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Original article

Comparative nestmate recognition in Asian honey bees, *Apis florea*, *Apis andreniformis*, *Apis dorsata*, and *Apis cerana**

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Abstract – In nestmate recognition bioassays, *Apis florea*, *A andreniformis, A. dorsata* and *A. cerana* do not exhibit aggressive responses. These negative results were obtained using three distinct techniques: pairings of bees between colonies, switching nest box locations (*A. cerana* only), and treatment with compounds known to serve as nestmate recognition pheromones in *A. mellifera*. This is in sharp contrast to previously observed responses in *A. mellifera*, which displays strong aggressive responses to conspecific non-nestmates in the same types of bioassays. *A. cerana* expresses nestmate recognition, but only under limited circumstances – when robbing is precipitated by honey harvesting or the merger of colonies by a beekeeper. Our results suggest that robbing of stored food may be more characteristic of *A. mellifera* than other species in the genus *Apis*, and consequently *A. mellifera* displays a more strongly developed response to conspecific non-nestmates than other *Apis* species.

nestmate recognition / *Apis* / defensive behavior

1. INTRODUCTION

Nestmate recognition is a critical aspect of colony defense in many social insects (Breed et al., 2004a). Species such as the western honey bee, *Apis mellifera* L., which are subjected to social parasitism, robbing of food, and robbing of brood, are typically capable of discriminating nestmates from non-nestmates. Such discriminations, which are generally characterized as nestmate recognition, are expressed at the nest entrance with a defensive response, such as biting, stinging, or grappling (Breed, 1998b; Vander Meer and Morel, 1998; Lenoir et al., 2001a, b). Nestmate recognition is well known in all the major eusocial insect taxa (Vander Meer et al., 1998; Starks, 2004).

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To our knowledge, only one previous study addresses nestmate recognition in a species of *Apis* other than *A. mellifera* (Sasagawa et al., 2002; Oldroyd and Wongsiri, 2006). Most of our current knowledge of the comparative defensive behavior of *Apis* species comes from Seeley et al. (1982). One aspect of defense that they did not address was intraspecific nestmate recognition; studies of species other than *A. mellifera* will give insight into the ecological and evolutionary malleability of nestmate recognition. In this paper we report results suggesting a lack of expression of nestmate recognition in Asian species of *Apis*; publication of such results is important in developing an overall understanding of nest defense in social insects (Gamboa et al., 1991).

Many investigations have assumed that nestmate recognition is an extension of the ability to make fine discriminations concerning relatedness among nestmates (e.g. Greenberg, 1988; Bura and Gamboa, 1994, but

see Dani et al., 2004). This may be true in primitively eusocial insects, such as the halictid bees studied by Greenberg (1988). However, in the highly eusocial insects nestmate recognition may be more related to the ecological circumstances and evolutionary history of a species than to intracolonial discrimination of genetic groups. For example, Argentine ants (*Linepithema humile*) in California, whose colonies often comprise unrelated workers and queens, do not display nestmate recognition over a broad geographic range (Tsutsui et al., 2003), nor do many polygynous, polydomous ant species (e.g., Starks et al., 1998). The primitively eusocial bumblebee, *Bombus impatiens*, also appears not to exhibit nestmate recognition (unpubl. data). Additionally, resource abundance and associated foraging strategies may be important influences on nestmate recognition. Increased competition for resources and intercolonial interactions could influence selection for stronger nestmate recognition. Reeve (1989) made an important theoretical contribution, pointing out that thresholds for expressing behavioral responses to non-nestmates may be dependent on ecological conditions.

