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Research article

Queen and pheromonal factors influencing comb construction by simulated honey bee (*Apis mellifera* L.) swarms

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Summary. The influence of the queen and her pheromonal signal on comb construction was examined. We tested four treatments with newly hived packages of bees containing: 1) a mated queen, 2) a virgin queen, 3) no queen but with a dispenser containing synthetic queen mandibular pheromone (QMP), and 4) no queen and no pheromone. After 10 days, the comb produced by each colony was removed, comb measurements made, bees from the comb-building area collected, the size of the scales on the wax mirrors of the collected bees ranked on a scale of 0–4 and the queens removed and analyzed for QMP components. Queenless workers built substantially less comb and the comb they did build had significantly larger, drone-sized cells than for the other 3 treatments, indicating that both cell size and the quantity of comb built are mediated through the queen, particularly QMP. The observations of wax scale size suggested that QMP influenced comb building behaviour rather than wax scale production. These results support the idea that queenless honey bees can adopt a strategy of constructing drone-sized cells in order to increase reproductive fitness through male production following queen loss.

Key words: Apidae, honey bees, *Apis mellifera*, queen mandibular pheromone, comb construction.

Introduction

Most highly social insects are vulnerable to losing their queen, and must replace her quickly if colonies are to survive. New queens can arise following queen loss by extant virgin queen adults mating and replacing their former queen, by adult workers changing their status to become functional queens that can lay female eggs, or by brood being reared by adult workers into new queens. However, in some situations colonies are unable to rear a new queen, and the only reproductive option is for workers to develop ovaries and lay male eggs.

Honey bees (*Apis mellifera* L.) often can rear new queens from brood following queen loss, but there is one situation in which there is no reproductive option except for worker bees to produce male drones. Honey bees reproduce by swarming, in which the old mated queen or a virgin queen leaves the nest with many thousands of workers and seeks a new nest site (Winston, 1987). Occasionally the queen is lost from swarms, but the orphaned swarm can still reproduce if some worker bees develop ovaries and the swarm constructs comb in which the laying workers can deposit unfertilized eggs to rear into drones before the incipient colony dies out.

Comb construction is essential in that situation, to provide cells in which worker eggs can be laid. The wax used to build comb is produced by workers in modified epidermal cells located under the fourth to seventh ventral sternites, secreted from these wax glands, removed by the workers' legs, and manipulated by the mandibles to construct regular hexagonal-shaped cells. Comb consists of two cell types, the smaller worker-sized cells and larger drone-sized cells. Under normal conditions worker-sized cells are found in greater proportions than drone cells (reviewed by Hepburn, 1986 and Winston, 1987). Drones can be reared in worker comb, but they are smaller and less reproductively fit compared to the larger individuals normally reared in drone comb (Ribbands, 1953; Gary, 1975).

Comb construction in the queen's presence has been studied extensively, and there clearly are numerous demographic and population-dependent factors that determine when and how much drone comb colonies build (Lee and Winston, 1985, 1987; Pratt, 1998). However, there are hints in the literature that the queen herself may have a direct

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stimulatory effect on the amount of comb constructed and cell type, particularly through her pheromonal influence (Darchen, 1957; Taranov, 1959). Queen pheromones, particularly queen mandibular pheromone, mediate many aspects of worker behaviour and physiology, including swarming and the inhibition of new queen rearing, foraging, worker ovary development, and other aspects of colony functions (reviewed by Winston and Slessor 1992, 1998). To date, however, the influence of queen pheromone on comb construction has not been studied directly, but the absence of queen pheromone in queenless swarms could have profound effects on the extent and nature of comb construction. Also, there are anecdotal reports in early literature of queenless swarms building intermediate (Taber and Owens, 1970) and drone-sized cells.

The objective of the current study was to examine the influence of the queen and her pheromonal signal on comb construction in simulated swarms with and without queens. More specifically, we compared the amount of comb constructed and the proportion of worker and drone sized cells produced in newly hived packages of bees with a mated or virgin queen, without a queen but with synthetic queen mandibular pheromone, and with no queen or pheromone.

Materials and methods

Four treatments were tested: 1) mated queen, 2) a virgin queen, 3) queenless with synthetic queen mandibular pheromone (QMP), and 4) queenless. There were eight replicates per treatment, for a total of 32 test colonies.

