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Effects of Instrumental Insemination and Insemination Quantity on Dufour's Gland Chemical Profiles and *Vitellogenin* Expression in Honey Bee Queens (*Apis mellifera*)

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Abstract Honey bee queens (*Apis mellifera*) mate in their early adult lives with a variable number of males (drones). Mating stimulates dramatic changes in queen behavior, physiology, gene expression, and pheromone production. Here, we used virgin, single drone- (SDI), and multi-drone- (MDI) inseminated queens to study the effects of instrumental insemination and insemination quantity on the pheromone profiles of the Dufour's gland, and the expression of the egg-yolk protein, *vitellogenin*, in the fat body. Age, environmental conditions, and genetic background of the queens were standardized to specifically characterize the effects of these treatments. Our data demonstrate that insemination and insemination quantity significantly affect the chemical profiles of the Dufour's gland secretion. Moreover, workers were more attracted to Dufour's gland extract from inseminated queens compared to virgins, and to the extract of MDI queens compared to extract of SDI queens. However, while there were differences in the amounts of some esters between MDI queens and the other groups, it appears that the differences in behavioral responses were elicited by subtle changes in the overall chemical profiles rather than dramatic changes in specific individual chemicals. We also found a decrease in *vitellogenin* gene expression in the fat body of the MDI queens, which is negatively correlated with the quantities of Dufour's gland content. The possible explanations of this reduction are discussed.

Key Words Chemical ecology · Gene expression · Behavior · Social insects · Pheromone

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Introduction

Pheromones regulate many aspects of worker physiology and colony organization in honey bees (Slessor et al., 2005; Le Conte and Hefetz, 2008). Honey bee queens have a highly complex chemical communication system, and their pheromones are produced by a variety of glands, including the mandibular gland, Dufour's gland, Nasonov gland, and tergal glands. Moreover, the composition of queen pheromone differs between mated and virgin queens (Slessor et al., 1990; Plettner et al., 1997; Le Conte and Hefetz, 2008). Recent studies have suggested that insemination quantity can affect the chemical profile of the mandibular gland,

which in turn affects worker behavior (Richard et al., 2007). Honey bee queens typically mate with multiple males, but the number of matings is highly variable (Tarpy and Nielsen, 2002). Extreme polyandry in honey bees results in greater genetic variability within the colony, which has been associated with healthier colonies (Tarpy and Seeley, 2006), as well as greater productivity and fitness (Mattila and Seeley, 2007). However, according to kin selection theory, lower relatedness among colony members should reduce altruistic and social behavior (Bourke, 1988). Thus, it is expected that queen pheromone composition would reflect her mating history, and that workers should be able to detect these differences. The relationships among multiple matings by the queen, her pheromone composition, and worker behavior have important implications for the evolution of social behavior in honey bees.

The Dufour's gland, previously called the alkaline gland, is an abdominal gland ubiquitous in the Hymenoptera. The gland is associated with the venom, sting sheath, and Koschevnikov glands in the sting apparatus (Martin et al., 2005) (see Fig. 1). Despite several studies, the precise role of the Dufour's gland in honey bees remains unclear. The chemical composition of the gland is affected by caste, task, and age of the bees (Le Conte and Hefetz, 2008). While both queen and worker glands produce hydrocarbons, queens also produce esters (Katzav-Gozansky et al., 1997a). However, the Dufour's gland of workers with developed ovaries also synthesizes queen-like esters (Katzav-Gozansky et al., 1997a), suggesting that the chemical profiles are linked to ovary development and reproductive potential. Previous studies on the effects of mating and reproduction on queen Dufour's gland content have been inconclusive (Katzav-Gozansky et al., 1997b); although newly mated and one-year-old mated queens tended to have lower quantities of Dufour's gland components than virgins, the differences were not statistically significant. Similarly, glands of mated queens tended to contain higher proportions of esters (compared to hydrocarbons) than virgins, but these differences also were not statistically significant. Finally, and contrary to analyses of gland contents, *in vitro* assays of ester and hydrocarbon production by using radiolabeled precursors revealed that mated queens produced significantly higher proportions of hydrocarbons than esters compared to virgin queens. However, these assays likely were confounded by age differences among the queens; other studies have demonstrated that production of queen mandibular pheromone (QMP) varies greatly with age (Plettner et al., 1997).

