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Plasticity in caste-related exocrine secretion biosynthesis in the honey bee (*Apis mellifera*)

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Abstract

Plasticity of Dufour's gland secretion in the honey bee is correlated with the individual's plasticity. Queens and queenless (QL) egg-laying workers possess a bouquet of esters and hydrocarbons, whereas queenright (QR) workers produce exclusively hydrocarbons. The effects of social environment (QR vs. QL conditions) and possible physiological constraints on the gland were studied by following the biosynthesis of these classes of compounds in vivo and in vitro. Biosynthesis in vivo followed the prediction based on glandular chemistry. Queens and QL egg-laying workers, but not QR workers or QL foragers, showed incorporation of sodium acetate into both hydrocarbons and esters. In contrast, the in vitro studies revealed that, in addition to queens and QL egg-laying workers, QR nurses retained their ability to produce the queen characteristic esters. Although there was some ester production in foragers, it occurred to a lesser extent. It is possible that the glands in the older foragers undergo irreversible changes. The in vitro incubation also revealed a temporal activation of ester biosynthesis in QR workers. In these glands alcohols, corresponding to the alcohol moiety of the esters, predominated in short-term incubations but decreased as the amount of newly synthesized esters increased. In contrast, queens and QL egg-laying workers showed predominant incorporation into esters from the onset of incubation. Thus, expression within the workers' Dufour's gland is regulated. In the presence of a queen, ester production is inhibited. Once the queen is removed the physiologically unconstrained gland starts to biosynthesize the queen-specific esters after a certain lag needed for the build-up of precursors and the enzymatic machinery. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Dufour's gland; Esters; Hydrocarbons; Glandular plasticity; Honey bee

1. Introduction

Caste-specific chemistry of exocrine secretion has been documented in several social insects. In honey bees it occurs in at least two exocrine glands, the mandibular (Winston, 1987; Blum, 1992; Slessor et al., 1988) and Dufour's gland (Katzav-Gozansky et al., 1997a). However, processes leading to this differentiation seem to be at least partly reversible. Young queens' mandibular glands contain 10-hydroxy-2-decenoic acid (a typical worker compound), while workers' glands under some conditions produce 9-oxo-2-decenoic acid (a typical queen substance; Crewe and Velthuis, 1980; Slessor et al., 1990; Plettner et al., 1993). Although these compounds are produced by disparate biosynthetic pathways, both pathways exist in queens and workers but are apparently differentially expressed (Plettner et al., 1996). Similar caste specificity and plasticity are exhibited by the Dufour's gland of the honey bee. In workers the glandular secretion is composed of a series of odd *n*-alkanes ranging from C_{23} to C_{31}, whereas that of queens contains, in addition to hydrocarbons, long chain esters. However, the secretion in egg-laying workers, induced under queenless conditions, possesses the major queen esters characteristic of queens (Katzav-Gozansky et al., 1997a).

Biosynthetic studies performed with queen honey bees, using [1-^{14}C] sodium acetate as a precursor, revealed differences in Dufour's gland expression between the in vivo and in vitro experiments. The major de novo products in vivo were esters and hydrocarbons, whereas in vitro there was no incorporation into hydrocarbons, suggesting that these are biosynthesized elsewhere in the body and sequestered by Dufour's gland (Katzav-Gozansky et al., 1997b).

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The secretionary function of Dufour's gland in honeybees is still elusive, but since the gland opens into the vaginal wall (Billen, 1987), it was suggested as a potential source for substances that may be applied onto the egg, helping workers to discriminate between queen-laid and worker-laid eggs (Ratnieks, 1995). The induction of ester production in egg laying workers lends further credence to this hypothesis. Furthermore, our preliminary data (Katzav-Gozansky et al., unpublished data) confirmed the presence of major queen specific esters on queen-laid egg surfaces, as predicted from the hypothesis.

The chemical plasticity demonstrated by Dufour's gland of workers seems to rule out the occurrence of "fixed caste-specific biosynthetic pathways" and suggests an alternative model of a regulated totipotent glandular biosynthetic capability. According to this model, glandular expression is regulated by external social factors (i.e., the presence or absence of the queen or the interactions between nest members), but may also be constrained by worker physiology (i.e., caste, task and reproductive state of individual bees). We attempted to separate the effects of social regulation and possible physiological constraints on workers' Dufour's gland plasticity by studying the biosynthesis of the glandular constituents in vivo and in vitro. We predict that the in vivo expression of the gland in workers taken from different social environments reflects social regulation. Under in vitro conditions, on the other hand, we assume that the gland is freed from this regulation and its final products reflect the inherent constraints on its biosynthetic plasticity.

