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Research article

Queen avoidance and mandibular gland secretion of honeybee workers (*Apis mellifera* L.)

R. F. A. Moritz[1], R. M. Crewe[2] and H. R. Hepburn[3]

[1] *Institut für Zoologie, Molekulare Ökologie, Martin-Luther-Universität Halle-Wittenberg, Kröllwitzer Straße 44, 06099 Halle/Saale, Germany, e-mail: r.moritz@zoologie.uni-halle.de*
[2] *Department of Zoology and Entomology, University of Pretoria, Pretoria, 0012 South Africa, e-mail: robin.crewe@bioagric.up.ac.za*
[3] *Department of Zoology and Entomology, Rhodes University, Grahamstown, 6140 South Africa, email: R.Hepburn@ru.ac.za*

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Summary. Individually labeled newly emerged honeybee workers were introduced into three queenright host colonies. The host colonies were housed in observation hives with one brood frame. The location of the workers during their first eleven days of life was monitored. In the initial phase of the experiment, the queen was allowed to roam freely in the colony. In a second phase of the experiment the queen was caged on one side of the frame. In all three colonies workers were observed either being attracted to or avoiding the queen. The mandibular gland secretions of workers attracted to the queen more often had the typical worker like mandibular gland secretion whereas workers avoiding the queen, produced a mandibular gland secretion more similar to that of a queen. This suggests that the workers avoiding the queen are attempting to escape queen control which otherwise suppresses the secretion of queen like pheromones.

Key words: Apis mellifera capensis, spatial distribution, queen pheromone, worker reproduction.

Introduction

The queen is usually the only reproductive female in the honeybee colony (*Apis mellifera* L.). Although workers do possess developed ovaries, these are not activated because of the suppressive pheromone signals secreted by the queen. In many studies the queen's mandibular gland secretions were found to be instrumental in worker ovary suppression (Butler, 1959, Butler and Fairey, 1963; Velthuis, 1970; among others). The pheromone of the queen mandibular secretions (QMS) are a complex bouquet of compounds dominated by a variety of fatty acids most of which have been identified and studied in detail (Winston and Slessor, 1992). Workers also produce a mandibular gland secretion, but with a fatty acid composition that is distinctly different. The dominating compound of the QMS is 9-oxo-(E) 2-decenoic acid (9ODA) which makes up about 60% of the total secretion. The worker mandibular gland secretion however is dominated by 10-hydroxy decanoic acid (10HDAA) and 10-hydroxy-(E) 2-decenoic acid (10HDA) (Crewe, 1982). The underlying biochemical pathways have been studied in detail and the critical chemical difference between the major secretion products of queens and workers is the position of a single hydroxyl group (Plettner et al., 1996; 1998). Whereas the QMS has been shown to have many pheromonal functions, the worker's mandibular gland secretions are believed to primarily function as an antibiotic additive to the larval food (Blum et al., 1959).

The queen's pheromones are absent in queenless colonies and workers activate their ovaries. They eventually develop into so called false queens or pseudoqueens, producing a queenlike mandibular gland signal themselves (Plettner et al., 1993). These pseudoqueens develop particularly quickly in the Cape Honeybee, *Apis mellifera capensis*. Workers of this subspecies have been known for a long time to establish themselves as pseudoqueens in the colony (Onions, 1912, 1914). Within a week after queen loss, workers can be seen egg laying and produce a queenlike mandibular gland secretion (Hemmling et al., 1979; Crewe and Velthuis, 1980) .

Laying workers can also be found in queenright colonies of all *A. mellifera* subspecies (Visscher, 1989, 1996; Ratnieks, 1993). However, under queenright conditions worker laid eggs are usually removed by other workers (termed "worker policing"; Ratnieks and Visscher, 1989). As a result, virtually no worker laid eggs are reared in the colony (Visscher, 1996). Because laying *A. m. capensis* workers produce parthenogenetically female offspring, worker policing is considered to be not adaptive in this honeybee subspecies (Greeff, 1996). Indeed, empirical studies have shown that laying

workers' brood is reared in ample amounts in queenright *A. m. capensis* colonies (Moritz et al., 1999). The impact of laying *A. m. capensis* workers is such that it can be measured at the population level (Moritz et al., 1998) and they are an important factor in the life history of the Cape honeybee (Hepburn and Crewe, 1991).

