

Role of hydrocarbons in egg recognition in the honeybee

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Abstract. Despite worker-laid and queen-laid honeybee eggs having significantly different hydrocarbon profiles, bioassays and chemical supplementation studies show that changing the hydrocarbon profile does not affect egg identity. Furthermore, full-sized eggs that are tested just before being laid or just after being laid have similar hydrocarbon patterns but are treated differently in egg-discrimination bioassays with only the laid eggs surviving. This suggests that hydrocarbons play no role in the egg-recognition system in honeybees.

Key words. Alkanes, alkenes, *Apis mellifera*, honeybee, hydrocarbons, recognition.

Introduction

Communication is the common bond that holds all societies together, whether it is visual, oral or chemical. All social insects employ a complex chemical communication system, which mediates orientation, foraging, defence, brood-care, reproductive hierarchy and kin recognition. Many pheromones have been identified in honeybees (Free, 1987) and ants (Hölldobler & Wilson, 1990), but the nature of those responsible for individual recognition remains elusive (Vander Meer & Morel, 1998). Cuticular hydrocarbons consist of mainly long-chained linear alkanes and smaller amounts of alkenes and methyl branched alkanes. They are biologically stable, found in all life stages of arthropods and have the primary function of providing protection from desiccation (Lockey, 1988). However, in social insects, cuticular hydrocarbons are widely assumed to be used in both nest mate and kin recognition (Singer, 1998). In honeybees, cuticular hydrocarbon profiles are partly genetically based (Page *et al.*, 1991; Arnold *et al.*, 1996) but can be affected by reproductive status (Katzav-Gozansky *et al.*, 1997). Because cuticular hydrocarbons are highly variable

between the sexes and castes in honeybees (Page *et al.*, 1991; Arnold *et al.*, 1996), this makes them ideal candidates for use in chemical communication (Smith & Breed, 1995).

The ability of honeybees to discriminate between worker-laid and queen-laid eggs, known as worker policing (Ratnieks & Visscher, 1989), was used to investigate the role of cuticular hydrocarbons in the egg-recognition system. Correlational studies were combined with data from discrimination bioassays and chemical supplementation data (Martin *et al.*, 2002). In light of the findings obtained, the potential role that cuticular hydrocarbons play as recognition compounds in honeybees is discussed.

Materials and methods

Individual honeybee (*Apis mellifera*) queen-laid or worker-laid eggs were obtained from queen-right or queen-less colonies, respectively, maintained at the University of Sheffield. Eggs were collected just before being laid (i.e. full-sized eggs dissected from the ovaries), just after being laid (i.e. eggs collected from queens held in a cupped hand or removed from a brood cell immediately after a worker or queen was seen to lay them), and up to 24 h after being laid normally. Each egg was sealed in a small glass tube and stored in a freezer until analysed. Some egg samples became contaminated with squalene from being handled but, as levels were always small, any squalene was ignored for subsequent analysis. Treated eggs in the chemical

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supplementation study were also handled in the same way. All eggs were analysed by crushing them inside a gas chromatography–mass spectrometer (GC–MS) using a Keele injector (Morgan, 1990). GC–MS analyses were performed in a splitless mode on a 5890 Hewlett Packard GC (Wilmington, Delaware) coupled with a 5970 quadrupole MS (70 eV electron impact ionization). A 15 m × 0.25 mm inner diameter column with a 0.25 µm thickness bonded BP5 stationary phase was used. The GC oven was programmed with an initial temperature of 170 °C to 260 °C with a ramp rate of 10 °C min⁻¹, with a subsequent ramp rate of 20 °C min⁻¹ to a final temperature of 325 °C, which was held for 2 min. The injection port and transfer line were held at 250 °C and 300 °C, respectively. The carrier gas was helium at 1 mL min⁻¹. All compounds were identified by comparison of retention times and mass spectra with synthetic standards. The peak area of each compound was calculated for each sample and then normalized by calculating the percentage abundance for that sample, and the mean values were determined. Differences in hydrocarbons patterns between the various groups were investigated further using discriminant analysis (Monnin *et al.*, 1998). The details and data of the egg-discrimination bioassays have been published separately (see Martin *et al.*, 2002 together with unpublished data) but, in brief, eggs from various sources were transferred into a test frame from an unrelated honeybee colony. The speed of egg removal was recorded by inspecting the test frame after 1, 2 and 20 h.