Fig. 1 Dufour's gland location in honey bees (in red)

The Dufour's gland may serve different functions in different species (Hölldobler and Wilson, 1990). It can produce a trail-marking pheromone, recruitment pheromone, sexual pheromone, or queen control pheromone (Hölldobler and Wilson, 1990; Le Conte and Hefetz, 2008). In honey bees, it has long been debated whether or not the Dufour's gland produces an egg-marking pheromone (Ratnieks, 1995). Selective oophagy of worker-laid eggs (also called 'workers policing') strengthens the functional sterility of their nestmates (Ratnieks and Visscher, 1989). Thus, the switch to queen-like Dufour's gland composition by laying workers may be an attempt to mimic the queen. Indeed, Dufour's gland chemicals have been found on eggs (Katzav-Gozansky et al., 2001). Worker-laid eggs treated with either whole Dufour's gland extracts or the ester fraction—at significantly higher quantities than naturally occurring levels—were removed more slowly than untreated worker-laid eggs, and no difference was observed when eggs were treated with the hydrocarbon fraction (Martin et al., 2002). However, another study that used more natural levels did not demonstrate an effect of Dufour's gland extracts on egg-removal and worker policing (Katzav-Gozansky et al., 2001). Thus, it is unclear whether or not the Dufour's gland in honey bee queens is a significant source of egg-marking cues. However, the Dufour's gland secretions are attractive to workers, suggesting that this gland plays a role in modulating worker behavior and perhaps in colony organization. Attracted workers antennate and groom the queen (Pankiw et al., 1994, 1995), and subsequently spread the queen pheromone through the colony; thus, attraction to the queen plays a role in colony social organization. The Dufour's gland extracts of queens and laying workers are significantly more attractive to workers than extracts of non-reproductive worker bees (Katzav-Gozansky et al., 2001, 2003). Furthermore, the esters, rather than the hydrocarbons, appear to mediate this attraction (Katzav-Gozansky et al., 2003). Finally, exposing bees to both QMP and Dufour's gland extracts has a greater inhibitory effect on worker ovary development than QMP alone (Katzav-Gozansky et al., 2006), suggesting that these chemicals may also serve as a primer pheromone.

Previous studies have demonstrated that chemical composition of the queen mandibular gland is affected by reproductive state and insemination quantity (Richard et al., 2007; Kocher et al., 2008, 2009). Here, we examined the effect of both instrumental insemination and insemination with one or multiple drones on the Dufour's gland. We monitored the attraction of caged workers to the Dufour's gland extracts of virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens. The chemical profiles of the Dufour's glands of these queens were then characterized by using gas chromatography-mass spectroscopy (GC-MS). We analyzed the quantities and relative proportions of esters and hydrocarbons, since previous studies indicated that mated queens may have lower total quantities of compounds and synthesize higher proportions of hydrocarbons than virgins (Katzav-Gozansky et al., 1997b). Furthermore, since previous studies indicate that the Dufour's gland is linked to ovary development in workers and, thus, might be an indicator of fertility, we also monitored levels of *vitellogenin* expression in the fat body of queens. *Vitellogenin* is an egg-yolk protein precursor, and expression of this gene is higher in recently mated, laying queens than in same-aged virgins (Kocher et al., 2008).

Methods and Materials

General Bee Rearing Colonies headed by SDI queens (*Apis mellifera carnica*, Glenn Apiaries, CA, USA) were maintained at the NCSU Lake Wheeler Honey Bee Research Facility (Raleigh, NC, USA). These colonies were used to produce super-sister queens for the experiments. Due to the haplodiploid sex determination of Hymenoptera, female progeny of SDI-queens have a genetic relatedness of G=0.75. Additionally, colonies headed by naturally mated *Apis mellifera ligustica* or Buckfast-SMR queens (B. Weaver, TX, USA) were used to provide drones for the semen collections and workers for the cage experiments.

