Associations between reproduction and work in workers of the Asian hive bee *Apis cerana*

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ARTICLE INFO

ABSTRACT

If a honey bee (*Apis* spp.) colony becomes queenless, about 1/3 of young workers activate their ovaries and produce haploid male-producing eggs. In doing so queenless workers maximize their inclusive fitness because the normal option of vicarious production of relatives via their queen's eggs is no longer available. But if many workers are engaged in reproduction, how does a queenless colony continue to feed its brood and forage? Here we show that in the Asian hive bee *Apis cerana* hypopharyngeal gland (HPG) size is larger in queenless workers than in queenright workers and that bees undertaking brood-rearing tasks have larger HPG than same-aged bees that are foraging. In queenless colonies, workers with a smaller number of ovarioles are more likely to have activated ovaries. This reinforces the puzzling observation that a large number of ovarioles reduces reproductive success in queenless *A. cerana*. It further suggests that reproductive workers either avoid foraging or transition to foraging later in life than non-reproductive workers. Finally, our study also showed that ovary activation and larger-than-average numbers of ovarioles had no statistically detectable influence on foraging specialization for pollen or nectar.

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1. Introduction

The hallmark of insect societies is a caste of workers that generally forego reproduction and instead allocate their time and physiological resources to rearing the brood of their mother, the queen. Yet workers of many species retain ovaries and the capacity to lay eggs in the absence of a queen and queen-laid brood. In Hymenopteran social insects, the parthenogenetic eggs laid by unmated workers are viable and can produce normal males (Huber, 1792). This capacity for virgin birth provides workers with an (albeit limited) opportunity for direct reproduction after they have lost their queen (reviewed in Beekman and Oldroyd, 2008; Winston, 1987). Furthermore, whenever circumstances arise where workers can benefit from personal reproduction over indirect reproduction via their queen, workers can activate their ovaries to reproductively parasitize their own or other colonies (Châline et al., 2002; Chapman et al., 2009a,b, 2010; Lopez-Vaamonde et al., 2004; Nanork et al., 2007, 2005; Oldroyd et al., 1994; Takahashi et al., 2010).

When an insect colony becomes queenless or the queen is replaced, the relatedness coefficients among colony members are profoundly changed. When a queen is replaced, the existing workers have half the coefficient of relatedness to the brood of the new queen as they did to the brood of the old queen (e.g. Crozier and Pamilo, 1996). In a queenless colony the workers are related, on average, to the sons of other workers by a little more than 0.125 whereas they are related to their own sons by 0.5. Thus, based on inclusive fitness theory (Hamilton, 1964), queenless workers are expected to compete over reproduction while still cooperating to the extent necessary to raise a final batch of male brood (Beekman and Oldroyd, 2008; Inbar et al., 2008; Miller and Ratnieks, 2001). As predicted by kin selection theory (Nieh, 2012), the change in intra-colonial relatedness that results from queen replacement during swarming or queenlessness in the Western honey bee (*Apis mellifera*) is associated with the rearing of 'rebel bees' that have ovaries with more ovarioles, are more likely to reproduce, and have smaller hypopharyngeal glands (hereafter HPG) than workers reared in the continuous presence of a queen (Woyciechowski and Kuszewska, 2012).

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Ovary size, as measured by the number of ovarioles present within an ovary, is thought to have significant effects on worker behavior. Workers with more ovarioles are more likely to become reproductively active (Makert et al., 2006), transition to foraging tasks at an earlier age (Amdam et al., 2006), and tend to specialize in pollen foraging (Amdam et al., 2004; Wang et al., 2010). These studies suggest that there is a causal association between reproductive traits like ovariole number, hypopharyngeal gland size, ovary activation and task allocation, especially the age at which young workers transition to foraging, and the kind of foraging tasks they perform (reviewed in Page and Amdam, 2007; Page et al., 2012; Page, 2013). Thus, when worker reproduction is induced by queen loss or by reproductive swarming, we anticipate that there will be significant changes in worker behavior and physiology that are associated with changes in their reproductive status (Rahman et al., 2014). Despite this prediction, Naeger et al. (2013) found that in queenless colonies of *A. mellifera ligustica* there is little or no association between reproductive status of queenless workers and the tasks that they perform. In particular, Naeger et al. showed that egg-laying workers often have large hypopharyngeal and wax glands, indicating that they engaged in personal reproduction while simultaneously contributing to colony welfare by producing brood food and building comb. Furthermore Naeger et al. (2013) showed that foragers were as or more likely than non-foragers to have activated ovaries. This latter observation is particularly surprising because in queenright colonies there is generally a negative association between ovary activation and the task of foraging (Oldroyd and Beekman, 2008; Oldroyd et al., 1999; Roth et al., 2014), and because there is generally a negative association between reproductive behavior and task performance (Hillesheim et al., 1989).