Results

Odd chain-length linear alkanes (C23–C31) were the dominant hydrocarbons on the surfaces of both queen and worker-derived eggs (Table 1, Fig. 1). Significant differ-

ences were found in the profile of the linear alkanes between full-sized worker and queen eggs just before being laid (Figs 1 and 2) (i.e. those dissected from the ovary). However, egg-discrimination bioassays showed that only queen-laid eggs (just laid, $n = 4$; 0–24-h old, $n > 100$) were not removed, whereas all worker-laid eggs (just laid, $n = 22$; 0–24-h old, $n > 100$) and all full-sized eggs taken from the ovaries (queen, $n = 19$; worker, $n = 7$) were removed within 2 h. These results have been supported in similar studies conducted in Australia (M. Beekman, unpublished data). Thus, despite full-sized eggs in the queen's ovary having a similar hydrocarbon profile to those just laid, they were treated very differently. The initial caste differences in hydrocarbon profiles disappear as their profiles merge during the first 24 h in the colony (Table 1, Figs 1 and 2), although this fails to protect the worker-laid eggs because they are always removed. In addition, even when the hydrocarbon profile of worker-laid eggs was artificially changed to be queen-like (Fig. 2) by the addition of a hexane extract of a queen Dufour's gland, these treated eggs were still removed (Martin *et al.*, 2002).

Discussion

It is well established that honeybees can differentiate between worker-laid and queen-laid eggs. The recognition is certainly chemically based because no physical differences can be found between the two egg types (Martin *et al.*, 2002; Katzav-Gozansky *et al.*, 2003). The correlational studies suggest that fluctuations in the proportion of C25 and C27 (Figs 1 and 2b) may be critical in allowing honeybees to discriminate between egg types. However, this is not supported by the egg-discrimination bioassays or the chemical supplementation studies (Martin *et al.*, 2002). This strongly suggests that the cuticular hydrocarbons, and

Table 1. List of hydrocarbons detected on the surface of various classes of eggs produced by queens and workers. The table shows the mean percentage ± SD for each class of egg. Only compounds with >0.5% relative abundance are considered.

Peak no.	Compound	Queen-laid eggs			Worker-laid eggs		
		In ovary ($n = 19$)	Just laid ($n = 13$)	c. 24-h old ($n = 12$)	In ovary ($n = 7$)	Just laid ($n = 4$)	c. 24-h old ($n = 20$)
1	C21 heinicosane	–	–	2 ± 2	–	–	1 ± 2
2	C22 docosane	–	–	5 ± 3	–	–	3 ± 3
3	C23:1 tricosene	1 ± 2	2 ± 4	2 ± 1	–	2 ± 1	2 ± 1
4	C23 tricosane	45 ± 8	29 ± 10	22 ± 9	42 ± 15	23 ± 4	18 ± 3
5	C25:1 pentacosene	2 ± 1	3 ± 3	3 ± 2	–	4 ± 1	4 ± 8
6	C25 pentacosane	22 ± 3	21 ± 6	24 ± 3	23 ± 9	14 ± 2	13 ± 3
7	C26 hexacosane	–	–	1 ± 2	–	–	1 ± 1
8	C27:1 heptacosene	1 ± 1	2 ± 1	1 ± 1	–	3 ± 1	1 ± 1
9	C27 heptacosane	13 ± 3	18 ± 3	18 ± 3	33 ± 11	23 ± 7	25 ± 7
10	C28 octacosane	–	–	1 ± 1	–	–	1 ± 1
11	C29:1 nonacosene	2 ± 1	4 ± 3	2 ± 1	–	4 ± 1	6 ± 3
12	C29 nonacosane	2 ± 1	4 ± 2	4 ± 1	2 ± 2	7 ± 1	8 ± 4
13	C31:1 heintriacontene	7 ± 4	9 ± 5	7 ± 1	–	9 ± 3	3 ± 5
14	C31 heintriacontane	1 ± 1	2 ± 1	2 ± 1	–	2 ± 1	7 ± 3
15	MeC31 methyl-heintriacontane	–	–	–	–	–	2 ± 2
16	C33:1 tritriacontene	4 ± 3	6 ± 3	6 ± 1	–	9 ± 3	5 ± 3

