Responses of queenright and queenless workers of *Apis cerana* to 9-keto-2(*E*)-decenoic acid, a pheromonal constituent of the mandibular gland

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Abstract In dequeened honeybee colonies ovarian activation occurs in some workers, and the pheromonal bouquets of these laying workers become more queen-like. In the Asiatic honeybee, *Apis cerana*, we compared the amounts of 9-keto-2(*E*)-decenoic acid (9-ODA), a mandibular gland pheromone component, between non-laying workers from queenright colonies and laying workers from queenless colonies and, further, applied synthetic 9-ODA to workers to determine whether workers discriminate workers with activated ovaries based on the level of this compound. Levels of 9-ODA were higher in laying workers from dequeened colonies than in non-laying workers from queenright colonies. In both queenright and queenless colonies, more workers treated with 9-ODA were attacked by other workers than were control-treated workers. These results suggest that detection of pseudoqueens in *A. cerana* is mediated by changes in 9-ODA.

Key Words: Aggression · Asiatic honeybee · Laying workers · 9-keto-2(*E*)-decenoic acid · 9-ODA

Introduction

The mandibular gland pheromone of a queen honeybee, *Apis mellifera* L. contributes to the suppression of ovarian activation in workers (Hoover et al. 2003). In queenless colonies, some laying workers develop a pheromonal bouquet, similar to that of queens (Crewe and Velthuis 1980), which stimulates physiological responses in other workers similar to those evoked by queens (Velthuis et al. 1990). In the Cape honeybee, *A. m. capensis*, the increased level of the queenlike mandibular pheromone substance, 9-oxo-2-(*E*)-decenoic acid (9-ODA), in workers is correlated with an increase in ovarian activation (Hepburn 1992). High levels of 9-ODA elicit increases in aggressive attacks (Pettis et al. 1995), as well as inhibit ovarian activation in workers (Hillesheim et al. 1989). Also working against reproductive development of workers in colonies (both queenright and queenless) is that other workers can recognize nestmates with activated ovaries (Visscher and Dukas 1995).

Ovarian activation in workers also occurs in queenright colonies of the Asiatic honeybee, *Apis cerana* Fabricius, and at higher levels in queenless colonies (Sakagami and Akahira 1958). However, it is not known whether *A. cerana* workers can distinguish workers with developed ovaries. In this study, we measured the 9-ODA content of normal and laying workers, and tested whether 9-ODA elicits attacks against laying workers.

Methods and Materials

Measurement of 9-ODA content of mandibular pheromone

Bees were sampled randomly until six workers showing no ovarian activation on dissection were obtained from each colony. After this, all queens were removed from

the colonies, and two weeks later workers bees were sampled again until six workers showed fully developed ovaries. The heads of these bees were placed in 200 μ l dichloromethane in GC bottles for two months at -15°C.

The extracts were evaporated to dryness under nitrogen and the residues re-dissolved in 20 μ l of an internal standard, octanoic acid and tetradecane in dichloromethane, and 20 μ l of bis-trimethylsilyltrifluoroacetamide. Extracts were injected splitlessly into a Hewlett Packard 6890 gas chromatograph, equipped with a 25 x 0.32 mm i.d., methyl silicone column, programmed from 60°C (held for 1 min.) to 110°C at 50°C.min⁻¹, then to 220°C at 3°C.min⁻¹. Helium, at 1 ml.min⁻¹ was the carrier gas. The compound 9-ODA was identified, based on comparison of retention time with that of an authentic standard, and quantified in relation to the internal standards.

Observation of workers' responses to 9-ODA

Experiments were conducted at Yunnan Agricultural University, Kunming, China. Twenty worker bees from each of three queenright and queenless colonies of *Apis cerana* were anesthetized with CO₂ and marked. They were divided into equal groups: one treated with 10 μ g 1% w/w 9-ODA, similar to the quantity produced by a laying worker, another with 10 μ g 200% w/w 9-ODA, similar to that produced by a queen (Tan et al. 2009), and the third (control) treated with 10 μ g distilled water. When the anesthetized workers recovered, they were returned to their hives on a single frame and monitored for 3 min with digital video cameras, one on each side of the frame, to record any aggressive behaviour.