The Asian hive bee, *Apis cerana*, is a cavity-nesting species that shares many characteristics of the Western hive bee *A. mellifera* (reviewed in Oldroyd and Wongsiri, 2006). However in one important respect *A. cerana* differs from all other honey bee species studied thus far (*Apis dorsata* (Wattanachaiyingcharoen et al., 2002), *Apis florea* (Halling et al., 2001), *A. mellifera* (Ratnieks, 1993)) in that about 5% of *A. cerana* workers have activated ovaries despite the presence of a queen (Bai and Reddy, 1975; Holmes et al., 2014; Nanork et al., 2007; Oldroyd et al., 2001). In the other species the proportion of workers with activated ovaries is normally negligible – less than 1%. *A. cerana* therefore provides an interesting counterpoint to other species where ovary activation is rare.

Here we determine whether *A. cerana* workers with activated ovaries behave differently to those without activated ovaries, how the reproductive physiology of queenless workers differs from that of queenright workers and whether workers with larger numbers of ovarioles have different foraging preferences to workers with smaller numbers of ovarioles. We contrast our findings from the more reproductive species, *A. cerana*, with those reported from *A. mellifera*, where the frequency of workers with activated ovaries is much lower under queenright conditions.

2. Materials and methods

2.1. Experimental colonies

We placed eight queenright, wild-caught *A. cerana* colonies in an apiary on the campus of Yunnan Agricultural University, Kunming, China. All colonies were housed in standard Langstroth hives and each colony comprised two frames of brood and two of honey and pollen. (This is typical for a full-sized *A. cerana* colony.) At the beginning of the experiment we caged each queen on an empty comb to lay eggs, thus providing us with a comb in which all brood emerged within 12 h. 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 33 °C. The next day, we paint

marked the emerged workers, about 500 from each colony, and transferred them back into their original colony. We then dequeened four randomly-selected colonies to produce four queenless colonies and four queenright control colonies. We retained the queenless state of the queenless colonies by removing any queen cells that developed.

2.2. Ovary activation and hypopharyngeal gland size

We sampled marked workers 22 days after emergence, at least 14 days after the youngest brood cells had been sealed and were no longer producing brood pheromone (Arnold et al., 1994; Mohammedi et al., 1998; Traynor et al., 2015). At this age, a significant proportion of workers had transitioned from brood-care tasks within the colony to foraging tasks outside the colony. From each of the eight experimental colonies we caught 10 marked workers from the brood combs, 10 marked foragers that carried pollen and >10 putative nectar foragers, also marked. Nectar-carrying bees were confirmed during dissection (see below) by showing that the sucrose content of the crop contents exceeded 15% as checked with a refractometer (Seeley, 1986), and the volume exceeded 18 μl. The volume cutoff was empirically determined by a sample of 40 returning foragers from four colonies unrelated to this experiment. The minimum crop volume was 18 μl, range 18–42 μl. Only bees ($n = 10$ per colony) that met these combined criteria were retained as nectar foragers.

Samples were frozen alive shortly after collection. We dissected workers as in Dade (1977). First we determined the degree of ovary activation according to the five point scale 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 and summed them for analysis. We then made an incision through the eye of the worker using dissecting scissors, pulled out the hypopharyngeal gland using forceps, and transferred it to a microscope slide. We then measured the cross-section diameter of the gland at its widest point under a microscope fitted with an ocular micrometer under 40 times magnification.

2.3. Statistics

For the main analysis we used generalized linear models based on restricted maximum likelihood to assess the relationship between task group (in-nest, pollen forager, nectar forager), and queen state (queenless or queenright colony) on ovariole number, ovary activation score and HPG size. We nested the effect of colony within task group and queenstate. We included all main effects, and the two-way interaction between task group and queen state. We used a normal distribution and linear link function for HPG size, and a Poisson distribution and linear link function for ovariole number and ovary activation score. All convergence criteria were satisfied.

Post hoc comparisons of means were based on least significant differences using the residual variance from the generalized linear models.

