Studies on Mixed–Species Colonies of Honeybees,

Apis cerana and *Apis mellifera*

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ABSTRACT

The honeybees *Apis cerana* and *Apis mellifera* are derived from the same ancestral base about two million years ago. With speciation and evolution, they have acquired many advanced living skills in common, but have also evolved very different living strategies due to different distributions. This thesis is an intensive study of the biology of the mixed-species colonies of these species, the aims of which were to investigate their behavioural relationships and uncover the evolutionary conserved features of their behaviours subsequent to speciation.

The results show that the two species can form a stable society to perform normal tasks. First, workers of both species in the mixed-colonies could form the typical retinue behaviour to hetero-species queens, thus indicating that queen pheromones could be spread to and by both species. Secondly, both species did not show significantly different ovarian activation under hetero-species queens, suggesting that the queen pheromones more likely play a role of "honest signal" rather than a "repression" substance in the honeybee colonies. Thirdly, both species could mutually decode each other's waggle dances, with unexpectedly low misunderstanding; revealing that the dance language in a dark environment is quite adaptive for cavity-nesting honeybees. Fourthly**,** workers of both species could cooperate with each other in comb construction, although the combs they built contain many irregular cells. Interestingly, *A. cerana* workers could be stimulated by *A. mellifera* workers to perform this task, thus confirming self-organization theory in the colony. Fifthly**,** *A. mellifera* workers behaved more "defectively" in thermoregulation, but perhaps because *A. cerana* workers are more sensitive to changes in hive temperature. Given these differences in strategy, *A. mellifera* workers' performance might in fact reduce conflicts. Lastly, when faced with threats of predatory wasps, both species engaged in aggressive defence. Although they did not learn from each other's responses, species-specific strategies were adopted by each of them so that the defence of the mixed-colonies is very effective.

I conclude that the two species can adapt to each other's efforts and task allocation is reasonably organized allowing mixed-species colonies to reach stability. These results suggest that all of the social behaviours discussed here were highly conserved following speciation. This thesis could provide some clues for the study of honeybee evolution from open-nesting to the transition of cavity-nesting.

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CHAPTER 1

GENERAL INTRODUCTION

Interspecific interactions among honeybee species

The Asian continent is the richest in the world in honeybee diversity and includes a number of indigenous species: *Apis cerana*, *Apis florea*, *Apis andreniformis*, *Apis dorsata*, *Apis laboriosa*, *Apis nigrocincta*, *Apis nuluensis* and *Apis koschevnikovi* as well as the introduced *Apis mellifera* which is widely used for honey production (excluding a recently discovered population in far northwestern Asia). When these *Apis* species occur sympatrically, they can interact in various ways (Koeniger, 1982). Worker bees of different species may rob each other's nests and compete for food or for nesting sites, while drones may interfere with each other during mating flights. Besides, a parasite or disease of one species may transfer to another to which it is not resistant.

Interspecific interactions among the *Apis* species have no doubt played a role in their evolution. Even though interspecific interactions of the present may not be like those of the past, before or during the process of speciation, it is still an interesting and potentially important topic that deserves investigation. Since the male genitalia (which are regarded as one of the most important factors in reproductive isolation and speciation) among some species are not completely distinct, the possibilities of food and/or nest competition might make more sense in considering speciation in the genus *Apis.*

1.1 Nest site competition

In Asia, the honeybee species have adopted different evolutionary strategies to adapt to their environments and, according to body size and nesting habits, they can be divided into three groups: dwarf honeybees, giant honeybees and cavity-nesting honeybees (Arias and Sheppard, 2005; Oldroyd and Wongsiri, 2006). Given that each of them has a distinct nesting behaviour, nest site competition between them can rarely be observed, so in this section only competition within each group is discussed.

1.1.1 Nest site competition in the dwarf honeybees

The dwarf honeybees include two species, *A. florea* and *A. andreniformis*, and both naturally occur in tropical and sub-tropical regions of Asia (Wongsiri et al., 1996). *A. florea* extends from the Middle East eastwards to peninsular Malaysia, whereas *A. andreniformis* is distributed from the Philippines to China and Myanmar, but they overlap in Southeast Asia (Otis, 1996; Wongsiri et al., 1996; Hepburn and Radloff, 2009). So our interest lies in whether they compete for nest sites in the limited areas where they overlap.

These two honeybee species are superficially similar in many respects and it took a number of years for honeybee biologists to define them as unequivocally separate species (Smith, 1858; Maa, 1953; Wu and Kuang, 1987; Ruttner, 1988; Wongsiri et al., 1990; Hepburn et al., 2005). As for nest sites, both species build single, exposed combs on the thin branches of bushes, shrubs or small trees (Wongsiri et al., 1996) and, in western Asia, often nest in small caves or in sheltered areas of buildings (Dutton and Free, 1979; Whitcomb, 1984). Although it has been reported that the two species may also nest at different altitudes: *A. andreniformis* in high mountainous and forest areas at about 1 600 m altitude, while *A. florea* is common in lowlands below 1 000 m (Wongsiri et al., 1996), an analysis of the complete distribution of the species shows that there is no significant difference in their altitudinal distributions (Hepburn and Radloff, 2009).

However, the nests of *A. andreniformis* appear higher (about 6 m from the ground) than those of *A. florea* (about 4m) (Rinderer et al., 2002), so that nesting competition between them can be inferred to happen only occasionally. In addition, Rinderer et al., (2002) did find that when these two species occur together in the same area, they tend to avoid each other. Such avoidance between these two species, although still controversial (Koeniger, pers. comm.), may make sense if the two species evolved the ability to recognize each other during the course of speciation and mutual adaptation. Interestingly, Rinderer et al. (2002) reported that both species of dwarf honeybees have a tendency to form aggregations of colonies in spatial distribution, but not as intensely as colonies of *A. dorsata* (see details below).

1.1.2 Nest competition in the giant honeybees

Two species, *A. dorsata* and *A. laboriosa*, form the group *Megapis*, or giant honeybees. *A. dorsata* is distributed mainly in tropical areas while *A. laboriosa* naturally occurs in mountainous regions, particularly the Himalayas, at altitudes between 1500 m to 4 000 m (Sakagami et al., 1980; Ruttner, 1988; Underwood, 1990a,b). The former species has a tendency to be highly aggregated, 100 or even more colonies crammed onto a single tree (Deodikar et al., 1977; Seeley et al., 1982; Dyer and Seeley, 1991b), and has a habit of seasonal migration. The latter also has a tendency for colony aggregations but only on cliffs (Roubik et al., 1985; Kuang and Kuang, 2002; Joshi et al., 2004). According to Underwood (1990a,b), *A. laboriosa* never nests on the limbs of trees. And although they also have seasonal migration behaviour, which results in a temporary sympatry with *A. dorsata*, it occurs during the non-nesting phase of *A. laboriosa*. So, we can safely conclude that nest competition between these two giant honeybees does not occur today and can only speculate as to the past.

1.1.3 Nest competition in the cavity-nesting honeybees

Cavity-nesting honeybees include *A. mellifera*, *A. cerana*, *A. nigrocincta*, *A. nuluensis* and *A. koschevnikovi*, all of which are native to Southeast Asia except *A. mellifera*. The four Asian cavity-nesting honeybees began their divergence from a presumed cosmopolitan *A. cerana* proto-type some 2 million years ago (Smith, 1991; Arias and Sheppard, 2005). Even so, the habitats of each species are very different. For example, *A. nuluensis* is confined to the highlands on the island of Borneo (Malaysia), and it is only known from the Crocker Range in Sabah (Tingek et al., 1996). The Sulawesian honey bee, *A. nigrocincta*, is confined to the islands of Sulawesi, Sagihe and Mindanao (Otis, 1996). *A. koschevnikovi* has a comparatively wider distribution area: from Java, Sumatra, peninsular Malaysia to southern Thailand, however, since this bee requires rainforest habitat, it is now rare outside of Borneo owing to deforestation (Hadisoesilo et al., 2008). *A. nuluensis* is confined to mountainous regions above 1500 m only on the spectacular Mount Kinabalu, in the Malaysian state of Sabah in Borneo (Tingek et al., 1996). *A. cerana* occurs on the mainland of Asia as well as the islands of the South China Sea (Radloff et al., 2009). For these combined reasons, we have not seen many reports about nesting competition among these cavity-nesting honeybees. Interestingly,

all these cavity-nesting honeybees, except *A. cerana*, mainly occur on islands in the South China Sea, the islands providing perfect geographic isolation, which undoubtedly has played a very important role in the speciation of these honeybees. It seems that the only practical place to investigate possible nest competition among these species is Borneo, where three cavity-nesting bees: *A. koschevnikovi*, *A. nuluensis* and *A. cerana* coexist or Sulawesi where *A. cerana* and *A. nigrocincta* are sympatric.

1.1.4 Social parasitism

Social parasitism in honeybees is generally understood to mean the phenomenon of worker bees joining neighbouring colonies by drifting or direct invasion (Neumann et al., 2001a). Social parasitism is widespread in social insects but has been studied only in *A. cerana* and *A. florea* amongst Asian honeybees. Nanork et al. (2006a) found that in queenright *A. cerana* colonies, 2-6% of workers are non-natal, but these drifted workers do not have active ovaries, suggesting that in queenright colonies social parasitism is not pervasive. However, in queenless colonies, there were significantly more non-natal workers (72.7%) with activated ovaries than natal workers (36.3%). Non-natal workers also had a significantly higher reproductive success than natal workers. The same phenomenon has been observed in the dwarf honeybees, *A. florea* (Nanork et al., 2006a; Chapman et al., 2009). In *A. florea* colonies, when a colony becomes queenless, workers bees have a higher tendency for parasitizing other colonies, preferring queenless to queenright colonies as their hosts for reproduction; and, as a result, queenless colonies are heavily parasitized with the eggs of non-natal workers (Nanork et al., 2006b). It has been suggested that social parasitism is present more or less in all honeybees species: 2-4% of the workers are non-natal, although these unrelated workers are thought to arise via orientation errors while retuning from foraging trips (Chapman et al., 2009).

Although social parasitism has only been observed intraspecifically in honeybees, interspecific parasitism has yet to be investigated. However, *A. cerana* was observed in a colony of *A. mellifera* for a short period but subsequently flew away (Denis Anderson, pers. comm.) and *A. cerana* workers have been seen on nests of *A. florea* (Duangphakdee, Hepburn, Phiancharoen, pers. comm.). The same phenomenon has been reported in *A. mellifera capensis* invading colonies of *A. m. scutellata* by Neumann

et al. (2001b). And, during the long history of evolution, parasitism might have played a role in nest competition and/or nest avoidance in speciation.

1.2 Food competition

Besides possible competition for habitat and reproduction, the species also compete for food resources when they occur in the same area. The performance of different bees in competition is of significance in speciation and/or coevolution. When different honeybees compete for food, body size is an important factor and the smaller bees are usually more aggressive in defending floral resources, probably because smaller bees have more restricted foraging ranges than the larger ones (Ruttner, 1988).

Koeniger and Vorwohl (1979) investigated the interactions of three honeybee species: *A. florea*, *A. cerana*, and *A. dorsata* and stingless bees *Trigona* by using an artificial feeding dish. They found that small bees generally attacked larger ones, but, *A. dorsata* was attacked only by *A. cerana*, never by the other two species. At times, only one species remained while the others stayed away, but a final "winner" was unpredictable. Ruttner (1988) concluded that honeybees with larger bodies enjoy more choices, usually avoid disastrous fighting and shift to other, more distant food resources.

In Nepal, Partap (1998) investigated the impact of the introduction of *A. mellifera* colonies on the foraging behaviour of a local honeybee, *A. cerana*. Foraging competition was studied by counting the number of foragers of *A. cerana* on several flowers during the presence of and after removal of *A. mellifera* colonies (Table 1.1). The results indicated that *A. cerana* foragers spend more time visiting flowers in the absence of *A. mellifera*. They also spend more time on flowers, visit more flowers per trip, collect more pollen, and more *A. cerana* foragers were seen on the flowers when the competition from *A. mellifera* was removed.

Table 1.1 Mean number (± S.D.) of *A. cerana* foragers during the presence of and after removal of *A. mellifera* (Partap, 1998)

Crop	Number of *A. cerana* foragers		Difference significance
	During the presence of *A. mellifera*	After the removal of *A. mellifera*	
Mustard	12.6±1.2	20.8±1.3	$p<0.01$
Broadleaf mustard	12.3±1.3	18.3±2.1	$p<0.01$
Cauliflower	18.4±1.1	28.3±0.8	$p<0.01$
Radish	11.7±0.9	16.2±1.2	$p<0.01$

Similarly, Dhaliwal and Atwal (1970) studied food competition between *A. cerana indica* and *A. mellifera* at feeding dishes. Firstly, the two species were fed at their own respective feeders, not mixing with each other, and showing no hostile behaviour, but as the feeders were brought nearer to each other, the bees became more and more aggressive. When *A. mellifera* workers were freely alighting on both feeders, *A. cerana* workers were hesitant to do so, and the latter were often stung by the former, some dying, but no *A. mellifera* died. Finally, *A. mellifera* workers formed a ring around the feeder while *A. cerana* workers could not alight to feed. The results indicated that *A. mellifera* was more successful in eliminating *A. cerana*.

Interestingly, as suggested by this experiment, honeybees can distinguish their nestmates outside of the hive, and so they can jointly compete for food. Kalmus (1941) found that even different strains of the same species can distinguish each other. Two colonies of differently coloured *A. mellifera* bees, Caucasians and Italians, were trained to feeders and behaved aggressively towards each other. So we can infer that during speciation, the newly forming species could probably recognize their own nestmates and fight others, which in turn could be expected to facilitate speciation. Stout and Goulson (2001) found that bumblebees (*Bombus* spp.) and honeybees (*A. mellifera*) are both able to use odour cues deposited on flowers by previous visitors. Both bumblebees and honeybees avoided flowers previously visited by each other when foraging on *Melilotus officinalis*, that is, bumblebees avoided flowers recently visited by honeybees and vice versa.

How do honeybees avoid serious competition among different species? Different species have different strategies. When we discuss this topic, two important decisive factors must be mentioned: energy consumption and body temperature of foragers. Firstly, body size and the length of the proboscis surely play an important role in competition, and the size of a forager may determine which floral resources are

available to it. For example, *A. dorsata*, one of the giant honeybees, can fly further than smaller honeybees and so enjoy larger foraging areas. Because they have a longer glossa, they may able to collect nectar from some deep flower corolla tubes, but not able to gain access to some very small flowers with deeply hidden nectaries. So in the history of co-evolution, flowers of highly specialized morphology have developed nectaries for specific pollinators, and foragers of the different species specialize on particular floral resources (Oldroyd and Wongsiri, 2006).

Secondly, different species have different flight designs. Some researches have intensively investigated the flight designs of honeybees (Hepburn et al., 1998a,b; Hepburn et al., 1999; Radloff et al., 2003), and have suggested that several factors can integrate into an excess power index (EPI) that determines the flight ability of honeybees. These factors include: whole body mass, thoracic mass, thorax/body mass ratio, wing surface area, and wing loading. The excess power index (EPI) is defined as (r^2/W) where W is the wing loading and r is the ratio of the thorax mass to total mass (Hepburn et al., 1998b). According to this index, the drones of Asian honeybees can be statistically divided into two groups: dwarf honeybee drones form one group and the other species belong to the other group. As for workers, the EPI can divide the Asian honeybees into three groups. It is suggested that prowess of flight in drones is driven by the need to compete and mate with queens flying high in the air while worker bees forage nectar and pollen on flowers (Radloff et al., 2003).

Dyer and Seeley (1991b) reported that among Asian species, *A. cerana* show a disproportionately high mass-specific metabolic rate, their foragers make many more trips per day in the same habitat than do foragers of the other species.

Last but not least, different species differ in both the times and temperatures to initiate their collecting trips. *A. cerana* colonies start their work earlier in the day than *A. mellifera* workers and can endure lower ambient temperatures and are more industrious in collecting nectar from scattered flowers, while *A. mellifera* workers tend to prefer big flower patches (Kuang and Kuang, 2002). Oldroyd et al. (1992) investigated foraging competition among four species: *A. dorsata*, *A. cerana*, *A. andreniformis*, and *A. florea* in Thailand on inflorescences of the king palm *Archontophoenix alexandrea*, which produces copious quantities of pollen overnight. Only the earliest visitors can collect the large amount of nectar available just before dawn. In order of appearance, *A. cerana* comes first, followed by *A. dorsata* shortly

after dawn, and less than an hour later they are replaced by *A. florea* and *A. andreniformis* and some stingless bees.

It may be difficult to understand these above mentioned phenomena without some analysis of two important factors: body temperature and energy consumption. Honeybee biologists have noticed that a thoracic temperature threshold is absolutely crucial for a forager to initiate a flight trip (Dyer and Seeley, 1987). A forager can increase her thoracic temperature by producing metabolic heat if the colony temperature is lower than the ambient temperature. The cavity-nesting species have the advantage of maintaining a higher nest temperature, which explains why *A. cerana* foragers begin collecting before dawn and earlier than other open-nesting bees. Fighting and food searching are a high energy consumption tasks, and the bigger the body size, the more energy required. This may be the reason why the giant honeybees can fly further and exploit other flowers rather than fighting against the smaller bees.

1.3 Robbing

Robbing is an act, or a series of acts, by which bees from one colony pilfer or steal honey from other colonies (Ribbands, 1953; Atwal and Dhaliwal, 1969). This differs fundamentally from food competition, which happens outside the nests, because robber bees enter the nests of other colonies, kill bees and take the stores. The smaller the colony the more susceptible it is to the loss of the stores and death of the workers (Hepburn and Radloff, 1998). Usually every colony has some guard bees at the entrance to fight against intruders, and these guard bees are able to distinguish their nestmates by their colony specific odours (Ribbands, 1954, 1955).

Robbing usually occurs in times of dearth when there is not enough available nectar (Hepburn and Radloff, 1998). However, robbing may occur at any time when the nectar flow is interrupted or the colonies become weak or diseased (Atwal and Dhaliwal, 1969). Atwal and Dhaliwal (1969) investigated robbing behaviour between *A. cerana indica* and *A. mellifera* and found that *A. cerana indica* bees are more prone to robbing than *A. mellifera*. But Breed et al. (2007) suggested that robbing may be more characteristic of *A. mellifera* than other species. They compared nestmate recognition in several Asian honeybee species, *A. florea*, *A. andreniformis*, *A. dorsata* and *A. cerana*,

and found that none of these species displayed strong aggressive responses to conspecific non-nestmates. This result indicates that *A. mellifera* has a more strongly developed response to conspecific non-nestmates than other *Apis* species. This conclusion explains what happens in China, when *A. cerana* and *A. mellifera* colonies are kept at the same apiaries. They rob each other during times of dearth, and it has been reported *A. cerana* is more likely to initiate robbing, but they usually lose when *A. mellifera* robs back (Yang, 2001a). Numerous *A. cerana* colonies were killed in this way and lost territory in some areas (Yang, 2001a). Other interspecific instances of robbing were reported by Koeniger (1976a) between *A. florea* and *A. mellifera*, and by Atwal and Dhaliwal (1969) between *A. dorsata* and *A. cerana indica*.

Robbing can also be a means of transmitting bee diseases and parasites, as shown by Atwal and Dhaliwal (1969) who reported that at a mixed apiary in India, under natural conditions, *A. mellifera* were free from acarine disease, but after robbing some weak *A. cerana* colonies, 70-80% of two *A. mellifera* colonies were infested. They also found that the acarine mite could be transmitted under experimental conditions from diseased *A. cerana* colonies to healthy *A. mellifera* colonies.

Although mites occur on several *Apis* species, (Koeniger et al., 1983; Delfinado-Baker et al., 1985; Kuang and Kuang, 2002), interspecific transmission has seldom been reported, except the *Varroa* mites from *A. cerana* to *A. mellifera* (Crane, 1990), and *Neocypholaelaps indica* from *A. cerana* to *A. florea* and *A. dorsata* via foraging on the flowers (Koeniger et al., 1983).

1.4 Intervention of mating

Among the most interesting of the interspecific interactions between the Asian *Apis* species are those arising from the numerous attempts to investigate the intervention of mating. Because queens of all the honeybee species have similar queen pheromones by which drones locate the virgin queens, 9-oxo-2-decenoic acid, or commonly abbreviated as 9-ODA (Butler et al., 1967; Shearer et al., 1970; Koeniger and Wijayagunesekera, 1976), drones from one species might fly after queens of another species and try to mate with them. How queens avoid interspecific mating and therefore evolved into different honeybee species has long been a puzzle.

Intervention of mating among the Asian honeybee species has been widely investigated, and it has been suggested that three factors can lead to mating isolation: differences in male genitalia (Koeniger and Koeniger, 1991), different drone congregation areas (DCA) (Koeniger and Koeniger, 2000), and different mating times (Koeniger and Koeniger, 2000). All of these factors are prezygotic barriers against interspecific mating, and if interspecific mating really occurs or was achieved by artificial insemination, there are also postzygotic barriers that prevent the appearance of hybrid offspring.

1.4.1 Male genitalia

There is an obvious difference in structure of endophalli among the drones of *Apis* (Fig. 1.1), which can undoubtedly lead to reproductive isolation.

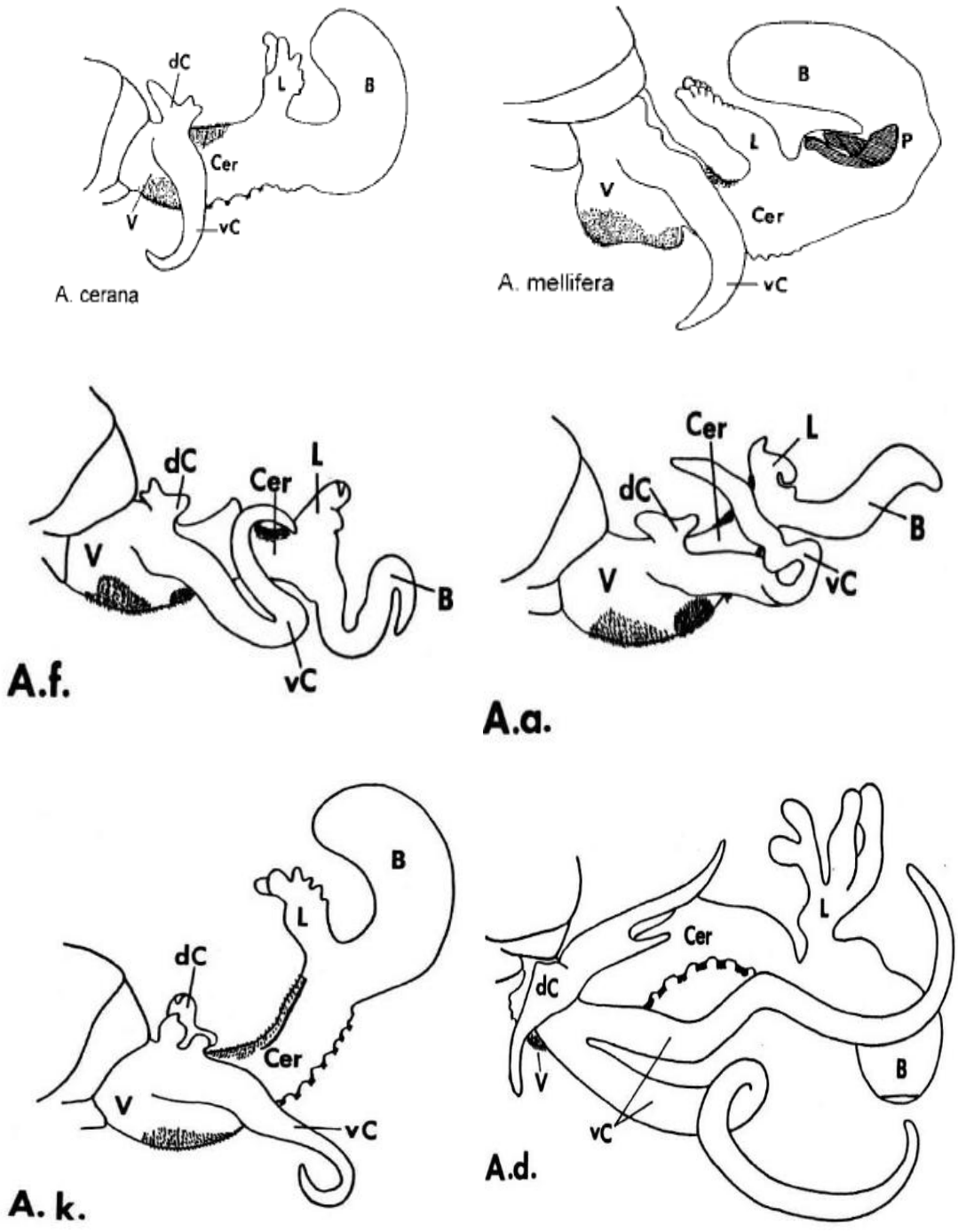


Fig. 1.1 Everted endophalli of *Apis* drones (Koeniger and Koeniger, 1991; Koeniger and Koeniger, 2000). Species: **A.f.** = *Apis florea*; **A.a.** = *Apis andreniformis*; **A.k.** = *Apis koschevnikovi*; **A.d.** = *Apis dorsata*. **B**: bulbus; **Cer**: cervix; **P**: chitinous plates of bulbus; **dC**: dorsal; **L**: lobe; **V**: vestibulum; **vC**: ventral cornua

Differences in body size between queens and drones and differences in drone genitalia among species also occur. When mating occurs in the air, drones have to fly fast enough to catch the flying virgin queen. Queens and drones from different species cannot mate with each other because of their body size differences. The weights of drones and queens of the nine species of honeybee are listed in Table 1.2. There are some crucial species-specific factors that determine the failure of interspecific mating as is shown in Table 1.2 below.

