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Honeybee workers (*Apis mellifera capensis*) compete for producing queen-like pheromone signals

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Physical fights are the usual means of establishing dominance hierarchies in animal societies. This form of dominance behaviour is most strongly expressed in honeybee queens who engage in fights to the death to establish themselves in the colony. Workers can also compete for reproductive dominance resulting in the establishment of stable hierarchies. They do not engage each other physically, but use pheromones that mimic those produced by queens. The dynamics of pheromone production in paired workers suggests that they engage in a pheromonal contest. Because queen pheromones suppress ovary activation, the contest results in the sterility of the loser.

Keywords: *Apis mellifera capensis*; dominance; queen pheromone; laying workers

1. INTRODUCTION

The honeybee colony is often referred to as a society where individual workers collaborate in a coordinated fashion for the good of the colony but at the expense of their own direct fitness (Seeley 1995). The cost of sterility for the individual worker is thought to be more than compensated for by the indirect fitness benefits each worker obtains through the enormous reproductive capacity of the queen (Hamilton 1964). This, however, assumes that all workers are sterile and there is no cheating. Indeed, honeybees have evolved several control mechanisms that prevent worker reproduction. Best studied are the queen's mandibular gland pheromones (QMP). Together with brood pheromones and other queen signals, they prevent ovary activation in workers (Slessor *et al.* 1988; Winston *et al.* 1989; Winston & Slessor 1992). However, as with most control mechanisms for complex processes, they occasionally fail, and in most queenright colonies there are a few workers that regularly lay eggs (Ratnieks & Visscher 1989). The survival of these worker-laid eggs is very limited, because 'policing' workers (Ratnieks 1988) most effectively remove eggs laid by other workers (Visscher 1996). Nevertheless, there are laying workers that can escape both the control systems, the pheromones and the worker police (Oldroyd *et al.* 1994; Moritz *et al.* 1999; Martin *et al.* 2002). Often these laying workers not only produce offspring but also a mandibular gland secretion with a composition very similar to that of the queen (Crewe & Velthuis 1980). As a consequence they release behavioural patterns and prime physiological responses in other subordinate workers that otherwise would only be observed in response to the presence of the queen (Velthuis *et al.* 1990). This is why these workers have been termed 'false queens' (Sakagami 1958) or 'pseudoqueens' (Velthuis *et al.* 1990). Pseudoqueens can suppress ovary activation in other workers, release retinue behaviour and escape worker policing. They also suppress the production of a queen-like pheromone signal in other workers (Moritz *et al.* 2000).

Pseudoqueen development is particularly frequent in the Cape honeybee (*Apis mellifera capensis*) (Neumann & Moritz 2002). Workers of this subspecies can develop a queen-like mandibular gland secretion, which, within a few days, is dominated by the queen substance (9-oxo-2-(E)-decenoic acid, or 9ODA) (Simon *et al.* 2001). However, not all workers in an *A. m. capensis* colony develop into pseudoqueens. Only a few successfully establish themselves after a phase of intense intracolonial competition (Moritz *et al.* 1996) resulting in a clear-cut reproductive hierarchy among workers. Since both queens and workers use the same biochemical pathways to produce either the so-called 'queen' (9ODA) or the 'worker substances' (10-hydroxydecanoic acid, 10HDAA; and 10-hydroxy-(E) 2-decenoic acid, 10HDA) (Plettner *et al.* 1996, 1997), this is also detectable at the level of pheromone production. Dominant workers have been shown swiftly to develop a 9ODA-dominated mandibular gland signal even in small experimental groups of as few as two worker bees (Moritz *et al.* 2000). In contrast to queens, which engage in lethal fights to settle dominance (Gilley 2001), aggression among pseudoqueens has not been observed. They do not kill nor are they killed by other pseudoqueen workers (Velthuis *et al.* 1990). Although there is no visible aggression between pseudoqueens of *A. m. capensis*, we show that they nevertheless have intense contests in the form of a pheromonal arms race during the first few days after emergence.