3. Results

3.1. Relationships between ovariole number, ovary activation and task group

Ovariole number ($P = 0.39$) and ovary activation score ($P = 0.89$) were uniform across task groups pooled across queenless and

Table 1
Effects of queen state, task and colony on ovary activation score, number of ovarioles and diameter of the hypopharyngeal gland (HPG) in *Apis cerana* workers in queenless and queenright colonies. Tests show the effect of adding the particular term to the generalized linear model.

Model effect	d.f.	Ovary activation score		Number of ovarioles		Diameter of the HPG	
		Wald χ^2	P	Wald χ^2	P	Wald χ^2	P
Queenstate (Q)	1	38.8	<0.001	65.6	<0.001	255.1	<0.001
Task (T)	2	1.9	0.39	0.24	0.89	176.5	<0.001
$Q \times T$	2	0.8	0.67	1.5	0.47	6.9	0.032
Colony ($Q \times T$)	18	2.0	>0.99	4.9	>0.99	27.4	0.072
Overall fit[*]	23	43.8	0.006	72.8	<0.001	258.9	<0.001

[*] Compares the overall fit of the model to the intercept only model.

queenright colonies and there was no significant interaction between task group and queen state ($P > 0.5$) for either measure (Table 1). However queenright workers had significantly more ovarioles ($P < 0.001$) and lower ovary activation ($P = 0.001$) scores than queenless workers (Table 1, Figs. 1 and 2).

There was no significant difference in the ovary activation score between pollen- and nectar-collecting workers in either queenright ($P = 0.91$) or queenless ($P = 0.87$) colonies (Fig. 1). Similarly, there was no significant difference in the number of ovarioles between the two types of foragers in either queenright ($P = 0.69$) or queenless ($P = 0.91$) colonies (Fig. 1) and brood nest workers had similar ovariole numbers to both types foragers in both queenless and queenright colonies (Fig. 1). However when queenless colonies were considered separately from queenright colonies, broodnest workers had higher ovary activation scores ($\bar{x} = 2.4 \pm 0.14$ s.e.) than the mean of pollen and nectar foragers combined (($\bar{x} = 1.9 \pm 0.10$) (general linear model, colony a random effect, orthogonal contrast, $F_{1,108} = 5.11$, $P = 0.03$). This was not the case for ovariole number ($F_{1,108} = 1.96$, $P = 0.16$). However a *post hoc* power analysis ($\alpha = 0.05$) shows that the power of this test is only 0.28, which is insufficient be confident that there was indeed no difference.

### 3.2. Relationships between task group and hypopharyngeal gland size

HPG were significantly larger in workers from queenless colonies than from queenright colonies ($P < 0.001$, Table 1 and Fig. 1). There was a highly significant association between task group and HPG size ($P < 0.001$, Table 1), with workers sampled from the brood nest having larger HPG than foragers (Fig. 1). There was a small but significant interaction between queen state and task ($P < 0.03$, Table 1), which arose because the difference in HPG size between brood nest workers and foragers was greater in queenless colonies than in queenright colonies (Fig. 1).

3.3. Associations between ovary and hypopharyngeal gland activation and ovariole number

In queenless colonies, and across all task groups, bees with more ovarioles had lower ovary activation scores (Spearman's rank correlation, $\tau = -0.84$, $P < 0.001$, $n = 120$, Fig. 2). This effect was absent in the queenright colonies where ovary activation scores were lower ($\tau = -0.15$, $P = 0.11$, $n = 120$, Fig. 2). There was no significant correlation between ovary activation and HPG size in either queenless colonies ($\tau = 0.16$, $P = 0.081$, $n = 120$, Fig. S1), or queenright colonies ($\tau = 0.04$, $P = 0.69$, $n = 120$, Fig. S1).