Comparative studies of nestmate recognition also may give insight into the diversity of chemical cues used in nestmate recognition. Nestmate recognition cue chemistry is well known for some social insects. In *Camponotus fellah* ants, the predominant nestmate recognition signals are alkenes, which are distributed among colony members via the postpharyngeal organ (Lenoir et al., 2001b). However, in Argentine ants, *Linepithema humile*, environmentally-derived cues are strongly implicated (Liang and Silverman, 2000). Nestmate recognition signals in *Polistes* wasps are methyl-branched alkanes (Dani et al., 2001). In the western honeybee, *Apis mellifera*, fatty acids play an important role, as do alkenes (Breed, 1998a). Differences in nestmate recognition among *Apis* species could correlate with differences in cue chemistry, sensitivity, or diversity, or could be affected by differences among species in response thresholds (Reeve, 1989).

Given the strong nestmate recognition response of *A. mellifera* (Moore et al., 1987; Downs and Ratnieks, 2000; Guzman-Novoa et al., 2002) we hypothesized that other species of *Apis* would display similar responses. We tested nestmate recognition in *A. cerana*, *A. florea*, *A. andreniformis* and *A. dorsata* using three different nestmate recognition bioassays (Breed, 2003) in order to better understand interspecific differences in expression of nestmate recognition and defensive behavior.

2. METHODS

2.1. General

Colonies were located in and near the Xishuangbanna Tropical Botanical Garden (XTBG), Yunnan Province, People's Republic of China, 21.41° N lat., 101.25° E long. XTBG is at 570 M elevation, on the Luosuo tributary of the Mekong River near the border of the PRC, Laos and Myanmar. This evergreen seasonal tropical forest habitat (1560 mm annual precipitation) has a long hot dry season, mid-March to May, and a cool dry season from November to February; the balance of the year is characterized by frequent rains. *A. florea*, *A. dorsata* and *A. andreniformis* are seasonal migrants in this habitat, moving to higher elevations during the rainy season. These experiments were conducted from October 2004 to July 2005.

For the first set of experiments, in October 2004, six *A. cerana* colonies, located in the XTBG and maintained by a beekeeper in moveable frame hives, were used. The *A. cerana* colonies were roughly equal in size, each in a box approximately the size of standard Langstroth hive. We also used three *A. florea* colonies, located on a farm several hundred meters higher in elevation than the XTBG, 20–30 meters apart from each other. The three *A. florea* colonies were roughly equal in size as well, as judged by the mass of bees on the nest. Additionally, two *A. dorsata* colonies, 30 kilometers separated, were used in these experiments. One was located near XTBG, and one 330 meters higher in elevation; both were typically-sized colonies for this area. The season when our study was conducted is apparently unfavorable at the elevation of the XTBG

for *A. dorsata* and *A. florea*. However, the *A. cerana* colonies at that elevation were actively foraging for both pollen and nectar, and showed no signs of the cessation of foraging that is characteristic of dearth conditions.

2.2. Bioassay 1. Intra- and intercolonial aggression

Aggression bioasssays, following methods used with *A. mellifera* (Breed, 2004a; Downs and Ratnieks, 2000) and the general principles described by Breed (2003) were conducted within and between colonies of each of the three species. In these bioassays, bees were captured and paired in 5 mL glass vials. The interactions between the bees were observed for 5 min; in each pairing the observer was blind with respect to whether the bees were nestmates or non-nestmates. In *A. cerana*, bees for the bioassays were collected from near the nest entrance. Although guards are not as easily identified in *A. cerana* as in *A. mellifera* (Moore et al., 1987), most of the bees used in the experiments were seen shimmering (see Results) and could therefore be considered guards. Identifying guards in *A. florea* and *A. dorsata*, was more difficult. *A. florea* and *A. dorsata* workers for bioassays were removed from the outer layer of bees blanketing the comb. These bees have defensive, as well as thermoregulatory, functions (Breed et al., 2004a), but we could not be sure that the bees used in the experiments were primed for colony defense.

