eggs. We can also exclude the possibility that discrimination is possible because queen eggs have higher amounts of esters than worker eggs. Although the queen gland has about seven times more secretion than the gland of an egg-laying worker, she lays far more eggs per day (more than tenfold). Although the queen's rate of ester biosynthesis is higher, workers should be able to apply comparable amounts of secretion per egg as queens. This is confirmed by the quantitative estimation of esters on the egg surface. Queen eggs contained far lower amounts of esters than found in Dufour's gland of egg layers. We therefore suggest that egg policing is indeed based on differential marking of queen eggs, but from a source other than Dufour's gland.

Considering the fact that under the QR condition, Dufour's gland secretion is caste specific, we suggest that it constitutes a component of the complex queen signal. Several exocrine secretions that are caste specific, such as the QMP (Slessor et al. 1988) and the queen tergal gland secretion (Blum 1992; Wossler and Crewe 1999) serve as queen signals that control, directly or indirectly, colonial activities. Moreover, some pheromones may have closely linked or overlapping effects. Using either the glass slide or the live-bee bioassays, we demonstrated that Dufour's gland secretion is attractive to workers, and that they form a retinue around the signal source. No balling behavior was observed when live bees were applied with Dufour's gland components. Moreover, there were no traces of QMP in Dufour's gland extracts that could account for the attraction of the workers toward the extract. This is comparable to the retinue behavior that was found for other caste-specific glands, i.e., mandibular glands (Slessor et al. 1988), tergal glands (Wossler and Crewe 1999), and the head gland (Slessor et al. 1998). As a queen signal, Dufour's gland secretion may play a role in the maintenance of reproductive dominance by the single queen, a system that is more complex than a one-pheromone one-signal system. It may, for example, inform the workers about queen quality/queen fecundity. Supersedure of an old, less fecund queen is common in honeybees (Winston 1987), as in other eusocial Hymenoptera. Preference for the most fecund queen by workers has previously been demonstrated in a number of ant species (Bartz and Holldobler 1982; Sommer and Holldobler 1995). The possible pheromonal mediation of the process became evident with the demonstration of fertility signaling in queens of *Leptothorax* sp. and of gamergates in *Hapegnathos saltator* (Ortius and Heinze 1999; Liebig et al. 2000). In honeybee colonies, queen quality has been suggested to be monitored using a complex of several signals. QMP acts to indicate queen presence, while combs with eggs and young larvae provide additional fecundity signals for inhibition of egg rearing (Pettis et al. 1997). The presence of Dufour's gland secretion both on the queen's abdomen and the egg surface may serve as a part of such a signal. Evidence in support of this possibility is the fact that mating affects biosynthesis of the Dufour's gland component in honeybee queens (Katzav-Gozansky et al. 1997b). The cooccurrence of ovarian development in workers and a queen-like Dufour's gland secretion may have an interesting outcome under QR conditions. Visscher and Dukas (1995) have shown that workers are able to detect certain characteristics in nestmates, most likely olfactory cues, that are correlated with ovarian development, and selectively attack them. The apparent correlation between ovarian development and Dufour's gland composition in workers (Katzav-Gozansky et al. 1997a), along with the fact that the secretion oozes out of the gland and contaminates the cuticle of the abdominal tip, may betray the egg-laying worker and result in worker molestation and inhibition from actual egg laying. Thus, Dufour's gland may be involved in worker policing not by protecting worker eggs, but by rendering rebelling workers subject to aggression. Under QL conditions on the other hand, workers that signal with Dufour's gland secretion to other nestmates that they possess developed ovaries may gain dominance and thus an advantage in the race for reproduction. Because social cohesion and brood care diminish rapidly, the majority of drones reared to maturity in a QL colony are laid within the first few days of worker oviposition (Page and Erickson 1988). Signaling by ready-to-lay workers may

not only attract attendance to them, but also result in reproductive self-restraint by other workers. In this way, more drones can develop to maturity and contribute to the inclusive fitness of the QL workers in general. Thus, the expression of Dufour's gland caste-specific chemistry may reflect an interplay between queen dominance and worker dominance in the race for reproduction.

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