

Intra- and interspecific brood recognition in pure and mixed-species honeybee colonies, *Apis cerana* and *A. mellifera**

Ken TAN^{1,2}, Mingxian YANG^{2,6}, Sarah E. RADLOFF³, Yushen YU⁴,
Christian W.W. PIRK⁵, H. Randall HEPBURN^{2,6}

¹ Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science, Kunming, Yunnan Province, China

² Eastern Bee Research Institute, Yunnan Agricultural University, Kunming, China

³ Department of Statistics, Rhodes University, Grahamstown 6140, South Africa

⁴ Bee Research Institute of Yunnan Agricultural Academy of Science, Mongzi, Yunnan, China

⁵ Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa

⁶ Department of Zoology and Entomology, Rhodes University, Grahamstown 6140, South Africa

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Abstract – We studied the effects of mixed honeybee colonies of *Apis mellifera* and *Apis cerana* on the intraspecific and interspecific recognition of female brood stages in the honeybees *A. cerana* and *A. mellifera* by transferring brood combs between queenright colonies. In the intraspecific tests, significantly more larvae were removed in *A. cerana* than in *A. mellifera*, whilst significantly fewer eggs and pupae were removed in *A. cerana* than in *A. mellifera*. In the interspecific tests, *A. cerana* colonies removed significantly more larvae and pupae of *A. mellifera* than the same brood stages of *A. cerana* were removed by *A. mellifera*. We show there are highly significant differences in both intraspecific and interspecific brood recognition between *A. cerana* and *A. mellifera* and that brood recognition operates with decreasing intensity with increasing developmental age within species. This suggests that worker policing in egg removal is a first line of defense against heterospecific social parasites.

Apis mellifera / *Apis cerana* / nestmate recognition / social parasitism

1. INTRODUCTION

Intraspecific and interspecific brood recognition is fundamental to reproductive security and often to colony integrity among sympatric social insects (Breed, 1998; Moritz and Neumann, 2004); and, indeed, is fundamental in the context of social parasitism (Nanork et al., 2007b; Neumann and Moritz, 2002). Nonetheless, honeybee workers are able to discriminate the degree of relatedness of larvae to themselves and to preferentially rear queens

from related larvae (Visscher, 1986), and this appears to operate in slave-making ants as well (Mori et al., 1996). To evaluate the relative significance of such differences in honeybees, one can raise the intracolony diversity in varying stages to reach an extreme in nests consisting of workers from more than one species. In other words, composing colonies in which the individuals are increasingly distantly related.

While the conspecific introduction of combs of immature stages between colonies is routine apicultural practice (Grout, 1946), heterospecific introductions have usually failed (Inoue, 1962; Oschmann, 1965; Dhaliwal and Atwal, 1970; Adlakha and Sharma, 1971; Oku

Corresponding author: S.E. Radloff,
s.radloff@ru.ac.za

* Manuscript editor: Stan Schneider

and Ono, 1990; Potichot et al., 1993; Koeniger et al., 1996) because of putative interspecific differences in brood pheromones (Potichot et al., 1993), which have now been experimentally demonstrated for eggs (Ratnieks, 1992; Katzav-Gonzansky et al., 2003; Sasaki et al., 2004; Martin et al., 2005) and larvae (Le Conte et al., 1994, 1995; Ayasse and Paxton, 2002). The pheromones of the immature stages differ between species and also change during development (Le Conte et al., 1994, 1995). However, there must be some degree of overlap in brood recognition cues among these sister-species because, otherwise, brood rearing in the mixed-species colonies would not have occurred.

Based on analyses of previously reported failed heterospecific introductions and the recent development of a method for producing mixed-species colonies of *Apis cerana* Fabricius and *A. mellifera* L. workers (Tan et al., 2006), we hypothesize that there are gradual but significant differences between intraspecific and interspecific transfers and controls; and, that recognition will be more strongly expressed towards certain immature stages in intra- and interspecific contexts. This approach provides a gradual experimental transition from limited genetic variation within the same colony, through moderately increased genetic differences in intraspecific transfers, and finally to very large, interspecific genetic differences. Here we report the results of field experiments with *A. cerana* and *A. mellifera* colonies to test these hypotheses.

