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Plasticity of Caste-Specific Dufour's Gland Secretion in the Honey Bee (*Apis mellifera* L.)

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Pheromonal communication is fundamental in social insects for regulating the multitude of intracolonial activities. The pheromones are produced by a plethora of exocrine glands and control most of the behavioral repertoires exhibited by these insects. Honey bees are especially rich in exocrine glands, possessing 15 known glands that produce a dazzling array of compounds [1, 2]. Noteworthy are the mandibular glands that produce caste-specific compounds and play a major role in queen-worker interactions [3], and the alarm pheromone producing tissue that is associated with the sting apparatus [4]. Another abdominal gland, Dufour's gland, has remained virtually unexplored with respect to its chemistry and function in this species.

Dufour's gland in bees is often well developed and is rather diverse in its biosynthetic capabilities. Chemical characterization of the glandular secretions reveals that while some classes of compounds such as macrocyclic lactones are characteristic of the lower bee families, i.e., Colletidae and Halictidae, aliphatic esters of various types and chain lengths as well as hydrocarbons are widely distributed among most bee families [5–11]. In many solitary bees the gland is the source of the brood cell lining, but in some species the glandular secretion has been shown to mediate behavior. These include sex pheromones [12, 13], nestmate discriminators [14], nest entrance markers [15], and trail pheromone [8]. Ultrastructural studies reveal that in the Apidae (*Apis mellifera* and *Bombus* species) the gland opens into the dorsal vaginal wall ([16], J. Billen, personal communication), suggesting it to be the source of substances that may be applied onto the egg before deposition. In the honey bee and in *B. terrestris* the gland is more developed in queens than in workers, suggesting caste specificity.

Caste specificity in pheromonal composition seems to be the rule in honey bees. Mandibular gland exudates differ drastically between queens and workers, in accordance with their role as a queen pheromone. Caste specificity is also expressed in the composition of the secretion produced by tissues associated with the sting apparatus. Queens possess long-chain esters that are missing in workers' secretions [17]. Likewise, queens' feces extract contains esters that are not found in those of workers. It has been suggested that these caste specific esters enable workers to recognize their queen or are used by the queen to scent-mark her colony [18].

The morphological and physiological differences between the castes in honey bees are already evident upon adult emergence, suggesting that regulation of caste specificity occurs during the process of caste differentiation in the preimaginal state. However, recently accumulating data indicate some caste plasticity with respect to the biosynthetic capability in the imago. Young queens' mandibular glands contain 10-hydroxy 2-decenoic acid (10-HDA, a typical worker compound), while workers' glands under some conditions produce 9-oxo 2-decenoic acid (9-ODA, a typical queen substance) [19–21]. It has been further demonstrated that these compounds are produced via disparate biosynthetic pathways [22], suggesting that both biosynthetic pathways exist in queens and workers but are differentially expressed.

The objectives of this study were to elucidate whether the chemical composition of Dufour's gland in *A. mellifera* is caste specific, and whether the secretionary composition of workers is affected by the presence of the queen.

Workers and queens (virgin, newly mated, and 1-year-old mated queens) of *A. mellifera ligustica* were obtained from the apiaries at the Experimental Station in Tzrifin and at Kibbutz Yad Mordechai, Israel. Young workers (nurses) were collected from the brood area of the hive while foragers with pollen loads were collected at the hive entrance. Egg-laying workers were collected from queenless colonies that were established according to Robinson et al. ([23], modified) as follows: Queenless colonies were obtained by shaking workers from two honey combs of the mother colonies into a hive containing four combs: two combs of honey, one comb of pollen, and one empty comb with mostly drone-sized cells. Each queenless colony was created from a different mother colony. Eggs were noted about 1 week after establishing the queenless colonies. Individuals observed with their abdomen inserted into a cell, often with wings folded, were assumed to oviposit and were collected as "egg-laying workers." Most egg-laying workers were collected during the first 11–15 days after establishing the queenless colony. Since it was not certain whether all workers that displayed oviposition behavior had indeed laid eggs, their ovarian state was also determined during the dissection of their Dufour's gland.

Ovarian development was classified according to Velthuis [24]: class I, ovaries resting and almost inactive; class II, early stages of development in which the eggs are still fairly round to bean-shaped form; class III, eggs become elongated. For extracting Dufour's gland we selected only egg-laying workers with ovaries at class III. Of the 70 nurses and foragers that were dissected, all but one had ovaries at class I. Only these workers were used. Dufour's glands were cleanly separated from the sting and the poison gland and extracted in dichloromethane. Pools of at least five glands from workers or queens were utilized for gas chromatography – mass spectrometry (GC/MS) analyses. Quantification of workers' secretion was also based on pooled samples of five bees, but queen's glands were analyzed individually. The extracts were analyzed by combined capillary GC/MS (EI ionization at 70 eV), using a DB-5 capillary column temperature programmed from 80° to 200°C at 15°C/min with an initial hold of 5 min and then to 300°C at 3°C/min. Compounds were identified by their GC retention time and mass spectra as compared to synthetic reference samples and published spectra [25]. The position of the double bond of the esters was determined by alkylthiolation using dimethyl disulfide (DMDS) [26]. The GC/MS analyses of the DMDS derivatives was carried out by using the above mentioned DB-5 column starting at 200°C for 3 min, then programmed to 300°C at a rate of 10°C/min, then to 320°C at a rate of 1°C/min. Quantification of the glandular secretion was performed by GC on a 30-m SE 54 capillary column, temperature programmed from 60° to 100°C at the rate of 20°/min and then programmed to 270°C at the rate of 5°/min. Eicosane (0.5 and 5 μg for the worker and queen samples, respectively) was added to the samples as an internal standard.