Colony set-up

New hive components (hive bodies, bottom boards, frames and inner covers) were used to prevent any residual pheromones from confounding the results. Each hive contained 10 wooden frames, with each frame having a beeswax strip 2 cm wide and 42 cm long attached to the top bar using hot wax. Two holes were drilled into each inner cover for water (1.3 cm in diameter) and sucrose (6.5 cm in diameter) feeders.

Colonies were placed on eight pallets, one colony from each treatment per pallet, in a shady location on the Simon Fraser University campus in Burnaby, B.C. The top and bottom entrances were screened using wire mesh to prevent drift and loss of bees from colonies.

Collection of bees

Bees on brood frames from source colonies were mixed in a large cage and then shaken into individual package boxes weighing 1.2 ± 0.2 kg of bees (approximately 10,000 bees) per package. The weight of the bees was re-assessed after introducing them into their test colonies by weighing the package boxes prior to and after shaking.

Introduction into test colonies

Bees were collected on 10 June 1999 and placed into hives that evening. The mated queen treatment included a queen caged with a candy-plugged exit hole to allow emergence into colonies. The virgin treatment had a queen cell close to emergence placed gently between two frames. The QMP treatment consisted of a slow release QMP dispenser (Bee Boost®, Pherotech, Delta, B.C., 10 Qeq per dispenser, 1 queen equivalent (Qeq): 200 µg (*E*)-9-oxodec-2-enoic acid (ODA), 80 µg (*E*)-9-hydroxydec-2-enoic acid (9-HDA), 20 µg methyl p-hydroxybenzoate (HOB) and 2 µg 4-hydroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996)) tacked onto the top bar. The queenless treatments had no queen or pheromone.

Once the bees were introduced, water and sugar feeders were inverted and the screens checked to ensure bees were not escaping. On the evening of 11 June 1999 the mated queen treatments were opened quickly to make sure that all queens had been released from their cages. Water and sugar feeders were refilled when necessary.

Dismantling colonies and comb collection

The experiment ended on 21 June 1999. Queens were placed in individual labeled vials and kept on dry ice until transferred to a –20 °C freezer. Samples of 20–30 bees from frames with comb from each colony also were taken and placed on dry ice, then transferred to the freezer. Finally, the frames from each colony were shaken to remove bees and the frames placed into an empty box.

Analysis

Each comb was cut from its frame and the diameter of ten cells across the top, middle and bottom sections of each comb was measured. The diameter of five cells was measured if the piece of comb was not large enough to make measurements of ten cells, and no measurements were made if there were fewer than five cells. The mean cell diameter for each comb, colony and treatment was determined from these measurements.

The area of comb produced was measured with a plastic grid; each square unit of the grid measured 25 cm^2. The areas of worker, intermediate and drone-sized cells were measured by recording the number of squares of each type of comb and converting into the actual areas in cm^2. Worker-sized cells were considered those with diameters of ≈ 5.6 mm, drone-sized cells ≈ 6.6 mm and intermediate-sized cells ≈ 5.9 mm.

All of the comb produced from each colony was collected, rendered in order to remove impurities such as honey, brood etc., and the final weight of comb produced from each colony recorded.

Fifteen bees from each colony were analyzed for wax scale production. The wax mirrors of these bees were examined for the presence of scales, as described by Jordan (1962):

0 = no wax
1 = small scale
2 = medium scales, not extending beyond the overlapping sternite
3 = large scales, extending beyond the overlapping sternite
4 = very large scales
The mean wax scale size/ bee/ treatment was calculated.