Queen Rearing Super-sister queens were produced by grafting young larvae (<24 h) from a single source colony headed by an SDI queen, and rearing them as queens in a queenless colony (Laidlaw, 1977). Capped queen cells were transferred to a dark incubator at 33°C and ~40% relative humidity. One to two days prior to emergence, each capped queen cell was placed into an individual Plexiglas cage (10×10×7 cm) with ~100 1-d-old workers and fed a 50% sucrose solution and honey/pollen paste (45% honey, 45% pollen, 10% sucrose) *ad libitum*. Five days after emergence, some queens (those that were to be inseminated) were treated with CO_2 for 4.0 min (Laidlaw and Page, 1997), while others (those that were to remain virgins) were left untreated. Two days later, queens were treated again with CO_2 for 4.0 min, during which time they were inseminated with semen from either one drone (SDI) or 10 brother drones (MDI) following standard insemination protocols (Laidlaw, 1977). The average drone produces approximately 1 μl of semen, and thus the total insemination volume of each queen was approximately 1 μl for SDI queens and 10 μl for MDI queens. There is obviously variation between different drones, however, and thus volume was not precisely controlled in this study. Note that we are studying the effects of the instrumental insemination procedure (which includes CO_2 treatment, manipulation with the insemination device, and insemination with semen), and the effects of insemination with different numbers of drones. Additional studies on the specific effects of CO_2 and physical manipulation are in preparation. Queens were returned to their respective cages and collected 5 d later onto dry ice and stored at −80°C. A total of 12 virgin, 12 SDI, and 9 MDI queens were produced. Queens were reared in two separate cohorts from the same colony, approximately 1 wk apart (Cohort 1: 7 SDI and 4 MDI; Cohort 2: 12 virgin, 5 SDI, and 5 MDI), but queens from different cohorts were combined in behavioral assays, gene expression studies, and chemical analyses.

Dufour's Gland Extraction Abdomens were dissected in ice-cold RNAlater (Qiagen, Valencia, CA, USA). The Dufour's glands were dissected (Fig. 1) and extracted in 50 μl of pentane containing 0.4 μg/μl of hexadecane (Sigma). Gland extracts were stored at −20°C until they were used for GC analysis or behavioral assays (see below). Internal organs of the abdomen were removed, and the carcass (with attached fat body) was stored at −80°C for gene expression analysis.

Worker Attraction to Dufour's Gland Extracts We compared the responses of adult workers to Dufour's gland extracts of virgin, SDI, and MDI queens. The glands were extracted from each super-sister queen produced as outlined above. Since the internal standard was introduced to each sample, the hexadecane was not expected to contribute to differences in behavioral responses among the sample groups. Previous studies also demonstrated no worker attraction to hexadecane (data not shown). Frames of brood were removed from a single colony headed by a naturally mated queen and incubated at 33°C. One day old workers were collected from the brood frames, and 30 bees were placed into Plexiglas cages (10×10×7 cm). Bees were provisioned with food as described above. Cages were kept in a 33°C incubator with ~40% relative humidity, and manipulations and observations were performed under red light to mimic natural conditions in the hive. In total, 34 cages were maintained for 5 d.

Since worker maturation is altered in the absence of queen pheromone (Morgan et al., 1998; Grozinger et al., 2003), we reared these workers with QMP to mimic natural colony conditions. Bees were exposed to 0.1 queen equivalents (Qeq) of synthetic QMP (Pherotech, Canada). Every day, 10 µl of QMP (0.1 Qeq) in hexane was placed on a glass microscope slide, and the solvent was allowed to evaporate before the slide was placed into the cage. This amount of QMP mimics a live queen in assays of worker behavior and physiology (Slessor et al., 1998, 2005; Hoover et al., 2003).

On the fifth day of the experiment, 30 workers were presented with two microscope slides containing equal quantities of pooled Dufour's gland extract (0.33 Qeq) to compare their responses in binary choices of virgin vs. SDI queens (N= 13 replicates), virgin vs. MDI queens (N= 10 replicates), or SDI vs. MDI queens (N= 11 replicates). The number of workers contacting each slide was counted every 5 min for 15 min after slide presentation. Each cage was only tested once, and the 3 data points per slide were summed as a cumulative measure of worker response.

Data are presented as mean ± SEM. The worker preferences between the two slides of the 34 cages were evaluated with a non-parametric Wilcoxon test.

Chemical Analysis of Dufour's Gland Extracts A 2 µl portion of the 50 µl Dufour's gland extract from each individual queen was analyzed by using GC on an HP 5890 equipped with a capillary DB-5 column (30 m×0.25 mm ID×0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) in splitless mode. Helium was used as carrier gas at a head pressure of 124 kPa (1.3 ml/min). The GC temperature was held at 150°C for 2 min and then increased at 15°C/min to 250°C (5 min), followed by an increase of 3°C/min to 300°C (30 min). Injector and FID were both set at 300°C. We analyzed the Dufour's gland extracts from each of the queens in the study (12 virgin, 12 SDI, and 9 MDI queens).