However, the presence of laying workers in presence of the queen is puzzling. The queen pheromones clearly suppress the development of pseudoqueens and Moritz et al. (2000) showed that 9ODA suppresses the development of a queenlike mandibular gland secretion in *A. m. capensis* workers. How can the workers escape the queen's signals? Here we study how workers achieve the development of a queenlike mandibular gland signal in spite of the presence of the queen in the colony. We use workers of the Cape honeybee to take advantage of the high potential to produce queenlike mandibular gland secretions, and compare these workers with workers of the adjacent subspecies *A. m. scutellata* and workers of colonies from the hybrid zone between the two subspecies ("natural" hybrid colonies).

Material and methods

Our experiment was designed to observe the spatial distribution of a large number of focal workers in the colony in respect to the queen's position. In a subsequent chemical analysis we quantified the mandibular gland pheromones of these workers to reveal potential correlations between the pheromones produced and the distance towards the queen.

Observation hives

Six queenright observation colonies (two each of *A. m. capensis, A. m. scutellata* and their natural hybrids) were established at Rhodes University (Grahamstown, South Africa). About 2500 honeybee workers were housed on a single brood frame containing freshly sealed pupal brood on both sides. A grid on the side perspex panes of each observation hive divided the frame into 54 squares (5 × 5 cm) with 9 columns and 6 rows. The workers of the colonies were allowed to forage freely but received supplementary feeding with sugar candy if necessary to prevent absconding which is frequent in African subspecies of *Apis mellifera* (Hepburn et al. 1999). Focal workers of the three racial groups were introduced into the observations hives. In order to avoid potential biases due to nest mate recognition in our experiment, we introduced workers of three colonies that were unrelated to all observation colonies. Brood frames of the *A. m. capensis*, the *A. m. scutellata* and the natural hybrid colony were each placed into an incubator (35 °C, 60 % r.h.). Emerging workers were collected daily and labeled individually with small colored and numbered tags (Opalithplättchen). The tags were glued to the thoraces of the workers with the top of the number in walking direction to avoid identification errors during later observations. 100 workers of each type were simultaneously introduced into each observation colony. All focal workers where thus of the same age in the observation colonies.

Observations

No observations were done during the first three days to allow for an undisturbed adoption process of the focal workers in the host colony. Nevertheless one *A. m. scutellata* colony absconded in this period and could not be used for further analyses. The observations of the labeled workers in the remaining five colonies started on the fourth day after introduction. One observer read aloud the color and number of every bee tag in each square beginning in the top left corner of the frame. Another person wrote down the numbers on a prepared protocol sheet. A census took about 15 minutes per frame side depending on the number of labeled workers on the comb. Six observation sessions were conducted per day on each colony. If a worker was found more than once during a census, only its first occurrence was used for the further analysis.

After five days the queens in three observation colonies (one each of *A. m. capensis, A. m. scutellata* and their natural hybrids) were caged on grid 2/6 on the frame side termed "A". The cage was made of queen excluder screen allowing the workers access to the queen but confining the queen. The other two colonies (*A. m. capensis* and hybrid) served as controls with unconstrained queens. The spatial distribution of the focal worker bees was recorded for another three days in all five colonies. The experiment was terminated when the workers were 11 days old. All labeled bees were taken from the frame and immediately deep frozen at – 20 °C. The workers were decapitated and the heads were stored in 500 µl dichloromethane until further gas chromatographic analyses. The data were entered into a computer using standard spread sheet software (Microsoft Excel).

We calculated for each focal worker the relative change towards the frame side A (with the queen) from the worker distribution in the initial phase of the experiment before the queens were caged (detailed data is given in Moritz et al., 2001). Thus queen preference, Q_p, was computed as

$$Q_p = A_c/(A_c+B_c) - A_f/(A_f+B_f) \quad (1)$$

where

A_c = number of sightings of worker on frame side A with caged queen
B_c = number of sightings of worker on frame side B (with queen caged on A)
A_f = number of sightings of worker on frame side A with free queen
B_f = number of sightings of worker on frame side B with free queen

The parameter Q_p ranges from – 1 to 1. The value $Q_p = 0$ reflects that a given worker was found with equal frequency on either frame side before and after caging of the queen. Only workers with at least three sightings each, in both caged and free queen conditions were included in the analyses. In the control colonies with unrestrained queens, Q_p reflects the ratio between sightings of the workers on the same frame side as the queen and on the opposite side.