2. Material and methods

Workers and queens of *Apis mellifera ligustica* were obtained from the apiaries at the Experimental Station in Tzrifin and Kibbutz Yad-Mordechai (Israel). Queens and workers were obtained from various colonies. Young workers (nurses) were collected from the brood area of the queenright (QR) colonies, while foragers with pollen loads were collected at the hive entrance. Egg-laying workers and queenless (QL) foragers were collected from QL colonies that were established as previously described (Katzav-Gozansky et al., 1997a). About 7–10 days after establishing the QL colonies, eggs were observed. Individuals that were observed with their abdomen inserted into a cell were assumed to oviposit and were collected as "egg-laying workers". This was further confirmed by inspecting ovarian development during the dissection of Dufour's gland. All the bees had ovaries at their final stage of development (stage III according to Velthuis, 1970). Each gland was cleanly separated from the sting and the poison gland and its length and width were measured. Gland volume was calculated assuming it has the shape of a cylinder.

In vivo and in vitro assays were conducted on glands from mated queens, egg-laying workers from QL colony, nurses and foragers from QR colony and foragers from QL colony. For the in vivo studies, 1 μCi [1-^{14}C] sodium acetate (56 mCi/mmol, NEN) was injected into the hemolymph through the intersegmental membrane of the abdomen in 0.5 μl bee medium (Kaatz et al., 1985, modified). After injection, groups of six injected workers bees were placed in a Petri dish (9 cm diameter) supplemented with candy. Queens were placed with three non-injected workers. After 24 h of incubation at 34°C the bees were chilled and dissected. The glands were extracted in CH_2Cl_2, workers glands in pairs and queens glands individually. For comparison of biosynthesis between the workers and queens the amount of radioactive compounds synthesized by queens' glands was multiplied by two. The major classes of compounds were separated by thin-layer chromatography (TLC) using silica gel coated plates (polygram Sil G). The TLC was performed in two successive steps: first hexane and subsequently hexane:diethyl ether:acetic acid (70:30:1), as running solvents. The various lipid classes were identified by comparing their Rf values with those of co-chromatographed standards and visualized by iodine vapor. We verified that the relevant TLC fractions contained only a single class of compounds by re-extracting the appropriate band and subjecting it to GC/MS analysis. This was performed using a DB-5 fused silica capillary column that was temperature programmed from 120°C to 300°C at 3°C per minute with an initial hold of 3 minutes. The eluting compounds were identified by their fragmentation pattern and by comparison to standards. The radioactivity of the various TLC fractions was determined by a phosphor imager (IP Autoradiography system). Quantification of the radioactive fractions was achieved by comparison to a standard curve using radioactive standards.

For the in vitro studies, Dufour's glands were dissected under medium and washed twice in fresh medium. The glands were incubated in 80 μl medium supplemented with 1 μCi [1-^{14}C] sodium acetate at 39°C for 4–20 h. Workers glands were incubated in pairs while queen's glands were incubated individually. The glands and incubation media were extracted in CH_2Cl_2 for 24 h and subjected to TLC, as previously described. For the determination of alcohols biosynthesized in vitro, Dufour's glands were incubated as above, but cold sodium acetate (0.3 mg/ml) was used as a precursor. The alcohol TLC fraction was eluted with CH_2Cl_2 and subjected to GC/MS as described above.

3. Results

The Dufour's gland in queens is approximately 10 times greater in volume than a normal worker gland

(3.0±0.1 mm^3 vs. 0.28±0.004 mm^3 for queens and QR nurses, respectively (ANOVA $p<0.005$). The glands of QL workers were slightly larger (0.30±0.006 mm^3) than that of QR, but these differences were not statistically significant.

Fig. 1 depicts the in vivo incorporation of [1-^{14}C] acetate into hydrocarbons and esters by the Dufour's gland in queens and in workers reared under different social regimes. As predicted from its larger size, queens' glands showed a higher level of incorporation than those of workers (Mann-Whitney U test, p=0.0001). Queens and QL egg-laying workers were characterized by possessing both newly synthesized esters and hydrocarbons. In contrast, QR workers (nurses or foragers) and QL foragers produced hydrocarbons de novo but failed to produce the esters. Incorporation into the ester TLC fraction was at background levels.