Compounds were identified using selected samples by splitless capillary GC-MS using a Hewlett-Packard 6890 GC and a model 5973A MSD with an electron impact ion source and an HP-5 ms capillary column (30 m×0.25 mm ID×0.25 µm film thickness). Compounds were identified by using diagnostic ions, comparisons with standard MS databases, and by determination of Kovats indices (Lommelen et al., 2006).

For assessing profile similarity based on the relative proportion of the constituent compounds, a stepwise discriminant analysis was employed using only peaks that were reliably and reproducibly quantifiable (i.e., peaks that were consistently below 0.1% of the total were omitted; Statistica 6.0. StatSoft® Inc.).

Quantification of Vitellogenin RNA Levels by Quantitative Real-time PCR Total RNA was isolated from eviscerated abdominal carcasses (which contained fat bodies) from individual queens using an RNeasy RNA extraction kit (Qiagen, Valencia CA, USA), yielding 0.6–1.5 µg/individual. cDNA was synthesized from 150 ng RNA using Arrayscript reverse transcriptase (Ambion, CA, USA). Abundance of *vitellogenin* transcript was measured using quantitative real-time RT-PCR (qRT-PCR) with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA, USA). *eIF3-S8* and *actin* were used as loading controls; both have been used in previous studies of fat body *vitellogenin* expression (Amdam et al., 2006; Grozinger and Robinson, 2007). For each sample, triplicate qRT-PCR reactions were performed and averaged. A standard curve was generated for each primer using dilutions of genomic DNA to calculate the relative quantity of mRNA for each sample. A dissociation curve and negative control (cDNA reaction without RT-enzyme) were used to ensure primer specificity and lack of contamination. *eIF-S8* primers were as in Grozinger and Robinson (2007). *Vg* and *actin* primers were as in Amdam et al. (2006)

We evaluated *vitellogenin* expression in 9 virgin, 10 SDI, and 9 MDI queens. For each individual sample, the ratio of the expression level of *Vg* to that of the geometric mean of the quantities of both reference genes (*eIF3-S8* and *actin*) was calculated. For graphical representation, the samples were normalized to the mean levels in virgin queens. Data are presented as mean ± SEM. The expression level of *vitellogenin* was evaluated with a non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U*-test.

Results

Worker Attraction to Dufour's Gland Extracts Worker attraction to the Dufour's gland extracts of virgin, SDI, and MDI queens was tested in cages containing 5-d-old bees. Out of 30 bees only a small fraction were attracted by to the different Dufour's gland extracts, which is consistent with previous studies (Slessor et al. 1988; Richard et al., 2007). Worker bees simultaneously exposed to two different Dufour's gland extracts were more attracted to the gland extracts from SDI and MDI queens than to extracts from virgins (t=5.5, P=0.005 and t=1, P=0.027, respectively, Fig. 2), and in a binary choice, they preferred MDI extracts to SDI extracts (t=6, P=0.016).

Insemination Quantity Affects the Relative Proportion of Compounds in the Dufour's Gland The queen Dufour's gland contains a complex mixture of hydrocarbons and esters. Fifty seven compounds were identified, 32 of which were found at relative proportions greater than 0.1% of the total (Table 1). These 32 peaks were used for all of the

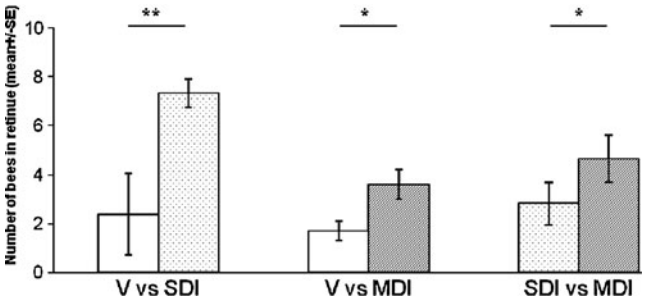

Fig. 2 Worker attraction to Dufour's gland extracts is affected by insemination and insemination quantity. Caged workers were exposed to Dufour's gland extracts of two different queens simultaneously, and the retinue response to each extract was monitored. Worker attraction was compared with a non-parametric Wilcoxon test. (*: $P<0.05$; **: $P<0.01$). *V* Virgin, *SDI* single-drone inseminated queen, *MDI* multi-drone inseminated queen

following analyses. We performed a forward stepwise discriminant analysis to determine if there was significant variation in the relative proportions of these compounds associated with insemination quantity. We also demonstrated a separation between virgin, SDI, and MDI queens ($F_{24,38}$=4.77, $P<10^{-3}$; Fig. 3). All three groups exhibited different chemical profiles (Mahalanobis distances, $P<0.01$).

