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Pheromones affecting ovary activation and ovariole loss in the Asian honey bee *Apis cerana*

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Signalling

ABSTRACT

The Asian hive bee *Apis cerana* has similar queen mandibular pheromones (QMP) to the Western honey bee *Apis mellifera*. However the effects of individual QMP components have never been tested to determine their effects on the reproductive physiology of *A. cerana* workers. We fed one queen equivalent of each of the major components of *A. cerana* QMP to groups of c.a. 500 day-old, caged, workers twice a day until the workers were 10 days old. Half of the cages were also provided with 10% royal jelly in the food. Workers were sampled each day and dissected to determine the number of ovarioles and the degree of ovary activation (egg development). In cages treated with 9-carbon fatty acids ovary activation was minimal, whereas the 10-carbon acids suppressed ovary activation very little. Royal jelly enhanced ovary activation, especially in cages treated with 10-carbon acids. The number of ovarioles declined with bee age, but the rate of decline was slowed by the 9-carbon acids in particular. The results show conservation of the composition and function of QMP between *A. cerana* and *A. mellifera* and support the hypothesis that QMP is an honest signal of queen fecundity rather than a chemical castrator of workers.

1. Introduction

Workers of eusocial Hymenopterans are generally sterile in the presence of a queen. However, in many species workers retain functional ovaries, which they activate if their queen is lost, producing viable offspring males by arrhenotokous parthenogenesis. Facultative worker sterility of this kind requires that workers can detect the presence of their queen, and modify their reproductive physiology appropriately if she is lost. In all species studied thus far, queens signal their presence and regulate worker ovary activation via primer pheromones (Dietemann et al., 2005; Hoover et al., 2003; Le Conte and Hefetz, 2008; Lim and Lee, 2005; van Oystaeyen et al., 2014; Vargo and Hulsey, 2000).

Cuticular hydrocarbons are long chain alkanes, alkenes and methyl-branched hydrocarbons whose primary and original function is to protect insects from desiccation (Blomquist, 2010). However in most social Hymenopterans cuticular hydrocarbons have been co-opted to play a role in queen signaling (Liebig, 2010; Nunes et al., 2014; van Oystaeyen et al., 2014). Queen and worker hydrocarbon profiles differ, and this provides workers with a mechanism to detect the presence of a queen in their colony (Liebig, 2010). A major exception to this generality is the honey bees, genus *Apis*, where the queen's signal is a mixture of long-chain fatty acids secreted from the mandibular glands (Butler, 1959; Plettner et al., 1997; Slessor et al., 2005). These pheromones inhibit worker ovary activation (Hoover et al., 2003), mediate worker retinue formation around the queen (Slessor et al., 1988), and act as a sex attractant (Gary, 1962).

Worker honey bees also produce pheromones from their mandibular glands, but these differ significantly from those of queens. In the cavity-nesting honey bees *Apis cerana* and *Apis mellifera*, mandibular gland secretions of queens have a high proportion of 9-keto-(*E*)-2-decenoic acid (9-ODA), whereas the secretions of queenright workers are richer in (E)-10-hydroxy-2-decenoic acid (10-HDA) and 10-hydroxydecanoic acid (10-HDAA) (Keeling et al., 2001; Plettner et al., 1997). Thus the ratio of 9-HDA + 9-ODA/10-HDA + 10-HDAA is a measure of the relative 'queenliness' of the pheromonal signal (Hoover et al., 2005; Moritz et al., 2000), and the more 9-ODA circulating in a colony, the less likely *A. mellifera* workers are to activate their ovaries (Hoover et al., 2003). The mandibular secretions of queenright *A. cerana* workers have extremely low amounts of 9-ODA and a relatively high proportion of 9-HDA (Plettner et al., 1997; Tan et al., 2012). However when *A.*

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cerana workers become queenless there is a 5–10-fold increase in the total amount of 9-ODA present in their mandibular secretions (Tan et al., 2010, 2012).