2.3. Bioassay 2. Treatment with *Apis mellifera* recognition pheromones

The second type of bioassay employed the same method of pairing bees used in the first bioassay, but in this case both bees came from the same colony. One bee in the pair was treated with a mixture of 18 carbon fatty acids, delivered as flax oil, which is a nearly pure mixture of oleic, linoleic and linolenic acids (Shim et al., 2003). In *Apis mellifera* treatment with these 18 carbon fatty acids results in aggression between the treated and untreated nestmate in a higher percentage of replicates (Breed et al., 2004b). This result confirms the activity of the 18 carbon acids as recognition pheromones. These same acids function similarly, and bees respond in the same type of bioassays, in a stingless bee, *Trigona fulviventris* (Buchwald and Breed, 2005). Unpublished results (Lyon, Breed and Buchwald) show that flax oil has the same effect as the pure fatty acids in *Apis mellifera*.

2.4. Bioassay 3. Colony transpositions in *Apis cerana*

A third bioassay approach was used in *A. cerana*, with the object of providing a more natural context for the expression of nestmate recognition. In this bioassay foragers from pairs of colonies were marked with enamel paint, each colony receiving a different color. We marked the bees from 1500 h to 1700 h one day, and then at 2000 h that evening (after sunset) the colony locations were transposed. The next day experienced foragers return to their colony's previous location, where they encounter workers from the other colony. We did four such switches, using six colonies. Colony entrances were observed for 1.0 hour beginning at 0700 h and the number of foragers entering the "wrong" colony was counted, as well as interactions between marked bees and resident bees. Not all foragers were marked; the marked individuals confirmed that bees from one colony are entering the other, but did not provide a complete count of transposed bees.

2.5. Seasonal variation

Bioassay 1 was used in tests for seasonal variability in expression of defensive behavior in *A. cerana*, *A. florea* and *A. andreniformis* (which was not available in the XTBG area for the tests in October 2004). These tests were conducted every other month over an eight month period.

Table I. Number of occurrences of aggression between pairs of bees in bioassays. The number before the slash is the number of replicates in which aggression was observed; the number after the slash is the sample size.

	Biting or Stinging observed
A. cerana between colonies	1/30
A. cerana within colonies (control)	0/20
A. florea between colonies	0/15
A. florea within colonies (control)	0/5
A. dorsata between colonies	3/20
A. dorsata within colonies (control)	0/10

Table II. Test for seasonal variation in results of nestmate recognition bioassays in three *Apis* species. The number before the slash is the number of replicates in which aggression was observed; the number after the slash is the sample size. At least three colonies were used for each set of tests.

	A. cerana	*A. florea*	*A. andreniformis*
Dec. 14, 2004	0/20		
Feb. 17, 2005		0/20	0/20
Feb. 19, 2005	0/20		
April 25, 2005		0/20	
April 30, 2005	6/20		
June 20, 2005		0/20	0/20
June 26, 2005	0/20		
Aug. 5, 2005	0/16		
Aug. 12, 2005		0/20	

2.6. Responses to alarm pheromone

Finally, we induced alarm pheromone release by grasping a bee with forceps. The bee would release alarm pheromone onto the forceps, which were then presented at the colony entrance (in the case of *A. cerana*) or near surface of the curtain of bees (in the other species).

3. RESULTS

3.1. Intra- and intercolonial aggression bioassays

Few instances of biting or stinging were recorded in the four species included in this study (Tabs. I, II). There was no significant difference among the between- and within-colony pairings in *A. cerana*, $P > 0.999$, df = 1 chi-square = 0.68, *A. florea*, $P > 0.999$, Fisher's exact test, or *A. dorsata*, $P = 0.52$, df = 1 chi-square = 1.67.

3.2. Treatment with *A. mellifera* recognition pheromones

Treatment of one nestmate in a pair with fatty acids also had no significant effect on nestmate recognition in the four Asian species of *Apis* (*A. cerana*, $P > 0.999$, Fisher's exact test; *A. andreniformis*, $P < 0.999$, Fisher's exact test; *A. florea*, $P < 0.999$, Fisher's exact test; *A. dorsata*, chi-square = 1.833, $P = 0.371$, df = 1 for all tests) (Tab. III).