4. Discussion

In our queenless colonies only, workers in the brood nest had higher ovary activation scores than workers that were engaged in
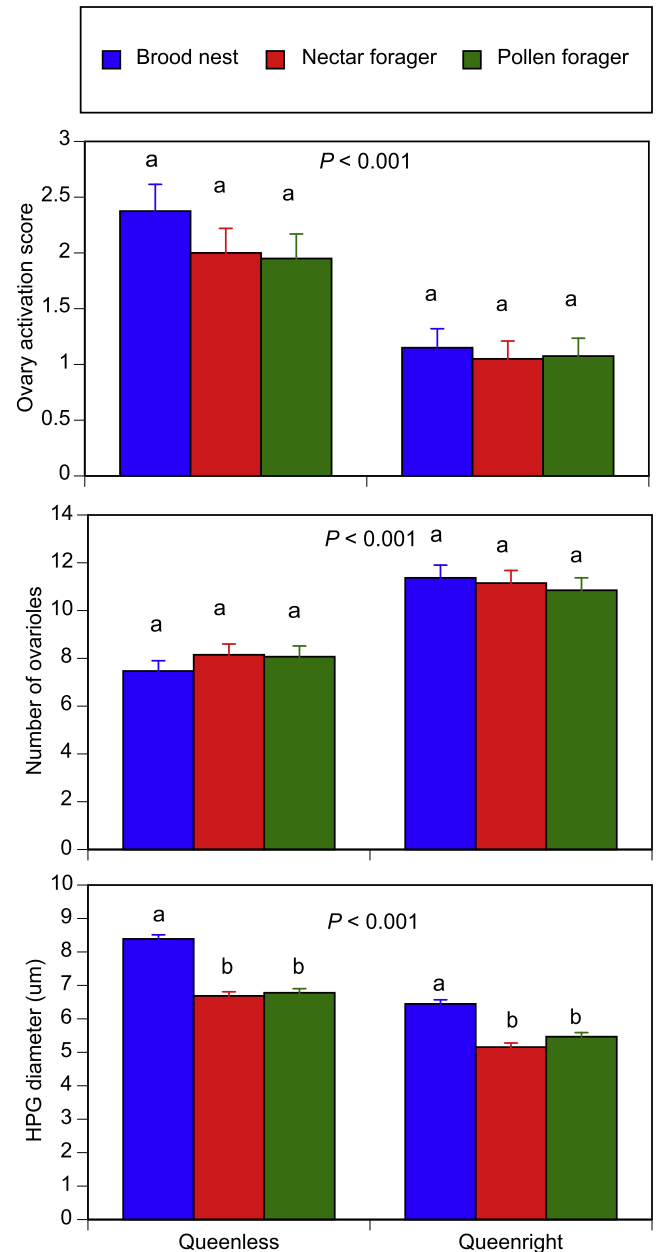
Fig. 1. The effects of task group and queen state on ovary activation scores, number of ovarioles, and hypopharyngeal size in *Apis cerana*. Within a bar cluster, means with a different letter are significantly different (5% least significant difference). n.s. = not significantly different. P values are a test of the difference between queenless and queenright workers from the generalized linear model (see Section 2.3 in Methods). Error bars are standard errors of the means. Each bar is the mean of 40 bees, 10 from each of four colonies.
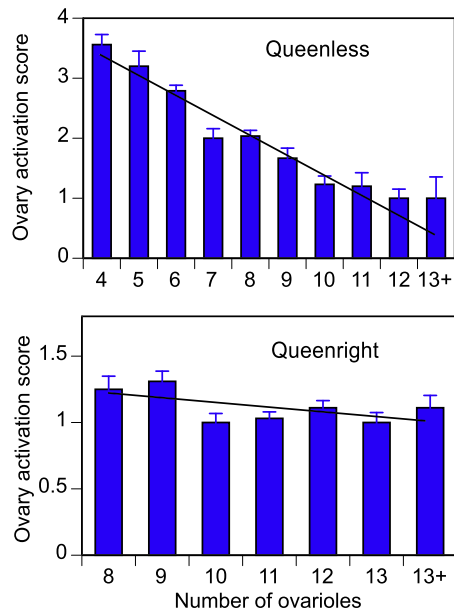
Fig. 2. The association between the number of ovarioles in 120 *Apis cerana* workers (pooled across task groups) and their ovary activation score in four queenless and in four queenright colonies. For illustrative purposes only, the lines are the linear regressions between ovary activation score and ovariole number. The Spearman rank correlation between the two variables is highly significant for queenless colonies, but not for queenright colonies (see text). Error bars are standard errors of the means.

foraging. (This conclusion should be treated with some caution because the difference was not significant in the overall analysis). Given this caveat, our observation supports the hypothesis of Schmid-Hempel (1990) that reproductive workers should avoid risky foraging. Similar phenomenon have been observed in the Cape honey bee *A. mellifera capensis* (Roth et al., 2014), 'anarchistic' *A. mellifera* (a strain selected for high rates of worker reproduction) (Oldroyd and Beekman, 2008), ants (Ito and Higashi, 1991), bumble bees (Jandt and Dornhaus, 2011) and social wasps (Cant and Field, 2001). Our findings with *A. cerana* contrast with those reported for wild type *A. mellifera* where it has been shown that actively-laying workers forage and that foragers and laying workers have similar ovary activation scores (Naeger et al., 2013). The difference between wildtype *A. mellifera* and other species and subspecies is intriguing and warrants further investigation.