Table 1.2 The key figures of reproduction in *Apis* species

Species	Drone weight mean±S.D.	Total number of sperm per drone ($\times 10^6$)	Queen weight	Sperm in spermatheca of queen ($\times 10^6$)	Sperm length	Mating frequency
A. andreniformis	70.8±3.0	0.13	112	1.3	—	10.5
A. florea	77.6±2.6	—	86	1.1	205.81	7.9
A. cerana	83.4±8.9	1.0±0.1	122	1.4	267.07	14.1
A. koschevnikovi	105.5±5.6	1.7±0.16	170	2.1	—	13.3
A. nuluensis	107.0±6.7	1.3±0.1	—	—	—	—
A. nigrocincta	—	—	—	—	—	40.3
A. mellifera	211.1±11.8	12.7	202	4.7	262.69	11.6
A. dorsata	155.7±8.5	—	290	3.9	218.69	44.2
A. laboriosa	—	—	—	—	—	28.4

Woyke, 1975; Ruttner, 1988; Koeniger et al., 1996a; Koeniger et al.,1996d; Koeniger and Koeniger, 2000; Baer, 2005

According to the data from available reports, as listed in Table 1.2, *A. mellifera* drones produce the greatest number of spermatozoa. It is somewhat strange that the drone of *A. mellifera* is heavier than that of the giant honeybee. Also, the spermatozoa of *A. mellifera* are longer than those of *A. dorsata*. The queens of *A. dorsata* and *A. nigrocincta* have higher mating frequencies than the queens of other *Apis* species.

Besides, there are some other species-specific organs that can prevent the interspecific mating between species. For example, the drones of dwarf honeybees, *A. florea* and *A. andreniformis,* have a basitarsus on their hind legs which serve to clasp the hind legs of the queen during mating. *A. koschevnikovi* drones have a specific sex characteristic of a hairy fringe on the margin of the tibia of the hind leg which also strengthens their connection with the queen during their copulation (Rinderer et al., 1989).

1.4.2 Drone Congregation Area differences

Without exception, all honeybee species mate on the wing. Drones from many colonies gather in a drone congregation area (DCA) to form a drone cloud waiting for virgin queens. Different species and even subspecies have different DCAs. *A. mellifera* drones form their clouds at heights between 5 m and 40 m according to the weather. *A. mellifera carnica* drones form their DCAs higher than those of *A. mellifera ligustica* (Koeniger and Koeniger, 2001). DCAs of *A. cerana* are usually near the top of big trees (Punchihewa et al., 1990). The locations of some species are shown in figure 1.2.

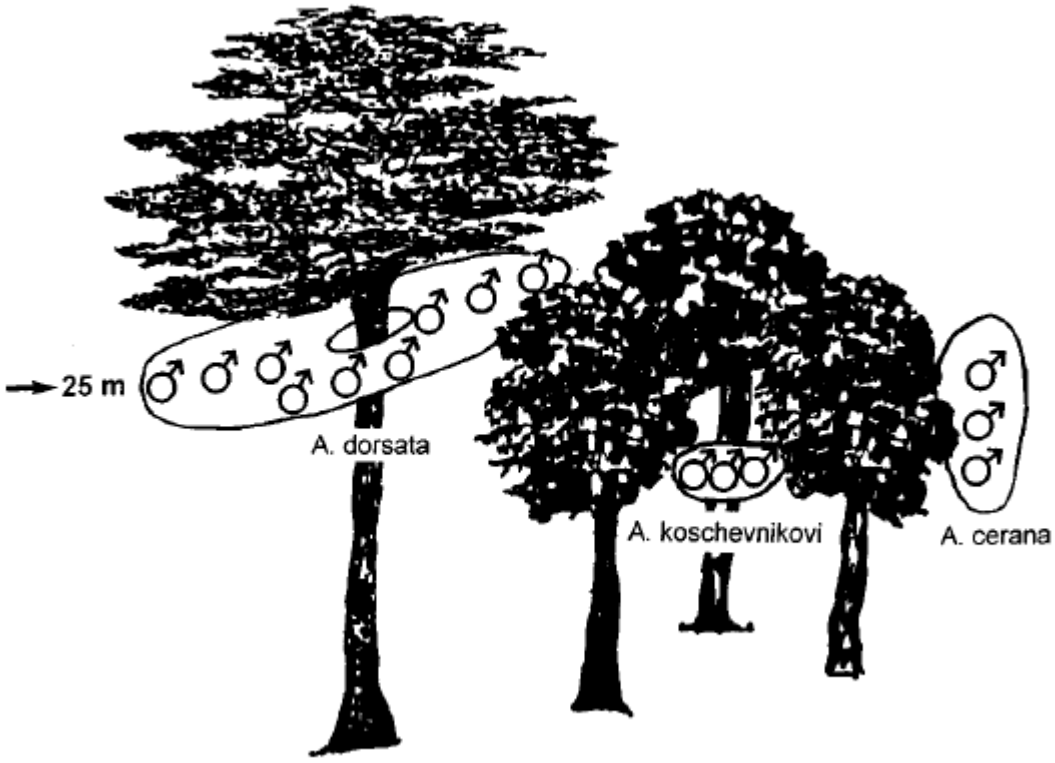


Fig. 1.2 Drone congregation areas (DCA) of three honeybee species: *A. cerana* drones congregate near branches, *A. koschevnikovi* under the thick cover of branches and trees, and *A. dorsata* drones congregate directly under the canopy of high emergent trees (From Koeniger and Koeniger, 2000, in Sabah, Borneo)

1.4.3 Mating times

Although different species of honeybees occurring in the same area tend to rear their new queens nearly at the same season given suitable weather and food resources, the

species have differing mating times. The mating times of several sympatric species in some areas are listed in Table 1.3. In Sri Lanka, where several honeybee species occur, *A. florea* mates earlier than *A. cerana* and *A. dorsata*, while in Thailand, *A. andreniformis* is the earliest, and on Sabah Province of Malaysia, several species there have mating times similar to those they have in Thailand.

Table 1.3 Mating time separation of sympatric honeybee species

Locality	**Sri Lanka**	**Thailand**	**Sabah, Borneo**
Author	Koeniger and	Rinderer et al.,	Koeniger et al.,
Species	Wijayagunesekera, 1976	1993	1996d
A. andreniformis	—	12.15-13.45	12.00-13.45
A. florea	12.00-14.30	14.00-16.45	—
A. cerana	16.15-17.15	15.15-17.30	14.00-16.15
A. koschevnikovi	—	—	16.45-18.30
A. dorsata	18.00-18.45	18.15-18.45	18.15-19.05

Koeniger and Koeniger, 2000

As we can see from Table 1.3, the same species in different locations may differ in mating times, but they do have a clear mating sequence when they are occur with other species: the dwarf species, *A. andreniformis* and *A. florea*, mate early, followed by cavity-nesting and middle size honeybees, *A. cerana* and *A. koschevnikovi*. The drones of *A. dorsata* perform mating flights at dusk at all locations (Koeniger et al., 1994b).

Different male genitalia, different DCAs, combined with different mating times strongly indicate that the Asian honeybees have solved the mating intervention problem in the process of speciation. However, the balance can easily be broken when *A. mellifera* is present, having the same flight time and the same reaction to the sex attractant at the same congregation areas.

It was shown that *A. mellifera* drones actually mate with *A. cerana* queens, though with a noxious effect on the queen. A young *A. cerana* queen was found with its damaged sting chamber firmly blocked by the mating sign of an *A. mellifera* drone (Ruttner and Maul, 1983). And thus it can be concluded that no pre-mating barrier exists between these two species as is the case between other species (Ruttner, 1988). Some researchers found that *A. mellifera* drones fly into the DCA of *A. cerana* and actually copulate with *A. cerana* queens (Yoshida, 1994). In China, it has been reported that when commercial *A. mellifera* apiaries arrived, there was a significantly higher loss rate of *A. cerana* virgin queens during their mating flights. Thus it has even been suggested

that these phenomena can be regarded as a not yet finished stage of speciation (Ruttner, 1988). Moreover, Koeniger (1976a) also inferred that the mating intervention from *A. mellfiera* may exist on *A. florea* in the tropic areas in Asia.

1.4.4 Artificial insemination

Now that interspecific mating can actually happen under natural conditions, one is prompted to pose two questions 1) what happens after such matings occur?, and 2) is there any hybrid offspring produced? None of the eggs hatch because of post-zygotic barriers between the species. Artificial insemination between *A. cerana* and *A. mellifera* has been applied by researchers (Ruttner, 1969, Ruttner and Maul, 1983; Woyke, 1973; Koeniger et al., 1996b; Koeniger and Koeniger, 2000; Phiancharoen et al., 2004), but no hybrids have been obtained thus far. Ruttner, (1988) described the detailed developmental process in eggs laid by the queen after hetero-specific instrumental insemination. The hetero-specific spermatozoa can enter the spermatheca, are able to survive there, and can fertilize eggs. Twenty-four hours after fertilization, cleavage is observed to the blastula stage of the zygote. Then, however, the cell walls start to disintegrate and nuclei migrate into the secondary periplasm to accumulate in the antero-ventral part of the zygote and then to degenerate completely later on. Thus no hybrid larva or imago ever develops.

Yoshida, (1994) used the mixed semen of *A. cerana* and *A. mellifera* drones to inseminate *A. mellifera* virgin queens. By using different mixed ratios of the two specific spermatozoa (approximate spermatozoa concentration ratio of 3 mm^3 of *A. mellifera* semen + 1 mm^3 *A. cerana* semen, 9:2, 2 mm^3 of *A. mellifera* semen + 2 mm^3 of *A. cerana* semen (6:4), 1 mm^3 of *A. mellifera* semen + 3 mm^3 of *A. cerana* semen (3:6) was 79.5%, 53.6% and 26.5%, respectively), he found the hatchability after the queen laid eggs produced only *A. mellifera* workers, interspecific fertilization resulted in non-viable larvae. Koeniger, (1996) reported interspecific hybrids between *A. cerana* and *A. koschevnikovi* produced by artificial insemination have low fertility and the hybrid colonies are probably nonviable.

Phiancharoen et al., (2004) used spermatozoa from drones of four species (*A. mellifera, A. cerana, A. florea* and *A. dorsata*) to respectively inseminate *A. mellifera* queens. They studied survival rate of each specific sperm type and the rate of eggs fertilized by each specific spermatozoon. The results showed that nearly 100% of *A.*

cerana and *A. mellifera* spermatozoa were still alive four weeks after insemination, but the motility of *A. florea* and *A. dorsata* spermatozoa decreased to a large extent, 83.4% and 61.2% respectively, after 3 days and only a small proportion were still alive in the queens' spermathecae. As for fertilization rate, 57% of *A. mellifera* eggs were fertilized by *A. mellifera* spermatozoa, 40% eggs fertilized by *A. cerana* and *A. florea* spermatozoa, while less than 20% by *A. dorsata* spermatozoa. The fluid in the queen's spermatheca played an important role in the survival rate and fertilization success rate of the hetero-specific spermatozoa, but no interspecific hybrid offspring emerged.

1.5 The impact of introduction of *Apis mellifera* to Asia

With the development of a beekeeping industry, honeybees, particularly *A. mellifera*, were introduced into many areas of Asia for such bee products as honey, pollen, royal jelly and propolis, etc. However, as the business benefits from the introduction of *A. mellifera* colonies grew, many problems emerged. As mentioned above, these included foraging competition, mating interference, robbing, and the transmission of disease. The introduction of *A. mellifera* colonies has also had an enormous impact on the native honeybee species in some areas of Asia (Japan: Sakagami, 1959; India: Atwal and Sharma, 1971; China: Ji et al., 2003; Yu and Han, 2003; Yang, 2005; Nepal: Partap, 1998).

A. mellifera was first introduced in China in the 1920s (Kuang and Kuang, 2002), On introduction, this western honeybee proved adaptable to a new environment and produced higher yields of bee products but also royal jelly, and propolis which can not be collected from *A. cerana* colonies because of their extreme low productivity. Since then, this productive species of honeybee began to be widely adopted in Chinese beekeeping.

While enjoying the high profits of these bees, the negative aspects have been widely neglected and few if any had realized the strong impact of *A. mellifera* on the environment and the local honeybees, especially *A. cerana,* until the 1980s. An investigation was launched and conducted by the *A. cerana* Association of China. The results showed that *A. cerana* has become extinct in the Daxing-anling forest areas in the northeast and in Xin Jiang province in the northwest. In the Northeast Plain and

North-China Plain areas, all of the *A. cerana* bees in manmade hives have absconded (Yang et al., 1982). In the whole northeast zone, only in the Changbai mountain areas can *A. cerana* bees be found in wild and man-made hives. The plain of drainage area of the Yangtze River where millions of *A. cerana* colonies were kept in the past are now hard to find. In the southern provinces such as Jiangxi, Hunan, Fujian, Guangdong, Guangxi and Hainan, there are still many *A. cerana* colonies but their distribution area has shrunk greatly. Compared with those areas above, *A. cerana* colonies in the southwest are in a better condition, particularly in mountainous areas where many *A. cerana* bees can be found living in tree holes, caves and man-made hives in Yunnan province and Tibet (Yang et al., 1982).

In conclusion, the introduction of *A. mellifera* caused great losses of *A. cerana* colonies. The population of *A. cerana* colonies is now estimated at not more than one million, a decrease of some 60% compared with the number before the introduction of *A. mellifera* and their distribution has shrunk by 75% (Yang, 2005).

In the case of the introduction of *A. mellifera* in Asia, as early as 1959, Sakagami had noticed the impact of *A. mellifera* on *A. cerana* in Japan. In Nepal, Partap (1998) reported that plants and fruits were in shortage of pollination because of the population decrease of *A. cerana* bees, which was caused by the introduction of *A. mellifera*. And even in Europe, with the rapid development of beekeeping at the beginning of 20th century, many beekeepers preferred to raise some subspecies such as *A. mellifera ligustica* and introduced them from other areas, which caused the local extinction of native subspecies (Ruttner, 1988).

Moritz et al. (2005) recognized the severe disaster caused by the introduction of *A. mellifera* to tropical ecological systems and pointed out that local honeybees or other pollinators suffered from the introduced species through food competition or diseases. This resulted in a reduction of biodiversity and an imbalance of the whole ecological system.

During the mating season, both the virgin queens of *A. cerana* and *A. mellifera* can attract heterospecific drones (Yang, 2001a; Ji et al., 2003; Wang et al., 2003). However, the *A. mellifera* drones, which are much stronger fliers than *A. cerana* drones, can trap the *A. cerana* queens, although they cannot always mate with them successfully because of the differences in copulatory organs (Fig. 1.1). Their encirclement behaviour can inhibit successful mating between *A. cerana* queens and drones. In some areas with very many *A. mellifera* colonies, most of the virgin *A. cerana* queens were trapped by *A.*

mellifera drones, and only 16% of *A. cerana* queens were able to mate successfully. More than 80% *A. mellifera* queens could successfully mate with conspecific drones although there was interference by *A. cerana* drones (Wang et al., 2003). This resulted in the population decline of *A. cerana* bees in some areas in recent years. In some areas they are threatened because their declining population is insufficient to support the community and honeybees are dying out. The decrease or extinction of the native honeybees is a definite threat to the balance of ecology and some plant species could also become extinct because of insufficient pollination (Yang, 2005).

1.6 Mixed-species colonies

The cavity-nesting honeybee species share several common morphological and behavioural characters and can be kept in the same colonies with heterospecific queens. Thus far, three types of mixed-species colonies: *A. cerana* with *A. koschevnikovi*, *A. cerana* with *A. nuluensis*, and *A. cerana* with *A. mellifera* have been successfully organized experimentally. Recently, in Thailand, a super-mixed colony of *A. florea*, *A. mellifera*, *A. cerana* and *A. dorsata* was set up, but only lasted several weeks and then absconded all together. No biological research has been done with this kind of super mixed colony (Phiancharoen, pers. comm.).

1.6.1 Mixed colonies of *Apis cerana* and *Apis koschevnikovi*

Mixed colonies of *A. cerana* workers with an *A. koschevnikovi* queen were organized by Koeniger et al. (1996c). They grafted young larvae of *A. cerana* and *A. koschevnikovi* simultaneously into artificial queen cells and inserted them into queenless colonies of either *A. cerana* or *A. koschevnikovi.* Not unexpectedly, all colonies preferred to rear conspecific larvae, but *A. cerana* colonies seemed more selective than *A. koschevnikovi* colonies against heterospecific larvae. Only 4% (4 of 102) *A. koschevnikovi* queens successfully emerged from *A. cerana* colonies, while 30 out of 140 (21%) *A. cerana* larvae developed into adult queens in an *A. koschevnikovi* colony.

To set up mixed colonies, nearly emerging virgin queens in queen cells of either of the two species were introduced into hetero-specific queenless colonies. In *A.*

koschevnikovi colonies, all of the *A. cerana* queen cells were destroyed and the queens were killed; while a few (4 out of 18) *A. koschevnikovi* queens were accepted by the *A. cerana* colonies and three of them succeeded in mating and laying eggs. Although these queens were in heterospecific colonies, they mated with their own specific drones. Interestingly, the drones of *A. koschevnikovi* can also find their own species-specific mating times even when they were reared in *A. cerana* colonies (Koeniger et al., 1994a). The mated *A. koschevnikovi* queens laid eggs and the emerged bees were successfully reared by *A. cerana* worker bees, thus the *A. cerana* host colonies were gradually transformed into *A. koschevnikovi* colonies.

1.6.2 Mixed colonies of *Apis cerana* and *Apis nuluensis*

de Guzman et al. (1996) set up a mixed colony of *A. cerana* and *A. nuluensis* containing brood combs and adult bees from one colony of *A. nuluensis* from one of the high mountains of Sabah, Malaysia in Borneo into a queenless of *A. cerana* colony 200km away. It was unusual that the adult workers did not attack each other. The authors investigated only the *Varroa* mites in this mixed colony and *Varroa jacobsoni* Oudemans and *Varroa underwoodi* were found. There have been no further reports about this kind of mixed colony.

1.6.3 Mixed colonies of *Apis cerana* and *Apis mellifera*

A. cerana and *A. mellifera* are very closely related and very similar both in morphology and behaviour to the extent that there was once doubt if they were distinct species (Ruttner and Maul, 1983). Researchers and beekeepers have long wanted to hybridize them. For example, Atwal and Sharma (1968) introduced *A. mellifera* queens into *A. cerana* colonies and found that the introduction was successful if the *A. cerana* workers were no more than a week old. The introduced queen could lay eggs in the host colonies and the eggs hatched into larvae and *A. cerana* workers attended them and they pupated and emerged as adults. Once the *A. mellifera* workers assumed field duties, they worked in harmony with the host *A. cerana* workers.

Studies show that young worker bees may lack pheromones and can be accepted by other colonies (Pham-Delegue et al., 1993; Laloi et al., 2001). So it is possible to exchange brood combs between colonies: firstly, previously prepared empty combs

were added to strong *A. mellifera* colonies which were then checked every day until the combs were filled with eggs so the emergence date for adults can be calculated. These brood combs are kept in nurse colonies until the adult bees are just about to emerge and then removed and introduced into queenright *A. cerana* colonies. When they emerge, the numbers of adult workers of the two species are about even, and no fighting was seen on opening the mixed-species colonies nor were dead *A. mellifera* workers found at the entrances.

Queen rearing

Tan et al. (2006) studied queen rearing in mixed colonies to assess the effect of food on the development of offspring. *A. cerana* larvae were grafted for queen rearing into two of these mixed-species colonies. Similarly, *A. cerana* larvae and *A. mellifera* larvae were also grafted conspecifically as controls. The success rate of *A. cerana* queen rearing in the test colonies was 64.5%, surpassing all previous attempts at interspecific queen rearing, in which single-species host colonies were used (Oschmann, 1965; Dhaliwal and Atwal, 1970; Oku and Ono, 1990; Potichot et al., 1993). After emergence, all virgin queens obtained from the three groups (N=90) were measured morphometrically. The *A. cerana* queens from the mixed-species colonies differed significantly in size and pigmentation from the *A. cerana* control queens and closely approximated the *A. mellifera* queens. It is inferred that these changes in the *A. cerana* queens reared in the mixed-species colonies can be attributed to feeding by heterospecific nurse bees and/or chemical differences in royal jelly, the data showed a strong impact of environment on the development of queens. The results further suggest that in honeybees the cues for brood recognition can be learned by heterospecific workers after eclosion.

Retinue behaviour

The retinue behaviour of worker bees of *A. cerana cerana* and *A. mellifera ligustica* in two types of mixed-species colonies was studied (Yang et al., 2009). In mixed colonies headed by an *A. cerana* queen almost equal numbers of *A. cerana* and *A. mellifera* workers attended the *A. cerana* queen; while in mixed colonies headed by an *A. mellifera* queen significantly fewer *A. cerana* workers were attracted than *A. mellifera*

workers. The pheromones 9-ODA, 9-HDA and 10-HDA of the queens were significantly different and the workers did not show avoidance behaviour to either hetero-specific queen. Both species of workers were attracted by the queens and engaged in retinue behaviour, suggesting that the retinue response was not related to a specific queen pheromone or colony environment. This non-specific queen retinue behaviour in the mixed colonies indicates that the queen pheromones can be transmitted among the workers from the two species without any obstacles. We conclude that retinue behaviour itself, as well as the pheromones of the queens, that induce this behaviour are both primitive, conserved traits that preceded speciation in apine bees.

Ovary activation

The workers in mixed colonies show different degrees of ovarian activation. *A. cerana* workers showed significantly greater ovarian activation in queenright mixed-species colonies than in conspecific queenright colonies. There was significantly greater ovary activation in *A. cerana* workers in mixed-species colonies headed by *A. mellifera* queens than *A. mellifera* workers in mixed-species colonies headed by *A. cerana* queens. *A. mellifera* workers in conspecific queenless colonies showed significantly greater ovarian activation than those in mixed-species queenless colonies. Quantification of the chemical components of mandibular gland pheromones of queens of the two species showed that they are similar. Combined, the results show that although queen signals have been preserved between the two species, the threshold of queen pheromone necessary to suppress ovary activation in *A. cerana* is higher than that for temperate *A. mellifera* (Tan et al., 2009).

Interspecific communication

Among the most interesting of the interspecific interactions between *A. cerana* and *A. mellifera* workers in the same colony is the mechanism of interspecific communication. Honeybees have a dance language by which information about food resources can be transferred from successful foragers to nestmates (von Frisch, 1967; Dyer, 2002).

The question arises: can the dance followers of one species understand the dances performed by the foragers of the other species although the structure of the dance language is very similar among species of honeybees? (Lindauer, 1956). Studies have

shown that the dance language not only differs among species in the genus of *Apis*, but different races of the same species may also have dialects (Steche, 1957; Sarma et al., 2004). For example, Lindauer (1956) observed the Asian species *A. cerana, A. florea,* and *A. dorsata* and reported that there were differences in the distance at which dancers changed from round dances to waggle dances. The transition distance was much closer for the Asian species, e.g. he reported that *A. florea* started waggling when the feeder was only 5 m from the hive. Lindauer (1956) and Boch (1957) also reported interspecific/inter-racial differences in the dance tempo (dance circuits per 15 s) at a given distance. For the same distance, different races or species would execute a different number of circuits per unit time.

Thus, the concept of dialects in the honeybee dance language was established which basically pointed to two differences in the dances by different species and races, firstly in the flight distance at which the dancers start performing waggle dances instead of round dances, and secondly in the circuit duration of the waggle dance performed by dancers for a given flight distance.

So we understand that although the structure of the dance language is very similar among species of honeybees, communication of the distance component of the message varies both intraspecifically and interspecifically. However, it is not known whether different honeybee species would attend interspecific waggle dances and, if so, whether they can decipher such dances. So far, two reports have tried to answer this question, and both found that *A. cerana* foragers could decode the dances of *A. mellifera* to successfully locate an indicated food source, by using mixed-species colonies of *A. cerana* and *A. mellifera* (Su et al., 2008; Tan et al., 2008). More recently, Tan et al. (2008) found that *A. mellifera* foragers can also be recruited to the experimental feeder by *A. cerana* dancers.

Comb building cooperation

Cooperation in comb building in mixed colonies has also been investigated (Yang et al., 2010a) Two types of cell size (*A. cerana* and *A. mellifera*) foundation made from wax of these two species were inserted into mixed colonies to study cooperation in comb construction. The mixed colonies did not discriminate between the wax types, but the *A. cerana* cell-size foundation was modified during comb building by the cooperative efforts of the workers of both species. In pure *A. cerana* colonies workers did not accept

any foundation, but were stimulated by *A. mellifera* workers to secrete wax and build on the foundation in mixed colonies. The task of comb building is actually performed by small groups of workers of the two species. In this way, the two species cooperate in comb building and can construct nearly normal combs, even though they contain many cells of irregular shapes (Yang et al., 2010a).

Thermoregulation

A. cerana and *A. mellifera* normally display different strategies in cooling their nests, raising the question whether they would coordinate their efforts to achieve stable thermoregulation in mixed colonies. The results show that the normal temperatures in the brood area in mixed colonies are more similar to those of pure *A. cerana* colonies than pure *A. mellifera* colonies. Under heat stress, *A. cerana* workers are more sensitive, and initiate fanning earlier than *A. mellifera* workers. In mixed colonies, the former become the main force for thermoregulation. When worker bees of both species were fanning together at the entrance, their own species-specific postures were adopted, but due to a significantly smaller number of *A. mellifera* workers engaged in fanning, the cooling efficiency of mixed colonies were closest to that of pure *A. cerana* colonies (Yang et al., 2010b).

