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Ultrastructural and chemical characterization of egg surface of honeybee worker and queen-laid eggs

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Summary. Worker policing in honeybees predicts the evolution of a mechanism to discriminate between queen- and worker-born eggs. Although it has been postulated that this discrimination is based on an egg recognition pheromone, neither the chemistry nor the glandular source were elucidated. To verify whether egg discrimination might be based on structural differences, we compared the ultrastructure surface of queen-laid diploid and haploid eggs to that of worker-laid eggs using SEM. Only small differences between the different types of eggs were found. Thus, at least based on the fine structure of the egg surface, queen eggs are indistinguishable from worker-laid eggs.

To explore the chemosensory hypothesis for egg discrimination, we conducted a detailed comparative chemical analysis of the different egg types. The coating of all egg types was dominated by linear alkanes, but queen eggs, diploid and haploid, differed from those of workers on two accounts: 1. The diversity of compounds found on queen-laid eggs was much greater than found on worker-laid eggs, mainly due to the number of hydrocarbons. 2. Acetates of some fatty alcohols, alkenes and especially monomethylalkanes were characteristic to queen eggs. The origin of the two latter substances and the acetates is still unknown. Whether these compounds constitute the signal that enables police workers to discriminate between queen- and worker-born eggs remains to be investigated.

Key words. Dufour's gland – methylalkanes – honeybee – egg coating

Introduction

An important outcome of multiple insemination in the queen honeybee was that of setting the conditions for worker policing and thence worker reproductive self-restraint (Woyciechowski & Lomnicki 1987; Ratnieks 1988). Consequently, the queen has almost absolute dominance over male production with only a small percent of males in the colony being worker-born (0.1%, Visscher 1989). Worker policing in honeybees is manifested through the selective elimination of worker-born, but not queen-born, eggs by other workers in the colony, a phenomenon called worker policing. While this suggests that workers have the ability to discriminate between these two types of eggs, the nature of the differences between them remains unknown. Initially it was suggested that this discrimination is chemically-based and that Dufour's gland is the source of the egg marking substances (Ratnieks 1995). This abdominal gland opens into the dorsal vaginal wall (Billen 1987), and contains a caste specific secretion (Katzav-Gozansky *et al*. 1997) that may be controllably applied onto the egg during oviposition. Preliminary analyses of the surface chemistry of queen-laid diploid eggs indeed revealed the presence of minute amounts of queen-specific esters (Katzav-Gozansky *et al*. 2001). However, recent bioassays with either Dufour's gland extracts or a synthetic blend of the queen-specific esters applied to worker-laid eggs did not protect them from worker policing (Katzav-Gozansky *et al*. 2001). This finding was further supported by Martin *et al*. (2002), refuting the hypothesis that Dufour's gland secretion is the source of the eggs' recognition signal. The fact that workers lay eggs in a disorderly fashion and often deposit more than one egg per cell may also assist police workers to recognize worker-laid eggs. However, there was no correlation between the number of eggs per cell or the way they were positioned in the cell and the efficacy of policing (Katzav-Gozansky *et al*. 2001).

In an attempt to disclose the egg discriminatory cues we compared egg surface morphology of queen-laid diploid and haploid eggs to that of worker-laid haploid eggs, as well as carrying out a detailed chemical analyses of the eggs' coating.

Materials and methods

Preparation of queen- and worker-laid eggs

All experiments were conducted with colonies of *Apis mellifera ligustica* between 1998 and 2002 at the Tzrifin apiary, Israel, and in experimental hives at the I. Meier Segals Garden for Zoological Research at Tel Aviv University. Egg surface analyses were performed using one-day-old queen-laid eggs (diploid or haploid) or worker-laid eggs that were individually collected.