The mandibular glands of the mated and virgin queens were analyzed by crushing the queen heads in ether. The final 500 µl extracts were analyzed by GC-Mass Spectrometry (Keeling et al., 2000). Each queen head was macerated in diethyl ether and centrifuged to remove solids, producing a 500 µl extract. An internal standard (undec-10-enoic acid) was added to each extract and a portion of each extract was derivatized with bistrimethylsilyltrifluoroacetamide (BSTFA) (Slessor et al., 1990) and then analyzed by splitless capillary gas chromatography-mass spectrometry (Varian 3400 GC with Varian 8100 Autosampler-Varian Saturn ion trap MS, J&W Scientific DB-5 ms column, 30 m × 0.25 mm ID × 0.25 µm film). The GC oven was programmed 100 °C (1 min), 10 °C/min to 200 °C (6 min), and 25 °C/min to 250 °C (21 min), head pressure 120 kPa helium. The SPI injector was programmed at 80 °C (0.1 min), 100 °C/min to 250 °C (38.2 min). The mass spectrometer was operated in electron impact mode at 70 eV with a target value of 10600, multiplier at 2300 V and an ionization current of 10 µA. Standard solutions of methyl *p*-hydroxybenzoate (HOB), 4-hydroxy-3-methoxyphenylethanol (HVA), (*E*)-9-oxodec-2-enoic acid (ODA), (*E*)-9-hydroxydec-2-enoic acid (9-HDA) derivatized with BSTFA were used to calibrate the response of the instrument with respect to the internal standard over a 500-fold range of concentration for each analyte. We used the mass spectrometer's software capability to integrate peaks over specific masses. Masses were chosen for each analyte based

on the fragmentation patterns of the trimethylsilyl derivatives and the absence of adjacent peaks with similar masses as follows: undec-10-enoic acid (241 and 257), HOB (193, 209 and 224), HVA (209, 297 and 312), ODA (241 and 257), 9-HDA (315 and 331). Retention indices (van den Dool and Kratz, 1963) for these derivatives on the DB-5 ms column were as follows: undec-10-enoic acid (1541), HOB (1492), HVA (1701), ODA (1704), and 9-HDA (1782).

Statistical analysis

Data for cell diameter, comb area, wax weights and wax scale size were transformed by log (x + 1) and data for percentages of intermediate and drone comb were arcsine square root transformed. The transformed data were analyzed by ANOVA (GLM procedure, SAS Institute, 1995). The Shapiro-Wilk test was performed to check for normality and the residuals were plotted to check for heteroscedasticity. The analysis of the QMP components was done using a two-sample *t*-test (TTEST Procedure, SAS Institute, 1995) for unequal variances. Regression analysis was done on cell size versus the individual QMP components (Sall and Lehman, 1996).

Results

The mean cell diameter of the mated queen treatment (Fig. 1, $\overline{x}$ = 5.56 mm) differed significantly from both the QMP ($\overline{x}$ = 5.72 mm) and the queenless treatment ($\overline{x}$ = 6.64 mm), in which 3 of 8 colonies constructed measurable numbers of cells. There was no significant difference between cell sizes in the mated and virgin queen treatments ($\overline{x}$ = 5.67 mm).

The mean area of comb constructed in the mated queen treatment (Fig. 2, 1611 cm^2) was significantly greater than the queenless treatment (271 cm^2). The QMP (869 cm^2) and the virgin queen treatments (421 cm^2) were not significantly different from either the mated queen or queenless treatments. A large percentage of the comb built by the queenless treatment ($\overline{x}$ = 84.6%) was intermediate or drone comb size, over half ($\overline{x}$ = 58.4%) was drone comb (Fig. 3A, 3B),

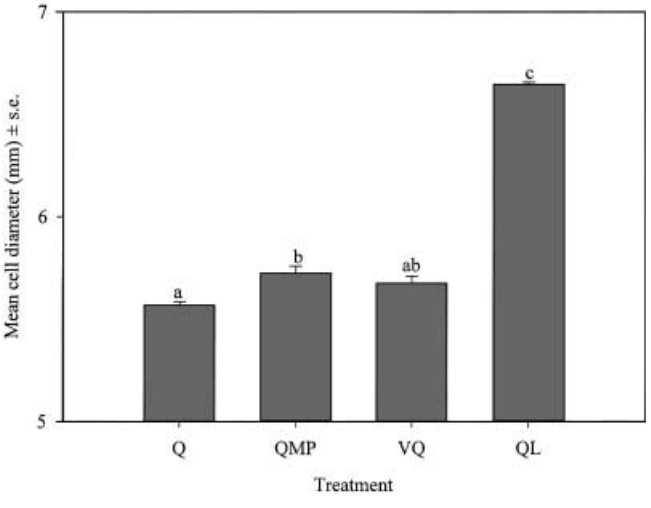

Figure 1. The variation in mean cell diameter between treatments. Q = mated queen, QMP = queen mandibular pheromone, VQ = virgin queen, and QL = queenless. Means with different letters were significantly different ($p < 0.05$, Ryan-Einot-Gabriel-Welsh Multiple Range Test)