Defense behaviour

When vespine wasps hawk honeybees at their nest entrances, alerted and poised guard bees of *A. cerana* and *A. mellifera* in the mixed colonies have average thoracic temperatures slightly above 24^{o}C. *A. cerana* workers assume their species-specific wing shimmering and raise their body temperature up to about 29^{o}C, while *A. mellifera* guard bees neither show significant body temperature increase nor wing shimmering. However, when faced with persist hawking wasps, guard bees of both species raise their thoracic temperatures and form a ball around it, the core temperature of the mixed-species balls were about 45^{o}C, which is not significantly different from the heat ball made up by only pure species. *A. cerana* bees engulf the ball tighter in the inner space while *A. mellifera* bees can be seen more likely roaming at the outer space. This result shows that the defense behaviours of the two species are based on their species-specific adaptations in the evolutionary background (Tan et al, 2010).

In conclusion, mixed colonies offer us a unique probe to study interspecific relations between species of honeybees. Behaviours in the mixed colonies confirm that these two species *A. cerana* and *A. mellifera* are indeed very closely related species. Also, it provides us more information about these two societies. It may also prove useful in finding a way to solve the problem following the introduction of western bees in Asia.

This thesis reports on experiments on some interspecific interactions between mixed-species colonies of the honeybees, *A. cerana* and *A. mellifera.* These include 1) retinue behaviour, 2) ovarian development; 3) communication; 4) comb building; 5) thermoregulation; and 6) defensive behaviour.

CHAPTER 2

Queen retinue behaviour of the workers in mixed colonies

Summary

In honeybee colonies, the queen is always circled by several workers, called retinue bees, and they perform two tasks: attending the queen, providing food and keeping her body clean, and grooming her with their antennae. Both of these tasks play a very crucial role in maintaining the harmony of honeybee colonies; the former keeps the queen healthy and the latter serves as the main way of transmitting queen pheromones to all individuals in the colonies. In this study, we investigated the retinue behaviour of workers to hetero-specific queens in mixed colonies. Such studies can give some indication whether observed behaviours are pre- or post-speciation developments. In *Apis cerana* queen-led mixed colonies, almost equal numbers of *A. cerana* workers (53.4±7.4) and *A. mellifera* workers (51.2±8.1) attended the *A. cerana* queen; while in *A. mellifera* queen-led mixed colonies, the *A. mellifera* queen attracted significantly fewer (47.8±5.9) *A. cerana* workers than *A. mellifera* workers (51.9±4.6). Thus about 100 workers in total were attracted to each queen. In pure *A. cerana* and *A. mellifera* colonies, the queen attracted 105.8±9.1 and 107.8±11.2 workers, respectively, there being no significant difference between them. Only the pheromones 9-ODA, 9-HDA and 10-HDA of the queens were significantly different and the workers did not show avoidance behaviour to either hetero-specific queen. Both species of workers were attracted by the queens and engaged in retinue behaviour, suggesting that the retinue response was not related to a specific queen pheromone or colony environment. This non-specific queen retinue behaviour in the mixed colonies indicates that the queen pheromones can be transmitted among the workers from the two species. We conclude that retinue behaviour itself as well as the pheromones of the queens that induce this behaviour are both primitive, conserved traits that preceded speciation in apine bees.

2.1 Introduction

Honeybees, like other social insects, have a definite reproductive division of labour among female members of a colony in which queens monopolise egg-laying while worker bees perform almost all of the other tasks within and outside of their colonies (Rösch, 1927; Strauss et al., 2008). Worker bees have only vestigial ovarioles which remain inactive in the presence of pheromonal signals of the queen (Winston 1987; Slessor et al., 2005) as well as from larvae (Winston 1987; Le Conte et al., 2001; Arnold et al., 1994). Almost all workers remain ovarially inactive and perform other non-reproductive tasks.

The queen's signal includes the queen mandibular gland substance or pheromone (QMS or QMP) and Dufour's gland secretions, both of which, when combined, are responsible for attracting worker bees to form a retinue around the queen (Slessor et al., 1988; Kaminski et al., 1990; Pankiw et al., 1995; Katzav-Gozansky, 2003). Both of these two queen substances are licked up from the queen by the retinue bees that surround her and then distribute them trophallactically among the bees of the colony (Butler, 1954; Sakagami, 1958; Velthuis, 1972; Seeley, 1979; Naumann et al., 1991; Pankiw et al., 1995), and in this way every worker bee in the colony can sense the presence of the queen.

Retinue behaviour is fundamental and crucial to the biology of social insects because queens must be attended to ensure functionality of colonies. "Retinue" behaviour [= "court behaviour", Allen, 1955, 1960; = "attending behaviour", Sakagami, 1958; Velthuis, 1972] refers to the behaviour of worker honeybees, *Apis spp.*, that form a loose circle facing in toward their queen. These bees feed and frequently lick her, but soon leave the circle to be replaced by others (Ribbands, 1953; Allen, 1955). As the queen moves over the comb, most of the workers who encounter her show a distinct interest, and extend their antennae and palpate her (Butler, 1954; Sakagami, 1958). Naumann et al. (1991) demonstrated that these retinue bees obtain pheromones from the body of the queen and pass them trophallactically to other workers. Although the wax combs in honeybee colonies also play a role in queen pheromone transfer (Hepburn, 1998), the retinue bees are the principal transmitters (Naumann et al., 1991). Consequently, as the first group of receivers and messengers, the retinue bees play a key

role in pheromonally transmitting the queen's presence throughout the colony (Seeley, 1979; Naumann et al., 1991; Pankiw et al., 1995).

The pheromones of a queen which attract workers and induce retinue behaviour include secretions from the mandibular glands and Dufour's gland (Slessor et al., 1988; Kaminski et al., 1990; Pankiw et al., 1995; Katzav-Gozansky et al., 2003). In retinue bioassays with *A. cerana* workers, only three constituents of the mandibular gland pheromones were sufficient to elicit full retinue behaviour (Plettner et al., 1997). Although *A. cerana*, is a sister-species of *A. mellifera*, having diverged only about 3 million years ago (Arias and Sheppard, 1996, 2005), some behavioural traits and morphological characteristics of the two species are very similar indeed, and, clearly are highly conserved, pre-speciation traits. Among them are pheromones of their respective queens, which share most, but not all, chemical constituents of the mandibular glad pheromonal bouquet (Plettner et al., 1997). For example methyl oleate, coniferyl alcohol, and linolenic acid appear unique to *A. mellifera* (Keeling et al., 2003). However, reciprocal assays to assess whether retinue behaviour can be induced within a heterospecific context, such that *A. cerana* queens attract *A. mellifera* workers and *A. mellifera* queens *A. cerana* workers remain to be performed.

Mixed-species colonies offer an intriguing model to investigate the behavioural relationships of the two species, and to suggest which features are ancestral to the common ancestor of *A. cerana* and *A. mellifera* and which may have preceded speciation. As examples, while there are dialectical differences in the waggle dances of different species (Lindauer, 1957; Dyer and Seeley, 1991a; Dyer, 2002), it has recently been demonstrated independently that heterospecific dance communication is operative in both *A. cerana* and *A. mellifera* (Su et al., 2008; Tan et al., 2008). Returning to pheromones, it remains to be seen whether heterospecific retinue behaviour is shared in *A. cerana* and *A. mellifera*, and if so, would such behaviour aid in the dispersal of the queen pheromones, or would the 'guest' species in such mixed colonies avoid the host queen in order to escape pheromonal control (Moritz et al., 2001; Neumann and Moritz, 2002). A plausible theoretical background for possible differences in heterospecific retinues would lead to the hypothesis that we would expect no differences in the proportions of *A. cerana* and *A. mellifera* workers attending heterospecific queens versus conspecific queens. In which case, a complete lack of differences would indicate that retinue behaviour had developed prior to speciation; and small differences would

indicate very recent changes in the system. The results could indicate whether any aspects of retinue behaviour are pre- or post-speciation developments.

2.2 Materials and methods

2.2.1 Honeybee colonies

The experiments and observations were conducted with colonies of *Apis cerana cerana* and *Apis mellifera ligustica* at an apiary of Yunnan Agricultural University, Kunming, China. In order to avoid differing amounts of queen pheromones owing to possible age effects, all queens tested were between 300-330 days old (Pankiw et al., 1995) and all queens had headed their colonies for 10 months.

2.2.2 Organization of the mixed colonies

Two types of mixed colonies were established: mixed colonies containing worker brood of both *A. cerana* and *A. mellifera* and were headed by *A. cerana* queens; and mixed colonies containing worker brood of both *A. cerana* and *A. mellifera* and headed by *A. mellifera* queens. Sealed brood of each species about to emerge as young adults was introduced into the colonies of the other species. Four colonies each of *A. cerana* and *A. mellifera* with an active egg-laying queen and populations of medium strength (4000-6000 workers for *A. cerana* and 6000-8000 individuals for *A. mellifera*) were chosen as parental colonies to maintain the sealed pupae until emergence. One empty comb and another one with pollen and honey were added to each of these colonies. The colonies were checked daily and the time when the empty combs had been filled with newly laid eggs was recorded so we knew when the developing bees would eclose as young adults. These combs were kept in the parental colonies until they developed into capped pupae and were then transferred into incubators.

Then the three *A. mellifera* and three *A. cerana* colonies were chosen as host colonies for establishing mixed-species colonies. These colonies were small, about 1500 individuals, mostly young adults (the older field bees having been eliminated by relocating the hives). These host colonies also had equal numbers of their own sealed pupae about to emerge, so a cohort of workers of the same age of both species could be

obtained at the same time. Three days before the young adults would emerge, these brood frames were introduced into hetero-specific host colonies i.e. one *A. mellifera* comb was put into each of the three *A. cerana* colonies and one *A. cerana* comb into each of the *A. mellifera* colonies. Newly emerged young adult bees are readily accepted by the host colonies and so the mixed colonies are constituted (Tan et al., 2006). Three pure *A. mellifera* colonies and three *A. cerana* colonies served as control groups and each contained comparable numbers of newly emerging adult workers of the same age as those which were introduced into interspecific colonies.

Although the mixed colonies were constituted by an unequal number of host (adult + emerging) and introduced (only emerging) workers, this ought not to have an effect on retinue composition because queen attendance by workers is strongly age-dependent, with 3–9 days being the age range for intense contact with the queen (Seeley, 1979).

2.2.3 Monitoring the retinue behaviour in the mixed-species colonies

Once the mixed colonies were settled, the introduced workers were adults about a fortnight old. In our observations, only those workers that attended a queen for at least 5 seconds were regarded as retinue bees (modified from Pankiw et al., 1995) because the queens were allowed to roam freely on the frames. Queen retinue behaviour of the workers was recorded with a video camera for five minutes in each of the mixed and control colonies once a day for seven days. Therefore, it was possible to very accurately count the numbers of bees of each species in a particular retinue at any given time. Using a 5 second contact paradigm for retinue bee recognition and a viewing window of 5 minutes over 7 days, the retinue data set was just about 420 observations per colony. We took the videos between 14:00-17:00 in the afternoon.

The queens were allowed to roam freely on the comb during which one group of retinue bees were left behind and new ones formed a new retinue circle. The colonies were kept in normal standard hives so that we were able to take videos only by opening the hive and taking out the combs carefully, but no matter how gentle we were, all the queens stopped egg-laying and were seen roaming in our video clips. So, the five minutes cumulative numbers are obviously greater than what one might see at any instant. Therefore numbers were derived from worker turnover around the queen. We did not mark the bees individually in the hive because we could not know which bees

would join a retinue, but we were able to eliminate pseudo-replication counts by replaying the video clips at a lower speed.

2.2.4 Pheromone analysis

After the experiment, the queens from the two types of mixed colonies were decapitated and the mandibular pheromones were extracted in 200 µl dichloromethane (DCM). The samples were then evaporated to dryness under a stream of nitrogen. The residues were re-dissolved in 10 µl of an internal standard solution (octanoic acid and tetradecane in dichloromethane; 0.38 and 0.25 mg/ml, respectively) and 10µl of derivatizing agent (bis-trimethylsilyltrifluoroacetamide). One µl of this solution was injected into a gas chromatograph (Hewlett Packard 6890) using routine analytical conditions (Dietemann et al., 2006). The following components, 9-keto-(E)-2-decenoic acid (9-ODA; "queen substance"), 10-hydroxy-(E)-2-decenoic acid (10-HDA; "worker substance"), methyl *p*-hydroxybenzoate (HOB), 10-hydroxydecanoic acid (10-HDAA), and 9-hydroxy-2-(e)-decenoic acid (9-HDA) were quantified using peak areas and the relative mass ratios calculated relative to tetradecane (Dietemann et al., 2006). The amount of 9-ODA relative to other components was quantified as 9-ODA / (9-ODA + 10-HDA + 10-HDAA). This ratio is an index of the 'queenliness' of honeybee mandibular pheromone: queens have a greater proportion of 9-ODA whereas workers have a greater proportion of 10-HAD (Hoover et al., 2005; Moritz et al., 2000).

2.2.5 Data analysis

Independent samples t-tests were used to compare the mean number and proportions of the retinue workers to different queens. Homogeneity of the variances between groups was checked using Levene's test. Differences in the proportions of each component of queen pheromones were tested using independent T-tests, and a multivariate ANOVA test was used to test for overall differences in mandibular gland components between *A. cerana* and *A. mellifera* queens. The means and standard deviations of each variable were calculated. All tests were performed using Statistica© (StatSoft, 2008).

2.3 Results

2.3.1 Queen retinue behaviour between colonies

In *A. cerana* queen-led mixed colonies, almost equal proportions of *A. cerana* workers (0.51±0.04) and *A. mellifera* workers (0.49±0.04) attended the *A. cerana* queens (Fig. 1) and the results were not significantly different (t = 1.32, df = 20, P = 0.202; Table 2.1). In *A. mellifera* queen-led mixed colonies a significantly smaller proportion of *A. cerana* workers, (0.48±0.04), than *A. mellifera* workers, (0.52±0.04), attended the *A. mellifera* queen (Fig. 2) (t = 2.71, df = 20, P = 0.014; Table 2.1).

Comparing the numbers of retinue bees on different types of queens, in *A. cerana* queen-led mixed colonies, 53.4±7.4 *A. cerana* workers were attracted by the *A. cerana* queen, whilst *A. mellifera* queens in *A. mellifera* queen-led mixed colonies attracted significantly fewer *A. cerana* worker bees (47.8±5.9, t = 2.74, df = 40, P = 0.009). As for the *A. mellifera* worker bees, 51.2±8.1 attended *A. cerana* queens and 51.9±4.6 attended *A. mellifera* queens, there was no significant difference (t = 0.33, df = 40, P = 0.744).

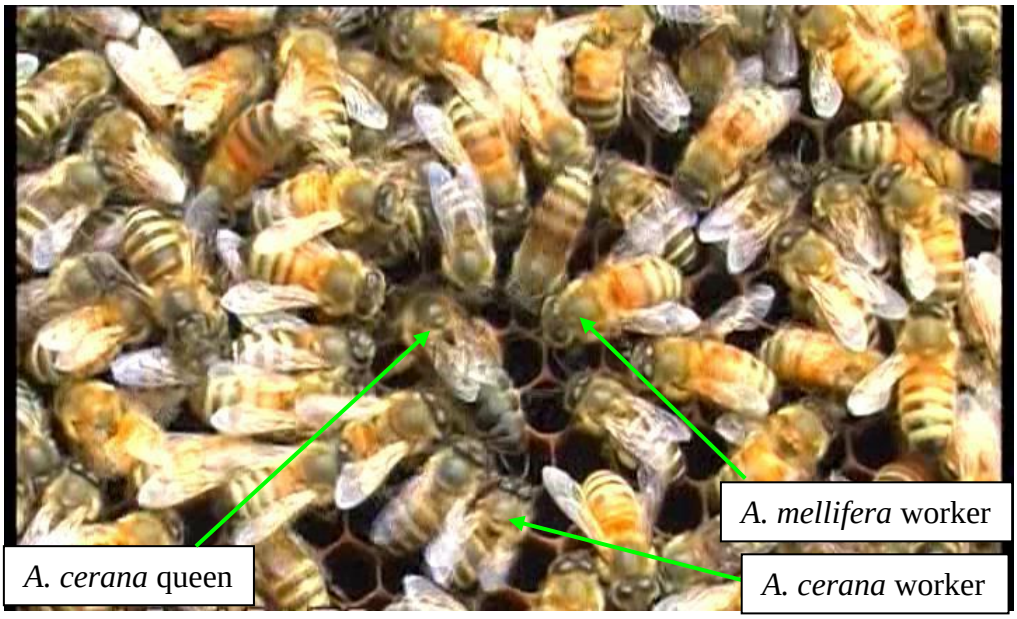


Fig. 2.1 *A. cerana* queen attended by *A. mellifera* and *A. cerana* worker bees in an *A. cerana* queen-led mixed colony

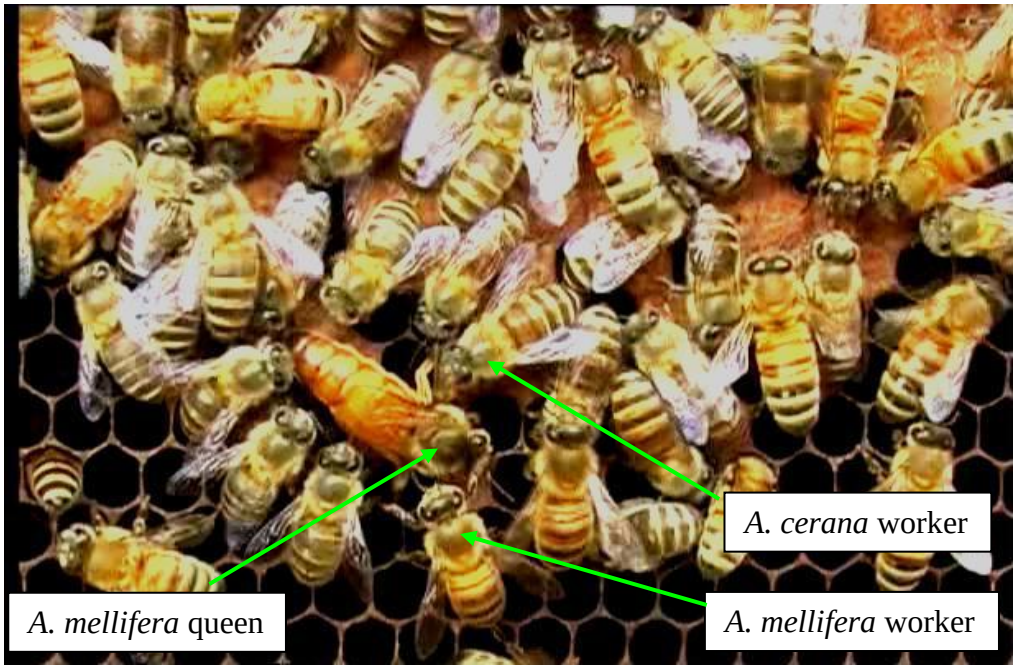


Fig. 2.2 *A. mellifera* queen attended by *A. cerana* and *A. mellifera* worker bees in an *A. mellifera* queen-led mixed colony

Table 2.1 Mean number and proportion (±S.D.) of retinue bees attracted to the queens for each group

Colony	***A. cerana* queen-led mixed colonies**		***A. mellifera* queen-led mixed colonies**		***A. cerana* pure colonies**	***A. mellifera* pure colonies**
	A. cerana retinue	*A. mellifera* retinue	*A. cerana* retinue	*A. mellifera* retinue	*A. cerana* retinue	*A. mellifera* retinue
1	56.4±6.4	54.9±6.4	49.4±6.6	50.4±3.1	101.9±4.9	103.9±7.6
2	46.6±4.6	42.9±4.7	44.4±5.7	51.0±5.0	109.1±10.0	107.3±12.5
3	57.3±6.0	55.9±5.9	49.4±4.6	54.1±5.1	106.4±11.0	112.1±12.6
$\bar{X}$ ± S.D.	53.4±7.4	51.2±8.1	47.8±5.9	51.9±4.6	105.8±9.1	107.8±11.2
Proportion	0.51±0.04	0.49±0.04	0.48±0.04	0.52±0.04	—	—

When the total numbers of workers (*A. cerana* + *A. mellifera*) attracted to a retinue of *A. cerana* queens were compared with those attracted to *A. mellifera* queens, in *A. cerana* queen-led mixed colonies, an average of 104.6±13.3 workers were observed in retinues around the *A. cerana* queen, while in *A. mellifera* queen-led mixed colonies, the *A. mellifera* queen attracted 99.7±7.8 retinue bees. The values are not significantly different (t = 1.49, df = 40, P = 0.145). In pure *A. cerana* and *A. mellifera* colonies, the

queen attracted 105.8±9.1 and 107.8±11.2 workers, respectively, there being no significant difference between them (t = 0.62, df = 40, P = 0.538).

There was a significant difference between the mean number of workers that *A. mellifera* queens attracted, 99.7±7.8, in *A. mellifera* queen-led mixed colonies and *A. mellifera* queen attracted in pure colonies, 107.8±11.2 (t = 2.74, df = 40, P = 0.009). There was no significant difference between the mean number of workers that *A. cerana* queens attracted, 104.6±13.3 in *A. cerana* queen-led mixed colonies and *A. cerana* queen attracted in pure colonies, 105.8±9.1, (t = 0.34, df = 40, P = 0.736). A final point of interest is that workers showed no ovarian activity or egg-laying under the host queens.

2.3.2 Pheromones

The proportional values of the pheromonal components of the queens in the two types of mixed colonies were analyzed, and the results are shown in Table 2.2. The results of multivariate ANOVA procedures to test for differences in proportional values of mandibular gland components between *A. mellifera* and *A. cerana* showed a significant overall difference (Wilk's lambda: F = 741.6, df = 4,1, P = 0.027; Table 2.2). Two of these components, HOB and 10-HDAA did not differ between species; however, 9-ODA, 9-HDA and 10-HDA differed significantly (9-ODA: t = 6.5, df = 4, P = 0.003; 9-HDA: t = 7.4, df = 4, P = 0.002; 10-HDA: t = 3.5, df = 4, P = 0.024). The ratio of pheromonal components 9-ODA/(9-ODA+10-HDA+10-HDAA) was significantly higher in *A. cerana* queens than in *A. mellifera* queens (t = 3.0, df = 4, P = 0.041; Table 2.2).

2.4 Discussion

2.4.1 Queen pheromones

Our results show that the proportional values of three of the pheromonal components from *A. mellifera* and *A. cerana* queens (9-ODA, 9-HDA and 10-HDA) differed significantly (Table 2.2). The proportional values for *A. mellifera* queens obtained here

are within the range of those reported in the literature (Crewe and Velthuis, 1980; Slessor et al., 1988; Naumann et al., 1991; Pankiw et al., 1995; Hoover et al., 2003)**.** Quantitative analysis of the amounts showed that *A. cerana* queens have significantly less of the QMS components than *A. mellifera* (Table 2.2). This result is consistent with previous investigations (Plettner et al., 1997; Free, 1987). These results confirm that there are high levels of variation between individuals, and possibly between different races. Possible environmental effects in the production of queens' pheromones are ruled out because comparisons of the heterospecific queen pheromones do not differ from those of normal queens for each species.

We argue that the basic queen signalling mechanism is conserved and queen pheromones and retinue formation preceded speciation in *Apis* because workers of both species respond to heterospecific queens. However, there is a pheromonal nuance because *A. cerana* workers responded less to *A. mellifera* queens and there are significant differences in the proportions of 9-ODA, 10HDA, 9HDA and in the ratio of 9-ODA/(9-ODA+10-HDA+10-HDAA) that could have led to differences in retinue responses. The queen pheromones appear to be quantitatively different between queens and could be 'interpreted' as different pheromonal "dialects". This would appear to be a parsimonious explanation for the differences in the attractiveness of queens for *A. cerana* workers, but begs the question for the *A. mellifera* workers. Nonetheless, this leaves unanswered questions such as 1) What does it mean if retinues of similar proportions are measured in the two species while the queens of one of these species produces more pheromone?, and 2) Why do *A. mellifera* queens attract fewer workers in mixed colonies compared to pure colonies?

2.4.2 Queen retinue behaviour

Workers form a retinue around the queen in all honeybee species thus far examined (Verheijen-Voogd, 1959; Free, 1987; Plettner et al., 1997). But, bioassay-guided identification of retinue-active compounds has only been done in *A. mellifera* (Kaminski et al., 1990; Plettner et al., 1997; Keeling et al., 2003). So, the exact compounds responsible for retinue behaviour in *A. cerana* are unknown (Plettner et al., 1997, Keeling et al., 2001). Under experimental conditions, Plettner et al. (1997) found that the retinue response of *A. cerana* workers to QMP blends with and without HVA did not differ significantly, suggesting that HVA is not required for maximal worker

attraction in *A. cerana*. However, this result can not exclude the possibility that this component is not necessary for *A. mellifera* workers to be attracted to exhibit retinue behaviour. Because cuticular hydrocarbons also play a role in the recognition systems of insects (Singer, 1998), and especially so in honeybees (Breed, 1998) this possibility must be addressed. We discount any importance of cuticular hydrocarbons in retinue behaviour in this case because queens being superseded do not attract retinues because of a pheromonal insufficiency (Slessor et al., 1988) while pheromonally queen-like workers (pseudoqueens) do (Moritz et al., 2000).