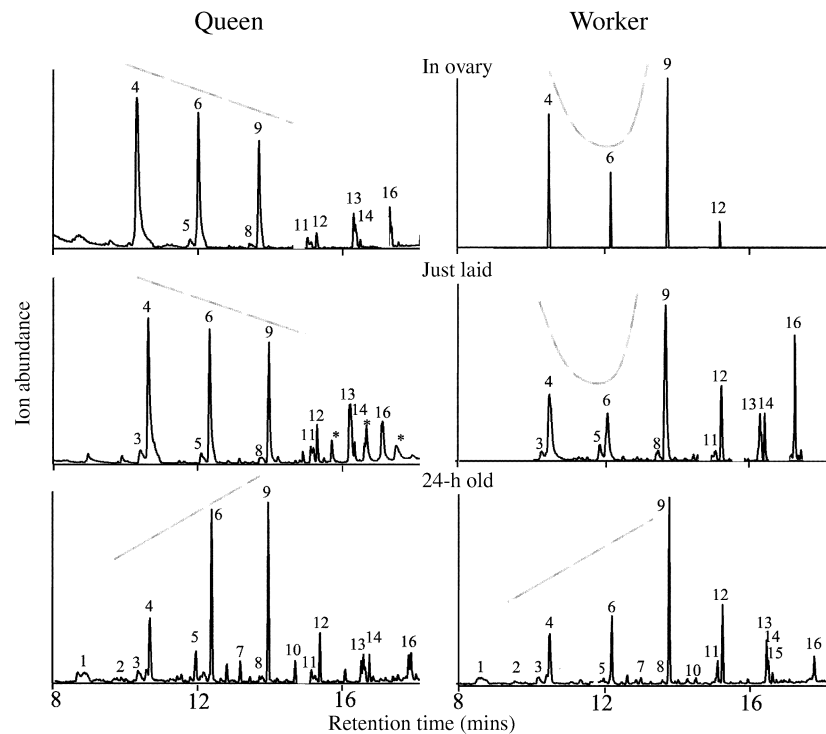


Fig. 1. Comparisons of typical chromatograms from the three egg types, full-sized eggs from the ovary just before being laid, eggs just after being laid and eggs laid approximately 24-h earlier. The asterisks represent esters that can contaminate the eggs when the queen is forced to lay an egg outside the colony.

specifically the linear alkanes, do not play a role in egg discrimination. This idea is supported by the findings of other studies, which have often been overlooked.

Pickett *et al.* (1982) found that fractions containing mainly the linear alkanes (C23, C25 and C27) or synthetic linear alkanes C23 and C25 gave no significant electroantennogram response, which measures receptor potentials of olfactory neurones in the honeybee antenna, whereas eicosenol, an alcohol-based worker alarm pheromone (Pickett *et al.*, 1982), gave a significant response. This would explain why honeybees discriminate between different waxes using esters and alcohols but not using cuticular hydrocarbons (Fröhlich *et al.*, 2001), based on experiments using the classic conditioning of the proboscis extension reflex response. Furthermore, no glomerular (neural) responses in the antennal lobe of honeybees were obtained for linear alkanes of less than C10 whereas a wide range of short-chained (C5–C10) alcohols, aldehydes and ketones elicited both strong neural responses (Sachse *et al.*, 1999) and can be discriminated by free-flying honeybees (Laska *et al.*, 1999). The reason why nonvolatile linear alkanes are not used as recognition cues may be because they have no electronegative atoms (such as oxygen or nitrogen) (Hauptmann, 1987), that their hydrophobic properties reduce interactions with the receptors, or that the alkanes could be coded in glomeruli not included in the study of Sachse *et al.* (1999).

By contrast to these findings are two often cited studies. Breed & Stiller (1992) demonstrated that honeybees only showed a response to the linear alkanes C16 and C18 but admitted that neither of these chemicals actually occur in wax or on honeybees, whereas linear alkanes that do occur (e.g. C29) had no significant effect. In addition, the concentrations of substances used in this study (10 µg/bee) are very high compared with natural abundance levels. Getz & Smith (1987) also demonstrated that honeybees appeared to be able to discriminate between two specific blends of the linear alkanes C23 and C25 (90% C23:10% C25 from 100% C25 or 10% C23:90% C25). However, they also found that honeybees were unable to discriminate between the two pure compounds (100% C23 from 100% C25) or several other various mixes of the two compounds. Getz & Smith (1987) stated that textural differences between the various mixes could have caused sensory modalities of touch to confound those of olfaction and that care must be taken in interpreting their results. The role of texture in determining the action of compounds has been suggested by the ant repellents used by wasps, where it appears that it is not the actual chemical but its physical state (liquid or solid) that is crucial to its function (Dani *et al.*, 2003).