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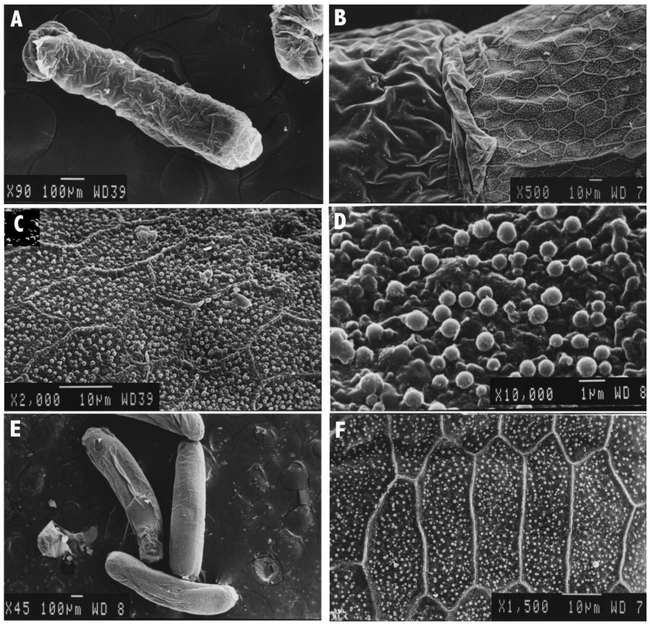

Fig. 1 Scanning electron microscope photographs of (A) queen-laid diploid egg, (B) the surface of an egg typified by hexagonal shapes and filled with small spheres, (C) the surface at larger magnitude, (D) the spheres in larger magnitude, (E) queen-laid diploid eggs washed with dichloromethane, (F) the surface of a queen-laid diploid egg washed with dichloromethane

Queens from 3 different colonies of at least 20,000 bees were induced to lay haploid or diploid eggs by inserting combs containing drone or worker cells into the hive. Worker-laid eggs were obtained from 2 different queenless broodless groups of about 2000 workers created from densely populated QR colonies (Katzav-Gozansky *et al*. 1997). Worker-laid eggs were observed about one week after the queenless colonies had been established.

Scanning electron microscopy

The external morphology of the different types of eggs was studied using scanning electron microscopy (SEM). Eggs were fixed with 2.5% glutaraldehyde in PBS. They were then washed, dehydrated in graded ethanol solutions, dried with CO_2 at critical point, coated with gold (Polaron sem coating unit E5100) and examined in a Jeol JSM 840A SEM. To investigate the effect of solvent wash on the fine structure of the egg surface, eggs were extracted for 5 min. in dichloromethane prior to fixation.

Extract preparation and chemical analyses

Egg surface chemistry was analyzed using 1000 eggs that were individually collected, then pooled and extracted for 1 min. in 5 ml dichloromethane (3 extracts for queen-laid and 2 for worker-laid eggs). The extracts were filtered through glass wool to remove solid particles and concentrated to 70 µl. Since preliminary analyses had revealed the presence of large amounts of hydrocarbons masking the presence of minor constituents, the extracts were fractionated on a florisil column (15 cm long and 0.8 cm wide; Merck, 0.150–0.250 mm). Hydrocarbons were separated from the more polar constituents (esters included) by stepwise elution with 6 ml pentane followed by 6 ml of ethyl acetate.

Structure elucidation of the soluble volatile compounds was carried out by GC/MS (Fisons MD 800 operated at 70 eV) using a 30 m × 0.25 mm id fused silica column, coated with DB5 and run under a temperature program from 60°C to 300°C at a rate of 5°C/min. The compounds were identified by their fragmentation patterns (Tengö *et al*. 1985; McLafferty & Stauffer, 1989; Doolittle *et al*. 1995; Francke *et al*. 2000) and by comparison with authentic samples. Positions of double bonds were determined as described earlier (Hefetz *et al*. 1996).

Results

Electron microscope scanning

The structures of all the different types of eggs are similar. The eggs are cylinder-shaped (Fig. 1A), and measure about 1.14 ± 0.04 mm (mean ± se) long ($n = 7$) for queen-laid eggs and 1.11 ± 0.03 mm ($n = 2$) for worker-laid eggs. The surface of the eggs is covered with an outer layer typified by hexagonal structures (Fig 1B-C) filled with small nodules about 0.5–1µm in diameter (Fig 1D) that are insoluble in dichloromethane (Fig 1E-F).