In our study, we tested the responses of workers of both species to hetero-specific queens, and found that three pheromonal components of the queens were significantly different, 9-ODA, 9-HDA and 10-HDA (cf. Table 2.2). The other compounds of the QMP are very similar, and the workers did not show any obvious avoidance behaviour to either of the hetero-specific queens. Both species were attracted by the queens, engaged in retinue behaviour, licked the queens and showed normal grooming and feeding behaviour. These results suggest that the retinue response was not related to a specific queen pheromone or colony environment, and this is consistent with the results of other investigations (Pankiw et al., 1994; Hoover et al., 2005). This non-specific queen retinue behaviour in the mixed colonies indicates that the queen pheromones can be transmitted among the workers from the two species without any obstacles, irrespective of possible "suppressive agents" (Fletcher and Ross, 1985) or "honest signals" (Peeters et al., 1999; Strauss et al., 2008). Workers showed no ovarian activity or egg-laying under the host queens (Tan et al., 2009). We conclude that retinue behaviour itself as well as the pheromones of the queens that induce this behaviour are both ancestral, conserved traits that preceded speciation in apine bees.

Table 2.2 Mean (± S.D.) weight (μg) and proportional values of mandibular gland components of mixed colonies *A. cerana* and *A. mellifera* queens (*N* = 3, each)

Component	***A. cerana***		***A. mellifera***		***P*** *
	weight (μg)	proportion	weight (μg)	proportion	
4-methyl-hydroxy-benzoate (HOB)	49.98±17.48	0.16±0.06	38.71±7.05	0.08±0.01	0.103
9 -keto-2(*E*)-decenoic acid (9-ODA)	232.07±27.55	0.71±0.04	244.13±30.27	0.52±0.03	0.002
9-hydroxy-2(*E*)-decenoic acid (9-HDA)	31.92±10.80	0.10±0.03	147.47±21.54	0.31±0.04	0.002
10-hydroxydecanoic acid (10-HDAA)	6.17±3.51	0.02±0.01	13.71±9.50	0.03±0.02	0.512
10-hydroxy-2(*E*)-decenoic acid (10-HDA)	4.26±0.99	0.01±0.00	25.91±10.29	0.06±0.02	0.024
Multivariate test of all components					0.027†
Ratio:9-ODA/9-ODA+10-HDA+10-HDAA	0.96±0.02		0.86±0.05		0.041

* Probability from univariate *t*-tests (*df* = 4)

† Wilk's lambda (*df* = 4,1)

CHAPTER 3

Ovarian activation of workers in mixed-species honeybee colonies (*Apis cerana* and *Apis mellifera*)

Summary

The ovaries of workers are inactive in the presence of a queen and brood; however, when the queen is lost, the workers can activate their ovaries. So it is widely believed that the pheromones of queens play a major role in regulating the ovarian activation of workers. *A. cerana* and *A. mellifera* queens have similar pheromonal components, but differ in quantity and the ratio of the components. Thus it is unknown if the ovaries of *A. cerana* workers would remain inactive under the headship of an *A. mellifera* queen in mixed-species colonies, and vice versa. In this chapter, this question is investigated, and we further studied the competition among the workers of the two species in the mixed colonies to activate their ovaries under queenless conditions.

We found that queens of both species could inhibit ovarian activation in conspecific workers to the same degree. In contrast, workers of both species showed significantly greater ovarian activation in queenright mixed-species colonies than in their respective conspecific queenright colonies. Moreover, there was significantly greater and faster ovarian activation in *A. cerana* workers in the mixed-species colonies headed by *A. mellifera* queens than of *A. mellifera* workers in mixed-species colonies headed by *A. cerana* queens. *A. mellifera* workers in conspecific queenless colonies showed significantly greater ovarian activation than those in the mixed-species queenless colonies containing *A. cerana* and *A. mellifera* workers, and conversely in queenless *A. cerana*. The rates and extent of ovarian activation in the two groups of queenless colonies, *A. mellifera* and *A. cerana,* differed significantly. Because *A. cerana* queens have a significantly stronger queen-biased signal than *A. mellifera* queens, we conclude that this interspecific bias of queen signals largely accounts for the greater rate and extent of ovarian activation in *A. cerana* workers in mixed-species colonies headed by *A. mellifera* queens. However, this does not preclude the possibility that interspecific worker-worker interactions in mixed-species colonies contribute to ovarian inhibition.

3.1 Introduction

In honeybee colonies, the queen is the only reproductive female with active ovaries that can lay eggs that develop into normal offspring, while the workers are infertile and have only vestigial ovaries which can only become active under queenless conditions (Winston, 1987) (Fig. 3.1).

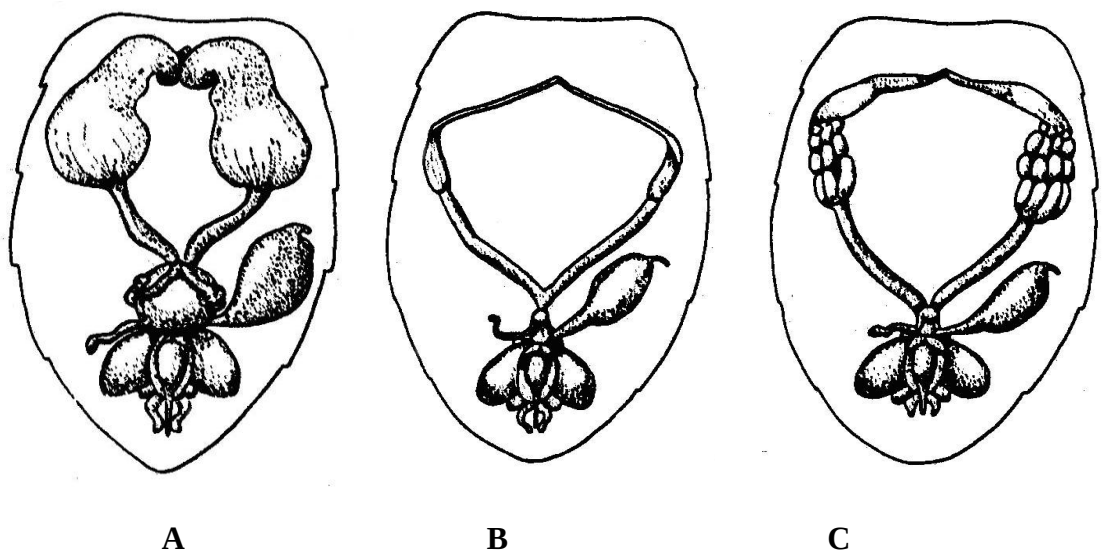


Fig. 3.1 Comparison of the ovaries of a queen and worker; A: ovaries of queen, B: ovaries of normal worker, C: ovaries of laying worker

Colony integrity is fundamental for a social insect colony to function as a single unit and is well referred to as a superorganism (Moritz and Southwick, 1992). To achieve this, social Hymenoptera exhibit an impressive self-organisation and is the basis for organising, in the case of honeybees, several thousands of individuals performing different tasks. A well-developed chemical communication system, including releaser and primer pheromones, plays a crucial role in social regulation (Le Conte and Hefetz, 2008). The mechanism of regulating worker fertility is in particular the focus of many studies and is related to the question whether the queen-borne primer pheromones affect the physiology of the workers directly (queen control) or indirectly serving as an informative signal (queen signal). The continuous emission of pheromones by the queen

in a honeybee colony produced in the mandibular glands results in the inhibition of ovarian activation in the worker bees (Free, 1987; Le Conte and Hefetz, 2008).

These physiological processes differ in degree, not in kind, and are essentially the same in both *A. cerana* and *A. mellifera* (for the former, Bai and Reddy, 1975; Rajashekharappa, 1979; for the latter, Müssbichler, 1952; Butler, 1966). Both species share all of the "essential" components of queen substance, but differ in the relative amounts, hence ratios in the bouquet (Free, 1987; Crewe, 1988; Keeling et al., 2001). However, there is evidence suggesting that the primer effects of synthetic queen pheromone are greater in *A. mellifera* than in *A. cerana* (Kuang et al., 2000), indicating that *A. mellifera* seems to have a higher sensitivity to the pheromones.

Indeed, significantly more workers with completely activated ovaries occur in queenright *A. cerana* colonies than in *A. mellifera.* That shows that in both species the queen-based pheromonal suppression of ovarian activity in workers is not complete (for the former, Sakagami, 1954; for the latter, Velthuis, 1970; Slessor et al., 1998) and that *A. cerana* appears to have a lower sensitivity to the pheromones. Furthermore, not only the queen-worker interaction plays a role in the suppression of ovarian activity but worker-worker interactions mediate ovarian activation. When the queen dies, the ovarian changes in worker bees co-varies with changes in the pheromonal blend of compounds in the mandibular glands from worker-like, through intermediates, to a very small percentage that is very queen-like (Crewe and Velthuis, 1980; Hepburn, 1992; Hepburn and Allsopp, 1994). The signal of the latter, a pseudoqueen or surrogate queen, mimics a queen-like pheromonal bouquet. Workers actually compete to produce the strongest signal (Velthuis et al., 1965; Moritz et al., 2000). Firstly, that signal also suppresses queen rearing and ovary activation in other workers (Velthuis and van Es, 1964; Velthuis, 1970) and secondly, it enables them to dominate the social system and receive protein rich food from subordinate workers (Schäfer et al., 2006) which is needed to activate their ovaries. Adding to the complexity of the regulation of ovarian activity is the fact that the brood is emitting primer pheromones which inhibits ovarian activation in workers (Fletcher and Ross, 1985; Mohammedi et al., 1996; Mohammedi et al., 1998).

On the one hand, the evolutionary distance between them is sufficiently small that heterospecific transfers of *A. cerana* and *A. mellifera* capped brood results in the ready acceptance of newly eclosed workers in their respective host colonies (Atwal and Sharma, 1967; Dhaliwal and Atwal, 1970; Tan et al., 2006). On the other hand, it is

evident that many similar physiological and behavioural elements occur in these two species that reflect their monophyletic origin.

To investigate the differences and similarities we tested the relative flexibility of ovarian activation/inhibition in both species under queenright and queenless conditions as well as in interspecific transfers of workers between species. This allows the quantitative measurement of the relative susceptibility of each species during reproductive competition in conspecific and interspecific contexts. The extraordinary complexity of pheromones regulating ovarian activation, the fact that in honeybees same primer pheromones are known (Le Conte and Hefetz, 2008), and the monophyletic relationship make the two sister species *A. mellifera* and *A. cerana* an ideal model system to test the underlying chemistry of primer pheromones and to broaden our understanding of the physiological aspects and the evolution of sociality.

3.2 Materials and methods

3.2.1 Composition of honeybee colonies

Twelve queenright colonies of *A. cerana* and twelve of *A. mellifera* were placed in an apiary on the campus of Yunnan Agricultural University, Kunming, China and disposed as follows: All colonies were equalized to contain two frames of brood and two of honey and pollen. Then, 1) Three queenright conspecific colonies each of *A. cerana* and *A. mellifera* served as positive controls to create a "base line" for both species (groups queen-right *cerana* and queenright *mellifera*, respectively). 2) Three more colonies each of *A. cerana* and *A. mellifera* were dequeened (groups queenless *cerana* and queenless *mellifera*, respectively). 3) In addition, six queenright colonies of *A. cerana* and six *A. mellifera* received a single frame of brood of the other species on the verge of eclosion. One frame from the *A. cerana* colonies was transferred to an *A. mellifera* colony and vice versa. These twelve colonies were therefore mixed-species colonies, six of which were headed by an *A. cerana* queen and the other six by *A. mellifera* queens (groups queenright mixed *cerana* and queenright mixed *mellifera*, respectively). 4) Six of the mixed-species colonies (three with an *A. cerana* queen and three with an *A. mellifera*

queen) were subsequently dequeened and thus became queenless mixed colonies (groups queenless mixed *cerana* and queenless mixed *mellifera*, respectively).

3.2.2 Measurements of ovarian activation

In all cases sampling began a fortnight after the sealed brood had begun to emerge thus providing a range of young adult worker bees. 10 worker bees were randomly collected from each of the single-species control colonies and 20 from the experimental mixed-species colonies (10 *A. cerana* and 10 *A. mellifera* workers) on a weekly basis for eight weeks. All worker bees were dissected at the time of collection and ovarian development was determined in five stages according to the system of Yang, 2001b (for *A. cerana*), and Hess (1942) for *A. mellifera*: Stage I – ovaries do not show any differentiation between eggs and nurse cells, 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 (Fig. 3.2).

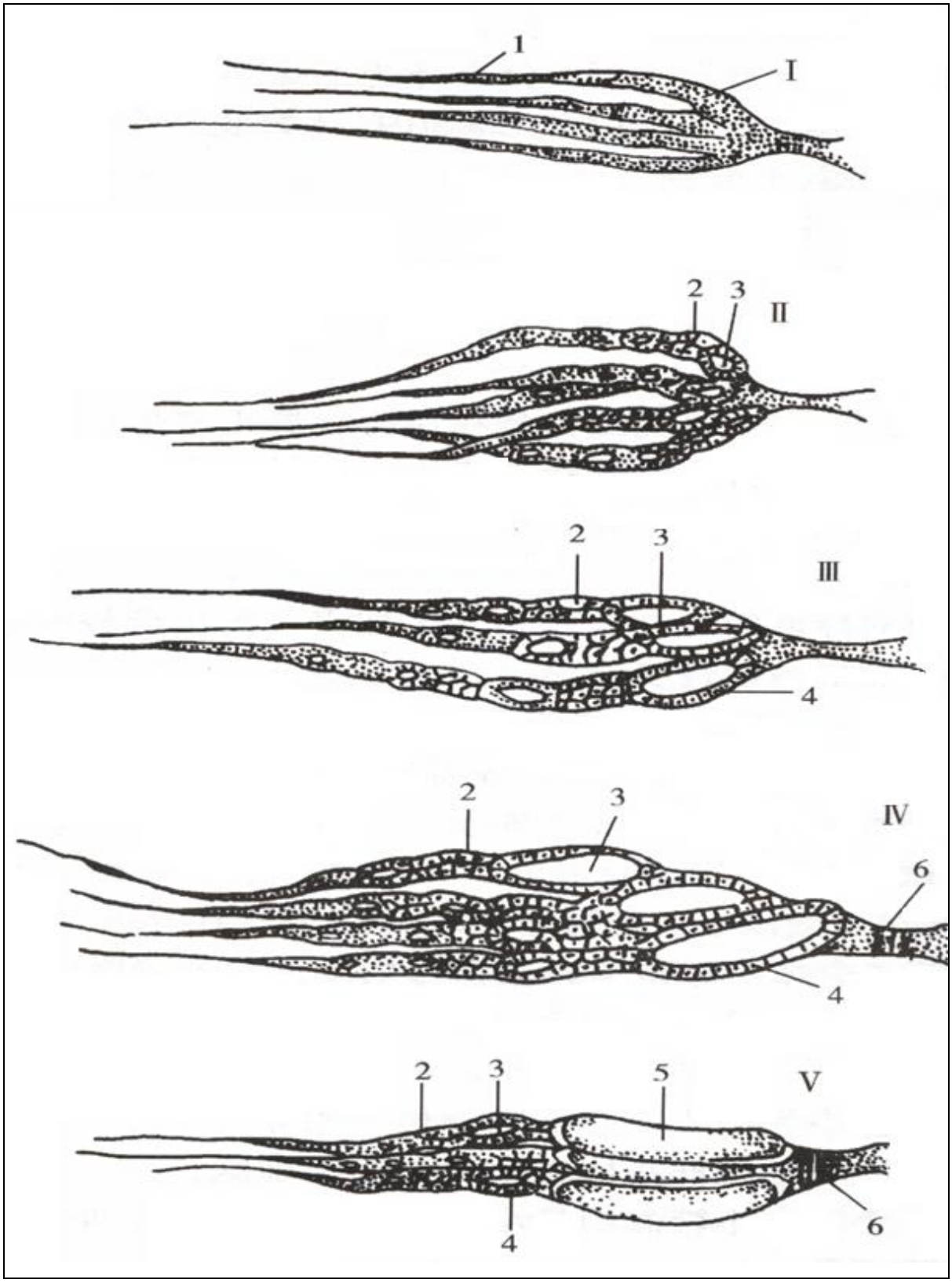


Fig. 3.2 The activation of workers' ovaries (Yang, 2001b)

Morphometrically, the ovaries of the workers of *A. cerana* are different from *A. mellifera* (Sakagami, 1959; Kuang and Kuang, 2002; Fig. 3.3). In this study, in order to make the data comparable, the same criteria were adopted to measure ovarian activation of the workers from the two species.

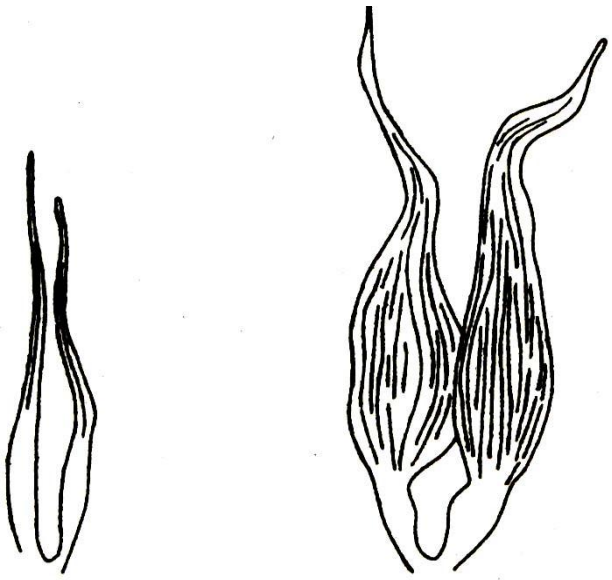


Fig. 3.3 Comparison of ovarian development between *A. cerana* (left) and *A. mellifera* (right). (Kuang and Kuang, 2002)

3.2.3 Pheromonal analyses

Immediately after the experiment was terminated the queens were decapitated. The heads were extracted in 200µl dichloromethane (DCM) for about a month. The samples were then evaporated to dryness under a stream of nitrogen. The residues were re-dissolved in 10 µl of an internal standard solution (octanoic acid and tetradecane in dichloromethane; 0.38 and 0.25mg/ml, respectively) and 10 µl of derivatizing agent (bis-trimethylsilyltrifluoroacetamide). One µl of this solution was injected into a gas chromatograph (Hewlett Packard 6890) using routine analytical conditions (Simon et al., 2001; Dietemann et al., 2006). The following components, 9-keto-(E)-2-decenoic acid (9-ODA; "queen substance"), 10-hydroxy-(E)-2-decenoic acid (10-HDA; "worker substance"), methyl *p*-hydroxybenzoate (HOB), 10-hydroxydecanoic acid (10-HDAA), and 9-hydroxy-2-(e)-decenoic acid (9-HDA) were quantified using peak areas and the

relative mass ratios calculated relative to tetradecane (Simon et al., 2001; Dietemann et al., 2006). The bias towards the queen substance (Plettner et al., 1998) pathway was determined by measuring the relative amount of 9-ODA, 'queen substance' as 9-ODA / (9-ODA + 10-HDA + 10-HDAA), which is a sensitive indicator of the biosynthetic investment in the queen substance (Moritz et al., 2004).

3.2.4 Statistical analysis

Pearson χ^2 tests were used to test for significant differences in the extent of ovarian activation among workers of queenright and queenless, conspecific and mixed-species colonies of the honeybees, *A. cerana* and *A. mellifera.* Bonferroni adjustment to the level of significance for multiple paired comparisons performed simultaneously on the same data set was used to ensure that the overall level of significance did not exceed 0.05. Therefore, the 28 paired comparison test results will be considered significant if $P = 0.05/28 = P < 0.00178$ with Bonferroni adjustment. Log-linear G-test analysis was used to test for homogeneity of the extent of ovarian activation among the colonies of each group (Sokal and Rohlf, 1995). Regression analysis procedures were used to test for differences in the rates of ovarian activation over time between the groups and t-tests for independent samples were used to test for differences in the amounts of the constituents in the pheromones between *A. cerana* and *A. mellifera* queens. All tests were performed using Statistica© (StatSoft, 2008).

3.3 Results

3.3.1 Pheromones of the mandibular glands of *Apis cerana* and *Apis mellifera* queens

The amounts of the principal constituents in the pheromone of the mandibular glands of the *A. cerana* and *A. mellifera* queens are shown in (Table 3.1). Under our rearing conditions, the relative amounts of 9-ODA, 10-HDAA and HOB did not significantly differ between species. However, *A. mellifera* queens had significantly greater amounts of 10-HDA and 9-HDA than did the *A. cerana* queens (t-test: 10-HDA: $t = 3.6$, $df = 4$, P

= 0.022; 9-HDA: t = 8.3, df = 4, P = 0.001). More importantly the [9-ODA / (9-ODA + 10-HDA + 10-HDAA)] ratios for *A. cerana* and *A. mellifera* were significantly different, the former having a ratio of 0.96±0.02 and the latter 0.86±0.05 (t = 3.0, df = 4, P = 0.041).

Table 3.1 Quantity of principal components of the mandibular gland pheromones of *A. cerana* and *A. mellifera* queens, µg/bee head

Species	HOB	9-ODA	9-HDA	10-HDAA	10-HDA
A. mellifera	38.71± 7.05	244.13±30.27	147.47±21.54	13.71±9.50	25.91±10.29
A. cerana	49.98±17.49	232.07±27.55	31.92±10.80	6.17±3.50	4.26± 0.99

3.3.2 Tests for homogeneity of the extent of ovarian activation among the colonies

Log-linear G-test analyses revealed no significant differences in the extent of ovarian activation among the colonies within each group (queenright *mellifera*: χ^2 = 3.48, df = 2, P = 0.176; queenless *mellifera*: χ^2 = 0.15, df = 2, P = 0.929; queenright mixed *mellifera*: χ^2 = 3.29, df = 2, P = 0.193; queenless mixed *mellifera*: χ^2 = 4.38, df = 2, P = 0.1118; queenright *cerana*: χ^2 = 0.07, df = 2, P = 0.966; queenless *cerana*: χ^2 = 3.19, df = 2, P = 0.203; queenright mixed *cerana*: χ^2 = 4.37, df = 2, P = 0.113; queenless mixed *cerana*: χ^2 = 4.16, df = 2, P = 0.125).

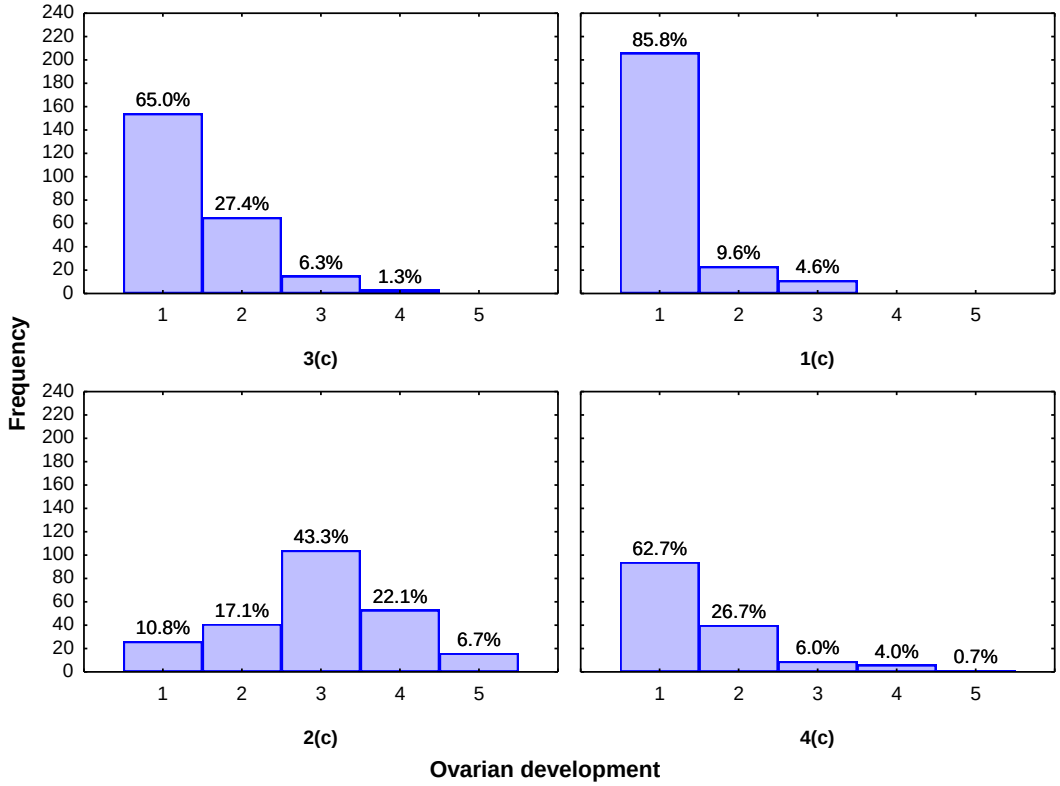
3.3.3 Ovarian activation of *Apis mellifera* workers in queenright, conspecific and mixed-species colonies

Less than 11.7% of the *A. mellifera* workers in colonies headed by *A. mellifera* queens showed ovarian activation whereas the number of workers with activated ovaries (21.7%) was significantly higher in queenright mixed-species colonies (χ^2 = 9.7, df = 2, P = 0.008; Fig. 3.4). The rate of ovarian activation in the mean values over time for *A. mellifera* workers in queenright mixed-species colonies was significantly greater than that of *A. mellifera* workers in colonies headed by *A. mellifera* queens (t = 4.5, df = 13, P < 0.001; Fig. 3.4).