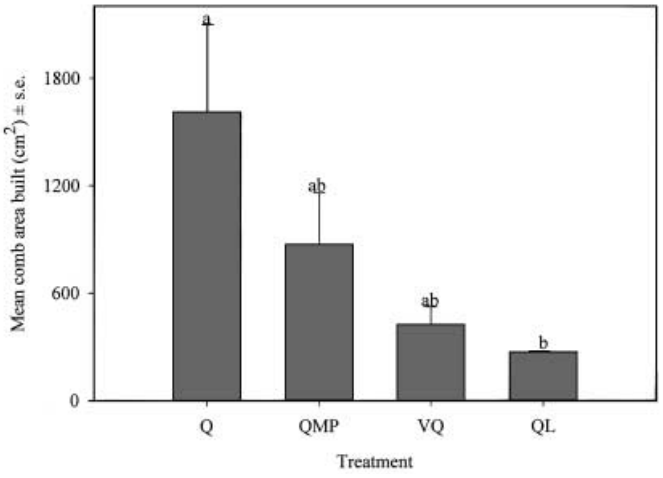

Figure 2. The mean comb area by treatment. Q = mated queen, QMP = queen mandibular pheromone, VQ = virgin queen, and QL = queenless. Means with different letters were significantly different ($p < 0.05$, Ryan-Einot-Gabriel-Welsh Multiple Range Test)

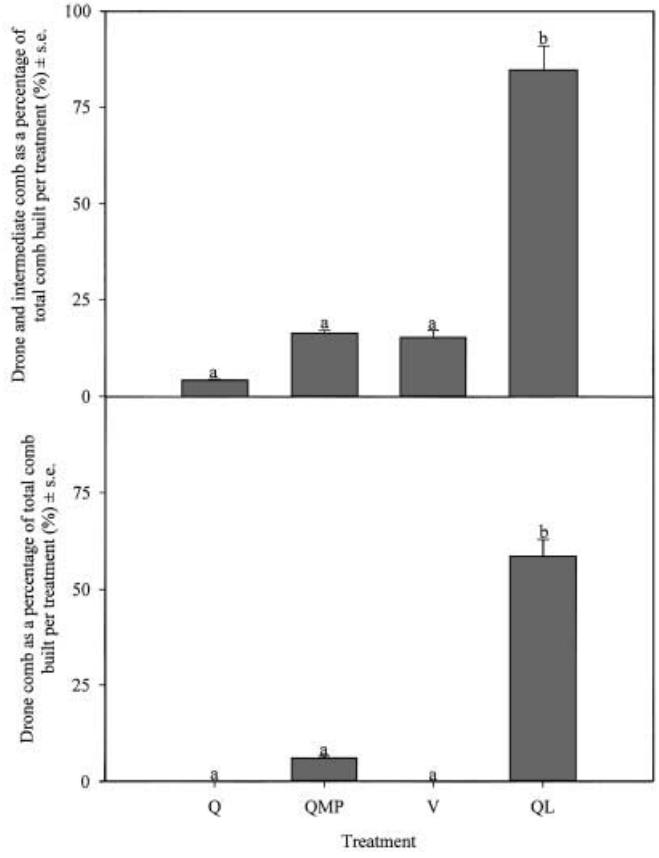

Figure 3. A: The mean drone and intermediate comb as a percentage of all comb per colony (%). B: The mean drone comb as a percentage of all comb per colony (%). Q = mated queen, QMP = queen mandibular pheromone, VQ = virgin queen, and QL = queenless. Means with different letters were significantly different ($p < 0.05$, Ryan-Einot-Gabriel-Welsh Multiple Range Test)