Statistical Analyses Independent *t*-tests compared the amounts of 9-ODA between queenright *A. cerana* workers and queenless laying workers. *Chi-square* tests were used to compare the aggression behaviour, directed to treated workers, between test and control groups, and between queenright and dequeened colonies. Count data were used in the *chi-square* calculations.

Results

Mean 9-ODA content in extracts of *A. cerana* workers was significantly different ($t_4=4.8$, $P<0.009$) between queenright [0.014 ± 0.005 μg (SD)] and queenless [0.096 μg ±0.029 (SD)] colonies. In queenright colonies, within 3 min. after workers treated with 9-ODA (either 0.1 μg or 0.2 mg/bee) were released, greater percentages (0.1 μg : $\chi^2_1=25.5$, $P<0.001$; 0.2 mg: $\chi^2_1=38.6$, $P<0.001$) of treated workers were attacked compared to the control groups (Table 1). In queenless colonies, greater percentages (0.1 μg : $\chi^2_1=18.3$, $P<0.001$; 0.2 mg: $\chi^2_1=21.8$, $P<0.001$) of treated workers were also attacked after release, compared to the control groups (Table 1). However, overall, there were fewer attacks against treated workers in queenless colonies than in queenright colonies. This effect was significant for treatment with 0.2 mg of 9-ODA ($\chi^2_1=12.3$, $P=0.001$), but not quite significant for treatment with 0.1 μg ($\chi^2_1=3.4$, $P=0.067$). In both queenright and queenless colonies, workers were more sensitive to the higher dose of 9-ODA. The difference was significant for the queenright colonies but not significant for queenless colonies (queenright: $\chi^2_1=5.5$, $P=0.019$; queenless: $\chi^2_1=0.3$, $P=0.606$) (Table 1).

Discussion

Within a week of being dequeened, some 40% of workers in an *A. cerana* colony have activated ovaries (Tan et al. 2009). We found that pheromonal changes corresponded with this ovarian activation, with the mandibular gland extract of a laying worker containing approximately seven times as much 9-ODA as a worker without activated ovaries.

In our behavioral tests, we showed that other workers attacked workers with developed ovaries and a queen-like 9-ODA bouquet. It is likely the detection of these pseudoqueens is mediated by the pheromonal cue (Hepburn 1992). Although laying workers increased their 9-ODA content, it was still considerably less than that produced by queens. In *A. mellifera*, such a difference in mandibular gland content is sufficient

for laying workers to be perceived as such by their nestmates and be attacked (Plettner et al. 1995).

Interestingly, more workers treated with 9-ODA were attacked in queenright colonies than in queenless ones. This effect may, in part, be due to greater numbers of attacking bees lacking ovarian activation in queenright colonies than in queenless ones (Tan et al. 2009). However, it may also be due to an increased tolerance of laying workers in queenless colonies over that in queenright colonies, perhaps mediated by prior 9-ODA levels in the respective colony types.

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Table 1 Number of treated *Apis cerana* workers attacked by nestmates (Mean±SD%), (N=10 workers per colony, 3 colonies)

Treatment 9-oxo-2-(<i>E</i>)-decenoic acid amount	Colony	Queenright		Queenless	
		Control	Test	Control	Test
0.1 µg	1	0	6	0	4
	2	1	6	0	4
	3	1	9	0	6
	Total	2 (6.7±5.8%) ^c	21 (70.0±17.3%) ^a	0 (0.0±0.0%) ^c	14 (46.6±11.5%) ^a
0.2 mg	1	1	9	0	5
	2	1	9	0	5
	3	2	10	0	6
	Total	4 (13.3±5.8%) ^c	28 (93.3±5.8%) ^b	0 (0.0±0.0%) ^c	16 (53.3±5.8%) ^a

^aMean percentages within one column or one row followed by a different letter are significantly different