The total amount of the glandular secretion of the queens and workers are presented in Table 1. The glands of queens are much larger in volume than those of workers and on the average contain 12 times more secretion (17.6 ± 3.3 for all queens n = 18 vs. 1.44 ± 0.48 for all workers n = 17; Mann-Whitney, P<0.0001). In newly mated, egg-laying queens the gland content tended to be greater, although not significantly so, than in either virgin or older queens. Among the queenright workers both behavioral castes had low amounts of secretion in the glands. Although foragers tended to have higher amounts of secretion than nurses, this difference was not significant. Likewise, foragers had similar low amounts irrespective of their social environment (queenright vs. queenless). In contrast, egg-laying workers formed under queenless conditions had significantly greater amounts of secretion in their Dufour's gland. The latter results can also be revealed by the following observation: on the average the glands of queenright and queenless foragers and those of queenright nurses combined contained 0.56 ± 0.14 μg/gland, whereas those of egg-laying workers contained 3.23 ± 1.16 μg/gland (Mann-Whitney, P = 0.0027).

Table 1. The total amount of volatile compounds from Dufour's gland secretion of queens and workers honey bees at various physiological states and under different social regimes.

Experimental group	Number of replicates	Total amount of secretion (μg/gland) mean±SE	Statistics*
Queenright workers (nurses)	7	0.32 ±0.069	b
Queenright workers (foragers)	2	0.47 ±0.049 (sd)	b
Queenless workers (forages)	3	1.17 ±0.383	bc
Queenless workers (egg laying)	6	3.23 ±1.159	c
Virgin queens	6	15.51 ±4.353	a
Newly mated, egg laying queens	5	20.90 ±5.560	a
One year old mated queens	7	16.951±6.955	a

* Statistical analysis was performed using ANOVA, followed by Fisher's PLSD at P<0.05. Different letters denote statistical differences

Caste specificity and the effect of social environment are definitely expressed in the chemical composition of the glandular secretion (Table 2). The secretion in queenright workers was composed exclusively of odd-numbered straight-chain alkanes ranging from C_{23} to C_{31}. These hydrocarbons were omnipresent in the bees examined, irrespective of their caste, behavioral task, and social environment. Hydrocarbons constituted only 30–40% of the secretion in queens, but their diversity was higher than in the workers' secretion. These included odd- and even-numbered alkanes, alkenes, alkadienes, and alkatriens, and one monomethyl alkane. Most of the queens' secretion was fortified by long-chain, saturated and unsaturated wax-type esters. The predominant esters were tetradecyl-tetradecanoate, tetradecyl-(Z)-9-hexadecenoate and tetradecyl-(Z)-11-hexadecenoate, tetradecyl-hexadecanoate and its complement hexadecyl-tetradecanoate (ratio 1:6), and tetradecyl-(Z)-9-octadecenoate. MS fragmentation patterns followed a known principle [27]. In contrast to the underivatized (E)-/(Z)-isomers of the unsaturated wax esters, the DMDS derivatives could be cleanly separated by GC. The addition of dimethyl disulfide was found to be stereo selective [28, 29], and in a variety of compounds showing different oxygen functions, the derivatives of the (Z)-isomer generally eluted earlier then those of the (E)-isomers [28–31]. Under the above conditions the DMDS derivatives of tetradecyl-(Z)-9-hexadecenoate eluted at 29.6 min while the derivative of the (E)-isomer eluted 0.2 min later. Using authentic reference samples, careful GC/MS-SIM analysis revealed that all unsaturated esters identified here show (Z)-configured double bonds.

Dufour's gland composition in queenless workers differed according to behavioral castes. Under queenless conditions egg-laying workers (with ovaries development at class III), but not foragers, produced several of the esters that are typical to queens. The six main esters found in the queens' secretion were also present in the egg-

Table 2. Chemical composition of Dufour's gland secretion of queens and workers of *Apis mellifera L.* of different physiological states and under different social regimes. To obtain an appreciable signal, analyses were carried out on pooled glands. At least 10 glands were used for each group. The results are presented as relative proportions: – not detected, * <1%, ** 1–5%, *** 5–10%, **** 10–15%, ***** above 15%, t = traces