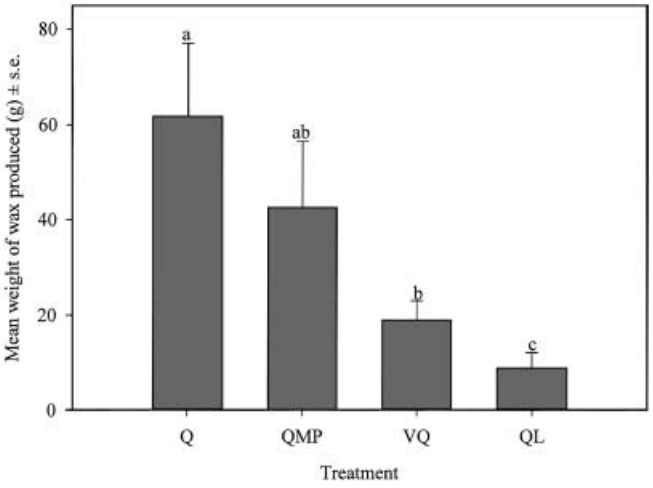

Figure 4. The mean weight of comb produced by treatment. Q = mated queen, QMP = queen mandibular pheromone, VQ = virgin queen, and QL = queenless. Means with different letters were significantly different ($p < 0.05$, Ryan-Einot-Gabriel-Welsh Multiple Range Test)

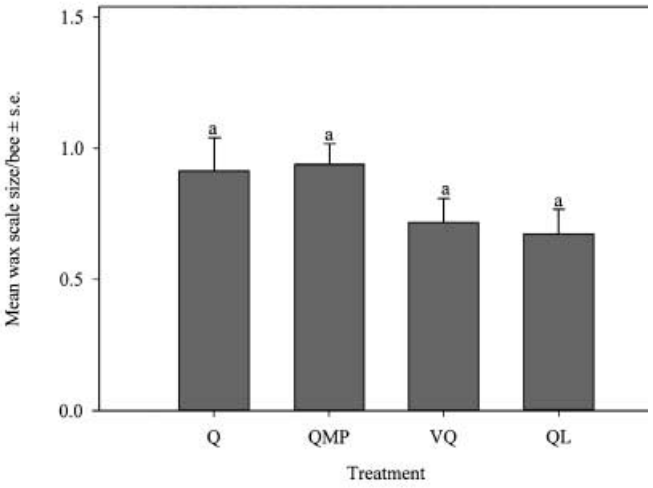

Figure 5. The mean wax scale size/bee by treatment. Q = mated queen, QMP= queen mandibular pheromone, VQ = virgin queen, and QL = queenless. Means with different letters were significantly different ($p < 0.05$, Ryan-Einot-Gabriel-Welsh Multiple Range Test)