3.3. Colony transpositions in *Apis cerana*

In contrast, aggression was observed in only one of the four transpositions of colony locations (Tab. IV, trial 3). In trials 2 and 4, marked bees were observed entering and exiting the other colony with no apparent aggression or reaction by the resident bees. In trial 1, few bees were marked, and although it is likely that foragers were transposed between the colonies, the marking did not confirm this.

Table III. The effect of treatment with a mixture of 18 carbon fatty acids on nestmate recognition in four species of Asian *Apis*. Each sample was a pairing between two bees from the same colony The number before the slash is the number of replicates in which aggression was observed; the number after the slash is the sample size. For controls refer to Tables I and II. All samples are not significantly different from the controls.

Apis cerana	0/98
Apis andreniformis	0/30
Apis florea	0/81
Apis dorsata	2/10

3.4. Seasonal variation

This pattern held for two of the three species reported in Table I in tests in subsequent time periods (Tab. II) with the exception of *A. cerana* in late April, when 30% of the replicates involved aggression.

3.5. Responses to alarm pheromone

Presentation of alarm pheromone in *A. cerana* stimulated bees to fly at the investigators, rather than at the source of the pheromone. The *A. cerana* colonies were quite docile, with no bees flying from the entrance at the investigators, even when prodded with fingers or forceps, until alarm pheromone was presented. After alarm pheromone presentation (N = 3 colonies), bees flew at, and stung, the investigator's faces.

In *A. florea*, forceps were used to remove bees from the outer blanket of bees covering the comb. The forceps, if unwashed, stimulated alert postures and movement among bees in the outer blanket on the colony, but not flight or shimmering; washing the forceps in water extinguished this response.

3.6. Presence of the Asian hornet

During our experiments, Asian hornets (*Vespa mandarinia*) were observed approaching the *A. cerana* and *A. florea* colonies, but not the *A. dorsata* colonies. All of the *A. cerana* and *A. florea* colonies were harassed by Asian hornets during the experiments. In *A. cerana*, small groups, up to approximately twenty-five individuals (guard bees), near the nest entrance synchronously flared their wings and shook their abdomens (shimmered) when a hornet approached. In *A. florea*, the outer layer of bees shimmered with a wave of wing-flares that started from a central point, usually on the lower portion of the comb, and progressed until the wave dissipated. In more intense shimmering responses the wave of movement continued until they reached the comb's margins.

4. DISCUSSION

Three independent bioassays for the expression of nestmate recognition in four Asian species of *Apis*, *A. cerana*, *A. florea*, *A. andreniformis*, and *A. dorsata*, yielded negative results. While negative results must always be viewed as tentative, as a different experimental design might give a positive result, the uniformity of outcome across bioassay design and across season strongly suggests that our findings are meaningful with regard to nestmate recognition in these species. The only other formal test of nestmate recognition in one of the non-*mellifera* species of *Apis* was performed by Sasagawa et al. (2002) on *Apis cerana japonica*; their results were consistent with ours, with 96% acceptance of foreign conspecific foragers by *A. cerana* colonies.

As Gamboa et al. (1991) strongly point out, ignoring, or failing to report, negative results in kin or nestmate experiments biases the literature on these subjects, giving a false impression of the generality of the expression of nestmate or kin recognition. Breed (2003) and Roulston et al. (2003) discuss in detail the cautions and values associated with interpreting negative results in nestmate recognition bioassays. An added caution is that Koeniger and Vorwohl (1979) observed fighting among bees, including these species, at feeding stations (but not on flowers); how their result might extend to colony defense is unknown. Speculatively, our results suggest that nestmate recognition behavior is not expressed in these species with nearly the frequency or

Table IV. Responses of *A. cerana* workers when colony locations were switched.