Chemical analysis of egg surface

Table 1 presents a comparative chemical analysis of the compounds found in both fractions of the three egg types as well as queen Dufour's gland secretion. Queen-laid eggs (diploid and haploid) showed a much more complex blend of volatiles (82 and 80, respectively) on the surface as compared to worker-laid eggs, which contained about 40 components. Among the chemical classes of compounds identified, hydrocarbons were responsible for most of the diversity of queen-laid eggs (53 and 65 for queen diploid and haploid eggs, respectively compared to 23 components found on worker-laid eggs). Although the most abundant volatiles on both queens' and workers' eggs were straight chain alkanes ranging from C_{12}–C_{35}, the principle diversity can be attributed to alkenes and monomethylalkanes. Removal of the abundant hydrocarbons by column chromatography revealed the presence of small amounts of wax-type esters characteristic of the queen's Dufour's gland secretion (found in all egg types), along with long chain aldehydes, isopropyl tetradecanoate and hexadecanoate as well as acetates of unknown origin. Apart from octadecyl acetate, the latter group of compounds proved to be absent in worker-laid eggs. A major difference between queen- and worker-laid eggs lies in the monomethylalkanes. Queen-laid eggs showed 13 and 23 monomethylalkanes (for diploid and haploid eggs, respectively), while worker-laid eggs contained very small amounts of only two, 11- and 13-methylpentacosane (Table 1). These were also present in the queen-laid haploid eggs as well as queen's Dufour's gland secretion. The origin of the other monomethyl alkanes remains unknown. Since the eggs for analysis were 24 hrs. old or less we can not exclude the possibility that some of the substances were added or removed by the nurse bees that frequently check the state of the eggs.

There were slight variations between the egg profiles from the different colonies, but the occurrence of the different classes of compound was consistent. Some samples obtained from egg surfaces contained rather large amounts of the amide of 13-docosenoic acid (not shown in Table 1) that was possibly acquired from environmental sources (Crucifera – Holde & Wilke 1923).

Discussion

Queen-worker and/or worker-worker competition over reproduction in many social insects becomes manifest by extensive oophagy. This may involve both active oophagy of workers' eggs by the queen and vice versa, as in the bumblebee *Bombus terrestris* (Van Doorn & Heringa 1986), or exclusively through worker policing as in the honeybee (Ratnieks 1995). Selective oophagy indicates the evolution of egg discrimination mechanisms, in particular between queen- and worker-born eggs. Such a discrimination was shown by behavioral assays in *Diacamma sp*. (Kikuta & Tsuji 1999; Nakata & Tsuji 1999), and also chemically in *Dinoponera quadriceps,* where gamergate eggs are marked with 9-hentriacontene that protects them from oophagy by ordinary workers (Monnin & Peeters 1997). In honeybees, worker-laid eggs are differentially removed when inserted into either a queenright or queenless colony along with queen-laid eggs (Ratnieks 1995; Katzav-Gozansky *et al.* 2001). It was suggested that Dufour's gland serves as a source of the egg recognition signal, but recent studies have refuted this hypothesis (Katzav-Gozansky *et al.* 2001; Martin *et al.* 2002).

In previous policing experiments we had verified that neither the number of eggs laid per cell nor egg position in the cell, both indicative of worker-laid eggs, affect policing (Katzav-Gozansky *et al.* 2001). The finding that there is no size differences between worker- and queen-laid eggs (Martin *et al.* 2002) is corroborated in the present study. The smaller egg size reported here can be explained by the dehydration procedure needed for egg preparation for scanning microscopy. To eliminate the possibility of structural differences between eggs, we examined the ultrastructure of the surfaces of all egg types using SEM. There were only small differences between the different types of eggs. The surface of the outer layer of all kinds of eggs was covered with hexagonal shapes filled with small spherical structures (Figure 1). A similar hexagonal pattern embossed on the outer surface of the chorion occurs in various insect species, reflecting the shapes of the follicle cells that secrete it (Chapman 1998). The nature of the nodules in these hexagons is not clear. They might be part of the exochorion or, alternatively, exude from the oocyte that pushes the chorion envelope outward. Washing the eggs with an organic solvent did not change the surface structure, suggesting that none of the extracted chemicals contribute to this structure. This is consistent with the hypothesis that this is a basic structure of the egg chorion. Thus, at least based on the fine structure of the egg surface, queen eggs cannot be distinguished from worker-laid eggs.