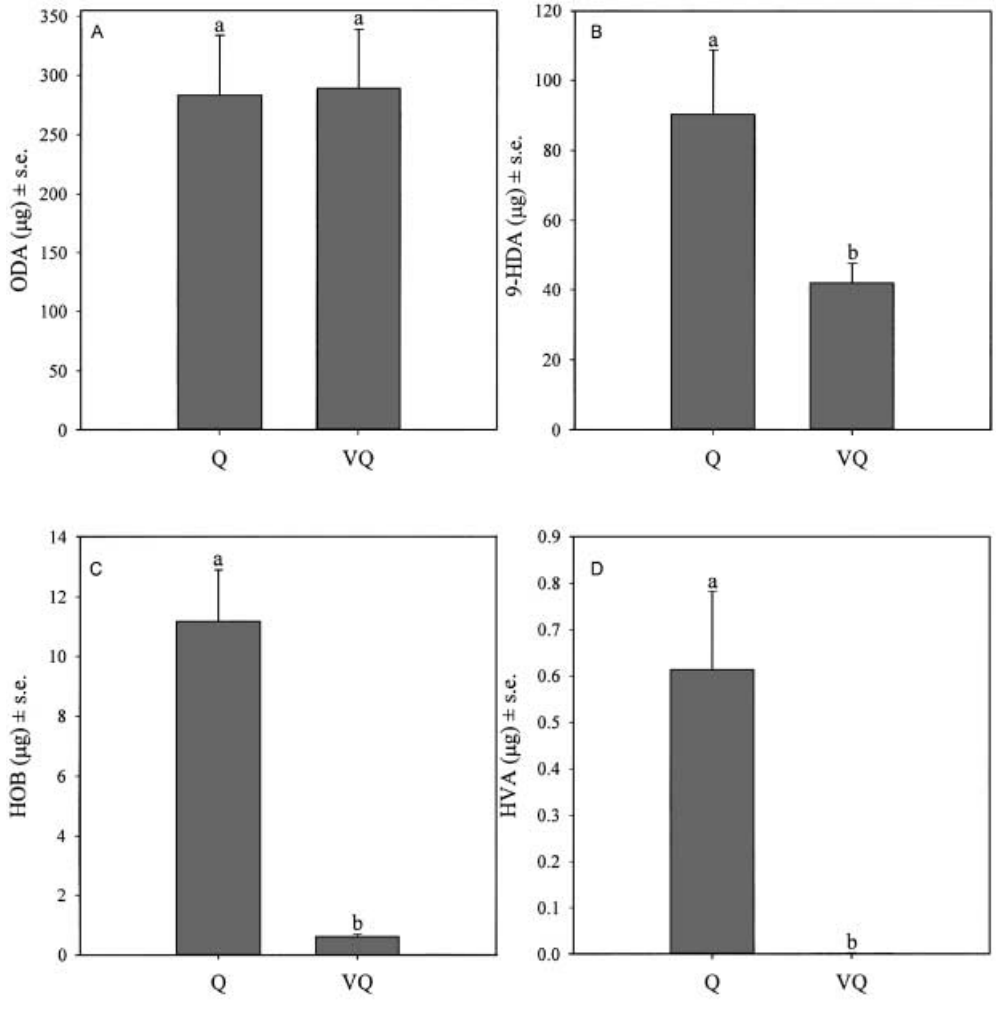

Figure 6. Comparison of QMP components between mated and virgin treatments. Q = mated queen and VQ = virgin queen. A: ODA B: 9-HDA C: HOB D: HVA. Means with different letters were significantly different ($p < 0.05$, TTEST procedure)

and these values were significantly different from the other three treatments. The mated and virgin queen treatments did not construct any drone comb and only a small proportion ($\bar{x}$ = 4.9%) of the comb built by the QMP treatment was drone sized (Fig. 3B).

The mated queen treatment produced the largest mean wax weight (Fig. 4, $\bar{x}$ = 61.8 g) but was not significantly different from the QMP treatment ($\bar{x}$ = 42.6 g). The virgin queen treatment ($\bar{x}$ = 18.8 g) was significantly lower than the mated queen treatment and the queenless treatment ($\bar{x}$ = 8.8 g) was significantly different from the other three treatments.

The mean size of wax scales per bee per treatment was not significantly different between treatments (Fig. 5). The QMP treatment had the largest mean wax scale size/bee ($\bar{x}$ = 0.94), followed by the mated queen ($\bar{x}$ = 0.92), the virgin queen ($\bar{x}$ = 0.72) and the queenless treatment ($\bar{x}$ = 0.67).

Analysis of mated and virgin queen mandibular gland components (Fig. 6) indicated that all QMP components were significantly higher in mated versus virgin queens except for ODA.

Regression analysis demonstrated no significant relationship between cell size and any individual component (Fig. 7).

Discussion

Our study indicates that queenless honey bees can adopt a strategy of constructing drone-sized cells in order to increase reproductive fitness following queen loss. Further, the queen's pheromonal message, particularly QMP, is the major signal that mediates the type and quantity of comb constructed. Finally, QMP unexpectedly appeared to act as a releaser pheromone to alter comb constructing behaviour of the bees rather than as a primer pheromone affecting wax production directly.