2. METHODS AND MATERIALS

2.1. Honeybees, *Apis mellifera* and *Apis cerana*

Nine unrelated queenright colonies each of *A. c. cerana* Fabricius (colonies 1–9) and *A. m. ligustica* Spinola (colonies 10–18) were placed at two different apiaries (~500 m apart) in Yunnan Province, China. At the same apiaries, three mixed-species colonies of both *A. cerana* (19–21) and *A. mellifera* (22–24) were established according to standard protocols (Tan et al., 2006). For that purpose, two frames of sealed worker brood (~1500 cells) of *A. mellifera* or *A. cerana* were introduced into

each of three queenright *A. cerana* or *A. mellifera* colonies respectively. Each colony had a young, naturally mated, laying queen and was equalized with four frames of combs and bees, each colony containing about 10 000 bees, as well as a frame of honey. Prior to the experiments, all colonies were given at least four days to settle to limit disturbance-induced absconding (Spiewok et al., 2006). The mixed-species colonies were allowed to settle for four weeks before they were used in the brood recognition test and their work forces consisted of ~50% *A. cerana* and *A. mellifera*.

2.2. Control, intraspecific and interspecific experimental groups

One brood frame with eggs, larvae and pupae was taken out of each colony and they were counted and their physical positions were marked for each individual on transparent plastic sheets. Each experimental colony was provided with equal amounts of brood (eggs, larvae and pupae). Then, the queen of each colony was caged on a comb and all frames were re-introduced. Only one frame was introduced in each colony as follows: (1) one frame from each of the three *A. mellifera* and three *A. cerana* colonies was removed and reintroduced in its own mother colony (*A. mellifera* and *A. cerana* controls); (2) one frame from each of three *A. cerana* and three *A. mellifera* colonies was placed in three colonies of their own species (intraspecific experimental groups); (3) frames from three *A. cerana* and *A. mellifera* colonies each were placed in three colonies of the other species (interspecific experimental groups); (4) one frame from each of three *A. cerana* colonies was placed in three mixed-species *A. mellifera* colonies and (5) one frame from each of three *A. mellifera* colonies was placed in three mixed-species *A. cerana* colonies. 72 hours after the introductions were made all introduced frames were inspected and eggs, larvae and pupae were counted using the transparent sheets as a reference base. No manipulations beyond that actually required for measuring the brood were performed. Nonetheless, other factors such as comb odour or variations in the proportions of nurse bees to field bees in each experimental colony may have influenced the results of these experiments.

Table I. Comparison of removal rates of eggs, larvae and pupae in pure and mixed-species honeybee colonies, *Apis cerana* and *Apis mellifera*.

1. Intraspecific exchanges of worker brood				
	<i>A. cerana</i>	<i>A. mellifera</i>	$\chi^2(1)$	<i>P</i> -value
	Mean \pm sd	Mean \pm sd		
<i>A. cerana</i> and <i>A. mellifera</i> controls				
Eggs	3.6 \pm 1.8	2.2 \pm 2.8	1.0	0.3134
Larvae	1.6 \pm 0.3	0.7 \pm 0.3	2.3	0.1290
Pupae	1.7 \pm 1.5	0.6 \pm 0.6	1.7	0.1918
<i>A. cerana</i> and <i>A. mellifera</i> brood in other conspecific colonies				
Eggs	3.9 \pm 0.5	24.3 \pm 3.9	14.8	< 0.0001
Larvae	13.8 \pm 4.8	8.5 \pm 4.7	9.8	0.0020
Pupae	2.5 \pm 2.3	9.5 \pm 1.8	6.7	0.0098
2. Interspecific exchanges of worker brood				
	<i>A. cerana</i>	<i>A. mellifera</i>	$\chi^2(1)$	<i>P</i> -value
	Mean \pm sd	Mean \pm sd		
<i>A. cerana</i> brood in <i>A. mellifera</i> colonies vs. <i>A. mellifera</i> brood in <i>A. cerana</i> colonies				
Eggs	100.0 \pm 0.0	100.0 \pm 0.0		100% removed
Larvae	100.0 \pm 0.0	67.9 \pm 10.6	272.4	< 0.0001
Pupae	85.8 \pm 4.9	46.9 \pm 4.7	84.1	< 0.0001
<i>A. cerana</i> brood in mixed-species <i>A. mellifera</i> colonies vs. <i>A. mellifera</i> brood in mixed-species <i>A. cerana</i> colonies				
Eggs	33.9 \pm 19.7	93.5 \pm 11.3	159.5	< 0.0001
Larvae	37.8 \pm 8.3	49.5 \pm 13.2	7.701	0.006
Pupae	10.5 \pm 0.7	4.3 \pm 1.0	5.4	0.0199

2.3. Data analysis

Differences both within and between species and between mixed-species colonies and single-species colonies in the numbers of eggs, larvae and pupae removed/accepted from the combs ($n = 3$ colonies each) for 72 hours were checked using chi-square tests of proportions (Johnson and Wichern, 2002). All tests were performed using Statistica[®] (StatSoft, 2007).