	First colony in pair, number of bees marked	Second colony in pair, number of bees marked	First colony in pair, number of bees observed entering or leaving second colony in pair	Second colony in pair, number of bees observed entering or leaving first colony in pair	Number of fights observed at first colony in pair	Number of fights observed at second colony in pair
Trial 1	30	30	0	0	0	0
Trial 2	200	75	22	0	0	0
Trial 3	150	150	3	0	13	8
Trial 4	300	200	27	7	0	0

intensity that it is in their congener, *Apis mellifera* (Downs and Ratnieks, 2000; Breed et al., 2004b), but this conclusion needs to be tested when all the species are available at the same time and place.

The lack of nestmate recognition expressed by the tested bees, except for *A. cerana* under limited circumstances, does not mean that workers of these species cannot perceive differences between nestmates and nonnestmates; discrimination may exist but may not be acted upon. The finding that aggression occurred between workers from one of the transposed pairs of *A. cerana* colonies, coupled with casual observations of beekeepers, suggests that the ability to recognize nonnestmates exists in *A. cerana*, but that behavioral expression of nestmate recognition is largely absent. Thus the question addressed by our findings is one of expression, not of signal production or perception.

Large quantities of stored food and the presence of robbing behavior seem to have favored the expression of nestmate recognition in *A. mellifera*. In many ways, this is an extension of the argument made by Downs and Ratnieks (2000) that temporal changes within a season in defensive behavior of *A. mellifera* colonies is related to ecological changes that affect risk factors to colonies (Reeve, 1989).

Experimental context is extremely important in designing and interpreting nestmate recognition bioassays (Breed, 2003; Roulston et al., 2003). Possibly, a different bioassay could be designed which would elicit stronger discrimination responses; the presence of a defensive response to Asian hornets showed that the colonies were primed for defense in one context, and had individuals that were active in colony defense. Both *A. cerana* and *A. florea* shimmer in response to the frequent approaches of Asian hornets, *Vespa mandarinia* (Pirk et al., 2002; Breed et al., 2004a). The value of our experiments lies in the comparison of these results with those already obtained for *A. mellifera*. Thus, the failure of these species to respond in this bioassay context does not preclude response in another type of test.

Of the species studied, *A. cerana* is the only one managed by beekeepers. Two beekeepers, one who currently manages the six colonies used in our study and another who had managed *A. cerana* colonies in the past, had never observed workers fighting at nest entrances or attempts by *A. cerana* workers to rob honey from other colonies under undisturbed conditions. On the contrary, both beekeepers had observed fights as the result of attempts to merge weak colonies with strong colonies, however. Another beekeeper had observed robbing attempts and fights during honey harvesting activities, when broken combs were exposed.

Colony defense against conspecifics carries with it considerable potential costs. These include expenditure of time and energy in guarding activities and loss of workers due to injury or death in conflicts. Without selective pressure for expression of this type of colony defense, it is not surprising that these investments would be minimized. The lack of known intraspecific or interspecific social parasites in *Apis* – as exemplified by the *Bombus*/*Psithyrus* relationship – removes another

possible selective pressure for closure of the colony. The appearance of aggressive colony defense in *A. mellifera* may then reflect substantial potential costs if stored food reserves were undefended. This raises the question of whether *A. mellifera* is unique among *Apis* species in its expression of defensive behavior, or if other cavity nesting *Apis*, such as *A. koschevnikovi*, *A. nuluensis* and *A. nigrocincta* (Koeniger et al., 1996; Tanaka et al., 2001; Smith et al., 2003) also aggressively defend their nest when confronted with non-nestmate conspecifics.

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Étude comparative de la reconnaissance des membres de la colonie chez les abeilles asiatiques *Apis florea, A. andreniformis, A. dorsata* et *A. cerana*.