In our experiment we placed honey bee workers in a situation analogous to queen loss following swarming, and

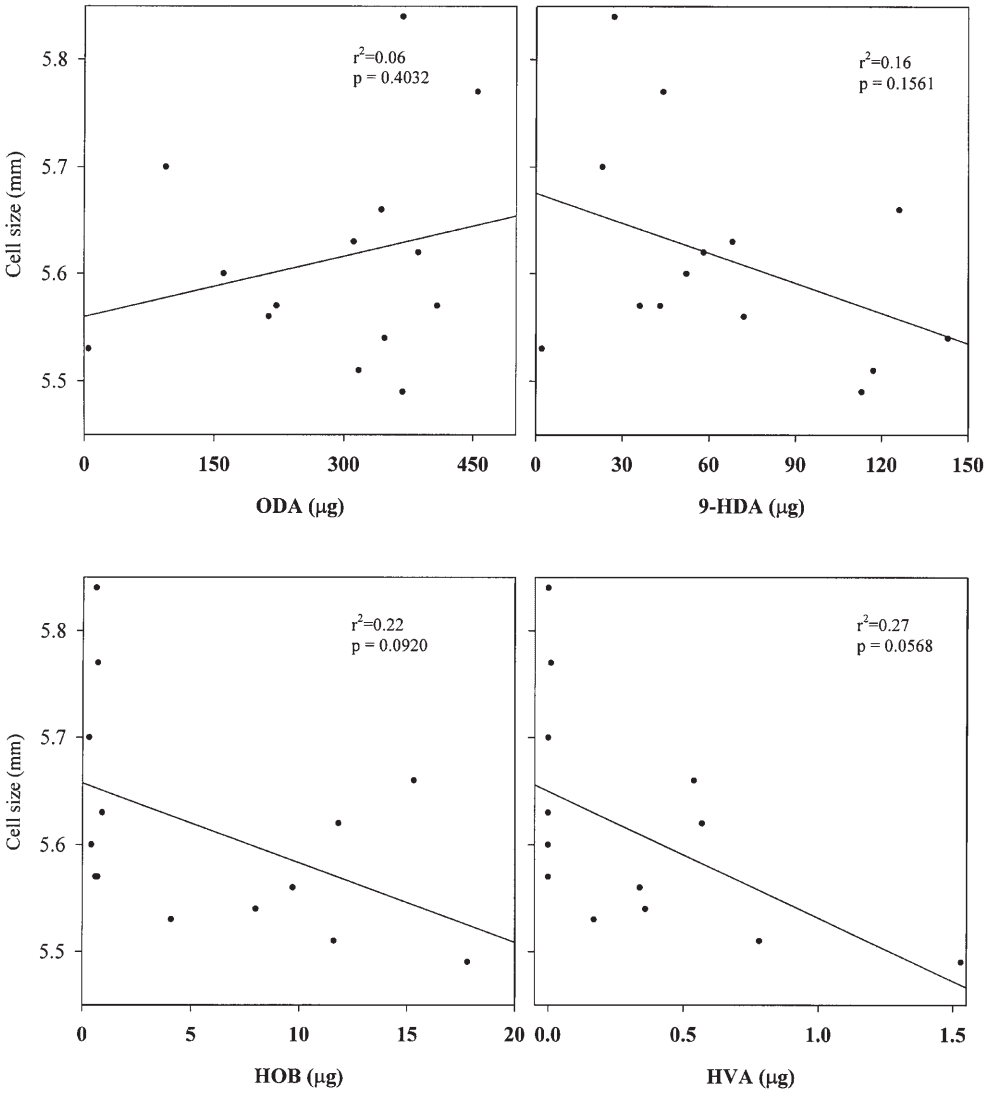

Figure 7. The results of the regression analysis, cell size vs. the QMP components of queens in mated and virgin queen treatments. The treatments were pooled and regressed (note: virgin and mated queens were analyzed separately and no significance was found)

these queenless bees constructed primarily drone-sized cells when they built comb (Figs. 1 and 3). The presence of a queen and brood within a colony normally inhibits egg-laying workers from developing. When queen loss removes this inhibition, workers can develop ovaries and begin to lay unfertilized eggs that develop into drones (Winston, 1987). This drone production is the only means of reproduction for a queenless, broodless colony, which will dwindle and die within a few months without the production of new worker bees to maintain normal colony functioning. Drones can be reared in worker-sized cells, but these smaller-sized males have a lower probability of mating with a virgin queen (reviewed by Ribbands, 1953; Gary, 1975), and thus construction of drone-sized cells substantially improves the diminished fitness of queenless bees.