In our queenless colonies we found a negative association between ovariole number and fecundity as has been previously observed in caged *A. cerana* (Tan et al., 2015) and *A. mellifera* (Velthuis, 1970) and field colonies of *A. m. capensis*. It may be that it is less metabolically expensive to activate ovaries with a small number of ovarioles than it is to activate ovaries with a larger number of ovarioles, providing an advantage of speed-of-activation in workers with a small number of ovarioles (Velthuis, 1970). Nonetheless, in a Brazilian population of *A. mellifera* (Makert et al., 2006), and in the anarchistic strain (Oldroyd and Beekman, 2008) workers with more ovarioles were more likely to have activated ovaries, and so the association between ovariole number and ovary activation is complex and cannot be generalized (Velthuis, 1970).

In this study we found no significant association between ovariole number and the foraging tasks undertaken by workers in either queenright or queenless colonies. In particular, we found no tendency for pollen foragers to have a greater number of ovarioles than nectar foragers, contradicting a major prediction of the

Reproductive Ground Plan – forager hypothesis (as classified by Oldroyd and Beekman, 2008), which holds that workers with large numbers of ovarioles preferentially forage for pollen (Amdam et al., 2006; Page, 2013). This association between ovariole number and foraging preference is not observed in *A. mellifera* strains with inherently high rates of worker reproduction (Oldroyd and Beekman, 2008; Roth et al., 2014) or in *A. cerana* (this study), so the association between foraging preference and ovariole number may be limited to populations where very few workers are actually reproductive. Nonetheless, Rueppell et al. (2008) found that a sample of 17 pollen foragers from one colony of *A. cerana* had significantly more ovarioles than a sample of 16 foragers that returned without pollen. We acknowledge that the absence of a significant correlation in a particular data set does not mean that no relationship exists.

It is thought that the number of ovarioles is fixed during the pupal stage, and is therefore unalterable by the environment experienced by the adult worker (da Cruz-Landim et al., 2006). It was thus surprising that workers sampled from queenless colonies had a smaller number of ovarioles than did workers with a queen. Woyciechowski and Kuszewska (2012) have shown that *A. mellifera* workers reared in a queenless colony have more ovarioles than workers reared in queenright colonies, a phenomenon that they attributed to a hypothesized ability of pupae of queenless colonies to alter their developmental trajectory in ways that enhance the reproductive capacity of the subsequent adult. Woyciechowski and Kuszewska argued that the change in ovariole number is not due to additional food – workers reared in queenright and queenless colonies had equivalent body mass. Rather, it seems that pupae reared in queenless conditions allocate resources to building ovaries at the expense of other tissues (Woyciechowski and Kuszewska, 2012). However in our study, workers in queenless and queenright colonies had all been reared in queenright colonies. It may be that workers with large numbers of ovarioles are at some sort of selective disadvantage in queenless colonies and have a shorter lifespan because they forage earlier (Amdam et al., 2006). It is also possible that the assumption that the number of ovarioles is fixed for the life of the adult *A. cerana* worker and is unaltered by environmental effects is in error (Tan et al., 2015).

There was a positive association between ovary activation and hypopharyngeal gland (HPG) size in queenless workers and HPG were larger in queenless workers than in queenright workers regardless of whether the worker was undertaking foraging or brood-rearing tasks. This suggests that there is no tradeoff between egg production and brood food production. Naeger et al. (2013) similarly found that laying *A. mellifera* workers have larger HPG than non laying workers, suggesting that reproductively active workers still make significant metabolic investments in colony maintenance and cooperative brood rearing. Thus although there is increased conflict in queenless colonies (e.g. Chapman et al., 2009a,b; Malka et al., 2008; Nanork et al., 2005; Visscher and Dukas, 1995), queenless honey bees continue to act altruistically, and seem to revert to a 'quasisocial' state in which individuals cooperate to rear brood, but reproduce individually (Michener, 1974; Naeger et al., 2013).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.08.003.

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