Gas chromatography

The heads were removed from the vials and the dichloromethane was evaporated under a stream of nitrogen just to dryness. The residue was redissolved in 30 µl internal standard (1mg octanoic acid and tetradecane in 4 ml dichloromethane) and 30 µl (bis-trimethylsilyl) trifuoroacetamide was added to the sample. 1 µl was injected into a gas chromatograph (HP 5890) with a split-splitless inlet and a 25 m × 0.32 mm methyl silicone coated fused silica capillary column. Helium was used as carrier gas at a flow rate of 1ml/min. The oven temperature was controlled as follows. The temperature was kept for 1 min at 60 °C; followed by a heating phase of 50 °/min to 110 °C, and subsequently another of 3 °C/min to 220 °C. Finally the temperature was held at 220 °C for 10 min. Peak areas were determined using HP Chemstation software and the mandibular gland compounds were identified based on the retention times of synthetic compounds, their retention time relative to the internal standards, and were quantified using peak area and the relative mass ratios (for methodological details see Simon et al. 2001). The following two fatty acids were quantified with this technique: 9-keto-(E)-2-decenoic acid (9ODA, "queen substance"), and the "worker substance" 10-hydroxy-(E)-2-decenoic acid (10HDA). These two compounds have been shown to characterize the two alternative biosynthetic pathways

for a queen and a worker mandibular gland secretion in European honeybees (9ODA being the ultimate product of the biosynthetic queen pathway and 10HDA the penultimate product in the worker pathway). Because we analysed extracts only containing a fraction of the actual mandibular gland secretion, the ratio between the two compounds was used for further statistical evaluations (Plettner et al. 1993, 1996). The more queenlike a mandibular gland secretion, the higher the ratio between 9ODA and 10HDA.

Results

Worker position

In all three racial groups there were workers changing their preferred position on the frame in the second phase of the experiment after the queens were caged on frame side A.

Fatty acid extracts

There were no significant differences for the amounts of fatty acids produced within a racial worker group among the three host colonies with the caged queens. We therefore pooled the data of the three observation colonies. Because of the large variance within the racial groups the mean amounts of the 9ODA and 10HDA did not vary significantly among *A. m. capensis*, *A. m. scutellata* and their natural hybrids (Table 1, ANOVA, $F < 1$ for 9ODA, 10HDA and 9ODA/10HDA ratio). Moreover, the amounts of 9ODA produced per worker were similar in colonies with free and caged queens. Similarly the production of worker substance (10HDA) was not significantly different between free and caged queen conditions (Table 1, t-test).

Queen preference was significantly negatively correlated to the queen substance in *A. m. capensis* and positively to the worker substance in the natural hybrid workers (Table 1). For all three tested racial groups we could detect a negative correlation between queen preference and the ratio between queen and worker substance under caged queen conditions. Figure 1 shows that this was most strongly expressed for *A. m. capensis* workers ($r = -0.54$, $p < 0.01$) but was also significant for the other two groups ($r = -0.24$ and $r = -0.41$ respectively). We found no effect of the host colony on this behavior and the correlations between queen preference and the queen – worker substance ratio were similar among the three observation colonies with caged queens (Fig. 2). In contrast, we could not find a significant correlation between queen preference and the QMS produced in the control colonies and in both cases the correlation coefficients were close to zero (Fig. 3).

Table 1. The amounts (µg) of the identified compounds of the mandibular gland secretions (mean ± se) of the focal workers and Pearson's correlation coefficients (r_{log}) between the compound amounts (logarithm) and the preference of the worker for the queen's frame side. The "queen/worker" values show the mean ratio between 9ODA and 10HDA and the correlation of the substance amounts to the queen preference queen/worker. significance levels: ** $p < 0.01$, * $p < 0.05$