To explore the chemosensory hypothesis for egg discrimination we conducted a detailed comparative chemical analysis of all egg types. Only a few studies dealing with the chemical composition of the egg coating in Hymenoptera are available. In the stingless bee *Melipona bicolor,* the egg surface contains large amounts of hydrocarbons accompanied by oleic acid (Jungnickel *et al.* 2001). Queen and worker reproductive eggs, but not trophic eggs, are rich in this coating that is concentrated on one tip of the egg and makes the eggs float on the liquid provision. In bumble bees, pentane extracts revealed the presence of linear hydrocarbons comprising approximately 95% of the extracted volatile material in addition to small amounts of methylalkanes (branching points not specified), esters and acetates (Ayasse *et al.* 1999). The composition is caste as well as colony specific, providing a chemical basis for an egg discrimination mechanism. The present analysis shows that the honeybee egg is coated primarily with linear alkanes, most of which occur in abundance also on the bee's cuticular surface (Arnold *et al.* 1996; Martin *et al.* 2001). After stripping these alkanes, however a pronounced chemical diversity was revealed. The relative amounts of the queen-specific esters on the eggs was minute as compared to those present in Dufour's gland (Katzav-Gozansky *et al.* 1997), and the blends were less complex. Though the qualitative composition of the wax-type esters were found to differ between the two queen-laid types of eggs and the worker-laid ones, no significant compound (or combination) could be identified that could account for the release of any policing behaviour. The possible role of some acetates should, nevertheless, be followed up. It is clear, however, that if police workers rely on chemosensory discrimination they cannot utilize wax-type esters as indicators.

In contrast, queen eggs, diploid and haploid, differed significantly from those of workers in showing a much larger diversity of alkenes and especially monomethylalkanes, rendering them good candidates for constituting an egg discrimination pheromone. These differences are apparently caste but not colony specific, which are consistent with previous findings that police workers do not harm queen-laid eggs, haploid or diploid, even if they originated from an alien colony (Katzav-Gozansky *et al.* 2001). Alkenes and methyl branched alkanes have been shown to serve as both pheromones and kairomones in many insect species (Howard & Blomquist 1982; Stanely-Samuelson & Nelson 1993). In the cockroach *Nauphoeta cinerea* differences in relative amounts of monomethylalkanes were found to be correlated with the male dominance status (Roux *et al.* 2002). In the wasp *Polistes fuscatus* three methylalkanes, constituents of a complex hydrocarbon blend, had properties postulated for a recognition pheromone (Espelie *et al.* 1994). In *Polistes dominulus* it was further demonstrated that application of methylalkanes or alkenes but not that of linear alkanes affected nestmate recognition (Dani *et al.* 2001). Using methylalkanes seems to be adaptive because they may be easier to distinguish due to differences in the branching points rather than relying on chain lengths as required for linear alkanes.

The origin of these methyl branched alkanes is still unknown. We tend to exclude the possibility that they are an integral part of the cuticular lipid that envelops the egg in favour of an active secretion. A detailed chemical analysis of adult workers has shown that all these monomethylalkanes

Table 1 Chemical composition of egg surface of queen diploid and haploid eggs and worker haploid eggs. The results are presented as relative proportions: ++++ 5–10%, +++ 2–5%, ++ 0.1–1%, + trace component < 0.1 %. In mixtures containing only few components the upper limit of each classification may reach 3 times of the given relative amounts

Substance	Queen Dufour's gland secretion	Queen-laid diploid eggs	Queen-laid haploid eggs	Worker-laid haploid eggs
Alkanes				
Dodecane		++	++	
Tridecane		+	+	
Tetradecane		++	++	++
Pentadecane	+	+		++
Hexadecane		++	++	+
Heptadecane	+	+	+	
Octadecane		++	++	
Nonadecane	+	+	+	
Eicosane		+	+	
Heneicosane	+	++	++	++
Docosane	+	+	++	
Tricosane	++++	++++	++++	+++
Tetracosane	++	++	+++	++
Pentacosane	++++	++++	++++	+++
Hexacosane	++	++	+++	++
Heptacosane	++++	++++	++++	++++
Octacosane	++	++	++	++
Nonacosane	+++	++++	++++	++++
Triacontane	++	++	++	++
Hentriacontane	+++	+++	+++	++++
Dotriacontane		+	++	+
Tritriacontane	++	++	++	++
Alkenes				
Tetradecene		+	+	
Hexadecene		+	+	
Octadecene		+	+	
9-Tricosene	++	++	+	+
8-Tricosene	++			
7-Tricosene	+			
10-Pentacosene	++	++	++	
9-Pentacosene	++	++	++	++
8-Pentacosene	++			
9-Heptacosene	++	++	++	+
9-Nonacosene	+	++	++	+
8-Nonacosene	++	++	++	
15-Hentriacontene	++	++	+++	
12-Hentriacontene			++	
10-Hentriacontene	++	++	++	+++
9-Hentriacontene		++	++	
8-Hentriacontene	++	++	++	+++
15-Tritriacontene	++	+++	+++	
12-Tritriacontene		+++	+++	
10-Tritriacontene	++	+++	+++	+++
9-Tritriacontene			++	
8-Tritriacontene		+++	++	
15-Pentatriacontene	++	++	++	
Methylalkanes				
11-Methyltricosane			+	
9-Methyltricosane			+	
2-Methyltetracosane			+	
2-Methylpentacosane			+	
3-Methylpentacosane			+	
13-Methylpentacosane	+		+	+
11-Methylpentacosane	+		+	+
9-Methylpentacosane			+	
2-Methylhexacosane			+	
3-Methylhexacosane			+	
13-Methylheptacosane	++	++	++	
11-Methylheptacosane	++	++	++	
9-Methylheptacosane		++	++	
7-Methylheptacosane		+		
3-Methylheptacosane		+	+	