3.3.4 Ovarian activation of *Apis mellifera* workers in queenless, conspecific and mixed-species colonies

Over 65.8% of the *A. mellifera* workers in queenless conspecific colonies showed ovarian activation (stage II and above) whereas it was significantly less, only 36.1%, of the *A. mellifera* workers in queenless mixed-species colonies (χ^2 = 39.9, *df* = 4, *P* < 0.001; Fig. 3.3). The mean values for the rate of ovarian activation over time for *A. mellifera* workers in queenless mixed-species colonies was significantly less than that of *A. mellifera* workers in queenless colonies (t = -5.9, *df* = 11, *P* < 0.001; Fig. 3.5).

A. cerana



A. mellifera

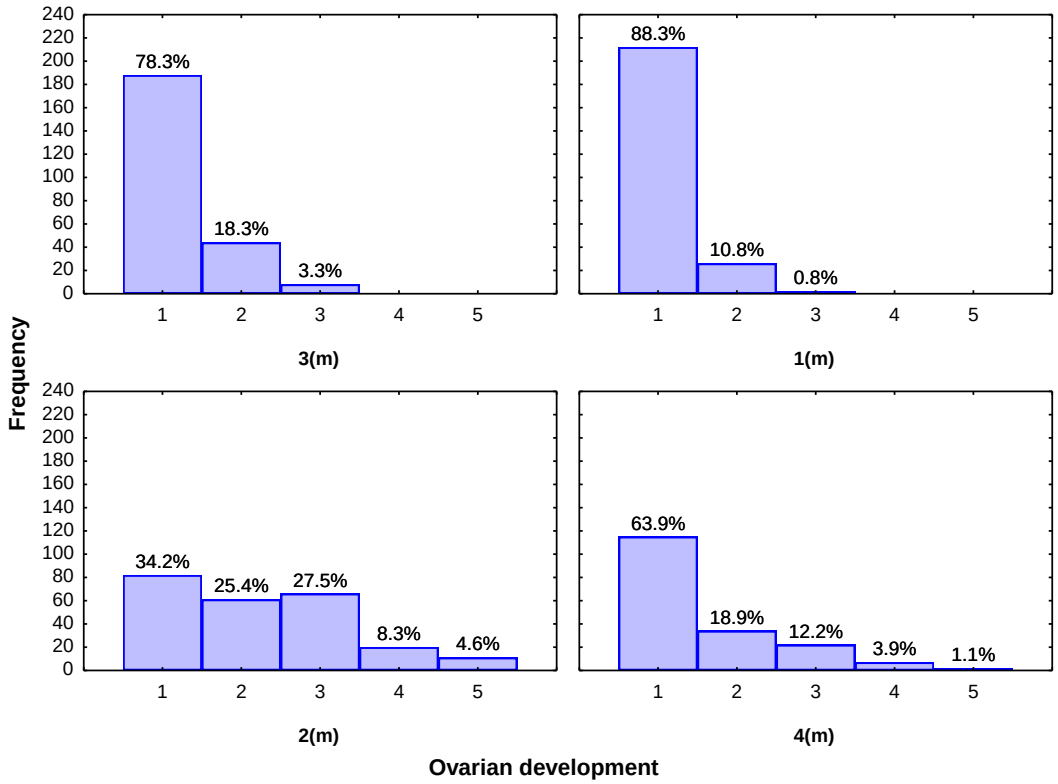
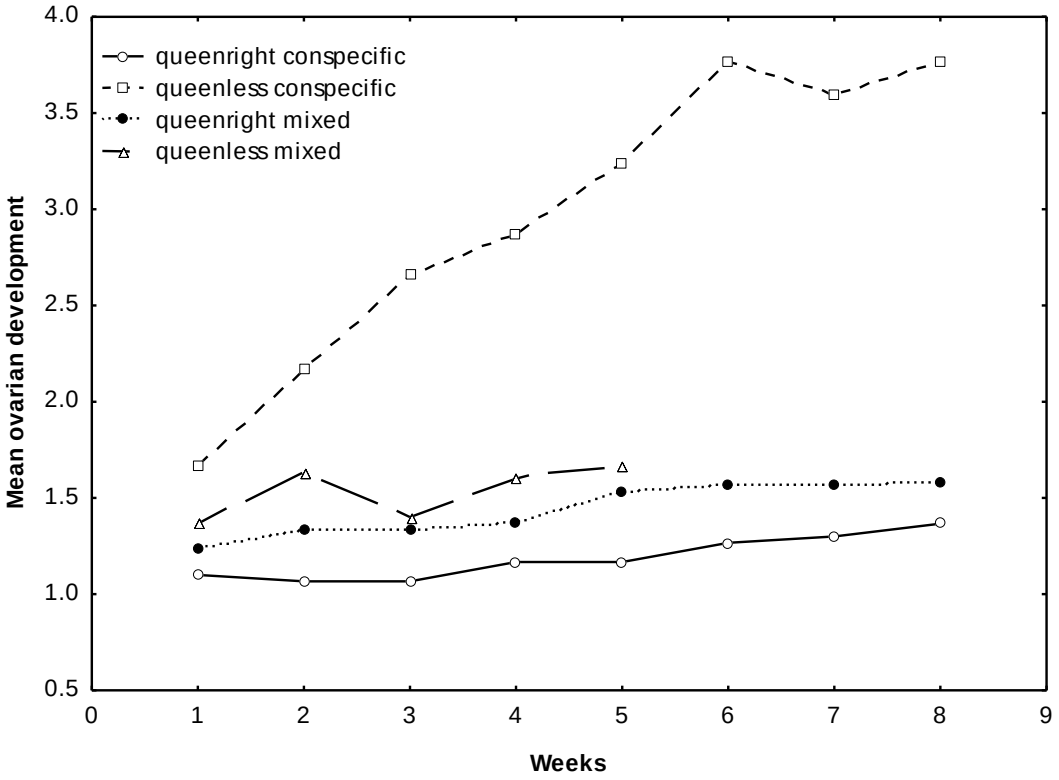


Fig. 3.4 Extent of ovarian activation in conspecific and mixed-species colonies of the honeybees *A. cerana* and *A. mellifera* at the end of the experiments

***A. cerana* groups**



***A. mellifera* groups**

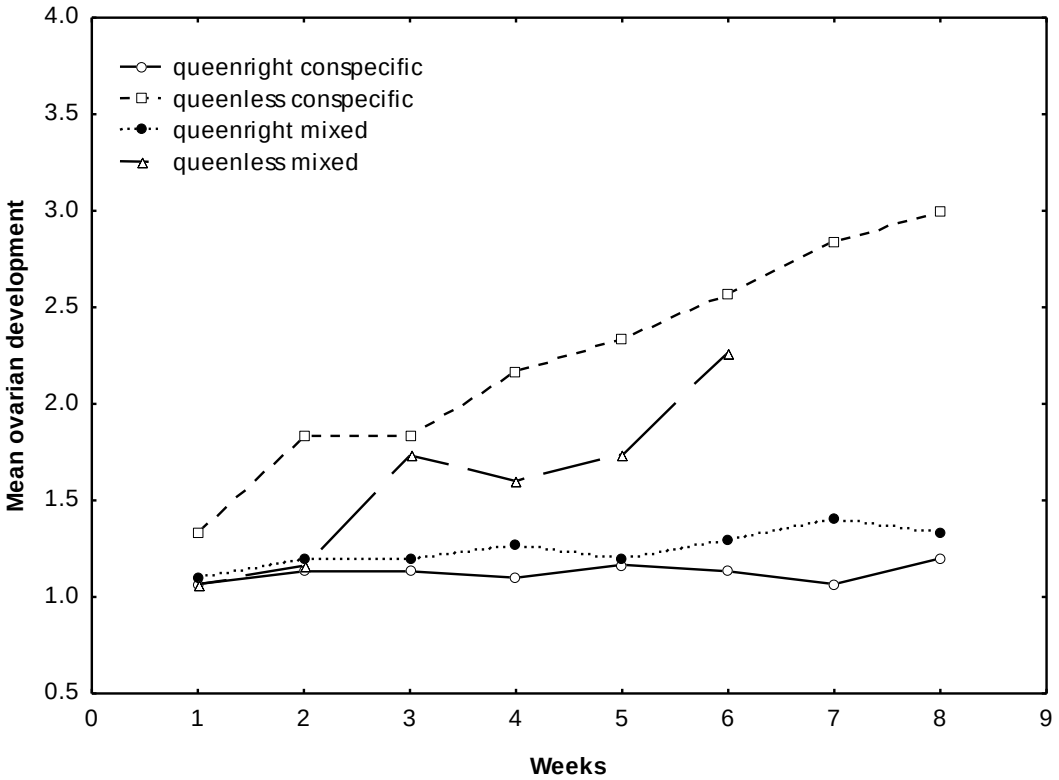


Fig. 3.5 Rates of ovarian activation in conspecific and mixed-species colonies of the honeybees *A. mellifera* and *A. cerana* over 8 weeks

3.3.5 Ovarian activation of *Apis cerana* workers in queenright, conspecific and mixed-species colonies

14.2% of the *A. cerana* workers in queenright colonies headed by an *A. cerana* queen, showed ovarian activation which was significantly less than the 34.6% of the bees in queenright mixed-species colonies (χ^2 = 31.2, *df* = 3, *P* < 0.001; Fig. 3.4, Table 3.2). The mean values of the rate of ovarian activation over time for *A. cerana* workers in queenright mixed-species colonies was significantly greater than that of *A. cerana* workers in colonies headed by *A. cerana* queens (t = 11.6, *df* = 13, *P* < 0.001; Fig. 3.4).

3.3.6 Ovarian activation of *Apis cerana* workers in queenless, conspecific and mixed-species colonies

Nearly 90% of the queenless *A. cerana* workers previously headed by an *A. cerana* queen exhibited some degree of ovarian activation and significantly more than the 37.3% in queenless mixed-species colonies previously headed by an *A. cerana* queen (χ^2 = 156.7, *df* = 4, *P* < 0.001; Fig. 3.4). The mean values for the rate of ovarian activation over time for *A. cerana* workers in queenless mixed-species colonies was significantly less than that of *A. cerana* workers in queenless colonies (*t* = -6.0, *df* = 10, *P* < 0.001; Fig. 3.4).

3.3.7 Ovarian activation of workers in queenright and queenless, conspecific and mixed-species colonies

The rate and extent of ovarian activation in all of the *A. mellifera* queenless colonies (queenless conspecific, 65.8% and queenless mixed, 36.1%) were significantly greater than in either of the *A. mellifera* queenright colonies (queenright conspecific, 11.7%, *t* = 7.8, *df* = 13, *P* < 0.001; χ^2 = 162.8, *df* = 4, *P* < 0.001; queenright mixed, 21.7%, *t* = 7.7, *df* = 13, *P* < 0.001; χ^2 = 120.8, *df* = 4, *P* < 0.001; Table 3.2).

Table 3.2 Pearson χ^2 test results for testing the extent of ovarian activation among workers of queenright and queenless, conspecific and mixed-species colonies of the honeybees, *A. cerana* and *A. mellifera*

	Queenright		**Queenless**		**Queenright mixed**		**Queenless mixed**	
Groups	*mellifera*	*cerana*	*mellifera*	*cerana*	*mellifera*	*cerana*	*mellifera*	*cerana*
	-	ns	*	*	ns	*	*	*
	ns	-	*	*	ns	*	*	*
	*	*	-	*	*	*	*	*
	*	*	*	-	*	*	*	*
	ns	ns	*	*	-	ns	*	*
	*	*	*	*	ns	-	ns	ns
	*	*	*	*	*	ns	-	ns
	*	*	*	*	*	ns	ns	-

* = significantly different $P < 0.0018$ with Bonferroni adjustment; ns = not significant

Comparing both queenright and queenless contexts for *A. cerana*, there was significantly less ovarian activation in the *A. cerana* queenright colonies (14.2%) than in either of the corresponding queenless colonies (queenless conspecific, 89.2%, $t = 9.7$, $df = 13$, $\chi^2 = 288.9$, $df = 4$, $P < 0.001$; queenless mixed, 37.3%, $t = 9.0$, $df = 10$, $\chi^2 = 34.7$, $df = 4$, $P < 0.001$). Furthermore, significantly fewer workers (34.6%) in mixed queenright *A. cerana* colonies had activated ovaries compared to worker in queenless conspecific *cerana* colonies (queenless conspecific, 89.2%, $\chi^2 = 225.9$, $df = 4$, $P < 0.001$). Interestingly, the rate and extent of ovarian activation in mixed queenright *A. cerana* colonies (queenright mixed, 34.6%) did not significantly differ from queenless mixed *cerana* host colonies (queenless mixed, 37.3%, $t = 3.8$, $df = 10$, $P = 0.004 > 0.002$ Bonferroni, $\chi^2 = 4.6$, $df = 4$, $P = 0.326$, Figs. 3.4 and 3.5).

In paired group comparisons of *A. mellifera* and *A. cerana*, there was a significant difference between *A. mellifera* (65.8%) and *A. cerana* (89.2%) queenless colonies for which *A. cerana* workers had the greater ovarian activation ($\chi^2 = 57.3$, $df = 4$, $P < 0.001$; Fig. 3.3). Also the mean values for the rate of ovarian activation over time for *A. cerana* workers in queenless colonies was significantly greater than that of *A. mellifera* workers in queenless colonies ($t = 7.6$, $df = 13$, $P < 0.001$). Although the extent of ovarian activation in mixed queenright *A. cerana* colonies (34.6%) did not differ significantly from mixed queenright *A. mellifera* colonies (21.7%, $\chi^2 = 12.5$, $df = 4$, $P = 0.014 >$

0.002 Bonferroni; Fig. 3.4), the rate of ovarian activation was significantly greater in mixed queenright *A. cerana* colonies ($t = 7.5$, $df = 13$, $P < 0.001$; Fig. 3.5).

3.4 Discussion

The fact that all queenright colonies, except for one comparison, showed significantly less ovarian activation in workers than the queenless counterparts in both *A. cerana* and *A. mellifera* demonstrates that the queens of the two species have pheromonal equivalence in the conspecific inhibition of worker ovarian activation (Fig. 3.4). Even the comparison of queenright mixed colonies headed by an *A. cerana* queen with its queenless counterpart, although not significantly different in the extent of the ovarian activation, showed a significant difference in the rate of activation, supporting the idea that queen presence affects workers of both species (Figs. 3.4 and 3.5). However, in none of the queenright colonies is the inhibitory effect complete as indicated by the proportion of workers with activated ovaries (Fig. 3.4). This partial ovarian activation is nonetheless sufficient to preclude reproductive competition by workers as none of the bees reached the laying worker stage, stage V.

The rates and extent of ovarian activation in the two groups of queenless conspecific colonies, *A. mellifera* and *A. cerana*, differed significantly. In the former, some 65.8% of workers exhibited some degree of ovarian activation and 4.6% reached the laying worker stage V; in the latter some 89.2% exhibited ovarian activation and nearly 7% reached laying worker stage V (Fig. 3.4).

Comparisons of the queenless colonies of *A. mellifera* show that *A. mellifera* workers in queenless conspecific colonies show significantly greater ovarian activation (65.8%) than those *A. mellifera* in the mixed-species queenless colonies containing *A. cerana* and *A. mellifera* workers (36.1%, Fig. 3.4). Clearly, *A. cerana* workers in mixed-species colonies exert a greater inhibitory effect on ovarian activation on *A. mellifera* than that achieved in queenless conspecific *A. mellifera* colonies. Somewhat unexpectedly, exactly the same trend occurred in the groups of queenless *A. cerana.* Conspecific, queenless *A. cerana* exhibited a significantly greater rate and extent of ovarian activation (89.2%) than those *A. cerana* in queenless mixed-species colonies (37.3%). That significantly fewer workers underwent ovarian activation in the mixed queenless

colonies is most parsimoniously explained as the effect of worker-worker pheromonal inhibition. Perhaps a much greater inhibition was derived from the presence of *A. cerana* workers in the same colonies as well as from other *A. mellifera* workers.

In contrast to this, *A. cerana* workers of queenright mixed-species colonies (34.6%) showed significantly greater ovarian activation than their workers in queenright colonies (14.2%). However, because queenless worker bees can also inhibit ovarian activation in other workers, comparisons among them in queenless, mixed-species colonies allow an estimation of the separate effects of queen-worker and worker-worker inhibition. There was no significant difference in the extent of ovarian activation between *A. mellifera* workers of queenright mixed-species colonies (21.7%) and their respective conspecific queenright colonies (11.7%).

In comparisons of conspecific and mixed queenless colonies of both *A. mellifera* and *A. cerana,* the only difference is the fact that mixed colonies contained workers of both species. We interpret this to mean that there is interspecific inhibition of workers between workers. The dead workers with activated ovaries recovered at the entrances of all the queenless colonies of *A. cerana* and *A. mellifera* are consistent with other reports that workers with developed ovaries are often attacked and killed by their nestmates (Sakagami, 1954; Anderson, 1963). This behaviour also explains the decline in the colonies of groups 4m and 4c between weeks 5 and 6 because during this period *A. cerana* bees were evicted and the *A. mellifera* bees were dwindling.

As we have shown in Chapter 2, the workers in the mixed colonies do not discriminate against heterospecific queens. When in heterospecific colonies, the workers normally form a retinue around the queen and touch her with their antennae, this behaviour no doubt serves to transmit queen pheromone (Naumann et al., 1991, 1992; Fig. 3.6, Table 3.3), and can partly explain why the workers in mixed colonies do not activate their ovaries.

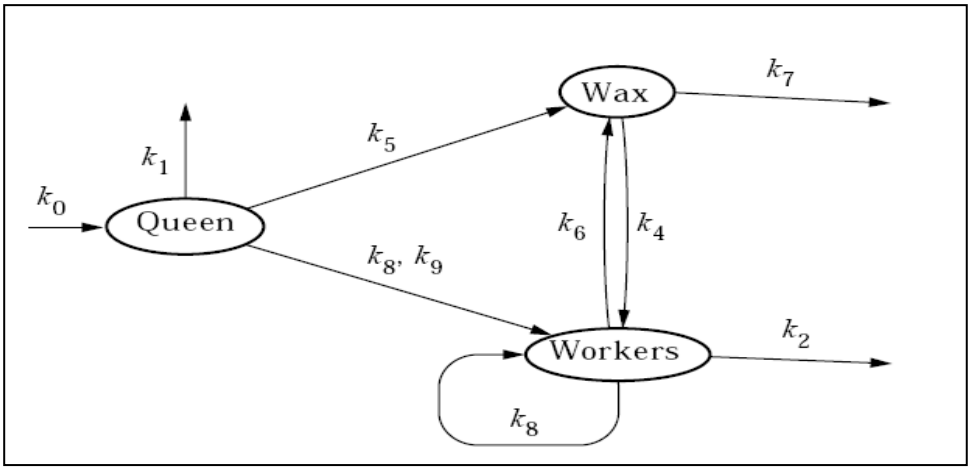


Fig. 3.6 Queen pheromone transmission in honeybee colonies (Naumann et al., 1991)

Table 3.3 Transmission rate of queen pheromones (Naumann et al., 1992)

Notation	Description	Range of values
K_0	production by queen	1 ngs^{-1}
K_1	absorption by queen (through cuticle)	$(8.0 \pm 2.4)\times10^{-4}$ s^{-1}
K_2	absorption by workers (through cuticle)	6.8×10^{-4} s^{-1}
K_3	mean transfer from queen to worker	$(3.5 \pm 1.4)\times10^{-4}$ s^{-1}
K_4	wax → worker transfer	$(2 \pm 1)\times10^{-4}$ s^{-1}
K_5	queen → wax transfer	$(8.0 \pm 2.4)\times10^{-5}$ s^{-1}
K_6	worker → wax transfer	$(1.3 \pm 0.1)\times10^{-4}$ s^{-1}
K_7	absorption into wax	$(1.0 \pm 0.2)\times10^{-3}$ s^{-1}
K_8	queen/messenger → antennating worker transfer	$(3.5 \pm 1.2)\times10^{-5}$ s^{-1}
K_9	queen/messenger → licking worker transfer	$(3.2 \pm 0.6)\times10^{-3}$ s^{-1}

These interpretations of ovarian activation are consistent with the results of the [9-ODA / (9-ODA + 10-HDA + 10-HDAA)] ratios. *A. cerana* queens have more strongly queen-biased signals than *A. mellifera* queens, results consistent with other published data (Keeling et al., 2001 for *A. cerana*; Crewe and Velthuis, 1980 for *A. mellifera*). Thus it is reasonable to conclude that this interspecific bias of queen signals largely accounts for the greater rate and extent of ovarian activation in *A. cerana* workers in mixed-species colonies headed by *A. mellifera* queens.

One could speculate that the stronger queen biased signal of the *A. cerana* is the result of a higher degree of social parasitism in natural populations of *A. cerana*. Indeed, the strong queen signal is comparable to queens of the African subspecies *A. m. capensis* (Wossler, 2002) in which workers can reproduce despite the presence of a

reproducing queen (Neumann and Hepburn, 2002; Wossler, 2002). Another similarity is that workers of *A. m. capensis* are less affected by the queens' pheromones of other *A. mellifera* subspecies, as were the *A. cerana* workers in the mixed colonies headed by the *A. mellifera* queen. Mandibular gland pheromones are likely to have played a central role in the evolution of social parasitism in honeybees (Dietemann et al., 2007). The importance of these pheromones is based on their multiple functions in determining reproductive status and allowing individuals to prevent reproduction by their nestmates (Velthuis et al., 1990; Simon et al., 2005).

CHAPTER 4

Dance communication of *Apis cerana* and *Apis mellifera* mixed-species colonies

Summary

Honeybee foragers use dance language to inform nestmates of the locations of food sites. Although the structure of the dance language is very similar among species of honeybees, communication of the distance component of the message varies both intraspecifically and interspecifically. However, it is not known whether different honeybee species would attend interspecific waggle dances and, if so, whether they can translate such dances. In this chapter, mixed-species colonies of *Apis cerana* and *Apis mellifera* were used to test interspecific communication between these two species. The results show that, despite internal differences in the structure of the waggle dances of foragers, both species attend, and act on the information encoded in each other's waggle dances but with limited accuracy. These observations indicate parsimony in communication that pre-dates speciation in honeybees.

4.1 Introduction

Waggle dance

Language is usually credited with being the major factor in making human society different from other higher animals. The fact that honeybees have a dance language that is unparalleled in the rest of the animal kingdom is therefore of great interest.

That honeybees use dance language to recruit nestmates to a food source has been known since Aristotle's time (Tautz, 1996). In the 'dance language' of honeybees, the dancer generates a specific, coded message that describes the direction and distance from the nest of a new food source, and this message is displaced in both space and time from the dancer's discovery of that source (von Frisch, 1967). von Frisch concluded that bees 'recruited' by such dances used the information encoded in it to guide them directly to a remote food source, and this Nobel Prize-winning discovery revealed the most sophisticated example of non-primate communication known. In spite of some initial skepticism, most biologists are now convinced that von Frisch was correct (Gould, 1975; Sherman and Visscher, 2002; Seeley, 1985). The dance behaviour of honeybees is also used for transmitting new nest site information during swarming (e.g. Lindauer, 1955; Seeley and Morse, 1978; Camazine, 1999; Seeley, 2003). With more and more information having been revealed, two lines of research gradually diverged, one dealing with the efficiency of this kind of recruitment, and the other with the mechanisms involved in this unique communication. The key question in dealing with the communication mechanisms focuses on the nature of the signals that are transmitted from dancer to follower bees. Optical signals can be ruled out because the dances take place in the darkness of the nest cavity. Mechanical and chemical signals remain as the most likely modalities. Tautz (1996) suggested that the dance floor of the comb plays a very important role in dance communication.

Dance dialect

In the process of "dancing", the distance and direction to a food source from the nest is encoded and conveyed to potential recruits (von Frisch, 1967; Dyer, 2002). Such messages are displaced in space and time from the actual discovery of the source (Riley

et al., 2005). Although the structure of the dance language is very similar among species of honeybees (Lindauer, 1956) communication of the distance component of the message varies both intraspecifically (Steche, 1957; Boch, 1957) and interspecifically (Lindauer, 1956). Dance dialects means the distances at which foragers of each *Apis* species make the transition between the "round" and "waggle" dance types and different distances encoded in the waggle runs if the "waggle" dances were performed. According to Lindauer's communication curve, *A. florea* and *A. mellifera carnica* display striking differences; however, Dyer and Seeley (1991a), reported that three Asiatic honeybee species, *A. florea, A. dorsata, A. cerana,* show very similar dance curves.

Some researchers have shown that the dance language could be influenced and affected by genetic factors (Oldroyd et al., 1991; Rinderer and Beaman, 1995; Johnson et al., 2002), while others have shown environmental parameters to also have a strong impact on foragers' dances (Srinivasan et al., 2000; Esch et al., 2001). Combining these findings, the dialects of honeybee species are rather complicated. For example, when based on the latter factor, Sen Sarma et al. (2004) found that *A. florea* and *A. mellifera carnica* showed quite similar dances. Unfortunately, all of these findings were not based on the same spatial route and same time parameters. Using mixed-species colonies of *A. mellifera ligustica* and *A. mellifera carnica,* Steche (1957) and Boch (1957) showed that the dance language includes 'dialects' such that foragers of both races of honeybees were recruited by each other's dances; but with consistent misinterpretations of the distance component of dances. Similarly, variations in the waggle dance among races of *A. cerana* have also been reported (Sasaki et al., 1993) but remain equivocal (Lindauer, 1956; Dyer and Seeley, 1991a).

However, it is not known whether different honeybee species would attend interspecific waggle dances and, if so, whether they can "translate" or interpret such dances. We tested mixed-species colonies of *A. mellifera* and *A. cerana* to establish (1) foraging intensity and waggle dance characteristics of both species, (2) whether translation of waggle dances could occur in an interspecific context, and (3) if so, then with what degree of precision.