There is good evidence that the above-mentioned fatty acids detected in mandibular glands of *A. cerana* queens constitute the functional queen mandibular pheromone. First, a mixture of 9-ODA, 9-HDA, and methyl p-hydroxybenzoate (HOB) is sufficient to elicit worker retinue behavior equaling that of total queen extract (Plettner et al., 1997). Second, in artificial mixed-species colonies of *A. cerana* + *A. mellifera* an *A. cerana* queen partially inhibits ovary activation in *A. mellifera* workers, suggesting that the well-characterized *A. mellifera* QMP is evolutionarily conserved in *A. cerana* (Ken et al., 2009). Finally, when 0.1 μg 9-ODA is placed on the dorsal surface of *A. cerana* workers they are often attacked by other workers, suggesting that they are recognized as being reproductive and are therefore policed (Tan et al., 2010).

A. cerana is unique among honey bees in that about 5% of workers have activated ovaries in colonies with a queen (Bai and Reddy, 1975; Holmes et al., 2014; Ken et al., 2009; Nanork et al., 2007; Oldroyd et al., 2001), significantly more than is seen in all other species in the genus (Halling et al., 2001; Holmes et al., 2013; Ratnieks, 1993; Wattanachaiyingcharoen et al., 2002). The high proportion of workers with activated ovaries is unexpected in a species like *A. cerana* where policing of worker reproduction is extremely efficient and virtually no males are sons of workers (Holmes et al., 2014; Oldroyd et al., 2001). This is because in species where worker reproduction is minimized, there are no fitness benefits to worker ovary activation (Wenseleers et al., 2004). Thus the proximate signals that regulate ovary activation in *A. cerana* are of considerable interest.

Which of the individual mandibular components are responsible for the suppression of worker ovary activation in *A. cerana* is currently unknown. Here we provide groups of queenless *A. cerana* workers with individual QMP components in their food and determine which of the components are capable of suppressing ovary activation.

2. Materials and methods

2.1. Treatments

During the period December 2013–October 2014 we caged up to 24 *A. cerana* queens at a time on an empty comb inside their colony overnight. All colonies were housed in standard Langstroth hives and each colony comprised two frames of brood and two of honey and pollen. Queens laid eggs on the caged comb for 12–18 h, thus providing us with a comb in which most bees emerged within 12–18 h of each other. Some queens failed to lay eggs or enough eggs, so the experiment was staggered as appropriate brood became available, but treatments were applied randomly to each set of progeny.

The day before the workers were due to emerge we placed the brood combs in individual boxes, and then placed the boxes in an incubator at 32 °C. The next day, when the newly emerged bees had emerged (n = c.a. 500 bees per box), we randomly allocated one of the feeding treatments to each box such that each feeding treatment was applied to bees sourced from 24 independent colonies in total. All diets were provided in 20 ml of 20% w/w sucrose solution, which we sprayed directly onto the bees and their comb through the screen mesh of the box twice per day.

The sucrose solutions contained either no fatty acid or one queen equivalent of one of the fatty acids found in the mandibular glands of *A. cerana* queens (9-ODA, 10 μg; 9-HDA 1.2 μg; HOB 1.2 μg; 10-HDA 0.04 μg or 10-HDAA 0.04 μg) or a mixture of all compounds as above in the same amounts, ratio 240:30:30:1:1 (Ken et al., 2009). Additionally, in half the cages treated with single components we added 10% v/v fresh royal jelly to the sucrose solutions. Royal jelly greatly enhances ovary activation in *A. mellifera* (Hoover et al., 2003; Lin and Winston, 1998). In total, there were three cages per treatment (fatty acid/royal jelly combination). Bees were able to obtain additional food, including pollen, that was present in their comb, and were able to store some of the sprayed food in their comb. All compounds were obtained from Aladdin Reagent Database Inc. (Shanghai, China) except for 9-ODA, which we obtained from Contech, Canada.