Caged queens				
Compound		*capensis* (n = 48)	hybrid (n = 68)	*scutellata* (n = 37)
9ODA	$\mu \pm$ se	1.43 ± 0.12	1.94 ± 0.15	1.66 ± 0.33
	r_{log}	– 0.43**	0.11	– 0.23
10HDA	$\mu \pm$ se	4.45 ± 0.43	5.54 ± 0.49	7.05 ± 0.66
	r_{log}	0.04	0.34**	0.28
9ODA	$\mu \pm$ se	0.33 ± 0.03	0.46 ± 0.05	0.18 ± 0.12
10HDA	r_{log}	–0.58**	– 0.24*	– 0.41*
control queens				
Compound		*capensis* (n = 35)	hybrid (n = 55)	*scutellata* (n = 9)
9ODA	$\mu \pm$ se	1.87 ± 0.28	2.28 ± 0.25	1.67 ± 0.47
	r_{log}	– 0.07	0.07	0.45
10HDA	$\mu \pm$ se	5.14 ± 0.75	6.49 ± 0.98	4.14 ± 1.38
	r_{log}	0.09	– 0.08	0.35
9ODA	$\mu \pm$ se	0.46 ± 0.23	0.64 ± 0.18	0.93 ± 0.34
10HDA	r_{log}	– 0.03	0.13	0.45

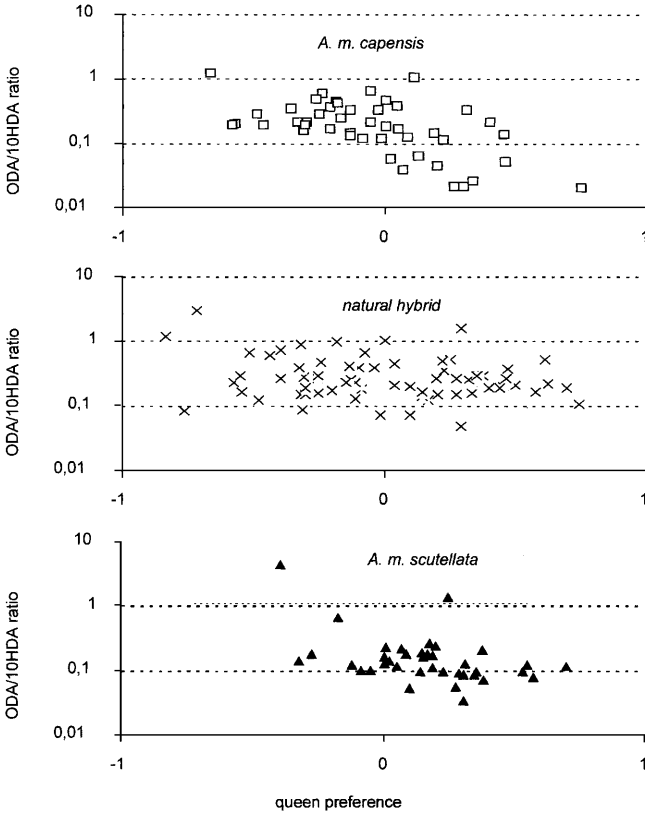

Figure 1. Scatterplot of the ratio of 9ODA and 10HDA in worker mandibular gland secretions of 11 day old workers and the queen preference observed between day four and day eleven. Data from workers of *A. m. capensis* (top), *A. m. scutellata* (bottom), and natural hybrids (center) pooled over all three observation colonies with caged queens