3. RESULTS

3.1. Intraspecific exchange colonies

The results showed that there were no significant differences in the percentages of eggs, larvae and pupae removed in *A. cerana* and *A. mellifera* control colonies (Tab. I, Fig. 1). When *A. cerana* and *A. mellifera* brood were placed in different but conspecific colonies, significantly fewer eggs and pupae were removed in *A. cerana* colonies than in *A. mellifera*, whilst significantly more larvae were

removed in *A. cerana* colonies than in *A. mellifera* (Tab. I, Fig. 1). The percentages of eggs, larvae and pupae removed in other conspecific colonies of *A. mellifera* were significantly higher than those in the *A. mellifera* controls (Fig. 1). Likewise the percentages of larvae removed in other conspecific colonies of *A. cerana* were significantly higher than those in the *A. cerana* controls but the percentages of eggs and pupae removed were not significantly different compared to the *A. cerana* controls (Fig. 1).

3.2. Interspecific exchange colonies

While *A. mellifera* and *A. cerana* colonies both removed all eggs of the reciprocal species, the latter removed significantly more larvae and pupae than the former (Tab. I, Fig. 1). In both species significantly more brood stages were removed than in intraspecific control and test colonies above. Mixed-species *A. mellifera* host colonies removed significantly more *A. cerana* eggs and larvae than

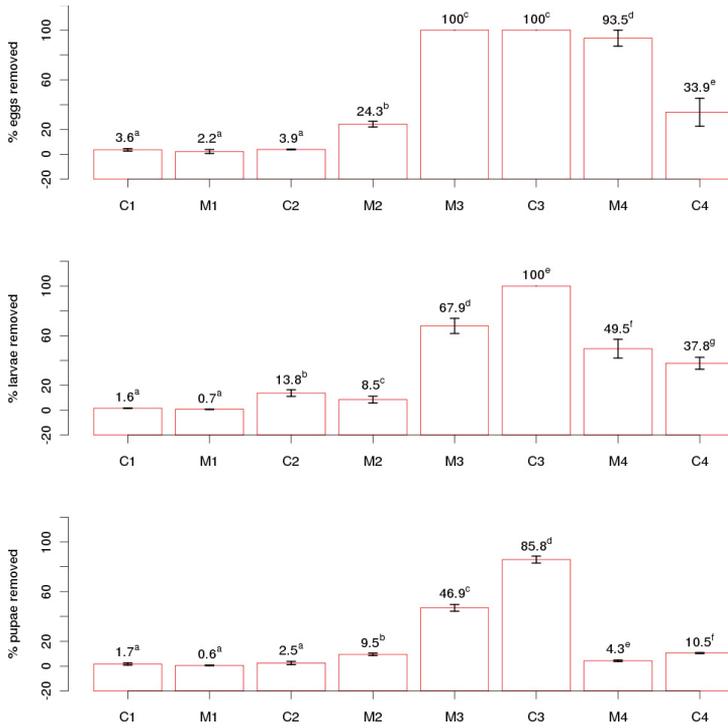


Figure 1. Removed percentage rates (mean \pm s.e.) of eggs, larvae and pupae in pure and mixed-species honeybee colonies, *Apis cerana* and *Apis mellifera*. C1: *A. cerana* control; M1: *A. mellifera* control; C2: *A. cerana* intraspecific; M2: *A. mellifera* intraspecific; C3: *A. cerana* interspecific; M3: *A. mellifera* interspecific; M4: *A. cerana* in *A. mellifera* hosted mixed colonies; C4: *A. mellifera* in *A. cerana* hosted mixed colonies. Different letters within each brood stage indicates significant differences ($P < 0.05$).

mixed-species *A. cerana* host colonies, but the latter removed significantly fewer pupae (Tab. I, Fig. 1). *A. cerana* mixed species host colonies removed significantly fewer *A. mellifera* eggs, larvae and pupae than *A. mellifera* offspring removed by pure *A. cerana* colonies (Fig. 1). Significantly fewer *A. cerana* eggs, larvae and pupae were removed by mixed-species *A. mellifera* host colonies than by pure *A. mellifera* colonies (Fig. 1).

3.3. Comparisons of control, intraspecific and interspecific exchange colonies

Significantly more *A. mellifera* eggs, larvae and pupae were removed by mixed-species *A. cerana* host colonies than were *A. mellifera* offspring in other *A. cerana* colonies (Fig. 1).