Compound	Queens			Queenright workers		Queenless workers	
	Virgins	Newly mated, egg-laying	One-year-old mated	Foragers	Nurses	Foragers	Egg laying
Hydrocarbons							
Heinecosane	*	*	*	–	–	–	–
docosane	*	*	*	–	–	–	–
Tricosene	*	*	*	–	–	–	–
Tricosane	***	***	***	***	***	**	**
Tetracosane	*	*	*	–	–	**	*
Pentacosadien	t	t	t	–	–	–	–
Pentacosene	**	*	*	–	–	–	–
Pentacosane	***	**	**	***	***	***	**
Hexacosane	–	–	–	–	–	–	*
Heptacosene (3 isomers)	**	*	*	–	–	–	–
Heptacosane	***	**	**	****	*****	****	***
13 Me heptacosane	*	*	*	–	–	–	–
Octacosane	–	–	–	–	–	**	**
Nonacosene	*	*	*	–	–	–	–
Nonacosane	**	**	*	****	*****	****	****
Triacontane	–	–	–	–	–	*	*
Heintriacontene (3 isomers)	*	**	*	–	–	–	–
Heintriacontane	**	**	**	*****	*****	****	****
Tritriacontadienes (2 isomers)	t	t	t	–	–	–	–
Tritriacontene (2 isomers)	**	**	**	–	*	–	–
Tritriacontane	t	t	t	–	–	–	–
Penatriacontane	t	t	t	–	–	–	–
Wax-type esters							
Tetradecyl dodecanoate	**	*	*	–	–	–	–
Tetradecyl-(Z)-9-tetradecanoate	t	t	t	–	–	–	–
Tetradecyl tetradecanoate	*****	*****	****	–	–	–	***
Tetradecyl-(Z)-9-hexadecenoate+ Tetradecyl-(Z)-11-hexadecenoate	***	***	***	–	–	–	***
Tetradecyl hexadecanoate+ Hexadecyl tetradecanoate	*****	*****	*****	–	–	–	***
Hexadecenyl hecadecenoate	–	–	**	–	–	–	–
Tetradecyl-(Z)-9-octadecenoate	***	***	**	–	–	–	***
Hexadecyl-(Z)-9-hexadecenoate	**	***	***	–	–	–	–
Hexadecyl hexadecanoate	**	**	***	–	–	–	–

laying workers, comprising about 32% of total secretion. Thus Dufour's gland of honey bees, as their mandibular glands, shows a certain plasticity in its biosynthetic capabilities that is apparently under queen control.

The hydrocarbon composition of queens' Dufour's gland is very similar to the composition previously reported for both tergal glands and cuticular lipids [32, 33]. On the other hand, with the exception of tetradecenyl dodecanoate, the esters identified in Dufour's gland differ from those that were previously identified in the tergal glands or feces of queens [17, 33, 34]. Also, our extraction of Dufour's gland showed none of the volatile components characteristic to the secretion emanating from the sting apparatus [35, 37], indicating that our preparation did not contain contamination from the adjacent glands. Wax-type esters made up of tetradecanol, hexadecanol, and fatty acids such as those identified here, have also been identified in bumble bees [9, 38], primitive social bees such as *Lasioglossum malachurum* [39], and solitary bees [40].

Both the caste specificity and the biosynthetic plasticity expressed in Dufour's gland raise questions concerning the function of this gland and the mechanisms regulating its activity. It was previously proposed by Ratnieks and Visscher [41], that honey bee workers discriminate between queen-laid and worker-laid male eggs, preferentially eating the latter. Discrimination of eggs necessitates a source of information by which police workers can recognize which of the eggs were laid by the queen and which by the workers. It was further postulated that Dufour's gland is the source of an egg marking pheromone [42]. The chemical caste specificity of the secretion presented here and the position of the gland near the opening of the oviduct [16] support this hypothesis.

Queenlike Dufour's gland secretion found in queenless egg-laying workers, but not in queenless foragers, further suggests a relationship between egg laying and glandular secretion. It can be hypothesized that queenlike Dufour's gland secretion might help egg-laying workers to mimic some of the queens' chemical signals and thus hide their eggs from egg policing. If Dufour's gland secretion is associated with egg laying, the glandular secretion is expected to be found on either the egg coating or the cell lining. The presence of Dufour's gland secretions in these locations is currently being investigated.

Dufour's gland might still have additional or other caste-specific pheromonal functions in honey bees. The apparent correlation between ovarian development and the amount and quality of Dufour's gland secretion may carry information on the reproductive state of the emitter. Since there is no correlation between mandibular gland pheromone production and ovarian development in queens [20], copious Dufour's gland secretion might constitute an additional queen signal. It might also act as a worker attractant. As previously described by Velthuis [24], egg-laying workers are capable of mimicking the queen and of attracting a small but recognizable retinue. It is well accepted that the queen affects her workers' behavior and physiology by a combination of pheromonal signals derived from a variety of glandular sources, and that the application of queen mandibular pheromone alone does not duplicate all her effects [43, 44]. Our findings suggest that Dufour's gland is the source of an additional queen pheromone. However, its effect on worker behavior and physiology remains to be investigated.

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