2.2. Sampling

Starting the day after the cages were set up we sampled 20 workers per day from each cage for 10 days. Workers were frozen at the time of collection until they were dissected (Dade, 1977). We determined the degree of ovary activation according to the system of Hess (1942): Stage I – ovarioles transparent with no sign of segmentation, hence no activation; II – ovaries slender, but differentiation between eggs and nurse cells visible; III – occurrence of a single egg cell; IV – eggs are bean-shaped; V – several eggs are fully mature and represent the stage at which workers can become laying workers. Following assessment of ovary activation, we counted the number of ovarioles in the two ovaries (e.g. Roth et al., 2014).

2.3. Analysis

We analyzed data by fitting generalized linear models using maximum likelihood. Bee age (1–10 days), pheromone treatment (9-ODA, 9-HDA, HOB, 10-HDA, 10-HDAA or the mixture), presence or absence of royal jelly and cage nested within pheromone by royal jelly combination were fitted as the independent variables, and ovary activation score or ovariole number as the dependent variables. We assumed the normal distribution and a linear link function for the ovary activation scores, and a Poisson distribution for the ovariole counts. These distributions and models provided the best fit to the data of any of the alternative models we tried. We did not use a repeated measures design since we sampled without replacement.

3. Results

3.1. Ovary activation

Ovary activation score was strongly affected by bee age, mandibular gland component and the presence of royal jelly (Fig. 1 and Table 1). Cage nested within treatments was also significant, suggesting some heterogeneity among cages of the same treatment combination.

In controls, ovary activation scores increased with age, in both the presence and absence of royal jelly (Fig. 1). The presence of royal jelly interacted significantly with pheromone treatment (Table 1). This interaction arises because in the presence of royal jelly all pheromone treatments strongly suppressed ovary activation (Fig. 1), whereas in the absence of royal jelly 10-HDA and 10-HDAA failed to inhibit ovary activation while 9-ODA and 9-HDA completely or almost completely inhibited ovary activation (Fig. 1).

3.2. Number of ovarioles

On average the number of ovarioles declined from 11.17 ± 0.12 (standard error) on day 1 to 9.63 ± 0.11 when the bees were 10 days old in cages without royal jelly and from 11.07 ± 0.11 to 9.55 on day 10 in cages with royal jelly. Therefore, overall, there

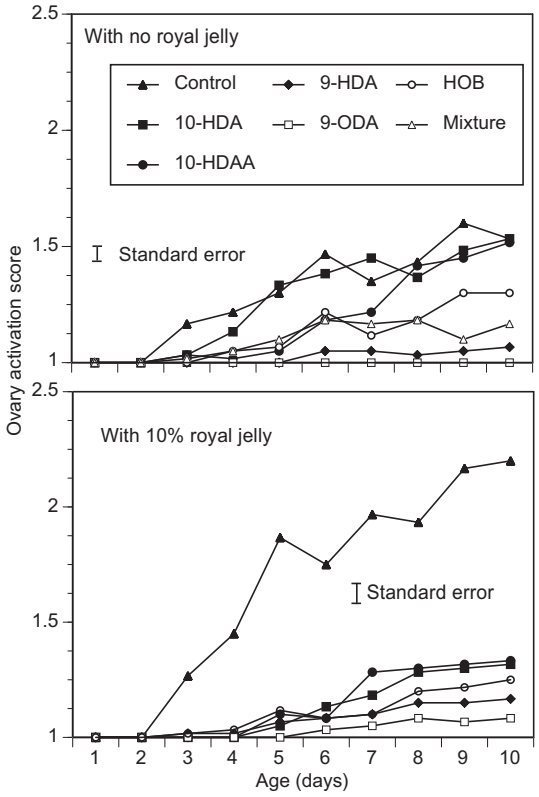

Fig. 1. Mean ovary activation scores (scale 1–4, see text) for *Apis cerana* workers exposed to queen mandibular gland components in the presence and absence of royal jelly. The standard error is based on the experiment-wide within-treatment variance and is the same for all days because the sample size is identical.