The study of the biosynthesis of these products in Dufour's gland in vitro (Fig. 2) revealed an appreciably higher incorporation compared to the in vivo case. This is expected since in vitro Dufour's gland is the only target tissue, whereas in vivo the non-specific acetate precursor is incorporated mostly into the general metabolic pool. In addition, there were striking differences in the end products synthesized de novo under in vitro conditions. First, the gland did not show any incorporation into hydrocarbons. Second, all the incubated glands showed incorporation into esters, irrespective of the social conditions under which the workers that contributed these glands were reared. The levels of incorporation into esters, however, differed according to the type of worker assayed. In queenright nurses and egg-laying workers the level of ester biosynthesis was the same (p=0.3, Mann-Whitney U test) and was appreciably higher than in foragers (queenright or queenless, p=0.024, Mann-Whitney U test). The degree of incorporation exhibited by the queens was significantly higher than incorporation by any of the workers (7308±3726 and 5563±2813 Dpm/2 glands in QR nurses and QL egg laying workers after 20 h of incubation vs. 63,300±4261 Dpm/2 glands in mated queens after only 4 h of incubation). Under in vitro conditions there was also a conspicuous incorporation into alcohols. Since the synthesis of monoesters probably occurs through the reduction of long chain fatty acids to primary alcohols followed by esterification with fatty acyl CoA (Stanley-Samuelson et al., 1988), we assumed that these alcohols represent precursors that accumulated during the first hours of incubation. We verified this assumption by repeating the 4 h incubation with cold acetate and subjecting the TLC alcohols fraction to GC/MS analysis. This revealed the presence of 1-tetradecanol, 1-hexadecanol, 1-octadecanol, and 1-eicosenol. The first two alcohols constitute the alcoholic moiety of the queen-characteristic esters. We do not know yet the function of the other alcohols found, but they may constitute additional precursors. The time course study presented in Fig. 3 demonstrates the transition in glandular activity towards ester biosynthesis. In queenright nurses, that normally do not produce esters, incorporation in the first 4 h of incubation was primarily to long chain alcohols. As incubation proceeded, the amount of radiolabeled alcohols decreased with a concomitant increase in radioactive esters. In egg-laying workers (that already possess esters in their Dufour's gland) incorporation into esters was always superior, with varying amounts of incorporation into alcohols.

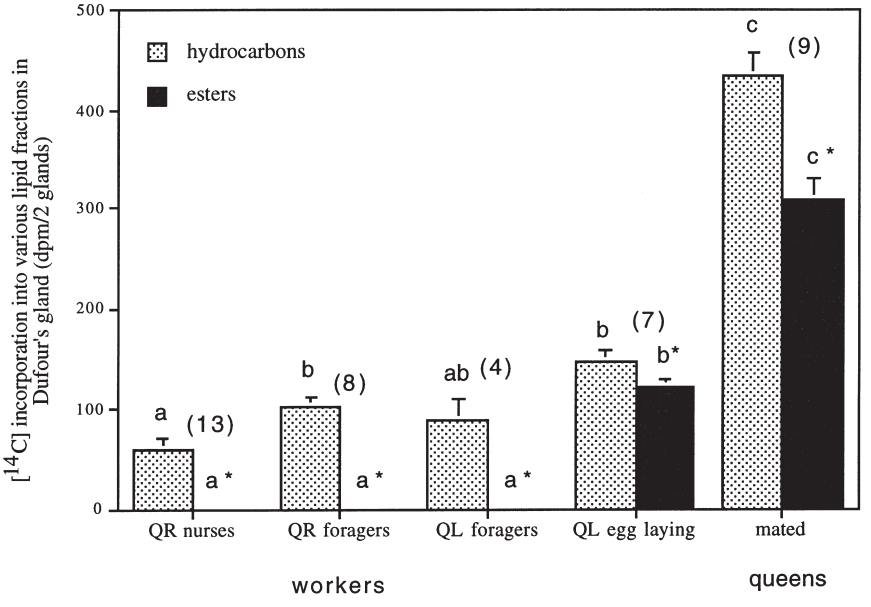

Fig. 1. Incorporation in vivo of [1-^{14}C] acetate into hydrocarbons and esters by Dufour's gland of queens and workers. Different letters indicate groups that are significantly different (Mann-Whitney U test, $p<0.05$). Letters accompanied by an asterisk denote the differences in ester biosynthesis between the groups. Numbers in brackets represent the number of replicates.