Detection of the queen's presence is essential to mediate the type of comb built, and our results demonstrate that QMP is a principal factor by which workers detect the queen's presence and thereby mediate initial cell size in newly founded colonies. While the influence of QMP was clear from the significantly smaller cell diameters between QMP and queenless treatments, queenright colonies produced cells significantly smaller than the QMP treatment, suggesting that QMP is not the only queen pheromone that influences cell size, or perhaps that a larger dose is needed to mimic the full queen effect. Studies of other reproductive functions of QMP, such as inhibiting queen rearing and worker ovary development, also indicate that QMP has an important but not complete queen-like effect (reviewed by Winston and Slessor, 1998).

Although the cell size effects of QMP mediate a reproductive function, QMP's mode of action may be releaser-like by acting through a behavioural rather than or in addition to a physiological mode. The mean wax scale size/bee (Fig. 5) did not vary significantly between treatments, suggesting that QMP did not have the physiological effect typical of a primer pheromone. However, QMP and virgin queen colonies constructed significantly more comb than queenless bees, and less comb than colonies with queens (Figs. 2 and 4), and the queen's presence was found to stimulate comb building in an earlier study (Darchen, 1957). Taken together, these results suggest that QMP influences comb building behaviour, a releaser function. This interpretation is supported by our observation that queenless colonies were heavily littered with wax scales that had not been used to construct comb, suggesting that the queen or QMP influenced comb construction behaviour more than wax production itself. Nevertheless, the lack of a statistically significant difference in wax scale size was an unexpected finding and deserves further research.

Queen mandibular pheromone consists of a blend of the aliphatic acids, ODA and 9-HDA (present as two enantiomers) and the aromatic compounds HOB and HVA (Slessor et al., 1988; Pankiw et al., 1996). We found that the quantities of 9-HDA, HOB and HVA present in virgin and mated queens were significantly different (Fig. 6). ODA levels present in virgin and mated queens were higher than in Pankiw et al. (1996), and in our study no significant difference in the levels of this compound were found between virgin and mated queens. The 9-HDA amounts we found also were higher than the amounts found by Pankiw et al. (1996), but the amounts of aromatic compounds tended to be lower than those found in their analysis. These results emphasize the considerable variation in mandibular gland components found in both virgin and mated queens.

Our regression analyses suggest that it is the blend of QMP components rather than individual components that influence cell size and comb type (Fig. 7). We did find relationships between cell size and HVA and HOB levels that were close to significance, indicating that these aromatic compounds may play a more important role in cell size than the aliphatic acids. Nevertheless, the full blend appears to be more active than any individual component in this function, as for other functions previously studied (Winston and Slessor, 1998).

The cell sizes of both worker and drone cells were larger than average values cited in previous studies or reviews (Taber and Owens, 1970; Winston, 1987; Pratt, 1998). This could have been due to various factors, particularly the genetic background of the bees or their confinement in test colonies.

Our results show that the queen's presence mediates comb type and QMP is the major signal for bees in swarm-like situations, but drone comb production in established colonies also might be mediated by QMP. Newly founded colonies initially build only worker cells (Free, 1967, 1987; Taber and Owens, 1970; Lee and Winston, 1985,1987) but as worker populations grow colonies begin to build drone comb on the periphery of nests. Naumann et al. (1993) examined the movement of QMP in populous and unpopulous colonies using radiolabelled ODA, and found that a significantly smaller proportion of the workers in more populous colonies received detectable amounts of the radiolabel compared to less populous colonies. Perhaps as colonies grow the level of QMP available to each bee diminishes, especially on the periphery away from the queen. As QMP influence diminishes, drone comb construction on the periphery may begin. This hypothesis is testable, and would be an interesting extension of the current study.

The phenomenon of queen loss occurs in virtually all social insects, and in this study we have demonstrated a mechanism for queenless workers to improve their fitness through pheromone-mediated construction of larger cells. A future study might take these findings further to establish the actual extent of drone production in these larger cells. One of us (M.L.W) has observed that queenless Africanized bees in South America do indeed produce drones in such situations and a comparison of European, African, and Africanized queenless swarms would be of considerable interest for future work. Also, the role of queen pheromones in mediating nest architecture in other social insects might be a fruitful line of investigation, but this and many other pheromone projects in social insects must wait until the chemical identity of queen pheromones has been established.

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