Table 1
Effects of QMP component (Q), royal jelly (RJ), bee age (A) and individual cage (C) on the number of ovarioles and ovary activation scores in caged *Apis cerana* workers. Tests show the effect of adding the particular term to the generalized linear model, and the overall fit of the model relative to the intercept only model.

Source	d.f.	Ovary activation score		Number of ovarioles	
		Wald χ^2	*P*	Wald χ^2	*P*
Q	6	1610.6	<0.001	342.27	<0.001
RJ	1	23.21	<0.001	1.55	0.21
A	9	1082.98	<0.001	216.49	<0.001
C(Q * RJ)	26	155.09	<0.001	46.34	0.008
RJ * Q	5	400.69	<0.001	78.97	<0.001
Q * A	54	721.09	<0.001	141.97	<0.001
RJ * A	9	24.96	0.003	3.72	0.93
A * RJ * Q	45	204.67	<0.001	39.66	0.70
Overall fit	155	3421.16	<0.001	830.07	<0.001

was no significant effect of royal jelly in the decline in ovariole number (Table 1). However this overall lack of difference masks significant interactions between royal jelly and pheromone treatments (Table 1). In the absence of royal jelly bees exposed to worker-associated fatty acids (10 carbons) and the control declined fastest, whereas in cages provided with queen-associated fatty acids (9 carbons) the decline was more muted (Fig. 2). In contrast, in cages provided with royal jelly, there was little decline in ovariole number in cages exposed to any kind of fatty acid, worker-associated or queen-associated, though there was still a marked decline in controls (Fig. 2).

Ovariole number was similar in those bees treated with a mixture of compounds as it was in bees treated with 9-ODA and 9-HDA alone. HOB also arrested the decline in ovariole number to a greater degree than the worker-associated fatty acids (Fig. 2).

3.3. Correlation between ovariole number and ovary activation

For 10-day old bees there was a significant negative correlation between ovary activation score and number of ovarioles (Spearman's $\tau = -0.73$, $n = 780$, $P < 0.001$).

4. Discussion

This study shows that the major components of the queen mandibular secretions 9-ODA and 9-HDA that are common to *A. cerana* and *A. mellifera* (Plettner et al., 1997) strongly inhibit worker ovary activation in young *A. cerana* workers. The minor components, 10-HDA and 10-HDAA have minimal effects on ovary activation, at least in isolation and at the concentrations we used here. Royal jelly enhances ovary activation, but ovary activation is still strongly suppressed by 9-ODA and 9-HDA in bees that have access to royal jelly. It is likely that young adult workers consume at least some royal jelly *in vivo* as part of their diet – it is certainly readily available in brood cells and in the mouthparts of other bees. So the effects of royal jelly are probably nutritional rather than pheromonal.

This study adds to the growing body of evidence that ovary activation is negatively correlated with ovariole number (Allsopp, 1988; Roth et al., 2014; Tan et al., 2015). It has been previously noted that ovariole number declines in adult workers to a greater extent in queenless *A. cerana* colonies than in queenright colonies (Tan et al., 2015). Here we have shown that ovariole number declines with age, and declines to a greater extent in the absence of queen-associated fatty acids. Roth et al. (2014) have speculated that there is greater decline in ovariole number in queenless colonies than in queenright colonies due to antagonism towards bees with more ovarioles and more queen-like pheromonal bouquets. Contrary to this view our present study shows that queen pheromones inhibit decline in ovariole number over the first 10 days of life. This suggests that ovary activation itself causes a decline in ovariole number, or that loss of ovarioles is required for ovary activation in queenless workers. However it is still possible that there is differential survival between workers with different numbers of ovaries. For example if bees with larger numbers of ovarioles activate their ovarioles at a younger age and are attacked and killed by other bees, then this could lead to the observed decline in ovariole number over time.