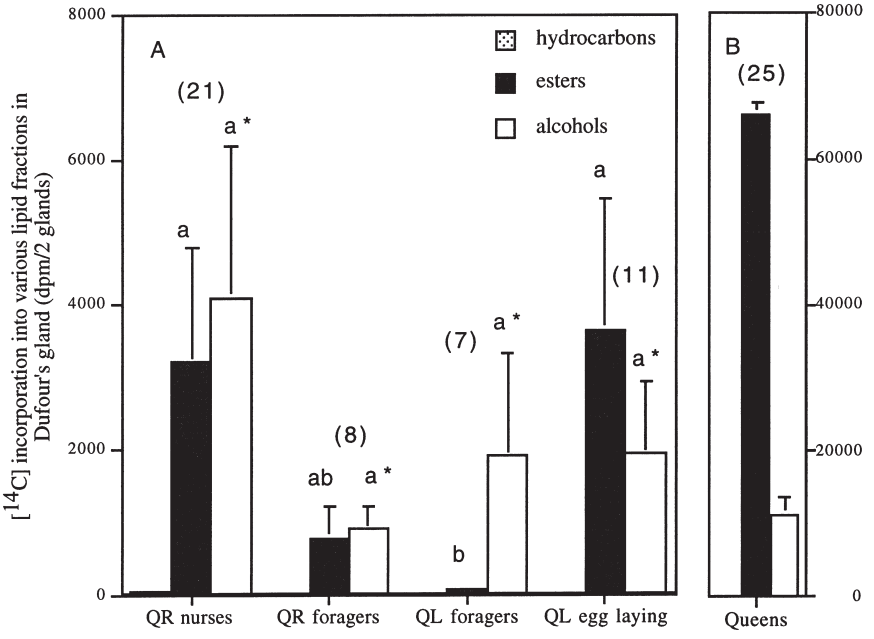

Fig. 2. Incorporation of [1-^{14}C] acetate into various lipid fractions in queen and worker: Dufour's gland incubated in vitro: Workers (20 h; A) queens (4 h; B). Note that the incorporation into hydrocarbons is at background level. Different letters indicate groups that are significantly different (Mann-Whitney U test, $p<0.05$). Letters accompanied by an asterisk denote the differences in alcohol biosynthesis between the groups. Numbers in brackets represent number of replicates.

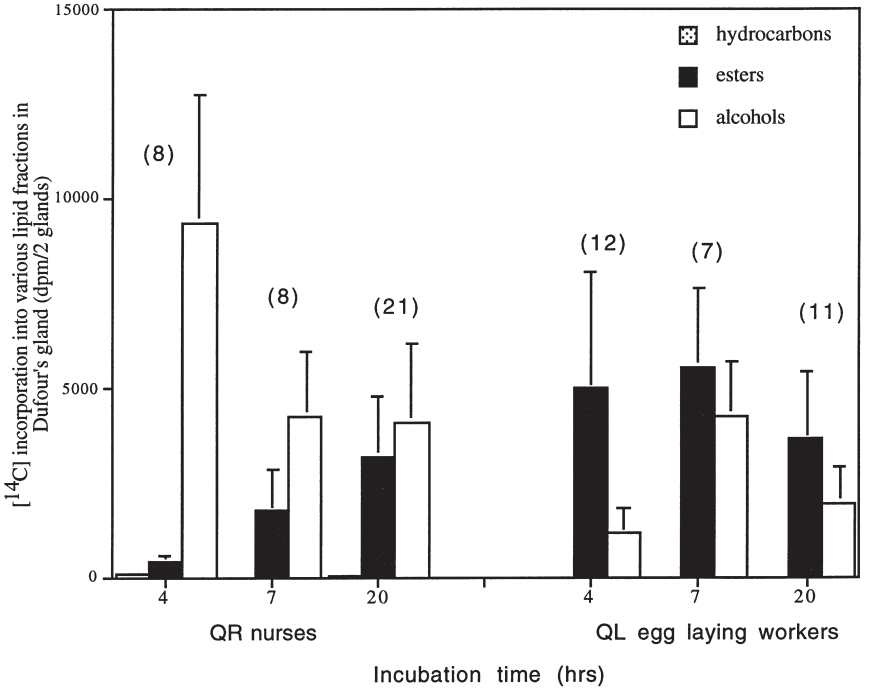

Fig. 3. Time course [1-^{14}C] acetate incorporation in vitro into various lipid fractions by Dufour's gland. Note that the incorporation into hydrocarbons is at background level. Numbers in brackets represent number of replicates.