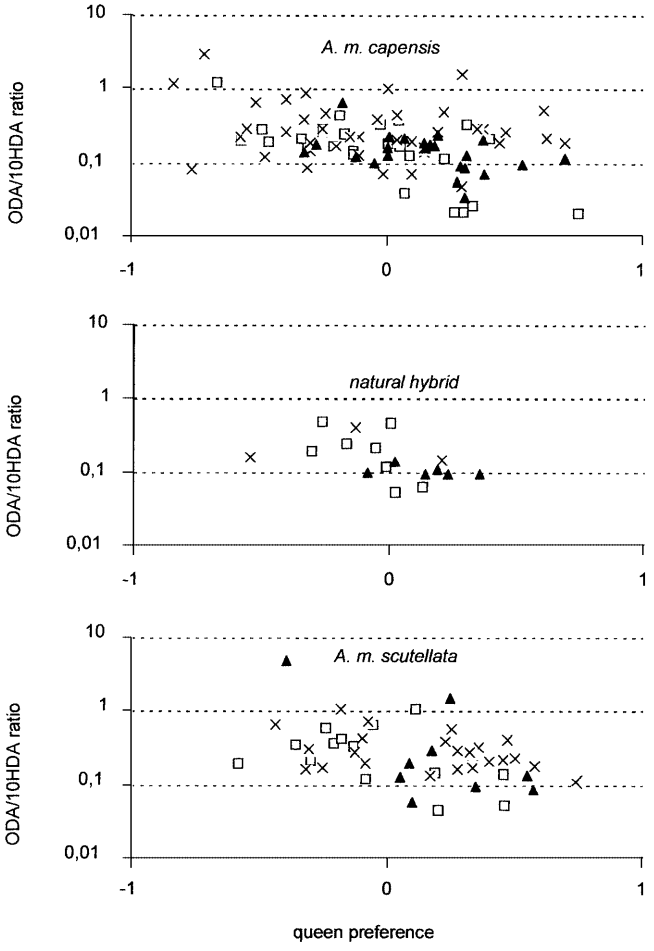

Figure 2. Scatterplot of the ratio of 9ODA and 10HDA in worker mandibular gland secretions and the queen preference of focal workers in the three host colonies with caged queens. *A. m. capensis* (top), *A. m. scutellata* (bottom), and natural hybrid (center). Note that samples sizes are unequal because the number of marked bees decreased to a different extent over the observation period in the observation hives. Open squares = *A. m. capensis* workers, triangles = *A. m. scutellata* workers, × = hybrid workers

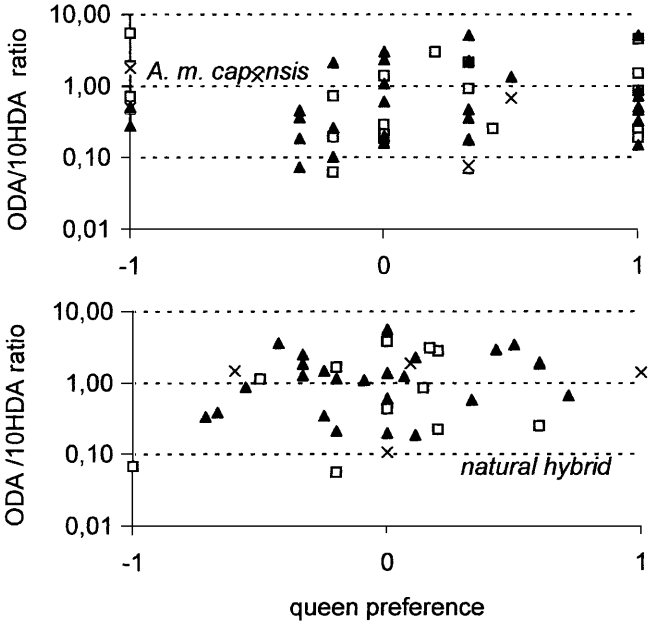

Figure 3. Scatterplot of the ratio of 9ODA and 10HDA in worker mandibular gland secretions and the queen preference of focal workers in the two control colonies with free-roaming queens. *A. m. capensis* (top), and natural hybrid (bottom). Open squares = *A. m. capensis* workers, triangles = *A. m. scutellata* workers, × = hybrid workers

Discussion

As shown previously (Moritz et al., 2001) some workers are attracted by the queen whereas others seem to actively avoid the proximity of the queen. In the control colonies with unconstrained queens this appeared to have no measurable effect on the typical fatty acid compounds of the pheromone secreted in the mandibular glands. The average 9ODA production of the focal workers in the control colonies was similar under caged queen conditions. The average queen/worker substance ratio was nevertheless higher in the control colonies because the production of worker substance was only about half the value found in workers with the caged queens. The ratio between worker and queen substance has been shown to be a reliable indicator of dominance among workers. The biosynthesis of fatty acids has been clarified in detail in honeybees (Plettner et al., 1996, 1998) and can alternatively result in either queen or worker compounds. The ratio between 9ODA and 10HDA is therefore a particularly sensitive reflection of investment in either the queen or the worker pathway. A queenlike mandibular gland signal can either result from an increased 9ODA production or from a reduced 10HDA production. From Table 1 we see that in *A. m. capensis* 9ODA production is significantly correlated to queen avoidance. In the hybrid workers it is an increased amount of worker substance in those individuals attracted to the queen that causes the negative correlation between queen preference and the queen/worker substance ratio.

The presence of the unconstrained queen did not prevent the expression of a queenlike mandibular gland signal in the workers, but it did affect the distribution of workers with a queenlike signal. The experiment with the caged queens suggests that workers with a more queenlike mandibular gland signal avoid the queen. With a free running queen this seems to be more difficult. The honeybee queen has been shown to be instrumental in affecting the movements of workers in the colony (Moritz et al., 2001) and her pheromones are certainly suitable signals of her presence.