Insemination Quantity Affects the Quantities of Hydrocarbons and Esters in the Dufour's Gland The total combined quantity of hydrocarbons and esters in the Dufour's glands in all three groups was compared; there were significant differences in total hydrocarbon and ester quantities ($F_{2,32}$= 2.1, $P<0.01$). Pairwise comparisons of the three groups revealed that virgin queens had higher quantities of hydrocarbons and esters than MDI queens (Least Significant Difference, LSD, $P<0.05$; Fig. 4), while SDI queens had intermediate amounts and were not significantly different from either virgin or MDI queens (LSD, $P>0.05$).

The total quantity of hydrocarbons and esters, tested by class, followed a similar pattern. When all three groups (virgin, SDI, and MDI) were compared separately, there were also differences in quantities (hydrocarbons, $F_{2,32}$= 4.23, $P<0.05$; esters, $F_{2,32}$=7.63, $P<0.01$). Pairwise comparisons revealed higher quantities in virgin than in MDI queens (hydrocarbons, LSD, $P<0.05$, esters, LSD, $P<0.05$) while SDI queens had intermediate levels for both hydrocarbons and esters, which were not different from total hydrocarbons in virgin and MDI queens (LSD, $P>0.05$).

Insemination Quantity Differentially Affects the Relative Proportions of Hydrocarbons and Esters in the Dufour's Gland The relative proportion of hydrocarbons in the Dufour's gland among virgin, SDI, and MDI queens revealed differences across the three groups ($F_{2,32}$=3.67, $P<0.05$). Pairwise comparison revealed a higher relative proportion of hydrocarbons in SDI queens than in virgin queens (LSD, $P<0.05$). However, the relative proportion of the hydrocarbons in MDI queens was intermediate and not significantly different from either virgin or SDI queens (LSD, $P>0.05$).

The relative proportion of esters in the Dufour's gland among virgin, SDI, and MDI queens revealed differences across the three groups ($F_{2,32}$=3.67, $P<0.05$). Pairwise comparisons revealed a lower relative proportion of esters in SDI queen Dufour's glands than in virgin queens (LSD, $P<0.05$). However, the relative proportion of esters in Dufour's glands of MDI queens were intermediate and not significantly different from either virgin or SDI queens (LSD, $P>0.05$).

Effect of Insemination Quantity of Vitellogenin Expression We used qRT-PCR to measure *vitellogenin* expression in the queens' eviscerated abdomens. *Vitellogenin* expression was higher in virgin than in MDI queens, using *eIF-S8* and *actin* as loading controls (H(2, N= 29)=10.78, P=0.004; Fig. 5). *Vitellogenin* expression was intermediate in SDI queens and not significantly different from that in either virgin or MDI queens.

Discussion

We instrumentally inseminated honey bee queens with semen from one or 10 drones and compared them to uninseminated virgin queens. These studies allowed us to examine the effects of the instrumental insemination procedure (which includes CO_2 treatment, physical manipulation, and insemination substance) as well as insemination quantity (via the comparison of the SDI and MDI queens) on Dufour's gland composition and gene expression under controlled environmental conditions. Our results clearly demonstrate that both the instrumental insemination procedure and insemination quantity alter queen Dufour's gland composition, which results in differences in worker behavior. Honey bee workers were more attracted to Dufour's gland extracts from inseminated queens than from virgin queens, and to extracts of MDI queens than to SDI queen extracts. Moreover, the overall comparison of Dufour's gland chemical profiles revealed a significant separation between virgin and inseminated queens and between SDI and MDI queens. A small number of esters and hydrocarbons were in higher proportions in MDI queens, while others were significantly lower. It remains to be determined which of the Dufour's gland constituents are most important for eliciting worker attraction to Dufour's gland extract in honey bee queens. Finally, we found a decrease in *vitellogenin* gene expression in MDI queens compared to

Table 1 Chemical composition of honey bees Dufour's gland contents in differentially inseminated queens: virgin, single (SDI), and multi (MDI) drone inseminated queens