The apparent inability of honeybees to use linear alkanes in recognition has also been found in the social paper wasp *Polistes dominulus* where linear alkanes were found to have

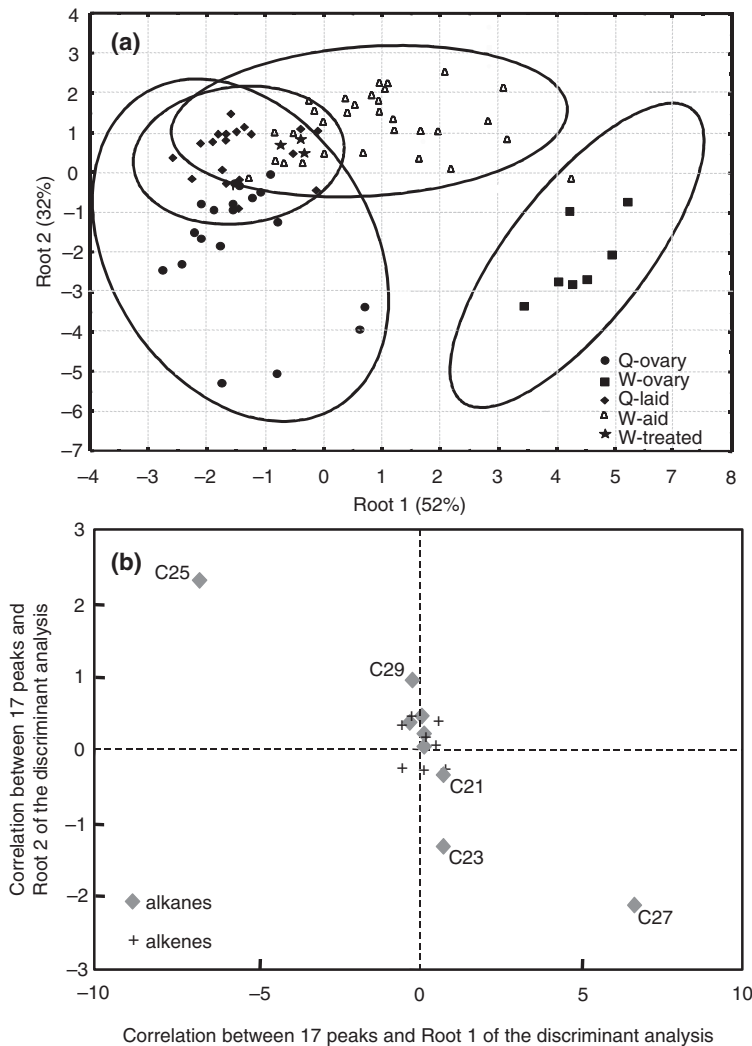


Fig. 2. (a) Discriminant analysis of seven full-sized worker (square) and 19 full-sized queen (circle) eggs just before being laid with 24 worker-laid (triangle) and 25 queen-laid eggs (diamonds) that were laid 0–24 h earlier. The three asterisks represent three worker-laid eggs treated with a queen's hydrocarbon fraction to make these eggs more queen-like. The analysis compared the 16 major peaks (after standardization) given in Table 1. Envelopes represent the 95% confidence ellipses. (b) Factor structure coefficients for the 16 peaks used in the comparison are given, with grey diamonds representing alkanes and crosses representing alkenes. Only the names of the key compounds responsible for group separation in the discriminant analysis are given. The eigen values are Root 1 = 6.1 and Root 2 = 2.2.

no effect upon their recognition response, whereas wasps treated with methyl-branched alkanes or alkenes were attacked (Dani *et al.*, 2001). Furthermore, differences between three termite (*Macrotermes falciger*) phenotypes were mainly based on alkenes, not alkanes, and these differences were associated with the level of aggression between the three groups (Kaib *et al.*, 2002). The branched alkanes and alkenes are far better candidates for recognition compounds than the linear alkanes because they possess a more defined conformation due to the double bond or branch position.

Despite the extensive research conducted on hydrocarbons during the past 20 years, there is still no unequivocal behavioural evidence in honeybees that cuticular hydrocarbons, especially the abundant linear alkanes, can be detected as recognition signals. Furthermore, no actual biochemical mechanism of recognition has yet been determined for any social insect (Breed, 1998). The discovery of specific kin-recognition chemicals would represent a major breakthrough in the field, but better bioassays, the testing of a

much wider range of compounds at biologically realistic levels and the use of a wider range of techniques, such as electroantennograms and the proboscis extension reflex, are required to select potential compounds from the vast number of possible compounds, rather than relying solely on correlational studies. Recently, a proteinaceous molecule was found in fire ant queens *Solenopsis invicta* that informs workers when to execute sexual larvae (Klobuchar & Deslippe, 2002) and may represent a major step forward in widening the search for recognition compounds.

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