The last common ancestor of *A. cerana* and *A. mellifera* lived 6 million years bp (Arias and Sheppard, 2005). Despite this distant relationship, the queen and worker pheromonal blends of the two species retain remarkable constancy in their effects and constituents. This conservation is incompatible with the idea that queen mandibular pheromones are a suppressive agent of ovary activation (e.g. Strauss et al., 2008). Suppression would result in an evolutionary arms race between castes in which mutations in workers to enhance their personal reproduction would be countered by changes to the queen pheromone to re-establish dominance (Hefetz and Katzav-Gozansky, 2004). Such an arms race is expected to lead to a proliferation of pheromonal compounds over time (Hefetz and Katzav-Gozansky, 2004), and loss of function (Keller and Nonacs, 1993). To us, the strongly conserved function and composition of queen and worker pheromones over evolutionary time points to the hypothesis that mandibular pheromones mediate a 'dialogue' between workers and queens and between workers (Kocher and Grozinger, 2011) about their fertility and their reproductive dominance. Nonetheless, it is unlikely that this question can ever be resolved empirically.

In this experiment we provided bees with one queen equivalent of each of the components of the *A. cerana* queen pheromone. Because 9-ODA is the greatest proportion of the mandibular

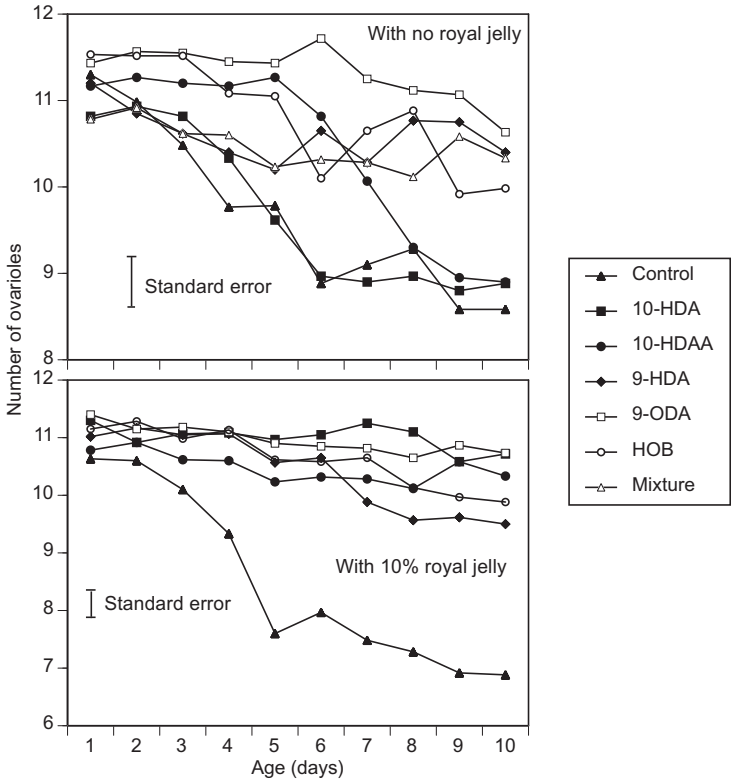

Fig. 2. Mean number of ovarioles in *Apis cerana* workers exposed to queen mandibular gland components in the presence and absence of royal jelly.

secretion of queens, we applied it in an amount that was an order of magnitude greater than the compounds that dominate the worker mandibular glad secretions, 10-HDA and 10-HDAA. It is possible that these worker-typical compounds would be more suppressive of ovary activation if applied in higher amounts. We therefore caution that it is premature to say that these compounds have no effect on ovary activation or ovariole number. However our results unequivocally show that 9-ODA alone suppresses ovary activation to a similar or greater degree than it does in a mixture of all compounds. It therefore appears that 9-ODA is the primary signal that mediates worker fertility in *A. cerana*.

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