	Virgin	SDI	MDI	Kruskal-Wallis H(2,*N*=33)	
Hydrocarbons					
Alkanes					
Pentadecane*	tr	tr	tr	NS	
Heptadecane	tr	tr	tr	NS	
Octadecane*	tr	tr	tr	NS	
Nonadecane*	tr	tr	tr	NS	
Eicosane*	tr	tr	tr	NS	
Heneicosane	+	+	+	NS	
Docosane	tr	tr	tr	NS	
Tricosane	+++	+++	+++	NS	
Tetracosane	tr	tr	tr	NS	
Pentacosane	+++	+++	++		*P*=0.006
Hexacosane	tr	tr	tr	NS	
Heptacosane	++	+++	++	NS	
Octacosane*	tr	tr	tr	NS	
Nonacosane	++	++	++	NS	
Triacontane*	tr	tr	tr	NS	
Untriacontane	++	+++	+++	NS	
Tritriacontane	tr	tr	+	NS	
Alkenes					
Tricosene	+	+	+	NS	
Pentacosene (2 isomers)	++	++	++	NS	
Heptacosene (3 isomers)	+	+	tr	NS	
Nonacosene (3 isomers)	+	+	tr	NS	
Hentriacontene (3 isomers)	++	++	++	NS	
Tritriacontene (2 isomers)	++	++	++	NS	
Alkynes					
Pentacosyne	tr	tr	tr	NS	
Methylalkanes					
9-, 11-, 13-, and 15-Methylpentacosane*	tr	tr	tr	NS	
11-,and 13-Methylheptacosane	tr	tr	tr	NS	
11-, 13-, and 15-Methylnonacosane	tr	tr	tr	NS	
13-, and 15-Methylhentriacontane	tr	+	tr	NS	
DiMethylalkanes					
3,9 Dimethylpentadecane	tr	tr	tr	NS	
Esters					
Tetradecyl dodecanoate	++	+	+		*P*<0.001
Tetradecyl tetradecanoate	++++	+++	+++		*P*=0.007
Tetradecyl (Z)-9-hexadecenoate+Tetradecyl (Z)-11-hexadecenoate	++	++	++	NS	
Tetradecyl hexadecanate+Hexadecyl tetradecanoate	++++	++++	++++		*P*=0.05
Tetradecyl (Z)-9-octadecenoate	++++	++++	++++	NS	
Hexadecyl (Z)-9-hexadecenoate	++	++	++	NS	
Hexadecyl hexadecanoate	+++	++	++	NS	
Hexadecyl (Z)-9-octadecenoate	++	+++	++	NS	
Octadecyl (Z)-9-hexadecenoate	+++	+++	++++		*P*=0.01
Octadecyl hexadecanoate	+	++	+	NS	

The results are presented as relative proportions: tr, traces; +, 0.5–1%; ++, 1–5%; +++, 5–10%; ++++, <10%. * = compounds which were not used in the analysis

virgin queens. However, additional studies are necessary to examine the effects of specific aspects of the instrumental insemination protocol (CO_2 anesthetization, physical manipulation of the reproductive tract).

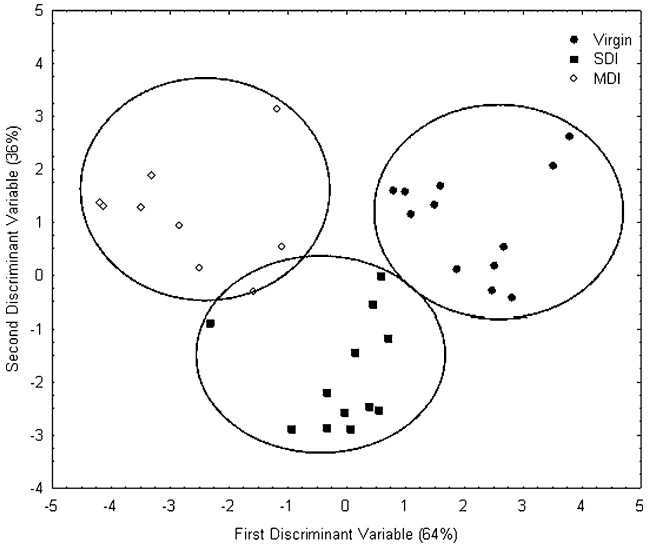

Fig. 3 Insemination quantity significantly alters the chemical profile of the Dufour's gland. Chemical composition of Dufour's gland extracts of virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens were analyzed using gas chromatography. Discriminant analysis was used to determine if there were significant differences in their Dufour's gland profiles, based on the relative proportion of each compound ($F_{24,38}$=4.77, $P<10^{-4}$). Mahalanobis distances, $P<0.01$. Ellipses were drawn to emphasize the categories, but have no specific statistical meaning