Significantly more *A. cerana* eggs and larvae were removed by mixed-species *A. mellifera* host colonies than in other *A. mellifera* colonies, but significantly fewer pupae were removed by mixed-species *A. mellifera* host colonies than in other *A. mellifera* colonies (Fig. 1).

4. DISCUSSION

Shortly after emergence, adult worker bees both acquire and learn the odours of their colony (Breed et al., 1988, 2004; Breed and Stiller, 1992) enabling guard bees to distinguish between nestmates and non-nestmates (Ribbands, 1954). While the underlying recognition mechanisms are well established for adult workers (Bethe, 1898; Breed et al., 1995; Breed, 1998), they are less well known

for the immature stages. Clearly, within the same colony brood must be recognized and accepted to ensure colony survival and for defense against heterospecific social parasites (Nanork et al., 2007a). Our data give strong support to earlier findings that *A. mellifera* are able to discriminate between nestmate and non-nestmate eggs (Pirk et al., 2007) as well as for larvae and pupae (Visscher, 1986). In contrast, nestmate recognition in *A. cerana* seems to be less developed and only occurs for larvae but not for eggs and pupae. This is consistent with recent findings on nestmate recognition for adults (Breed et al., 2004). In conspecific exchanges of worker brood, significantly fewer eggs and pupae of *A. cerana* were removed than in *A. mellifera*, but conversely for removal of larvae.

Our data confirm earlier studies on heterospecific brood transfers in honeybees that brood discrimination is well expressed for eggs and young larvae in *A. cerana* (Oschmann, 1965; Dhaliwal and Atwal, 1970; Adlakha and Sharma, 1971; Oku and Ono, 1990; Potichot et al., 1993) and in *A. mellifera* (Oschmann, 1965; Dhaliwal and Atwal, 1970; Adlakha and Sharma, 1971; Oku and Ono, 1990; Potichot et al., 1993). The pupal stage of the honeybee clearly represents a different context in heterospecific transfers of both *A. cerana* in *A. mellifera* because the removal rate for pupae was significantly slower than that for eggs and larvae. We suspect that less brood pheromone is produced by pupae and/or that diffusion of pheromones through the wax capping is reduced because of their lipophilic nature (Free, 1987), or that pupal removal is energetically more costly. No larvae of *A. mellifera* reached the sealed cell stage. The only immatures that eclosed as adults were those which had already been capped when frames were introduced into the *A. cerana* colonies.

Because *A. cerana* and *A. mellifera* are sister-species (Alexander, 1991) showing close similarities in a variety of features (behaviour – Sakagami, 1959; nuclear and mitochondrial DNA – Smith, 1991; Arias and Sheppard, 2005; morphology – Ruttner, 1988; beeswax composition – Aichholz et al., 2000), it is so not surprising that very young adult workers may be readily exchanged

conspecifically as well as heterospecifically. This has been shown for *A. mellifera*, *A. cerana*, *A. florea* and *A. koschevnikovi* (Atwal and Sharma, 1967; Dhaliwal and Atwal, 1970; Nakamura, 1994; Koeniger et al., 1994), probably because they have not yet acquired a colony odour themselves (Breed et al., 2004).

As expected, mixed-species colonies were intermediate in their brood removal compared to interspecific and intraspecific exchanges, possibly because of an intermediate degree of relatedness. They removed less heterospecific brood compared to pure species colonies but more compared to the intraspecific exchanges. Assuming similar foraging of *A. cerana* and *A. mellifera* (Devkota and Thapa, 2005), this suggests that the composition of the work force has an impact on brood recognition in honeybees. Genetic cues for brood recognition similar to nestmate recognition for adults (Breed, 1998; Moritz and Neumann, 2004).

Although both kinds of mixed species colonies were treated in exactly the same way and consisted of 50% *A. cerana* and 50% *A. mellifera*, we found significant differences. Assuming similar foraging of *A. cerana* and *A. mellifera* bees from both test and control groups were sympatric in foraging range (Devkota and Thapa, 2005), only the combs and the queens were different between the two types of mixed-species colonies. Thus, it appears as if either the role of combs for nestmate recognition is different and or the queen may play a role in brood recognition (D'Ettorre et al., 2006). One could also speculate that the higher overall intraspecific brood removal rate in *A. cerana* is the result of a higher degree of social parasitism in natural populations of *A. cerana* (Nanork et al., 2007b).