4.2 Materials and methods

Three queenright, mixed-species colonies of *A. cerana* and *A. mellifera*, each in a hive containing frames of sealed worker brood of the same age of both species were established in an apiary at Kunming, China. When this brood began to emerge as adults, each colony was placed in a two-frame observation hive headed by an *A. cerana* queen. About two weeks later, when the *A. mellifera* and *A. cerana* bees had reached foraging age, and foraging and waggle dance baselines, and species-specific characteristics of the waggle dances for both species were recorded.

4.2.1 Training of foragers

Foragers were collected in darkened tubes at the hive entrances and individually released at one of two feeders. One feeder 130 m south of the hives was reserved for training only *A. mellifera*, and the other, 130 m west of the hives, reserved for training only *A. cerana*. Feeders for *A. cerana* and *A. mellifera* were placed in different directions from the hives to unambiguously obtain species-specific waggle dance characteristics for each species. Foragers of the three mixed-species colonies were tested in five cycles during summer. The cycle sequence of testing was that the *A. cerana* foragers of colony 1 were tested on day 1 and foragers of colony 1 *A. mellifera* on day 2; the *A. cerana* foragers of colony 2 were tested on day 3 and foragers of colony 2 *A. mellifera* on day 4; the *A. cerana* foragers of colony 3 were tested on day 5 and foragers of colony 3 *A. mellifera* on day 6.

4.2.2 Recruitment and waggle dances

A foraging intensity baseline was established by counting departing and returning foragers of both species separately from each hive for 20 minutes in the morning on each test day. Similarly, a waggle dance intensity baseline was established for both species separately in each colony by video-recording the same side of a comb in the same position in each colony for 20 minutes around noon on each test day. Then the complete waggle dances of 6 individually marked foragers of each species were video-recorded on a CD-cassette for each colony and the numbers of waggle runs per

dance were counted. A random sample of the duration of 10 individual waggle runs was measured by replaying the video recording at 1/4 normal speed. Then, the numbers of both *A. cerana* and *A. mellifera* potential recruits following *A. cerana* dancers were counted; and, conversely, the numbers of both *A. cerana* and *A. mellifera* potential recruits following *A. mellifera* dancers were counted.

Translation of waggle dances

The procedure for the second experiment was the same as in experiment 1, but 4 feeders were used. Feeders containing a 30% sucrose solution scented with honey were put at 4 sites in a straight line 110 m, 130 m, 170 m and 210 m from the hive. Because in earlier experiments with two races of *A. mellifera* (*carnica* and *ligustica*) it was observed that a fixed distance from the hive to the feeder was interpreted consistently differently by the two races (Steche, 1957; Boch, 1957), in our experiment 4 feeding stations were set up to compensate for that possibility, but bees were only trained to the feeder set at 130 m from the hive. During the course of the six-day experiment in early autumn, the compass direction of the line of 4 feeders was changed every day but the distances of the 4 feeders remained unchanged. Each day only one species of foraging bees from each mixed colony were trained to the feeder for one day. In the course of a morning about 40 bees were released slowly. Once the bees were out of the tube at the feeder and began to imbibe the syrup, they were colour-marked. This procedure was continued until 20 individual workers had been colour-marked. When the marked bees returned to their hives and recruited new foragers, these new arrivals at the feeders were marked and counted for 2 h and the experiment concluded for that day.

4.2.3 Data analysis

Statistical significance was determined using two-tailed Student's t-tests at the 5% level of significance. Prior to analysis, homogeneity of variances and normality of the data were examined using Levene's tests and Shapiro-Wilk's tests (Johnson and Wichern, 2002). Heterogeneity was eliminated after a square-root transformation of the data. Statistical significance of the intra- and interspecific recruitment abilities of *A. cerana* and *A. mellifera* foragers was determined using chi-square tests of proportions at the 5% level of significance. All tests were performed using Statistica© (StatSoft, 2008).

4.3 Results

In the first experiment, the numbers of departing and returning bees per unit time (= foraging intensity base-lines) for both *A. cerana* and *A. mellifera* foragers were calculated and there were no significant differences between the numbers of either departing (*A. mellifera*: 179.0 ± 17.1; *A. cerana*: 174.7 ± 23.1, $N = 3$ colonies each, $P = 0.656$) or returning (*A. mellifera*: 165.1 ± 33.5; *A. cerana*: 182.2 ± 30.6, $N = 3$ colonies each, $P = 0.275$) foragers between species (Table 4.1). Waggle dance intensity base-lines and the mean number of waggle runs per dance were calculated for foragers of both species. *A. mellifera* bees performed an average of 23.9 ± 7.4 waggle dances which was significantly greater than the only 4.2 ± 1.2 performed by *A. cerana* over the same time period ($P < 0.001$). Likewise the mean number of waggle runs per dance for *A. mellifera* was 23.2 ± 7.9 which was significantly greater than that of 17.8 ± 5.4 for *A. cerana* ($P = 0.023$).

Table 4.1 Mean (± S.D.) waggle dance and recruitment intensities for *A. mellifera* and *A. cerana*

	A. mellifera	***A. cerana***	**t-value**	**df**	***P***
Waggle runs in each dance ($N = 36$)	23.2±7.9	17.8±5.4	2.39	34	0.023
Duration of each waggle run (1/100 s) ($N = 60$)	63.8±5.3	50.0±6.2	7.93	58	< 0.001
Follower bees of *A. cerana* dancers ($N = 36$)	0.8±0.7	5.7±1.1	16.14	34	< 0.001
Follower bees of *A. mellifera* dancers ($N = 36$)	1.4±0.9	7.6±1.7	13.27	34	< 0.001

When waggle dancers were performing in the mixed-species colonies, both species were attracted by the dancer (Figs 4.1 and 4.2), and then they were recruited to the feeders.

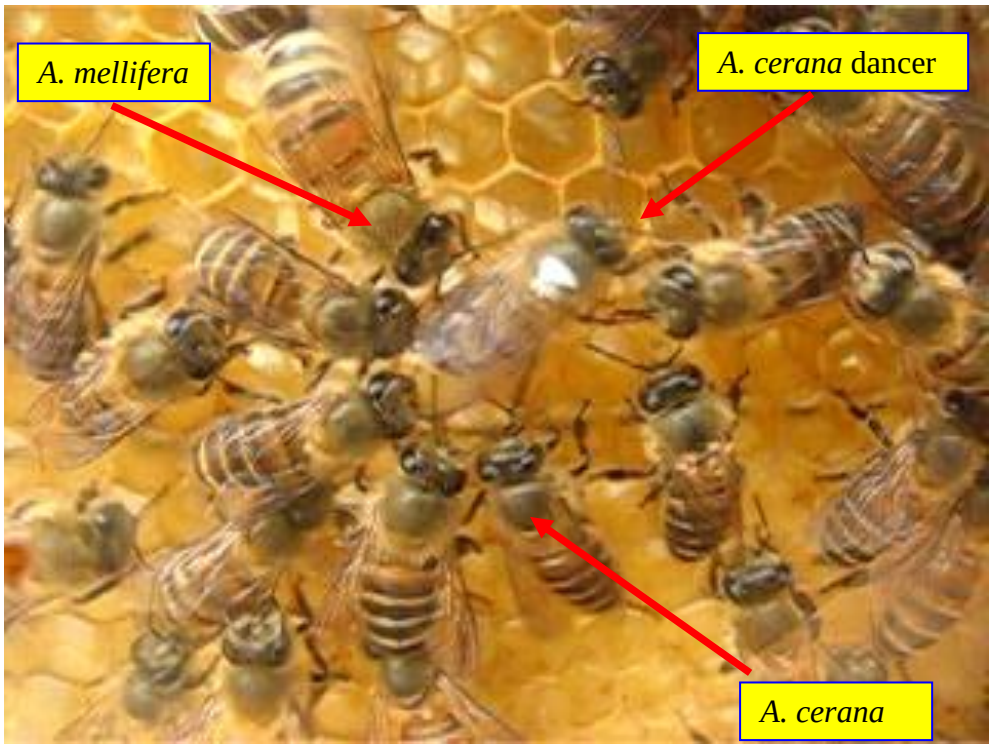


Fig. 4.1 Workers of both species attending an *A. cerana* dancer

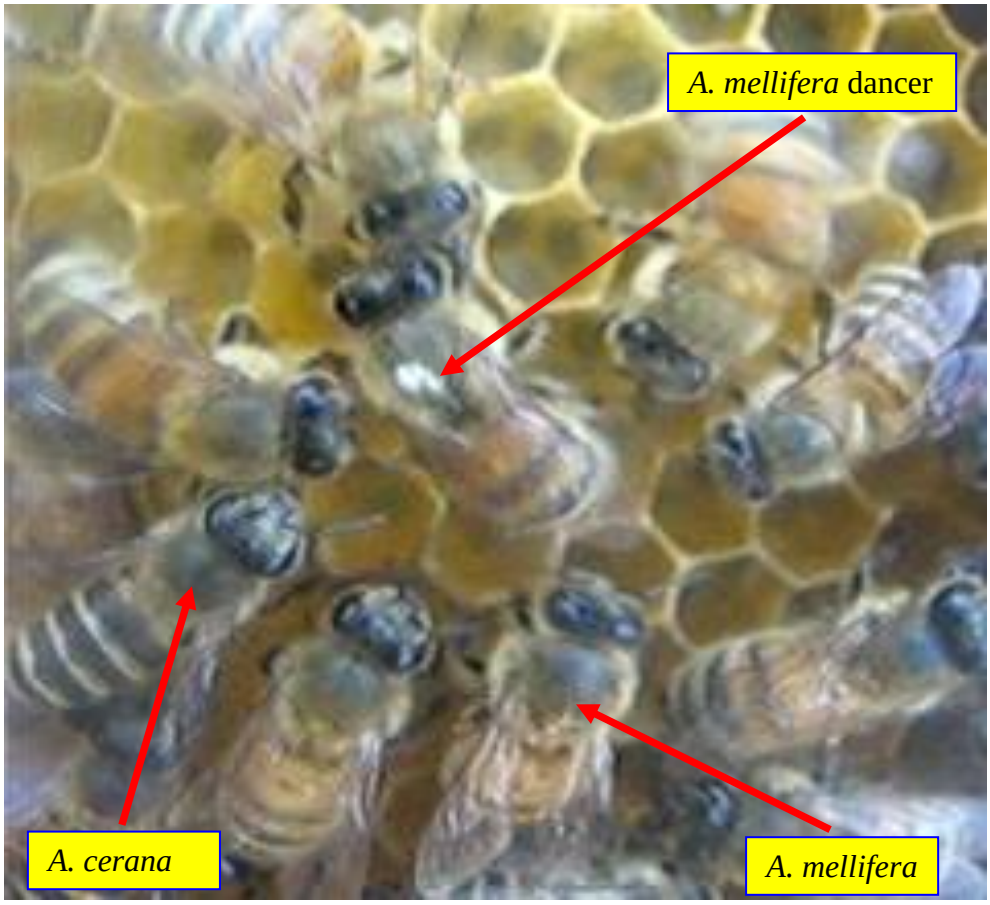


Fig. 4.2 Workers of both species attending an *A. mellifera* dancer

Turning to recruitment, the numbers of potential recruit bees that followed six individual dancers of each species were recorded and there was a highly significant difference in the mean numbers of *A. mellifera* (0.8 ± 0.7) and *A. cerana* (5.7 ±1.1) potential recruits following dances when performed by *A. cerana* dancers ($P < 0.001$). Most interestingly, given the converse situation, there was a highly significant difference in the mean numbers of *A. mellifera* (1.4 ± 0.9) and *A. cerana* (7.6 ± 1.7) potential recruits following dances when performed by *A. mellifera* followers ($P < 0.001$). The mean duration of waggle runs for *A. mellifera* was 63.8 ± 5.3 which was significantly greater than that of 52.0 ± 6.2 for *A. cerana* ($P < 0.001$). There were no significant differences in the three conspecific test colonies for the foraging and waggle dance baselines, and the species-specific characteristics of the waggle dances for both species.

The results of the second experiment are shown in Table 4.2. In terms of intraspecific recruitment, there was a highly significant difference in the total number of conspecific new recruits for *A. cerana* ($N = 131$) and *A. mellifera* ($N = 27$) ($P < 0.001$). In the case of interspecific recruitment, significantly more *A. cerana* foragers ($N = 235$) were successfully recruited by *A. mellifera* waggle dancers than *A. mellifera* foragers ($N = 33$) recruited by the *A. cerana* waggle dancers ($P < 0.001$). Interestingly, there was no significant difference in the numbers of *A. mellifera* recruited by either *A. mellifera* or *A. cerana* ($P = 0.439$). Oddly, significantly more *A. cerana* foragers were recruited by *A. mellifera* than by *A. cerana* ($P < 0.001$).

Table 4.2 Conspecific and heterospecific recruitment of new foragers of *A. cerana* and *A. mellifera*

	Feeder distance (m)	No. of new recruited bees to the feeder in 120 min			
		A. mellifera dancer		*A. cerana* dancer	
		mean±S.D.	Precision %	mean±S.D.	Precision %
A. mellifera recruited (*N* = 3 colonies)	110	2.0±1.0	22.2	3.7±3.5	33.3
	130	3.3±0.6	37.0	4.7±0.6	42.4
	170	2.3±2.3	25.9	1.7±0.6	15.2
	210	1.3±2.3	14.8	1.0±1.7	9.1
A. cerana recruited (*N* = 3 colonies)	110	25.0±8.7	31.9	9.0±2.6	20.6
	130	34.3±22.7	43.8	16.0±7.6	36.6
	170	10.0±7.6	12.8	16.0±13.4	36.6
	210	9.0±9.6	11.5	2.7±2.3	6.1

Turning to the accuracy with which the new recruits first reached the 130 m feeder, only 36.6% of *A. cerana* bees recruited by *A. cerana* waggle dancers reached the feeder. Of the balance, 20.6% went to the feeder at 110 m and 42.7% over-shot the 130m feeder and landed on the more distant feeders. Like *A. cerana*, only 37.0% of *A. mellifera* bees recruited by *A. mellifera* waggle dancers reached the feeder while of the balance, 22.2% found the feeder at 110 m and 40.7% over-shot and landed on the more distant feeders. There was no significant difference in the percentages of successful intraspecific recruits for *A. cerana* and *A. mellifera* ($P = 0.969$).

4.4 Discussion

Although waggle dances could provide a number of recruitment stimuli, it remains unknown which the bees actually use. And, indeed, those features of the dance that assist followers to stay with the dancers need not be the same as those that carry the direction and distance signal (Dyer, 2002). Although there are internal differences in the waggle dances of *A. cerana* and *A. mellifera* foragers, the basic structure of the waggle dance is the same in both (Lindauer, 1956). For the successful interpretation of the waggle dance of any group of honeybees, it is an a priori requirement that there must be a dancer with information to transmit. Such a dancer needs an audience to which it can deliver its information and members attending such dances must acquire and act on that information. Foragers of *A. cerana* and *A. mellifera* fulfil these conditions when each

performed waggle dances and successfully recruited foragers of the other species together in a mixed-species colony.

Thus, it is demonstrable that both species can acquire and act on information provided by each other's waggle dances in mixed-species colonies of *A. cerana* and *A. mellifera.* Inasmuch as the round dances change to waggle dances at different distances, target distance should be overshot in the one and undershot in the other. However, the same percentages of *A. cerana* and *A. mellifera* recruits both undershot and overshot the target, under both conspecific and heterospecific dance conditions. Towne and Gould (1988) showed that the spatial precision of the dance in *A. mellifera* is neither so accurate that they usually find areas which have already been depleted nor so inaccurate that they usually fail to find the advertised resources altogether. Moreover, the bees' distance errors decrease greatly with increasing distance to the target. It is just this pre-speciation flexibility in precision that allows about 40% *A. cerana* and *A. mellifera* recruits to accurately home into a target on the first time out.

CHAPTER 5

Comb construction in the mixed-species colonies

Summary

Comb building behaviour of mixed-species colonies of honeybees, *Apis cerana* and *Apis mellifera*, was studied. Two types of cell-size foundation were made from the waxes of both species and inserted into mixed-species colonies headed either by an *A. cerana* or *A. mellifera* queen. The mixed-species colonies did not discriminate between the wax types, but the *A. cerana* cell-size foundation was modified during comb building by the cooperative efforts of the workers of both species. In pure *A. cerana* colonies workers did not accept any foundation, but were stimulated by *A. mellifera* workers to secrete wax and build on the foundation in mixed-species colonies. Although the task of comb building requires the cooperation of many individuals, it is actually performed by small groups of workers through a mechanism of self-organization. In this way, the two species cooperate in comb building and can construct nearly normal combs, even though they contain many cells of irregular shapes. The utilization of the combs which were built on the two types of foundation differed. In pure *A. mellifera* colonies, the *A. cerana* cell-size was modified and the queens were reluctant to lay eggs on such combs. In pure *A. cerana* colonies, the *A. mellifera* cell-size was built without any modification, but these cells were used either for drone brood rearing or for food storing. The principal elements of comb building behaviour are common to both species which indicates that they evolved prior to and were conserved after speciation. The use of mixed-species colonies is established as an important probe to explain the social mechanisms driving comb construction and to illuminate behavioural traits that evolved prior to speciation.

5.1 Introduction

Nest construction behaviour in social insects is a very complex and highly cooperative phenomenon (Wilson, 1971; Belic et al., 1986). In honeybee colonies, the nests result from numerous kinds of operations performed by many individual bees (Hepburn, 1986). How so many individuals are able to cooperate in comb building has long tantalized researchers. For example, does any individual comb construction worker have some concept, some blueprint, for the whole comb that she is working on as would an individual human worker with access to a blueprint of a building being constructed? Although the task of comb construction, like many tasks performed in a honeybee colony, requires concerted actions by many nestmates, individuals are in fact very poorly-informed and lack a central controller. Coordination relies on subtle mechanisms combining individual decision rules with specialized signals and informative local cues (Pratt, 2004). It has been suggested that the comb building of honeybees can be interpreted as a model of self-organization (Belic et al., 1986; Bonabeau et al., 1997; Hepburn, 1998).

Theories of self-organization were originally developed in the contexts of physics and chemistry in order to describe the emergence of macroscopic patterns from processes and interactions defined at the microscopic level (Bonabeau et al., 1997). These theories have been introduced into ethological systems, particularly social insects, to show that complex collective behaviours may emerge from interactions among individuals that actually exhibit simple individual behaviours (Bonabeau et al., 1997). Over the last two decades, the concept of self-organization has dramatically changed our views on how collective decision-making, division of labour and structures may emerge in societies of ants, wasps, termites and honeybees (Boomsma and Franks, 2006; Detrain and Deneubourg, 2006). This enables researchers to map almost the whole image of insect societies: how regulation of internal conflicts and individual skills and collective intelligence in resource acquisition, nest building and defense, occur (Boomsma and Franks, 2006). Indeed, regulation of behaviour through self-organization, specifically in honeybee societies, can be used to interpret behaviours including comb construction (Belic et al., 1986; Hepburn, 1998), as well as the arrangement of food storing and brood rearing on the combs (Camazine et al., 1990; Camazine, 1991) and the regulation of food collection behaviour (Jenkins et al., 1992).

Several studies on comb building in *A. mellifera* have shown that some very simple building rules (Darchen, 1954; Hepburn and Whiffler, 1991) coupled to the physico-chemical properties of beeswax as a building material (Pirk et al., 2004; Buchwald et al., 2006; Hepburn and Pirk, 2009) can parsimoniously explain several aspects of comb building behaviour. However, the two sister-species, *A. cerana* and *A. mellifera*, not only differ in the chemical components of their waxes (Aichholz and Lorbeer, 1999), but also have different worker cell-sizes. *A. cerana* worker cells have a diameter of 4.4-5.1 mm (Kuang and Kuang, 2002), while those of *A. mellifera* are 5.2-5.4 mm and differ among the races of this species as well (Winston, 1987; Kuang and Kuang, 2002). In beekeeping practice, in order to induce the colonies to build combs more quickly and with more regularity, artificial beeswax foundation embossed with the average cell-sizes are commonly used for a particular species.

In a recent study, Hepburn et al., (2009) reported that when sheets of foundation made from *A. cerana* wax and *A. mellifera* wax were made available to *A. m. capensis* colonies, they tended to accept and construct on both of them, indicating that they do not exercise wax discrimination; but *A. cerana* colonies either gnawed the foundation or left the *A. m. capensis* wax untouched (Hepburn et al., 2009). Mixed-species colonies offer us a valuable opportunity for the integrative investigation of the relationships of the two species and provide us with a new perspective to study the theories of self-organization in honey bees. Studies have already reported that the workers of *A. cerana* and *A. mellifera* accept heterospecific queens and cooperatively coexist with bees of other species (Tan et al., 2006). They can even mutually understand the "dance language" irrespective of dialectical differences (Su et al., 2008; Tan et al., 2008).

However, division of labour in mixed-species colonies remains an intriguing issue not previously considered. In this chapter we report studies on comb construction behaviour of mixed-species colonies of *A. cerana* and *A. mellifera* to answer several questions: 1) Will the mixed-species colonies accept the waxes of both species? 2) Will pure colonies of *A. cerana* accept *A. mellifera* wax and vice versa? 3) Given that the bees are presented with beeswax foundation of different cell base sizes, are these accepted as such or are they modified? 4) Do *A. cerana* workers and *A. mellifera* workers co-operate heterospecifically in comb building, or do they form separate, conspecific festoons? 5) Under the various conditions above, what cell-sizes would result in the newly constructed combs? 6) Once constructed, how are these cells used in the economy of the nest.

5.2 Materials and methods

5.2.1 Organization of the mixed-species colonies

Mixed-species colonies of both *A. cerana* and *A. mellifera* were established. Three colonies contained worker brood of both *A. cerana* and *A. mellifera* and were headed by *A. cerana* queens; and, reciprocally, three contained worker brood of both *A. cerana* and *A. mellifera* headed by *A. mellifera* queens. Sealed brood about to emerge as young adults of each species was placed into the colonies of the other species (Tan et al., 2006). The wax building behaviours were investigated when the newly emerged workers of the two species were about 10-18 days old, the peak age of wax secretion (Hepburn et al., 1984; Seeley, 1995). Pure *A. cerana* and *A. mellifera* colonies with the same age cohort of workers were selected as control groups. Each of the colonies was equal in size.

5.2.2 Wax foundation

The experiments on the utilization of the newly built combs in the pure *A. cerana* and *A. mellifera* colonies were done at an apiary of Yunnan Agricultural University, Kunming, China to refine the final experimental protocol. In these experiments, beeswax was extracted from the combs of both *A. cerana* and *A. mellifera* and then used to make small sheets of beeswax foundation (about 25 x 80 and 2 mm thick) of two worker cell-sizes: *A. cerana*, about 4.75 mm diameter and *A. mellifera*, 5.35 mm using a silicon rubber mould (Hepburn et al., 2009). We inserted both *A. cerana* cell-size (4.75 mm diameter) foundation and *A. mellifera* cell-size (5.35 mm) foundation into pure *A. cerana* colonies and pure *A. mellifera* colonies.

The experiments on cell-size and wax discrimination, and comb building cooperation were conducted with colonies of *A. cerana cerana* and *A. mellifera ligustica* at an apiary at the Ratchaburi Campus of King Mongkut's University of Technology Thonburi, Thailand. The same four types of beeswax foundation sheets (2 cell-sizes and 2 wax types) were fixed on the top bar of a frame, their relative positions determined by random number assignment. They were then inserted into the centre of the hives.

5.2.3 Observations

We used a video camera to record the comb building behaviour of the test and control colonies for 10 sec intervals three times a day. On replay of the video clips, we were able to obtain detailed information on 1) how many workers of each species were engaged in which type of comb building; 2) how many starting sites were used to extend the building of new combs; 3) whether they formed a mixed-species building chain and cooperated with each other in comb building; 4) how many workers of each species were in each festoon; and 5) when building was complete. When the foundation sheets had been extended beyond their original lengths by the addition of several centimetres of new wax, the combs were removed from the hive and represented one sample for that colony. These combs were replaced by a new top bar with the same four kinds of foundation. The cell-sizes and cell patterns were measured.

5.2.4 Statistical analysis

Chi-square tests were used to test for differences in the numbers of modified cells and patterns of cell orientation between the four types of colonies of *A. mellifera* and *A. cerana* queen-headed mixed-species colonies, and pure *A. cerana* and *A. mellifera* colonies. To test for differences in the mean numbers of workers engaged in comb building and the mean cell size of the new built combs between the four types of colonies, we used ANOVA and Tukey post-hoc tests. Homogeneity of the variances between types of colonies was checked using Levene's test. Paired samples *t*-tests were used to compare the mean number and proportions of *A. cerana* workers to *A. mellifera* workers in the *A. mellifera* and *A. cerana* queen-headed mixed-species colonies. The means and standard deviations of each variable were calculated. All tests were performed using Statistica© (StatSoft 2008).

5.3 Results

5.3.1 Cell-size and wax discrimination

Pure *A. cerana* colonies ignored all sheets of beeswax foundation and began building new combs either from the top bar or from the lower edges of the foundation sheets (Fig. 5.1a). In contrast, the pure *A. mellifera* colonies accepted all sheets of both *A. cerana* and *A. mellifera* foundation and built cells on both cell-sizes as well (Fig. 5.1b). In the two types of mixed-species colonies, workers of both species were seen building cells on all the four types of foundations (Figs. 5.1c, 5.1d and 5.2; Table 5.1). None of these colonies showed any preference for a particular type of foundation with respect to wax type or cell size (Repeated measures ANOVA: $P > 0.05$).