The production of 9ODA in *A. m. capensis* workers has been shown to suppress 9ODA production in other workers (Moritz et al., 2000). Inhibitory effects of queen mandibular gland secretions have also been suggested by Huang et al. (1998) at the level of juvenile hormone production in worker bees. Pankiw et al. (1998) could show that synthetic queen mandibular gland pheromone had inhibitory effects on worker development. One way for a worker to avoid suppression could be to increase the spatial distance to the pheromone

source. Air borne 9ODA suppresses the 9ODA production in other workers (Moritz et al., 2000). We certainly expect a queen's pheromone gradient in the colony if the queen is constrained at a specific site in the colony, in spite of messenger workers distributing the signal (Seeley, 1979; Velthuis, 1972). If the queen is not constrained she roams through the colony thereby spreading her pheromones and giving potential workers less of a chance of evading her signal.

Our results may explain frequent observations of laying workers in queenright colonies (Visscher, 1996; Ratnieks, 1993). Beekeeping management may enhance laying worker activity. The honey combs are separated from the queen's brood nest by a queen excluder screen, allowing workers to pass but not the queen. Workers may escape the effect of the queen pheromones in these honey supers. Since worker policing is not well established in *A. m. capensis* colonies (Moritz et al., 1999) laying workers in this subspecies can establish considerable brood nests in queenright colonies (Hepburn and Radloff, 1998).

Clearly 9ODA alone is less efficient in suppressing worker reproduction than live queens (Pain, 1961; Butler and Farey, 1963; Velthuis, 1970). Compounds produced by larvae (Arnold et al., 1994) and tergal gland secretions have been found to affect ovarian development (Wossler and Crewe, 1999). However, this does not mean that the mandibular gland secretions have no impact at all on ovary suppression as suggested by Willis et al. (1990). The claim of these authors is in fact puzzling because their published results actually show suppressed ovary activation in workers when they used fixed queen pheromone lures in the colony. Worker ovary suppression required high pheromone doses and was less strong than by live queens, as was already observed by several others (Pain, 1961; Butler and Fairey, 1963; Velthuis, 1970). In the light of our results the reduced efficiency of pheromone lures in a honeybee colony do not seem to be surprising. The fixed lure may be very similar to the caged queen in our case. If the workers avoided the proximity of the lure this might well explain why ovary inhibition was not as strong as with a live queen or as expected from other studies where the workers could not escape the queen's pheromonal signal (Butler and Fairey, 1963; Butler et al., 1962; Pain, 1961; Velthuis, 1970). If workers can escape the queen's pheromones, and (as in our study) develop a queenlike mandibular gland secretion just on the other side of a frame, a relaxation of suppressive queen effects in larger colonies is certainly to be expected. The further a worker is away from the queen, the weaker may be the suppression. This does not exclude that also other components may be important in this phenomenon.

Overall the empirical evidence indicates that the mandibular gland pheromones, and in particular 9ODA, probably in combination with other queen pheromones are important for suppressing ovary activation and the regulation of mandibular gland secretion in workers. This however suggests, that workers by evading the queen signals in the colony, can establish themselves as pseudoqueens producing both a queenlike mandibular gland signal and eggs (although we did not test for the latter in this study). This behavior seems to be particularly strongly expressed in *A. m. capensis* workers where the correlation between developing a more queenlike secretion and the distance to the queen was most evident. However, both hybrid and *A. m. scutellata* workers also revealed a similar relationship between queen proximity and mandibular gland secretion, suggesting that this could be a general feature of African honeybees and not specific to the high reproductive capacity of *A. m. capensis* workers.

The highest amount of 9ODA produced under queenright conditions was less than half of that of a fully active pseudoqueen (Crewe and Velthuis, 1980) and in no case could we observe worker egg laying in the observation hives. Although we had no data on ovary development available, we nevertheless consider these differences in pheromone production to be of important selective significance for the workers. If the colony loses the queen, workers having had less contact with the queen, may have an important head start on queen substance production. This may give them an advantage in the intracolonial selection process for pseudoqueens. Only workers with the strongest signals can establish themselves as pseudoqueens (Moritz et al., 1996, 2000) and even minute initial advantages may turn out to be highly important for suppressing other workers producing a weaker signal.

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