Nestmate recognition for adults depends on both environment and genetics to varying extents (Breed, 1998), but our data show that it does not necessarily operate in the same way for eggs and immatures. Because previously suspected egg and brood recognition pheromones (Potichot et al., 1993) have been recently confirmed (Ayasse and Paxton, 2002; Sasaki et al., 2004), we surmise that specific egg and brood pheromones play crucial roles for the high removal rates of alien eggs and larvae observed in both the conspecific

and heterospecific transfers in both species. In these experimental contexts, brood recognition seems to operate with decreasing intensity with increasing developmental age, but at each level is amplified with increasing genetic distance.

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Reconnaissance intra- et interspécifique du couvain dans des colonies d'espèces pures et d'espèces mélangées, *Apis cerana* et *A. mellifera*.

***Apis cerana* / *Apis mellifera* / reconnaissance interspécifique / reconnaissance intraspécifique / couvain / parasitisme social / phéromone de couvain / colonie mixte**

Zusammenfassung – Innerartliche und zwischenartliche Bruterkennung in reinen und gemischten Honigbienenvölkern von *Apis cerana* und *A. mellifera*. Innerartliche und zwischenartliche Bruterkennung ist bei im gleichen Verbreitungsgebiet lebenden sozialen Insekten für die Sicherheit der Vermehrung und oft auch für die Integrität der Kolonien von grundlegender Bedeutung (Breed, 1998; Moritz and Neumann, 2004). Von besonderer Wichtigkeit ist sie im Kontext des Sozialparasitismus (Nanork et al., 2007b). Wir untersuchten die Auswirkungen gemischter Honigbienenvölker von *Apis mellifera* und *Apis cerana* auf die innerartliche und zwischenartliche Erkennung weiblicher Brutstadien der beiden Honigbienenarten mittels des Tauschs von Brutwaben zwischen weiselrichtigen Völkern. In den innerartlichen Tests wurden bei *A. cerana* signifikant mehr Larven entfernt als bei *A. mellifera*, während dagegen bei *A. cerana* signifikant weniger Eier und Puppen entfernt wurden als bei *A. mellifera*. Bei den zwischenartlichen Tests entfernten *A. cerana* signifikant mehr Larven und Puppen von *A. mellifera* als die gleichen Brutstadien von *A. cerana* durch *A. mellifera* entfernt wurden. Unsere Daten bestätigen damit frühere Untersuchungen mit zwischenartlicher Brutübertragung, dass

bei *A. cerana* und *A. mellifera* die Brutunterscheidung bezüglich Eiern und jungen Larven gut ausgeprägt ist (Oschmann, 1965; Dhaliwal and Atwal, 1970; Adlakha and Sharma, 1971; Oku and Ono, 1990; Potichot et al., 1993). Wie zu erwarten, verhielten sich die gemischtartigen Völker in ihrem Brutentfernungsverhalten zwischen dem innerartlichen und zwischenartlichen Austausch der reinen Völker. Sie entfernten signifikant weniger heterospezifische Brut im Vergleich zu reinen Völkern, aber mehr als bei innerspezifischem Austausch. Unter der Annahme von ähnlichem Sammelverhalten von *A. cerana* und *A. mellifera* (Devkota and Thapa, 2005) legt dies nahe, dass die Zusammensetzung der Arbeitsbienen einen Einfluss auf die Bruterkennung bei Honigbienen hat und die genetischen Auslöser der Bruterkennung denen der Erkennung von Nestgenossen bei Arbeiterinnen ähnlich sind (Breed, 1998; Moritz and Neumann, 2004).

Die Erkennung von Nestgenossen bei Arbeiterinnen hängt zu wechselnden Anteilen von der Außenumgebung und der Genetik ab (Breed, 1998), allerdings zeigen unsere Daten, dass diese sich nicht unbedingt in gleicher Weise auf die Eier und immature Stadien auswirken. Da bereits zuvor vermutete Pheromone zur Erkennung von Brut und Eiern (Potichot et al., 1993) kürzlich bestätigt wurden (Ayasse and Paxton, 2002; Sasaki et al., 2004), nehmen wir an, dass spezifische Pheromone der Eier und Larven eine grundlegende Rolle bei den sowohl bei gleichartigen als auch bei zwischenartigem Austausch beobachteten hohen Entfernungsraten der fremden Eier und Larven spielen. Unter diesen experimentellen Bedingungen schien die Bruterkennung mit steigendem Entwicklungsalter in der Intensität abzunehmen, aber in jedem Alter durch die genetische Distanz verstärkt zu werden.

***Apis mellifera* / *Apis cerana* / Nestgenosserkennung / Sozialparasitismus**

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