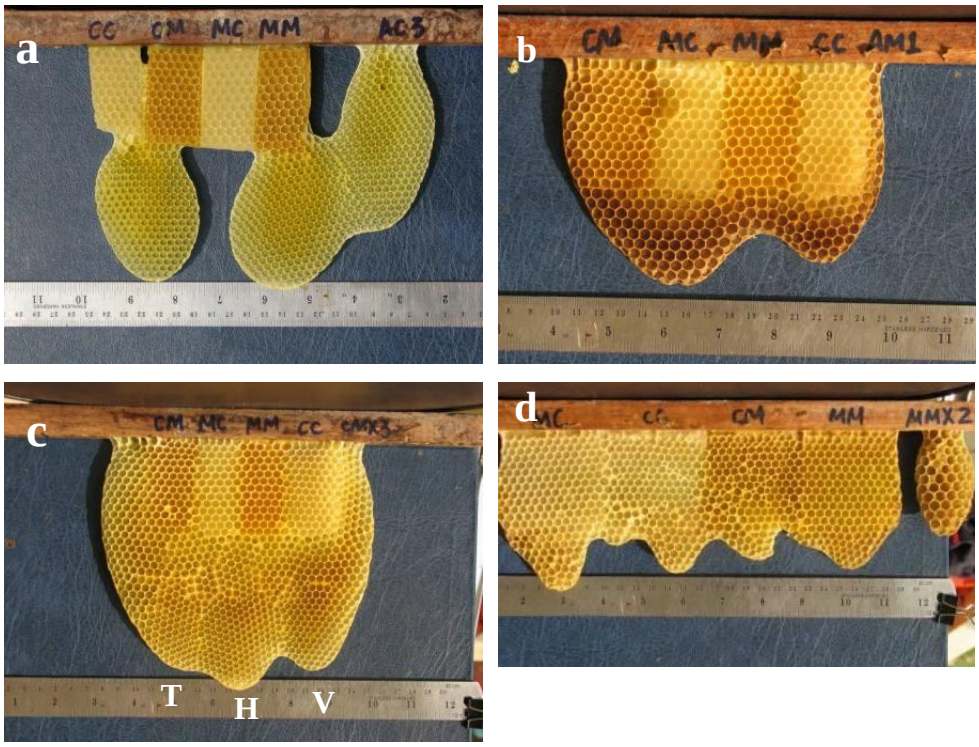


Fig. 5.1 Combs built in the four types of colonies. **a)** pure *A. cerana*; **b)** pure *A. mellifera;* **c)** *A. cerana* queen-headed; and, **d)** *A. mellifera* queen-headed colony. Abbreviations on the top bars are: **CC** = *A. cerana* cell-size foundation made from *A. cerana* wax; **CM** = *A. cerana* cell-size foundation made from *A. mellifera* wax; **MM** = *A. mellifera* cell-size foundation made from *A. mellifera* wax; **MC** = *A. mellifera* cell-size foundation made from *A. cerana* wax; cell direction patterns of newly built combs, **V** = vertical, **H** = horizontal, **T** = tilted

Fig. 5.2 Comb building by a mixed chain of *A. cerana* and *A. mellifera* workers

Table 5.1 Mean numbers (± S.D.) of worker bees engaged in comb building on the four types of foundation

Foundations		**Host colonies**					
Waxes	Cell-size	*A. cerana* queen-headed mixed colonies (*N*=3,14 replicates)		*A. mellifera* queen-headed mixed colonies (*N*=3,10 replicates)		Pure *A.cerana* colonies (*N*=3,12 replicates)	Pure *A.mellifera* colonies (*N*=3,12 replicates)
		A.cerana workers	*A.mellifera* workers	*A.cerana* workers	*A. mellifera* workers	*A. cerana* workers	*A. mellifera* workers
A.cerana	*A.cerana*	3.5±2.2	18.0±5.7	3.3±2.1	18.2±9.0	—	16.8±9.8
	A.mellifera	5.1±2.4	16.6±6.1	2.5±2.3	17.0±7.5	—	21.2±9.7
A.mellifera	*A.cerana*	4.1±2.4	17.0±3.3	1.4±1.2	18.1±8.2	—	19.3±10.4
	A.mellifera	3.4±3.3	16.5±4.9	1.9±2.0	19.2±4.5	—	15.8±10.6
	P-value	0.221	0.743	0.110	0.863	—	0.216

5.3.2 Comb building cooperation in the mixed-species colonies

Cell-size modification on the foundation sheets

All the *A. mellifera* cell-size sheets of foundation were built to their original size without any modification, but the *A. cerana* cell-size foundation sheets were modified in all colonies except the pure *A. cerana* colonies. Some of these cells were squeezed to make space for enlarging neighbouring cells. The percentages of combs which had modified cells in the test and control groups are shown in Table 5.2. In *A. mellifera* queen-headed mixed-species colonies, all the *A. cerana* foundation sheets were modified, as they also were in the pure *A. mellifera* colonies, which is significantly different from the *A. cerana* queen-headed mixed-species colonies and pure *A. cerana* colonies (Chi-square: $\chi^2_3 = 71.7$, $P < 0.001$).

Table 5.2 Percentages of *A. cerana* cell-size foundations with modified signs

Colony Type	*A. cerana* cell-size foundations	percentage of foundations with modified signs
Pure *A. cerana*(*N*=3,12 replicates)	24	0%
Pure *A.mellifera* (*N*=3,12 replicates)	24	83.3%
A. cerana queen-headed mixed (*N*=3,14 replicates)	28	10.7%
A. mellifera queen-headed mixed (*N*=3,10 replicates)	20	100%

Free built combs

On completion of the trials of comb building on the artificial foundation sheets (except pure *A. cerana* colonies), the workers from the four types of colonies started building new combs at several sites (Table 5.3). Pure *A. mellifera* colonies and *A. mellifera* queen-headed mixed-species colonies had significantly more festoons at new comb building sites than *A. cerana* and *A. cerana* queen-headed colonies (ANOVA: $F_{3,44}$ = 15.9, $P < 0.001$; Table 5.3). In *A. cerana* queen-headed mixed-species colonies, workers of both species were seen working together in festoons, although significantly more *A. mellifera* workers were involved (42.1±6.2% *A. cerana* workers, 57.9±6.2% *A. mellifera* workers; Paired *t*-test: t_{13} = 4.9, $P < 0.001$). Similarly, in *A. mellifera* queen-headed mixed-species colonies, significantly more *A. mellifera* workers than *A. cerana* workers were engaged in comb building in the festoons (32.5±4.8% *A. cerana* workers, 67.5±4.8%, *A. mellifera* workers; Paired *t*-test: t_9 = 9.8, $P < 0.001$; Table 5.3). In total, significantly more workers were engaged in comb building in the mixed-species colonies than in the pure *A. cerana* and pure *A. mellifera* colonies (ANOVA: $F_{3,44}$ = 11.3, $P < 0.001$; Table 5.3).

Table 5.3 Means (± S.D.) of characteristics of free built combs

	A. cerana queen-headed mixed colonies(*N*=3; 14 replicates)	*A. mellifera* queen-headed mixed colonies(*N*=3; 10 replicates)	Pure *A. cerana* colonies(*N*=3; 12 replicates)	Pure *A. mellifera* colonies(*N*=3; 12 replicates)
Number of festoons	2.3^{b} ±0.5	4.2^{a} ±1.4	1.9^{b} ±0.9	3.9^{a} ±1.1
Number of *A. cerana* workers on the festoons	61.4±13.4	36.8±10.7	108.0±29.1	—
Number of *A.mellifera* workers on the festoons	84.6±16.1	75.6±16.3	—	90.3±25.0
Total number of two species workers on the festoons	146.1^{a} ±22.0	112.4^{b} ±24.5	108.0^{b} ±29.1	90.3^{b} ±25.0
Percent of irregular cells	9.1^{a} ±3.6%	10.8^{a} ±4.7%	0.8^{b} ±0.5%	2.7^{b} ±1.7%
Patterns of the newly built combs: V= vertical H= horizontal T= tilted R= rosette	V+H: 29% V+H+T: 22% V+T: 21% V: 14%; T: 7% V+H+R: 7%	V+H: 60% V: 40%	V: 75% V+H: 17% T: 8%	V: 83% V+H: 17%
Cell-size of the new built combs (mm)	5.41^{b} ±0.27	5.93^{a} ±0.61	4.38^{c} ±0.06	5.74^{ab} ±0.61

Means within one row followed by the same letter are not significantly different (Tukey multiple comparisons: $P > 0.05$)

As for irregular cells on the new combs, pure *A. cerana* and pure *A. mellifera* colonies built significantly fewer irregular cells (0.8% and 2.7%, respectively), than did the mixed-species colonies (9.1% and 10.8%, respectively), most of which were located at the seams of combs which had been started at different sites (ANOVA: $F_{3,44} = 30.0$, $P = 0.003$; Table 5.3). The *A. cerana* queen-headed mixed-species colonies showed significantly greater variation in the patterns of cell orientation on the newly built combs than *A. mellifera* queen-headed colonies, pure *A. cerana* and *A. mellifera* colonies: different festoons on one comb may build patterns different from others (Chi-square: $\chi^2_6 = 27.9$, $P < 0.001$; Fig. 1c, Table 5.3). *A. mellifera* queen-headed colonies built new combs mainly in vertical and horizontal patterns (Fig. 5.1d); in pure *A. cerana* and *A. mellifera* colonies, the patterns of cell orientation were more homogeneous and mainly vertical (Figs. 5.1a, 5.1b, Table 5.3).

The different mixed-species colonies built significantly different sized cells (ANOVA: $F_{3,44} = 34.8$, $P < 0.001$; Table 5.3). The largest cells were built by *A. mellifera* queen-headed mixed-species colonies. The cells built in the pure *A. mellifera* colonies and *A. mellifera*-queen-headed mixed-species colonies were like those *A. mellifera* drone cells (6.0-6.3 mm; Winston, 1987), while in the *A. cerana* queen-headed mixed-species colonies, the cells had a diameter of 5.41±0.27 mm, which is like normal

A. mellifera worker size cells. The pure *A. cerana* colonies built cells of 4.38±0.06 mm size, which is the normal *A. cerana* worker cell-size.

5.3.3 Utilization of the newly built combs in the pure *Apis cerana* and *Apis mellifera* colonies

In these experiments, we inserted both *A. cerana* cell-size (4.75 mm diameter) foundation and *A. mellifera* cell-size (5.35 mm) foundation into pure *A. cerana* colonies and pure *A. mellifera* colonies with the following results. Pure *A. cerana* colonies accepted both foundation types and built cells without altering the original cell base. Pure *A. mellifera* colonies accepted both foundation wax types but changed the *A. cerana* cell-size to their normally larger cells with the inclusion of many irregular cells.

Once the control combs had been constructed, *A. cerana* colonies differed from *A. mellifera* colonies in the subsequent use of these cells. The pure *A. cerana* colonies used the *A. mellifera* size cells either for food storing (Fig. 5.3) or drone brood rearing (Fig. 5.4); while the *A. cerana* size cells were normally used for rearing worker brood. In pure *A. mellifera* colonies, the queens laid eggs into both *A. mellifera* size and *A. cerana* size cells, but they all showed a preference for *A. mellifera* size cells and laid eggs into the former cells first and more regularly (Fig. 5.5).

Fig. 5.3 Utilization of combs built on two types of cell-size foundation in pure *A. cerana* colonies: the *A. mellifera* size cells (left) were used for food storing while the *A. cerana* size cells (right) were used for brood rearing

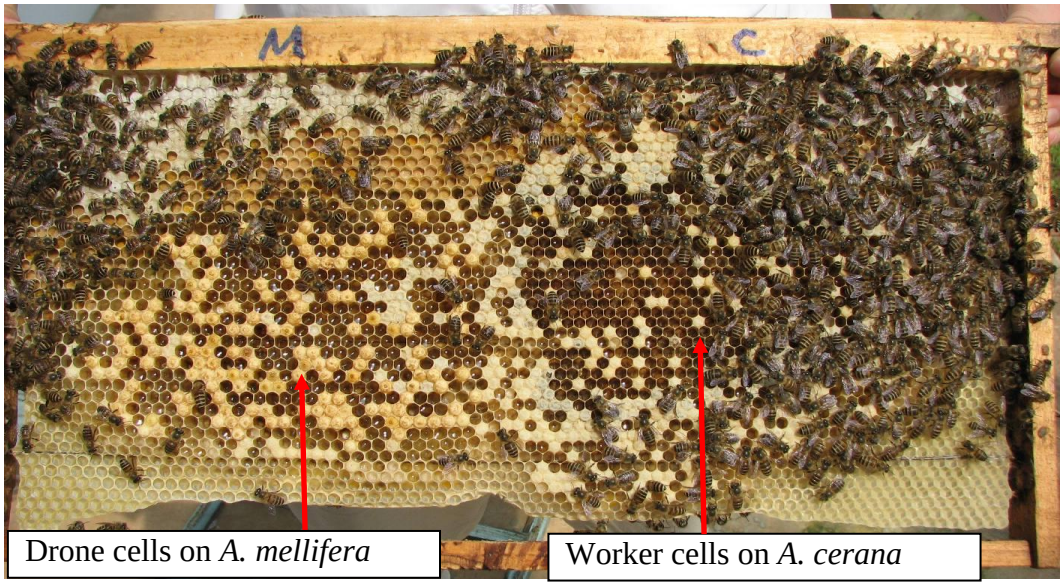


Fig. 5.4 Utilization of combs built on two types of cell-size foundation in pure *A. cerana* colonies: the *A. mellifera* size cells (left) were used for drone brood rearing (with typical capping apertures) while the *A. cerana* size cells (right) were used for rearing worker brood

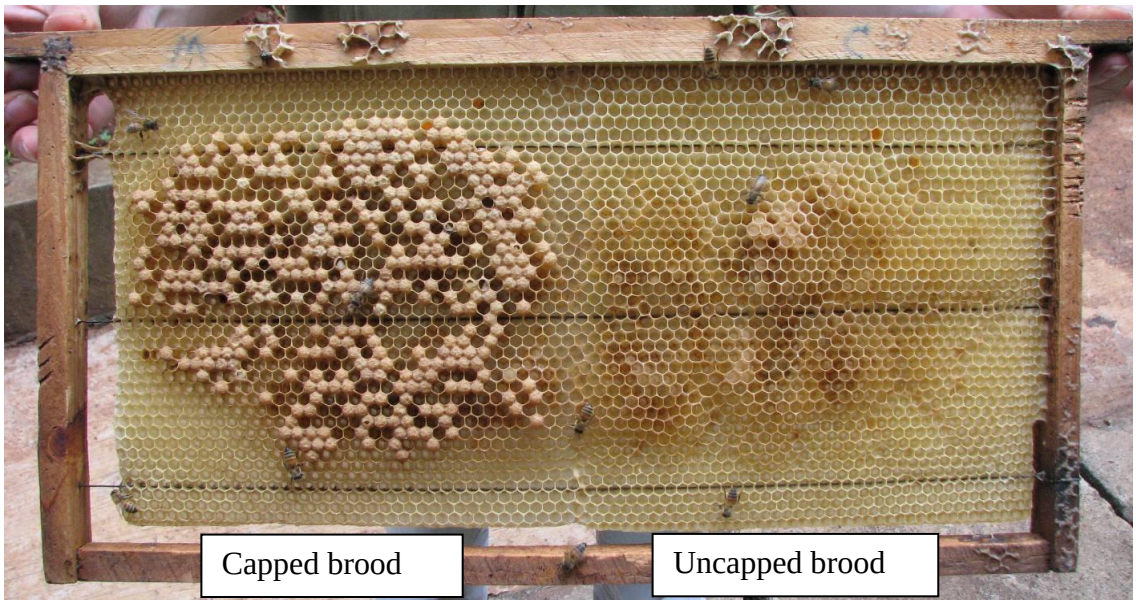


Fig. 5.5 Utilization of combs built on two types of cell-size foundation in pure *A. mellifera* colonies: the brood cells on the *A. mellifera* cells are capped already, but the larvae on the *A. cerana* side still need about 3 more days until capping, suggesting that the queens first laid eggs on the left side and only laid eggs in the *A. cerana* size cells somewhat later

5.4 Discussion

It is common knowledge that the cavity-dwelling honeybees build multiple, parallel combs (Crane, 1990), and that this parallelism is recognized as a building rule (Darchen, 1954; Hepburn, 1986; Hepburn and Muller, 1988). But to achieve parallelism of all the combs, each constructed at separate and independent starts, is not an easy task from a human perspective. Comb constructing bees are working in a dark cavity or hive where there is no central source of information. When construction begins, the workers cling together in elongated chains or festoons, forming a dense cluster which facilitates an equable temperature for wax secretion and manipulation (Hepburn, 1986; Winston, 1987). Numerous comb building workers, with active wax glands, engage in the task of comb construction. But, instead of building a single comb together, several festoons begin at independent sites and begin building several cells, hence combs, simultaneously and only later connect them with some irregular transitional cells (Hepburn, 1986; Winston, 1987; Hepburn and Whiffler, 1991). In this case, the parallelism rule can only be achieved indirectly at the finishing stage of comb building,

with many irregular cells and seam connections between several branches started from separate sites (Hepburn and Whiffler, 1991).

Beeswax is obviously a kind of "biological construction material" (Buchwald et al., 2006), which derives from the synthesis and secretion of beeswax (Ribbands, 1953; Hepburn, 1986). Although the beeswaxes differ somewhat in chemical composition, synthesis and secretion of the wax glands have been highly conserved features during honeybee evolution (Hepburn et al., 2009). Indeed, they all share a complex mixture of homologous neutral lipids in common: alkanes C25–C27, monoesters C40–C54 and diesters, hydroxymonoesters C40–C52, hydroxydiesters C50–52 and diesters C56–58 (Aichholz and Lorbeer, 1999). There are nonetheless notable species-specific differences in beeswaxes (Aichholz and Lorbeer, 1999). For example, waxes from *A. cerana* and *A. mellifera* differ in 27 chemical components, none of them present in both kinds of beeswaxes. The two honeybees species, *A. cerana* and *A. mellifera* are closely related sister species and it has been suggested that they diverged only about 3 million years ago (Arias and Sheppard, 1996, 2005). They share many common characteristics, and they can be reared in the same hive with special techniques (Tan et al., 2006).

It is somewhat strange that in the pure *A. cerana* colonies, none of the four types of foundations were accepted, although 2 of the 4 foundations were embossed with normal *A. cerana* cell size. In sharp contrast, in the pure *A. mellifera* colonies, the workers were seen building cells on both types of wax and both cell-sizes. These results indicate that *A. mellifera* workers are more tolerant of wax and cell-size factors. This contrast is revisited in both types of mixed-species colonies where more *A. mellifera* workers than *A. cerana* workers were seen building comb, irrespective of the host queen. However, interestingly, *A. cerana* workers did engage in comb building on foundations of both waxes and the two cell sizes in the both types of the mixed-species colonies (Table 5.1). This certainly suggests that *A. mellifera* comb building workers can stimulate *A. cerana* workers to start comb building. And, a comb building stimulus appears reciprocal because in pure *A. mellifera* colonies, while 83.3% of the *A. cerana* cell-size foundation sheets were modified and expanded to *A. mellifera* cell-size, only 10.7% were modified in mixed-species colonies headed by *A. cerana* queens. In the *A. cerana* queen headed mixed-species colonies, more *A. mellifera* workers were engaged in comb building in festoons, so it is not surprising that the cell sizes were similar to normal *A. mellifera* workers.

It is interesting to note that in this type of mixed colony, the festoons were formed predominately by *A. mellifera* workers with fewer *A. cerana* workers joining them. However, the combs built in the mixed-species colonies did have more irregular cells than were observed in any of the pure *A. cerana* or *A. mellifera* colonies. This seems to indicate that the *A. cerana* workers also play a role in determining final cell-size. Although they did cooperate with each other in festoons, the two species cannot really perform the comb building task harmoniously. That the combs in the pure *A. mellifera* colonies and *A. mellifera* queen-headed colonies were built into normal *A. mellifera* drone size cells, may be related to the season in which we conducted the experiment.

In conclusion, the *A. cerana* workers, as a colony did not accept any type of beeswax foundation, but as individuals, they can be stimulated by *A. mellifera* workers to engage in comb building. So, our results are consistent with the idea that honeybee comb building behaviour is an example of self-organization. We also confirm that in the mixed-species colonies, these two closely related honeybee species did in fact cooperate in comb building, even though irregular cells arise through their joint efforts. We can also infer that, although the comb building workers are poorly-informed and lack a central controller (Pratt, 2004), comb building is really a task which can only be finished by some smaller groups in which individuals closely cooperate to achieve progress.

CHAPTER 6

Thermoregulation in mixed-species (*Apis cerana* and *Apis mellifera*) honeybee colonies

Summary

Apis cerana and *Apis mellifera* normally display different strategies in cooling hive temperature, raising the question whether they would coordinate their efforts to achieve stable thermoregulation in mixed-species colonies. The results show that normal temperatures in the brood area in mixed-species colonies are more similar to those of pure *A. cerana* colonies than pure *A. mellifera* colonies. Under heat stress, *A. cerana* workers are more sensitive, and initiate fanning sooner than *A. mellifera* workers. In mixed-species colonies, the former become the main force for thermoregulation. When worker bees of both species fanned together at the entrance, they adopted their own species-specific postures; but, due to a significantly smaller number of *A. mellifera* workers engaged in fanning, the cooling efficiency of mixed-species colonies were closest to that of pure *A. cerana* colonies.

6.1 Introduction

Temperature regulation is regarded as one of the major innovations in honeybee biology (Seeley, 1981; 1985). While body temperature of individual honeybees is strongly dependent upon the ambient, honeybees are able to maintain a stable temperature at colony level quite independently of the surrounding environment (Dyer and Seeley, 1987; Ruttner, 1988). For cavity-nesting honeybees, environmentally induced temperature changes within the nest are compensated by individual honey bee workers via endothermic heat production (Kronenberg and Heller, 1982; Harrison, 1987) or evaporative cooling (Lindauer, 1954; Kleinhenz et al., 2003; Groh et al., 2004). Because temperatures above 36ºC for any appreciable time are harmful to brood (Winston, 1987), the workers collect water and fan their wings at the hive entrance to increase ventilation (Kuhnholz and Seeley, 1997) to prevent them from over-heating. Fanning workers line up in chains facing the same direction throughout the brood nest and at the nest entrance (Winston, 1987).

Interestingly, and also possibly of evolutionary significance, these closely related species differ in body posture when fanning their wings at the hive entrance (Sakagami, 1960; Verma, 1970; Ruttner, 1988), and adopt "opposite" strategies to cool their nests. *A. cerana* bees face away from the entrance and fan outside air into their hives while *A. mellifera* face the entrance and draw the inner, hot air out. So, it would be of interest to study whether bees of mixed-species colonies coordinate their efforts in cooling their nests. Does one species adopt the technique of the other, changing their fanning body posture to that of the other species? Is there a special division of labor such that one species mainly performs this task while the other simply does not fan? If this were the case, the mixed-species colonies might offer us some information about cooperation in the evolution of honeybee societies. Thus, in this chapter, we report investigations on the effectiveness of thermoregulation in the mixed-species colonies and test whether: 1) workers of both species engage in ventilating at the hive entrance? If so, 2) do they fan with their own species-specific body posture or does one species adopt that of the other? And 3) is ventilation efficiency improved or reduced?

6.2 Materials and Methods

6.2.1 Organization of mixed-species colonies

The experiments and observations were conducted with colonies of *A. cerana cerana* and *A. mellifera ligustica* during May to September, 2009 at an apiary of Yunnan Agricultural University, Kunming, China. In order to obtain workers of the same age cohort for ventilating at the entrance (5-25 days old, Winston, 1987), combs with adults near eclosion are prepared for the organization of mixed-species colonies and control groups (Tan et al., 2006).

Two types of mixed-species colonies were established: mixed-species colonies containing worker brood of both *A. cerana* and *A. mellifera* and headed by *A. cerana* queens; and conversely, mixed-species colonies containing worker brood of both *A. cerana* and *A. mellifera* and headed by *A. mellifera* queens. Four colonies each of *A. cerana* and *A. mellifera* with an active egg-laying queen and populations of medium strength (4000-6000 workers for *A. cerana* and 6000-8000 individuals for *A. mellifera*) were chosen as parental colonies to maintain the sealed pupae until emergence. One empty comb and another one with pollen and honey were added to each of these colonies. The colonies were checked daily and the time when the empty combs had been filled with newly laid eggs was recorded so we knew when the developing bees would eclose as young adults. These combs were kept in the parental colonies until they developed into capped pupae and were then transferred into incubators.

Then three *A. mellifera* and three *A. cerana* colonies were chosen as host colonies for establishing mixed-species colonies. These colonies were small, about 1500 individuals, mostly young adults (the older field bees having been eliminated by relocating the hives). These host colonies also had equal numbers of their own sealed pupae about to emerge, so a cohort of workers of the same age of both species could be obtained at the same time. Three days before the young adults would emerge, these brood frames were introduced into hetero-specific host colonies i.e. one *A. mellifera* comb was put into each of the three *A. cerana* colonies and one *A. cerana* combs into each of the *A. mellifera* colonies. Newly emerged young adult bees are readily accepted by the host colonies and so the mixed-species colonies are constituted (Tan et al., 2006). Three pure *A. mellifera* colonies and three *A. cerana* colonies served as control groups and each

contained enough newly emerging adult workers of the same age as those which were introduced into interspecific colonies.