It is important to note that significant changes in the chemical composition of both the Dufour's and mandibular glands occur relatively rapidly, within 5 days after insemination (Richard et al., 2007). While these changes

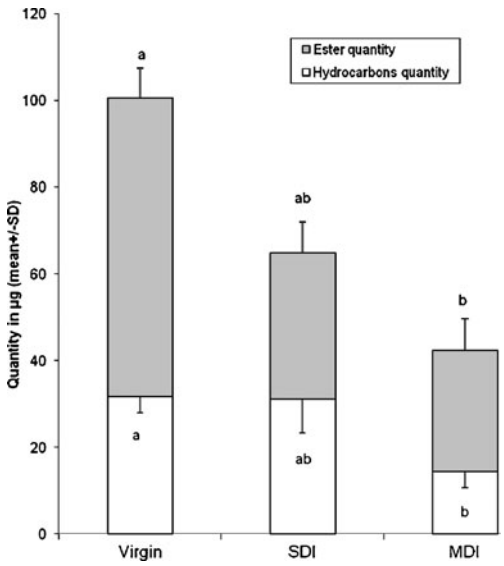

Fig. 4 Insemination quantity significantly alters contents of the Dufour's gland. The quantity of esters and hydrocarbons are significantly different among virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens (respectively: Kruskal-Wallis: $H_{2,32}$=7.63, p<0.01; $H_{2,32}$=4.23, $P<0.01$)

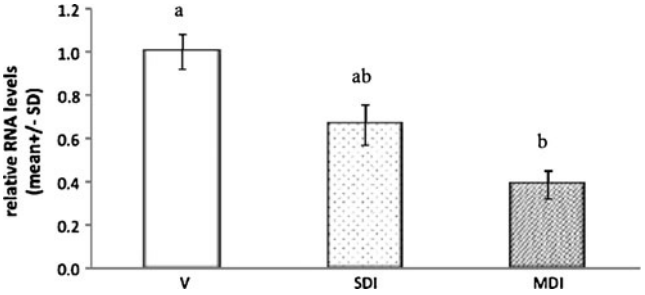

Fig. 5 Expression of *vitellogenin* in the fat body is affected by insemination quantity. Expression of *Vg,* using *eIF3-S8* and *actin* as controls, was monitored using quantitative real-time PCR using individual bees [virgin: $N=9$; single-drone inseminated (SDI): $N=10$; multi-drone inseminated (MDI): $N=9$]. Bars with different letters are significantly different (Kruskal-Wallis: $H_{2,29}$=10.79, P=0.004)

could be due to artificial aspects of the instrumental insemination protocol (such as treatment with CO_2), studies with naturally mated queens have demonstrated that mandibular gland pheromone profiles begin to change before behavioral changes are observed (Kocher et al., 2008). In other insect species, stretch receptors in the bursa, oviducts, or spermatheca, or seminal proteins in male semen are involved in stimulating post-mating changes in females (Sugawara, 1979; Wolfner, 2002, 1997). Both types of mechanisms appear to be operating in honey bee queens (unpublished data), but it remains to be determined if these rapid changes in pheromone production and gene expression are triggered by mechanosensory pathways, chemosensory pathways, or both. Recent studies suggest that insemination volume is an important cue that triggers immediate changes in pheromone production, while semen or seminal fluid may trigger behavioral and brain expression differences over a longer time scale (Kocher et al., 2008; Richard et al. unpublished). However, we note that our MDI queens differed from SDI queens in two regards: they were inseminated with a 10-fold greater volume than SDI females, and they received semen from more genetically diverse sources (ten drones rather than a single drone). Therefore, our experimental design does not allow us to separate the effects of semen volume, sperm number, quantity of semen constituents, or other possible effects of genetic diversity.