2. MATERIAL AND METHODS

Sealed worker brood frames of an *A. m. capensis* colony were placed into an incubator at 35 °C and 60% relative humidity. Freshly emerged workers were individually labelled and kept for 7 days in small arenas (a Petri dish placed on filter paper) and fed with pollen, honey and water *ad libitum*. Starting on day 2, the fatty acid secretions were removed *in vivo* from the mandibles by using (bis-trimethylsilyl) trifluoroacetamide-treated silicone tubing as a trapping technique (Crewe *et al.* 2003). In this way the mandibular gland secretions could be sampled on successive days from the same individual. The trapped signal was removed from the tubing by placing it in dichloromethane (DCM). The solvent was evaporated under nitrogen to dryness and 20 µl of internal standard (1 g of tetradecane in DCM) were added. One millilitre of this solution 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 the carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature was kept at 60 °C for 1 min. This was followed by a heating phase of 50 °C per minute to 110 °C and subsequently another of 3 °C per minute to 220 °C. Finally, the temperature was held at 220 °C for 10 min. 9ODA, 10HDA and 10HDAA were identified based on the retention times of synthetic compounds and their retention time relative to the internal standards. They were quantified using peak area and the relative mass ratios. The retention times of these compounds were verified by gas chromatography–mass spectrometry analysis.

We quantified the mandibular gland secretions daily of 18 pairs of worker bees for 7 days after emergence. For each measurement, we determined the bias towards the queen substance pathway by

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Table 1. Establishment of consistent hierarchies in the 18 worker pairs over a 5 day period of time.

(The number of pairs that had not established the final hierarchy decreased consistently over time (Kendall's τ, $\tau = -1.00$, $p < 0.01$). On days 6 and 7 all hierarchies were consistent in all pairs.)

	hierarchy	
after day	switched	consistent
2	11	7
3	8	10
4	6	12
5	3	15
6	0	18
7	dominance defined	

measuring the relative 9ODA amount as 9ODA/(9ODA + 10HDA + 10HDAA). We did not use the absolute values for further analyses, because the amounts extracted with the trapping technique may vary substantially and the ratio of 9ODA in the fatty acid blend is a more sensitive indicator of the biosynthetic investment in the queen substance. The dominant worker was identified based on this 9ODA ratio for each pair at the end of the observation period. The fatty acid data of all pairs on the previous days were analysed based on this classification. The 9ODA ratios were arcsine transformed for parametric statistics and analysed with STATISTICA 6.0.

3. RESULTS

The ratio of the 9ODA content in the fatty acid secretion resulted in clear-cut hierarchies at the end of the observation period. The 9ODA ratio in dominant workers was, on average, twice that of the subordinate workers. The hierarchies were initially not firmly established, and workers with a dominant signal on one day might be subordinate on the next day. The number of pairs with inconsistent hierarchies was highest on day 2 but then decreased significantly day by day (table 1). After 3 days, workers that were dominant at the end of the trials developed a more queen-like mandibular signal, whereas the subordinates maintained significantly lower 9ODA ratios throughout the remaining test phase ($n = 72$, paired t-test, $p < 0.01$). The enhanced 9ODA ratio of dominant workers was paired with a constant or decreased 9ODA level in the subordinates (figure 1). By contrast, however, there was a significant positive correlation between the 9ODA ratios of paired dominant and subordinate workers at the beginning of our experiment on day 2 (figure 2). Subordinate workers paired with workers expressing a high 9ODA ratio also had strong 9ODA signals, some close to the ratio (0.64) observed in European *A. m. mellifera* queens (Crewe 1982). It appears as if both workers compete for the strongest queen signals in this phase of the experiment. However, this potential competition rapidly decreased as the experiment proceeded, with the dominant workers producing more 9ODA, whereas the subordinate workers stalled (figure 1) and their 9ODA ratios did not increase.