(Continued)

Table 1 (*Continued*)

Substance	Queen Dufour's gland secretion	Queen-laid diploid eggs	Queen-laid haploid eggs	Worker-laid haploid eggs
2-Methyloctacosane		+	+	
15-Methylnonacosane		++	++	
13-Methylnonacosane		++	++	
11-Methylnonacosane		++	++	
9-Methylnonacosane			+	
15-Methylhentriacontane		+	+	
13-Methylhentriacontane		+	+	
11-Methylhentriacontane		+	+	
9-Methylhentriacontane		+	+	
Alcohols				
Tetradecanol	+++			
9-Hexadecenol	++			
Hexadecanol	+++	+		
Aldehydes				
Decanal		++	++	
Octadecanal		++	++	
Eicosanal		++		++
Docosanal		++	++	++
Tricosanal		+		
Tetracosanal		++	+++	++
Esters				
Hexadecyl acetate		++	++	
Octadecyl acetate		++	+	++
Eicosyl acetate		+		
Docosyl acetate		++		
Methyl tetradecanoate	+			
Methyl hexadecanoate	+			
Isopropyl tetradecanoate		++		
Isopropyl hexadecanoate	+	+	+	
Ethyl oleate	+			
Tetradecyl dodecanoate	++	+		
Tetradecenyl tetradecanoate	+			
Tetradecyl tetradecanoate	++++		++	++
Tetradecyl 9-hexadecenoate	++++	+	+	+
Tetradecenyl hexadecanoate	+++			
Dodecyl octadecenoate	++	+		
Tetradecyl hexadecanoate	+++	++	++	++
Hexadecyl tetradecanoate	++++	++	++	++
Dodecyl octadecanoate	++			
Tetradecyl octadecenoate	+++	+		++
Hexadecyl 9-hexadecenoate	+++	+		++
Tetradecenyl octadecanoate	++			
Octadecyl tetradecenoate	++			
Hexadecyl hexadecanoate	+++	+	+	++
Tetradecyl octadecanoate	++			
Octadecyl hexadecanoate	++	+		++
Dodecyl eicosanoate	++			
Hexadecenyl 9-octadecenoate	++		+	
Octadecyl hexadecanoate		++		++
Hexadecyl octadecanoate		+		++
Octadecyl octadecanoate		+		+++
Terpenes				
Geranylgeranyl acetate		++	+	++
Squalene		++	+++	++

are present as cuticular components (Martin *et al*. 2001), yet they are completely absent on the worker egg surface. Whether queen-laid eggs are actively marked with the methylalkanes and alkenes rather than being "contaminated" from the cuticle, and whether these compounds constitute the signal that enables police workers to discriminate between queen- and worker-laid eggs are still open questions. In such investigations, the stereochemistry of chiral methylalkanes should not be neglected. Only the (5*S*,9*S*)-stereoisomer of 5,9-dimethylheptadecane, the sex pheromone of the leaf miner moth *Leucoptera scitella* (Francke *et al*. 1987) has been shown to date to be significantly attractive to males (Tóth *et al*. 1989). This indicates that insects may well discriminate between stereoisomers of branched hydrocarbons.

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