4. Discussion

The present study aimed to unravel the biosynthetic basis of caste specificity in the chemistry of Dufour's gland secretion and unfold the physiological basis of its chemical plasticity. From earlier studies it was clear that the queen presence has an impact on the chemical composition of the gland in workers, but whether the worker gland is physiologically constrained was still unknown. By employing two methods of biosynthetic studies we

attempted to resolve this point. The clear distinction between the end products of worker glands (exclusively hydrocarbons) and those of queen glands (concomitant occurrence of hydrocarbons and esters) simplified this study and enabled us to investigate the specific occurrence of a whole class of compounds, rather than specific substances.

The results of the in vivo experiments are in agreement with the chemical composition of the glands in queens and the various types of workers (Katzav-Gozansky et al., 1997a). This indicates that the Dufour's gland, both in workers and queens, is metabolically active throughout the individual's life. The in vivo studies also disclosed an effect of the queen on glandular expression of QR workers. These workers, irrespective of task (nurses or foragers), produced de novo only hydrocarbons. On the other hand, glands of egg-laying workers obtained under QL conditions also produced esters de novo. This was not true for QL foragers that failed to show this reversal in biosynthetic capacity. We still do not understand the reason for these differences between workers, but they may be linked to either their age or task. These foragers were collected at the hive entrance with conspicuous pollen loads and are assumed to be among the oldest workers (Robinson, 1992). It is possible that some physiological changes that occur in old foragers are irreversible, as was shown for example for the hypopharyngeal glands (Huang and Robinson, 1996). The biosynthetic picture in vitro was drastically different from that in vivo. Like in queen's gland, we observed no in vitro incorporation of radioactivity into hydrocarbons by workers' glands. This suggests that the copious amounts of hydrocarbons that are present in the gland result from sequestration rather than de novo synthesis. It is unlikely that a deficiency in precursors for hydrocarbon biosynthesis in vitro was the cause for their absence, since there is a similar precursor requirement for the biosynthesis of esters (Stanley-Samuelson et al., 1988). However, we cannot exclude the possibility that under in vitro conditions Dufour's glands lack the activating factors needed for hydrocarbon, but not ester, biosynthesis. The fact that hydrocarbon sequestration by exocrine glands is known to occur in other insects, e.g. in ants (Soroker et al., 1994) and arctiid moths (Schal et al., 1998), support our sequestration hypothesis.

In contrast to the in vivo system, glands of QR nurses incubated in vitro synthesized esters in amounts that were not different from egg-laying QL workers. The situation with foragers was less clear. QR foragers had less incorporation into esters than either QR nurses or QL egg-laying workers, but these differences were not statistically different. QL foragers had very little de novo ester biosynthesis, significantly less than nurses (QR or QL egg-laying), but not different from QR foragers. This corroborates the above hypothesis that some glandular systems undergo irreversible changes when the bee becomes an old forager. The in vitro system also revealed possible temporal activation of the gland, once the queen effect is eliminated. In glands of QR workers there was a burst of radioactive alcohols in a short incubation period that declined as the incubation period was prolonged. The fact that this decline was accompanied by an increase in radioactive esters suggests that these alcohols are the precursors of the esters. Indeed, GC/MS analyses of the alcohol TLC fraction formed under these incubation conditions revealed the presence of the two primary alcohols that constitute the alcoholic moiety of the published esters (Katzav-Gozansky et al., 1997b), although their link to the ester biosynthesis remains speculative. The smaller amounts of alcohol detected in QL egg-laying workers as well as queens lend credence to our suggestion. It seems that all the intermediates and enzymes are already present in the glands of these bees, therefore enabling immediate ester biosynthesis.

The retention of the queen-like biosynthesis ability in the worker's Dufour's gland raises an interesting evolutionary question. Does it reflect an incomplete process of caste differentiation, or was it specifically selected for? It was previously reported that Dufour's gland secretion serves to discriminate between worker and queen-laid eggs, facilitating egg policing (Ratnieks, 1988). Recently, we were able to detect the presence of the major queen Dufour's gland esters on queen laid eggs, supporting in part the above function. If so, exocrine plasticity may have a social significance by enabling egg-laying workers (even under QR conditions) to mimic some of the queens' chemical signals and escape policing. Another possible explanation to the reversal in glandular expression is that it reflects worker–worker conflict over reproduction under a "hopeless queenless situation". We maintain that retaining Dufour's gland biosynthetic capability is therefore a prerequisite for successful worker reproduction, in addition to the retention of the egg production machinery, and it is likely to increase their direct fitness. From this perspective we can regard the retention of the potential of Dufour's gland biosynthesis as a retained component in the arms race between queens and workers over reproduction.

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