***Apis* / comportement défensif / reconnaissance intraspécifique / reconnaissance interindividuelle / membre de la colonie**

Zusammenfassung – Vergleichende Erkennung von Nestgenossinnen bei den asiatischen Honigbienen *Apis florea, Apis andreniformis*, *Apis dorsata* und *Apis cerana*. Die Erkennung von Nestgenossinnen ist bei vielen sozialen Insekten ein wichtiger Aspekt bei der Verteidigung des Volkes. Bienenarten wie *Apis mellifera* sind häufig einem Sozialparasitismus und der Räuberei von Futter bzw. Brut ausgesetzt und daher meist in der Lage, zwischen Nestgenossinnen und fremden Bienen zu unterscheiden. Diese Fähigkeit zur Unterscheidung zeigt sich am Nesteingang durch Abwehrverhalten wie Beißen, Stechen oder Umklammern. Territoriale Arten der Stachellosen Bienen zeigen aggressives Verhalten gegenüber fremden Bienen nicht nur am Nesteingang sondern auch an den Futterstellen. Mit einer Ausnahme wurden Untersuchungen zur Erkennungen von Nestgenossinnen bei Honigbienen ausschließlich bei *A. mellifera* durchgeführt. In den Arbeiten zur Nestverteidigung bei anderen Honigbienenarten wurde der Aspekt der intraspezifischen Erkennung von Nestgenossinnen dagegen nicht berücksichtigt. Die Untersuchung dieser Honigbienenarten sollte daher Aufschlüsse über die ökologische und evolutionsbiologische Bedeutung der Nestgenossinnen-Erkennung bringen. In Anbetracht der ausgeprägten Reaktionen von *A. mellifera* gegenüber Nicht-Nestgenossinnen erwarteten wir bei anderen *Apis*-Arten ähnliche Reaktionen. Wir prüften die Erkennung von Nestgenossinnen bei *A. cerana*, *A. florea*, *A. andreniformis* und *A. dorsata* mit drei Testsystemen: einem Standard-Biotest (zwei Bienen aus verschiedenen Völkern wurden in einem Glas beobachtet), Verstellen von Bienenvölkern (nur *A. cerana*) und Applikation von Pheromonen zur Nesterkennung.
Bei keiner der geprüften Bienenarten (mit Ausnahme von *A. cerana* in einem Fall) konnte eine Nestgenossinnen-Erkennung nachgewiesen werden. Möglicherweise werden Unterschiede zwischen Nestgenossinnen und Nicht-Nestgenossinnen wahrgenommen, aber Abwehrreaktionen gegenüber fremden Bienen der gleichen Art blieben aus. Auch saisonale Effekte wurden getestet, indem die Biotests in Abständen über das ganze Jahr durchgeführt wurden. Da keine saisonalen Schwankungen auftraten, kann das Fehlen einer spezifischen Nestgenossinnen-Erkennung bei den drei getesteten Arten nicht von saisonalen Faktoren abhängen.
Unsere Ergebnisse lassen sich unter ökologischen Gesichtspunkten wie folgt interpretieren: *A. mellifera* gehört zur hoch entwickelten höhlenbrütenden Gruppe innerhalb der Gattung *Apis*, während *A. florea* und *A. andreniformis* zu den primitiveren „Zwerghonigbienen" gehören. Die große Menge an Futtervorräten und die weit verbreitete Räuberei scheint bei *A. mellifera* die Entwicklung einer effektiven Erkennung von Nestgenossinnen begünstigt zu haben. Aus dem aggressiven Verteidigungsverhalten bei *A. mellifera* kann man demnach schließen, dass die Futtervorräte im Volk einen sehr hohen Wert darstellen und sich die Verteidigung dieser Vorräte lohnt. Damit erhebt sich die Frage, ob *A. mellifera* bezüglich des Verteidigungsverhaltens einzigartig innerhalb der Gattung *Apis* ist oder ob andere höhlenbrütende *Apis*-Arten wie *A. koschevnikovi*, *A. nuluensis* und *A. nigrocincta* ebenfalls ihr Nest aggressiv verteidigen, wenn sie mit Nicht-Nestgenossinnen konfrontiert werden.

Nestgenossinnen-Erkennung / *Apis* / Abwehrverhalten

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