While mating stimuli, such as stretch receptors or seminal proteins, may play a role in triggering changes in Dufour's gland composition, the pathways that translate these stimuli into physiological changes in the queen remain to be determined. We observed a quantitative decrease, but not statistically significant, in both *Vg* gene expression and Dufour's gland content in MDI compared to SDI queens, suggesting that these two phenotypes may be regulated by a common pathway. In *Bombus* and *Apis,* the Dufour's gland size and secretion activity in workers

increase with ovary activation (Abdalla et al., 1999a, b). In bumble bees, ovary activation is positively mediated by juvenile hormone (Bloch et al., 2000), but the role of JH in honey bee reproduction is not fully understood (Robinson and Vargo, 1997). In fact, *Vg* expression and JH levels are negatively correlated in honey bees (Pinto et al., 2000; Guidugli et al., 2005; Corona et al., 2007). Thus, while JH may mediate both Dufour's gland secretion and ovary activation in bumble bees (reviewed in Abdalla and Cruz-Landim, 2001), it is likely that an alternative mechanism is operating in honey bees.

The total quantity of compounds in the Dufour's glands decreased following insemination. This decrease may be simply an artefact of the instrumental insemination process. Since our study used same-aged queens, the changes in gland contents are directly linked to the effects of insemination on queen physiology. There are several possible explanations for why insemination would cause a reduction in gland quantities. A high quantity of compounds in the Dufour's gland of virgin queens could be involved in other aspects of reproduction, such as attracting drones during mating flights. Alternatively, the chemical profiles of virgins may deter workers from attacking the queen (see Gilley et al., 2003; Tarpy et al., 2004).

Assuming that instrumental insemination (which includes CO_2 treatment, physical manipulation, and the introduction of semen) mimics natural mating, a third hypothesis is that there is a negative impact of mating on the physiology of the queen. Indeed, in *Drosophila* females, mating stimulates an immune response, suggesting that the introduction of novel male-specific proteins or sperm has effects on the females. Baer et al., 2006 found that newly mated queens of leaf-cutting ants up-regulated their immune responses 9 days after their mating flight. Mating also could be metabolically costly because the female needs to activate processes involved in sperm storage and maintenance of live sperm in the spermatheca. For example, expression levels of antioxidant enzymes increase in the spermathecae of mated queens compared to virgins, suggesting that the mated queens must produce an additional repertoire of proteins to increase sperm longevity (Collins et al., 2004). The cost of mating could explain the short-term effect that we observed before long-term reproductive success. Indeed, we also observed a reduction in *vitellogenin* levels after insemination, despite the fact that *Vg* is necessary for egg-maturation, which suggests that inseminated queens may be negatively affected by mating. Alternatively, there may be a period of physiological remodelling after mating, before egg-production can be fully stimulated. In naturally mated queens (Kocher et al., 2009), these changes occur quickly, while in inseminated queens, changes may occur more slowly.

Previous studies have compared Dufour's gland compounds among non-reproductive virgin, newly mated, and one-year-old mated queens without finding a significant difference in the relative proportion of hydrocarbons and esters (Katzav-Gozansky et al., 1997b). However, analysis of the biosynthesis of Dufour's gland constituents have revealed that the relative proportion of newly synthesized hydrocarbons was significantly higher in mated queens than in virgins, while for esters the pattern was reversed (Katzav-Gozansky et al., 1997b). We found that the relative proportion of hydrocarbons in inseminated queens is significantly higher than in virgins. However, when the three groups of queens were compared separately, hydrocarbon levels of MDI queens were intermediate between the SDI and virgin queens. Thus, insemination appears to have a significant effect on the relative proportions of the hydrocarbons and esters, but insemination volume seems to have additional effects.

Interestingly, workers can discriminate between the Dufour's glands of queens in different mating states. They were more attracted to the gland extracts of inseminated queens than virgin queens, and more attracted to the glands of MDI queens than SDI queens. Previous studies found that queen-like esters, rather than hydrocarbons, seem to play an important role in eliciting this behavior. However, in our studies, the proportion of esters was *lower* in the more attractive blends; thus, the attractive cue may be more specific than a simple increased quantity or proportion of all esters. Some esters were more represented in MDI queens, while others were significantly lower in MDI queens. Similarly, significant differences were seen in individual hydrocarbon compounds.

In summary, both the instrumental insemination procedure and quantity have profound impacts on queen physiology, queen worker interactions, and gene expression in honey bees. Importantly, workers are more attracted to both mandibular gland (Richard et al., 2007) and Dufour's gland extracts (present study) from MDI queens compared to SDI or virgin queens. These behavioral differences are elicited by changes in the chemical profiles that appear to signal the reproductive state of the queens to the workers. Our results suggest that post-mating changes in honey bee queens involve complex processes that are regulated by multiple parameters.

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