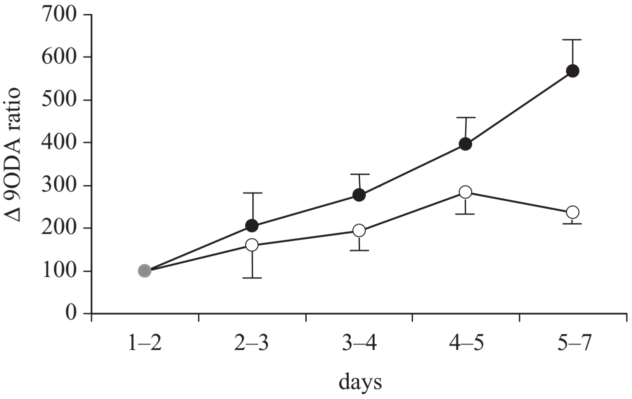

Figure 1. Change of 9ODA ratio (y-axis in % of ratio on day 2, mean ± s.e., value on day 2 = 100%) for the dominant (filled circles) and subordinate workers (open circles). The dominant workers steadily increase their signal over time (sixfold on day 7), whereas the subordinate ones do not further enhance the 9ODA ratio beyond a two- to threefold increase.

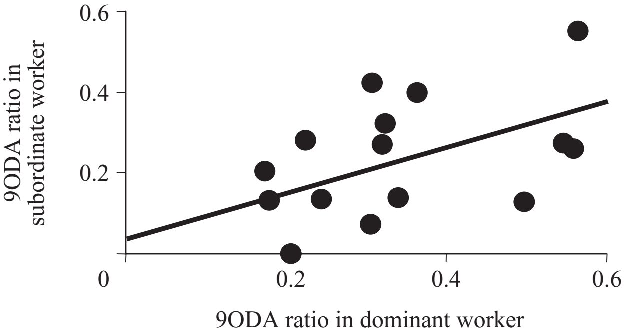

Figure 2. Competition between paired subordinate and dominant workers on day 2 after emergence. Subordinate workers produce a more queen-like signal when paired with a dominant worker ($r = 0.49$, $n = 18$, $p < 0.05$) of the corresponding queen substance ratios. Each data point indicates a pair of workers for which chemical analyses of both individuals were available.

4. DISCUSSION

Over the course of the experiment, the paired workers established consistent hierarchies. Such hierarchies have been previously reported based on aggressive and trophallactic interactions among older workers (Korst & Velthuis 1982; Moritz & Hillesheim 1985). We cannot exclude the possibility that subtle behavioural interactions among the very young workers, which escaped our attention, were involved in the development of hierarchies. However, the chemical data suggest that there is strong competition between the workers for the dominant position very soon after emergence. If one worker had a strong bias towards 9ODA production in its fatty acid signal on day 2, the companion worker also had elevated 9ODA levels. The possible transfer of the signal between the workers is unlikely to be the cause of this correlation, because otherwise signal similarity should increase rather than disappear over time. Subordinate workers are attracted towards dominant ones and have more, and closer, interactions (Moritz *et al.* 2001), which should allow for more signal exchange. It thus seems that workers actively increased their investment in the queen substance pathway when paired with a worker that had a strongly 9ODA-dominated signal. In addition, the high frequency of hierarchy switches in the first 3 days suggests competition for the dominant position. This competition is most strongly expressed on day 2 after emergence and declines thereafter as increasingly stable hierarchies are established.

We suggest that workers compete for the most queen-like signal, until one worker has exceeded a certain threshold of 9ODA production that suppresses the 9ODA production in the other (Moritz *et al.* 2000). Subsequently, the subordinate worker stalls at a more worker-like mandibular gland secretion and does not regain the dominant position. This mechanism could be important for establishing dominance hierarchies in natural colonies. Those workers with the strongest signal eventually suppress the more subordinate workers by producing a stronger 9ODA signal (Neumann & Moritz 2002; Wossler 2002). This explains why the number of pseudoqueens found per colony is limited, and only workers of relatively few subfamilies finally monopolize the colonies' resources for reproduction (Moritz *et al.* 1996).

Our results also suggest that the queen pheromone may not necessarily be an 'honest' signal, as previously suggested by others (Keller & Nonacs 1993). 9ODA appears to be an agonistic chemical weapon for establishing dominance rather than a label of fertility to which nest-mates respond by foregoing reproductive competition. It causes not the death, but the sterility of the individual that loses.

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