6.2.2 Testing the cooling behaviour and the cooling efficiency in the mixed-species colonies

Once the mixed-species colonies were settled and the introduced workers were adults about fortnight old, we checked and recorded the brood temperature of the colonies three times a day (8:00, 12:00 and 16:00) for a week. A test hive was altered to satisfy our experimental design (Fig. 6.1). This test hive would allow us to monitor the bees' behaviour from the upper side hole, and there is a small side door which can be used to remove the heater without disturbing the test colony. An electric heater was put in the hive to heat the colonies. A thermometer (Sensorted, BAT-12, made in U.S.A, 0.1ºC accuracy) with an external sensing probe was used to measure the fluctuation of the temperature of the brood area in the hive (Fig. 6.1). The test colonies of all colonies in our experiment were kept in moveable frame hives. We transferred bees into this test hive for the thermoregulation measurements, so that all colonies were tested in the same test hive. Because the bees might fan both inside the hives (Winston, 1987) and at the entrance, we monitored fanning behaviour both at the entrance and from the upper side hole. We determined how long after heating the workers started fanning, and the species of the workers observed as were the numbers of fanning bees. We stopped heating once the brood area temperature reached 38.0ºC, the heater removed immediately, and the temperature recorded whenever there was a fluctuation until it returned to normal. The time taken by the tested colony to regulate the brood area temperature from 38.0ºC back to normal was calculated. Each colony was tested at 15:00 in the afternoon for three successive days.

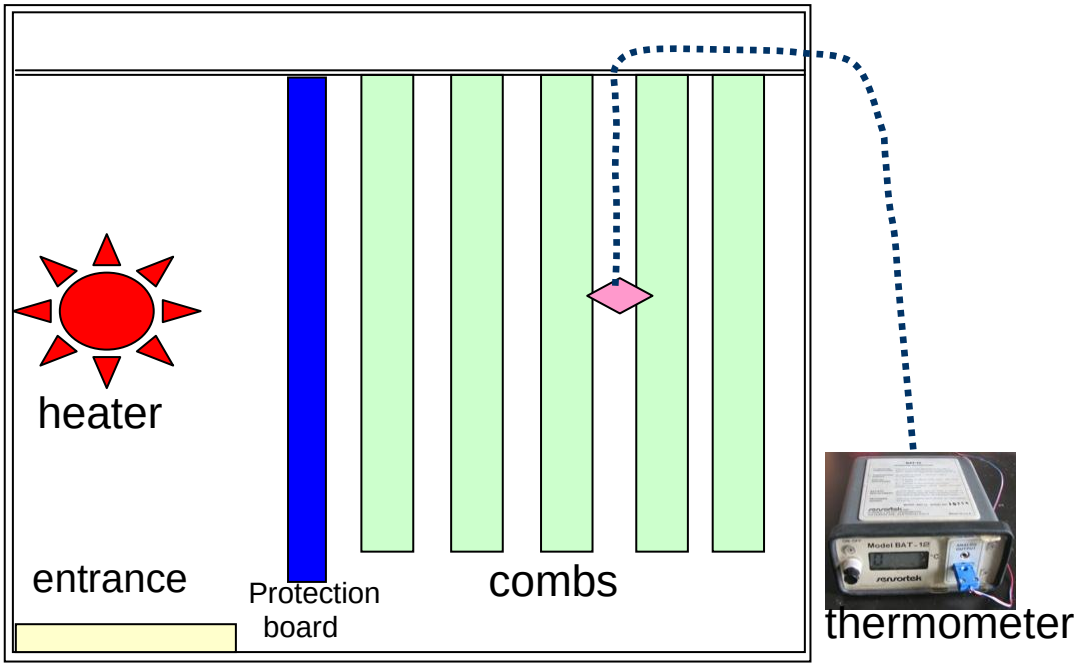


Fig. 6.1 Experimental set-up for heating the hive and monitoring temperature fluctuations of the brood area of honeybee colonies to investigate thermoregulation performance and efficiency

6.2.3 Data analysis

Repeated measures ANOVA tests were used to test for overall differences of normal brood area temperatures of the mixed-species colonies and control groups of pure *A. cerana* and pure *A. mellifera* colonies. The efficiency of thermoregulation differences were compared using ANOVA procedures. Tukey post-hoc multiple comparison tests were used for significant group effects. The means and standard deviations of each variable were calculated. All tests were performed using Statistica© (StatSoft, 2008).

6.3 Results

6.3.1 Normal brood area temperature of mixed-species colonies

The normal temperatures of the brood area at three different times during the day for seven days are listed in Table 6.1. The ambient, external temperature during the test period was between 16.9 ~ 27.4ºC. Pure *A. cerana* colonies had significantly higher

temperatures than pure *A. mellifera* colonies, while temperatures of mixed-species colonies were intermediate (species: $F_{3,80} = 25.1$, $P < 0.001$; time of day: $F_{2,160} = 15.5$, $P < 0.001$; interaction: $F_{6,160} = 4.3$, $P < 0.001$). The two types of mixed-species colonies did not significantly differ. The pure *A. mellifera* colonies had the most stable brood area temperature (varying by only about 0.3ºC, while mixed-species colonies and pure *A. cerana* colonies were quite similar, showing wider fluctuations in temperature.

Table 6.1 Means (± S.D.) of normal brood area temperature (ºC) of the colonies ($N = 3$)

	Normal brood area temperature (ºC)					
Colony type/time	08:00	12:00	16:00	highest	lowest	fluctuation
pure *A. cerana*	35.7^{a}±0.5	35.4^{b}±0.4	34.7^{a}±0.2	35.8^{c}±0.3	34.6^{b}±0.2	1.2^{b}±0.4
pure *A. mellifera*	34.6^{b}±0.1	34.7^{a}±0.1	34.5^{a}±0.1	34.7^{a}±0.1	34.4^{ab}±0.1	0.3^{a}±0.1
mixed (*A. cerana* queen)	34.9^{b}±0.7	35.0^{ab}±0.7	34.8^{a}±0.4	35.4^{b}±0.5	34.4^{a}±0.4	1.1^{b}±0.6
mixed (*A. mellifera* queen)	34.8^{b}±0.8	35.3^{b}±0.7	34.7^{a}±0.5	35.6^{bc}±0.5	34.3^{a}±0.5	1.3^{b}±0.7
ambient	17.8±0.6	25.7±1.1	23.7±0.8	27.4	16.9	10.5

Means within one column followed by the same letter are not significantly different (Tukey multiple comparisons: $P > 0.05$)

6.3.2 Thermoregulation of the mixed-species colonies

Table 6.2 Means (± S.D.) of thermoregulation behaviour and efficiency of the colonies (heating time: the time taken for heating a colony from its normal temperature to 38ºC; honey bee cooling time: the time taken a colony to cool the temperature from 38ºC to its normal temperature)

Colonies	**Workers**	number of bees fanning at entrance	bees fanning inside hive	start fanning when heated (min)	heating time (min)	cooling time (min)
pure *A. cerana*	*A. cerana*	149.4^{a}±15.0	+++	1.4^{a}±0.5	12.0^{a}±2.1	66.8^{a}±4.7
pure *A. mellifera*	*A. mellifera*	106.0^{b}±13.4	+++	3.8^{b}±1.2	12.0^{a}±1.2	54.9^{b}±1.8
mixed (*A. cerana* queen)	*A. cerana*	144.3^{a}±14.4	+++	1.4^{a}±0.5	11.2^{a}±1.9	67.9^{a}±2.9
	A. mellifera	26.7^{c}±10.0	+	5.2^{bc}±1.7		
mixed (*A. mellifera* queen)	*A. cerana*	136.6^{a}±12.1	+++	1.9^{a}±0.8	11.4^{a}±1.3	63.9^{a}±2.7
	A. mellifera	27.9^{c}±6.1	+	6.3^{c}±1.6		

Means within one column followed by the same letter are not significantly different (Tukey multiple comparisons: $P > 0.05$)

The number of fanning bees at the entrance was counted and there were about 130 to 150 *A. cerana* at the entrance of the mixed-species colonies, which did not differ from that of pure *A. cerana* colonies. In contrast, significantly fewer *A. mellifera* workers fanned in pure *A. mellifera* colonies compared to pure *A. cerana* colonies and significantly fewer *A. mellifera* workers engaged fanning in both types of mixed-species colonies (species(mixed *cerana* workers): $F_{3,32} = 18.0$, $P < 0.001$; species(mixed *mellifera* workers): $F_{3,32} = 244.8$, $P < 0.001$; Table 6.2). The bees fanning in the hives of these test colonies were also observed; however, because the bees were also fanning between the multiple combs, we only observed if workers of both species were engaged in fanning on the visible top bar of the hive. The numbers of each species were not counted, as they were at the entrance. *A. mellifera* workers were seen fanning among the combs in the hive, but their numbers were fewer than the fanning *A. cerana* bees.

As is shown in Table 6.2, there were fewer *A. mellifera* workers engaged in fanning either at the entrance or on the top bar of the combs in the hives in both types of mixed-species colonies; but, when workers of the two species fanned together, they retained species-specific poses, i.e., *A. mellifera* with their heads facing toward the hive entrance and *A. cerana* facing out (Fig. 6.2).

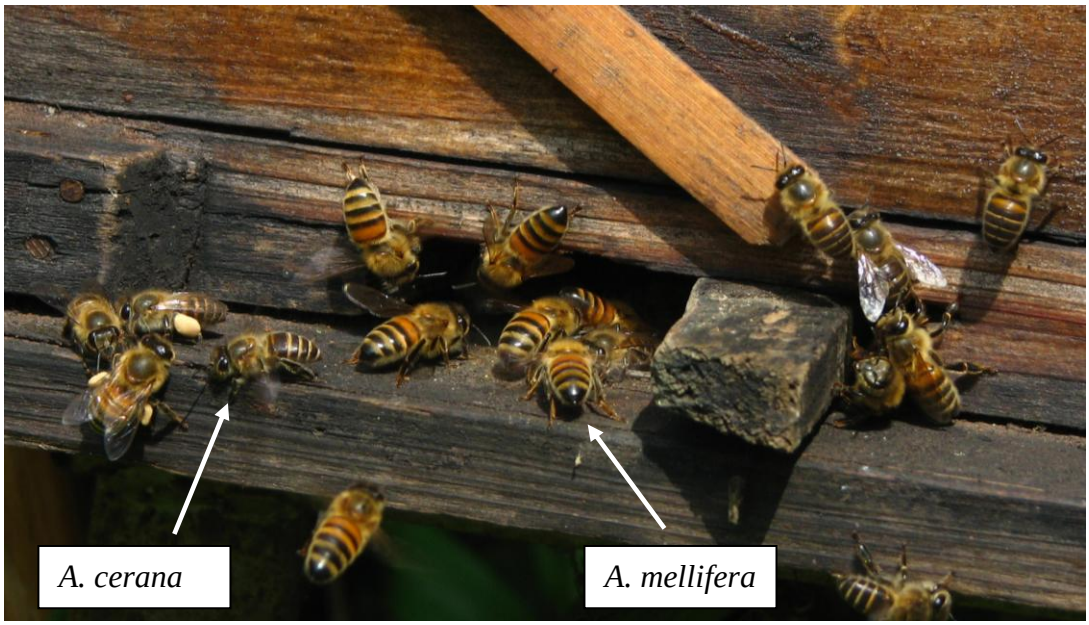


Fig. 6.2 Body posture of *A. cerana* and *A. mellifera* workers fanning at the hive entrance

Pure *A. mellifera* colonies showed the most effective thermoregulation and required only about 55 min to decrease their brood area temperature from 38ºC to normal temperature. The mixed-species colonies consumed significantly longer time to regulate the temperature and were similar to the pure *A. cerana* colonies ($F_{3,32}$ = 30.7, P < 0.001; Table 6.2).

The workers from all of the colonies did not differ significantly in fanning vigorously when their brood area temperatures had risen above 37 ~ 38ºC, but when they decreased it lower than about 37ºC , all colonies appeared to recruit fewer fanning bees. The temperature dropped very fast from 38ºC to 37ºC, and required 10 min for 38ºC to 37ºC, 20 min from 37ºC to 36ºC and 30 min 36 to normal respectively (Fig. 6.3).

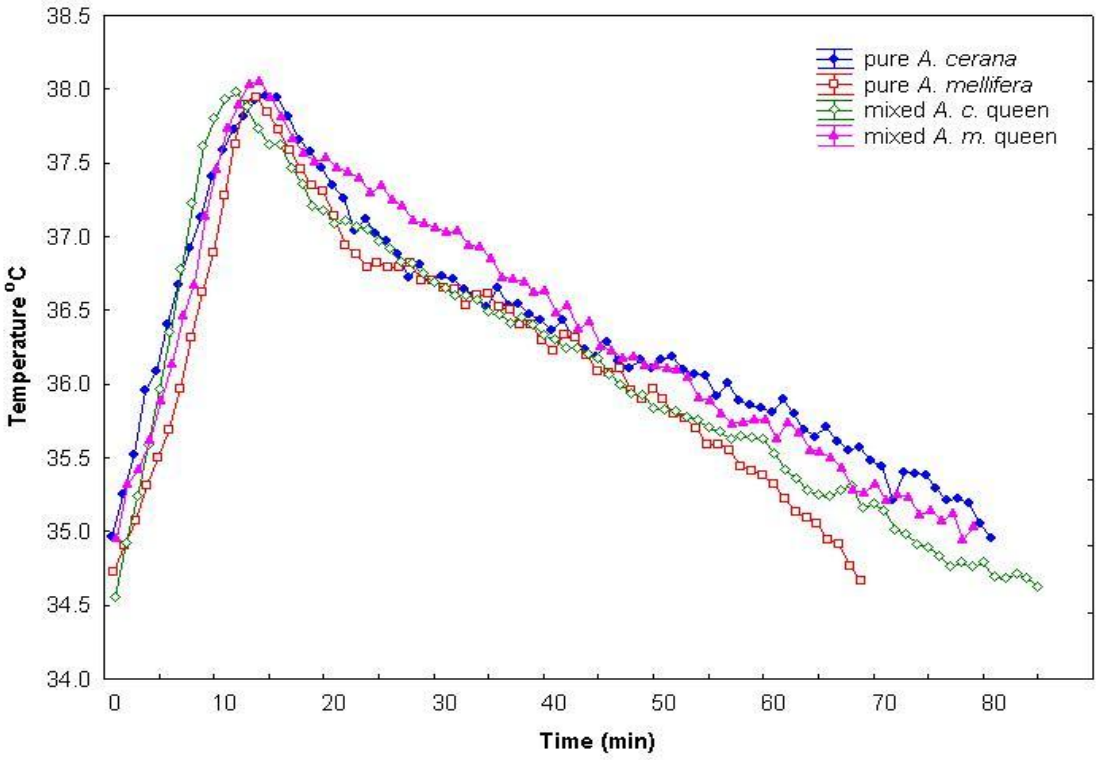


Fig. 6.3 Thermoregulation efficiency of the colonies

6.4 Discussion

The brood temperature of mixed-species colonies did not significantly differ from either of the pure species colonies (Table 6.1). In the morning, their temperatures were close to that of pure *A. mellifera* colonies, while at noon they were intermediate between the

two pure species colonies. But, similar to pure *A. cerana* colonies, both types of mixed-species colonies had greater fluctuations in temperature than pure *A. mellifera* colonies, which indicates that *A. cerana* workers in these mixed-species colonies played more active role than *A. mellifera* workers in thermoregulation.

Workers of all the test colonies were seen fanning both inside the hives and at the entrances. Because there were multiple combs in the test hives (Fig. 6.1), we were not able to accurately count how many workers were fanning between the combs, thus only the species of the fanning bees were distinguished, and their fanning postures noted. When the two species of workers were fanning together at the entrance, they maintained their species-specific postures, their heads facing in opposite directions (Fig. 6.2) as was also observed by Dhaliwal and Atwal (1970). However, it was strange that in both types of mixed-species colonies, *A. cerana* workers were the main force in ventilating the hive, while only few *A. mellifera* workers fanned. This probably explains why both types of mixed-species colonies have thermoregulation efficiency similar to pure *A. cerana* colonies.

Altruism and cooperation are the main factors that make honey bees different from solitary insects; but, cooperation in social organisms has been a difficult issue for evolutionary theory since the time of Darwin (Axelrod and Hamilton, 1981). At first sight, altruism would reduce fitness of the concerned individual and strengthen its opponent's fitness, but this theory is not compatible with individuals of social insets, which live together with hundreds to thousands of nestmates. At the colony level, some new ideas have been introduced such as the famous Kin selection theory (Hamilton, 1963) and the Prison's Dilemma of "game theory" (Chase, 1980) in order to interpret evolutionary history in social insects,).

Hamilton's Kin selection theory suggests that social animals only aid related animals to gain group genetic benefits, while the Prisoner's Dilemma game mainly deals with balance between cooperation and defection. Because two individuals can either cooperate or defect, the payoff to a player is in terms of the effect on its fitness (survival and fecundity). No matter what the other does, the selfish choice of defection yields a higher payoff than cooperation. But if both defect, both do worse than if both had cooperated.

However, in this paper, we studied cooperation of the mixed-species colonies of *A. cerana* and *A. mellifera* in thermoregulation. We found that *A. cerana* workers are more sensitive to temperature changes and initiated ventilation fanning earlier than *A.*

mellifera workers. On the other hand the *A. mellifera* workers abandoned fanning when they detected that the fanning task was been done by others. Thus *A. cerana* workers could be defined as more "cooperative" while *A. mellifera* workers as more "defective".

Before reaching a final conclusion, we must inspect it from some other perspectives: the evolution of hive ventilation at the entrance for captive honeybees and the division of labour mechanism of honeybees. Honeybee colonies usually contain several to tens of subfamilies due to polyandry of the queen. However, honeybee societies do benefit from this subfamily diversity because different subfamilies have different temperature response thresholds in modulating the hive-ventilating behaviour (Graham et al., 2006). So, diversity of subfamilies might well prevent excessive colony level responses to temperature fluctuations (Jones et al., 2004).

However, this idea of "diversity promotes stability" does not easily apply to mixed-species colonies of honeybees, because the workers from different species adopt different techniques in ventilating the hive at the entrance. If both of them fan using their own unique postures, it really reduces the effectiveness of the fanning effort. It would be of interest if one could answer the question why *A. mellifera* workers fan with their heads facing toward the entrance while *A. cerana* workers face out of the hives. Obviously, drawing warm air out of the hive would be more effective in cooling the hive than fanning cooler air into the hives. This idea has been confirmed by the fact that in our experiments, pure *A. mellifera* colonies have more stable hive temperature than pure *A. cerana* colonies.

Task allocation in honeybees is very complicated probably because there is in fact no central information source available and no "controller" bees engaged in task allocation. It has been suggested that each individual has to make its own decisions which could be produced as a self-organization mechanism (Page and Mitchell, 1998; Bonabeau et al., 1997). This mechanism is sufficiently effective when each work force is properly "arranged". Thus, if one task is being done by enough nestmates, newly recruited individuals might stop to perform other tasks, and this might explain the case of *A. mellifera* workers performances in our mixed-species colonies.

CHAPTER 7

Coordinating efforts in colony defense

Summary

In this chapter, the defensive behaviours of mixed-species honeybee colonies: *Apis cerana* and *Apis mellifera* were tested using a common Asian predatory wasp (*Vespa velutina*). When the vespine wasps hawk honeybees at their nest entrances, alerted and poised guard bees of *A. cerana* and *A. mellifera* in the mixed colonies have average thoracic temperatures slightly above 24^{o}C. *A. cerana* workers assume their species-specific wing shimmering and raise their body temperature up to about 29^{o}C, while *A. mellifera* guard bees neither show significant body temperature increases nor wing shimmering. However, when faced with persistent hawking wasps, guard bees of both species, raise their thoracic temperatures and form a ball around it, the core temperature of the mixed-species balls was about 45^{o}C, which is not significantly different from that of only pure species. *A. cerana* bees engulf the ball tighter in the core while *A. mellifera* bees can be seen more likely toward the outer edge. This result shows that the defense behaviours of the two species are based on their species-specific adaptations over evolutionary time.

7.1 Introduction

Vespa velutina, a vespine wasp endemic to Southeast Asia, preys on honeybees, both the native *A. cerana* as well as the introduced European *A. mellifera* (Matsuura and Yamane, 1990; Tan et al., 2005; Ken Tan et al., 2007). When faced with this wasp's attacks, the two honeybees adopt different strategies to defend their colonies. *A. cerana* workers have a quite unique behaviour to recruit additional nestmates by shaking their abdomens. They shake their bodies violently from side to side accompanied by a peculiar hissing (Sakagami, 1960) and then use heat balling (Ono, et al., 1987; Tan et al., 2005; Ken Tan et al., 2007) to kill the wasps if caught by the guard bees. Experiments show that the lethal thermal limit for the wasp *V. velutina* is 45.7^{o}C, which is lower than that of *A. cerana* and *A. mellifera* (50.7^{o}C and 51.8^{o}C, respectively). Thus the wasps can be killed through this thermal margin (One et al., 1987; Tan et al., 2005).

Workers of *A. mellifera*, on the other hand, usually do not show alarm shimmering. Rather, they recruit nestmates to block the entrances and then attack the wasp directly, biting and stinging. This is not as effective as that of *A. cerana* because the wasp's body is covered by a hard cuticle through which the stings cannot be easily inserted. However, *A. mellifera* bees do engage in balling non-nestmate bees or predators and kill them by raising the core temperature of the ball (Heinrich, 1979; Stabentheiner et al., 2002). However, it has not been reported that they have adopted this strategy to defend their colonies against the Asian wasps (*V. velutina*) because of a very short term of possible adaptive evolution after their introduction into this area. In addition, *A. cerana* workers have very unique flying patterns to avoid being seized by the predators: they fly rapidly, hastily, sashaying and unpredictably zigzagging, which is very different from that of the steady, rather clumsy flight of European *A. mellifera* bees (Sakagami, 1960; Ruttner, 1988). Due to these defensive limitations, *A. mellifera* suffers greater losses than *A. cerana* (Tan et al., 2005; Ken Tan et al., 2007). The typical defensive behaviour of *A. mellifera* workers has been reviewed by Seeley (1985). When a bee guarding her nest's entrance is struck by a wasp, she raises her abdomen and protrudes her sting, where upon the surrounding bees instantly go on alert, either standing ready for attack with wings and jaws spread wide, or launching into flight in search of the foe. If severely disturbed, the guards will retreat inside the nest, there exciting additional guards to join in defense. For example, when Maschwitz (1964) monitored a colony one

evening for 24 minutes without creating any disturbance, he observed just one bee patrolling the entrance opening, but when he pinched 8 bees at the hives' entrance, 140 guards boiled out in an aggressive frenzy.

In the former chapters, we have tested the stability of the mixed colonies of the two species with respect to colony cooperation of communication, queen retinue, comb building, thermoregulation and about ovarian activation caused by changing the reproductive environment. But it is still very important to investigate the defensive aspects to test how well the mixed colonies might cooperate. As listed above, due to differences in evolutionary backgrounds, the two species have quite obviously different defense behaviours, thus the wasps offer us a useful tool to test the defensive cooperation to answer the following questions: 1) Do the bees of the two species cooperate in defense of their hives at the entrance? If so, 2) How does each of them behave? Do they use species-specific methods, or do they learn from each other? For example, do *A. mellifera* workers shimmer at the entrance when shimmering is performed by *A. cerana* bees, and vice versa? 3) Do they form a mixed ball to kill the wasp? How do they behave on the ball? And 4) Do they raise their body temperature when facing different defense/attacking backgrounds?

7.2 Materials and Methods

The experiments and observations were conducted with colonies of *A. cerana cerana* and *A. mellifera ligustica* in autumn (September-October 2009) in an apiary at Yunnan Agricultural University, Kunming, China.

Mixed colonies of both *A. cerana* and *A. mellifera* were established. Three colonies contained worker brood of both *A. cerana* and *A. mellifera* and were headed by *A. cerana* queens; and, reciprocally, three contained worker brood of both *A. cerana* and *A. mellifera* headed by *A. mellifera* queens. The organization method is the same as that of chapter 2. Wasp-defending behaviour was investigated when the newly emerged workers of the two species were about 12-25 days old, the peak age of defending behaviour (Winston, 1987). Pure *A. cerana* and *A. mellifera* colonies with the same age cohort of workers were selected as control groups. Each of the colonies was equal in size.

In the bioassays, a live wasp, *V. velutina*, was collected with an insect net and then tied at the petiole with fine wire. It was held about 20 cm away from the entrance of a hive and could fly and move freely within the confines of its wire and its movements would alert the guard bees.

For each bee colony, the thoracic temperatures of 20 individual guard bees were measured in the absence of a wasp as the control group and 20 more bees measured after presentation of the live wasps as the test group in experiment 1. In a second experiment, the live wasps were put closer, about 5 cm from the entrance, to let the guard bees form a "ball". They were placed on the entrance board directly if some colonies under some circumstance could not form a ball firmly. A thermometer (Sensorted, BAT-12, with an accuracy of 0.1ºC) with an external sensing probe was used to measure the core temperature of the ball, and the performances of the workers of the two species were carefully observed. The balls were moved several meters away from the hives for measurements.

The thoracic temperatures of the guard bees both at the entrance of the hive and on the ball were measured about 20-30 cm away from the entrance with a hand-held laser infrared digital thermometer with a resolution of ± 0.1^{o}C (AZ$^{@}$ Model 8889, AZ Instrument Corp, Taichung City, Taiwan, China). During the tests ambient temperature was about 21-23^{o}C. Just when the guard bees were launching to strike the wasp, their thoracic temperatures were immediately measured.

Repeated measures ANOVA tests were used to test for overall differences in thoracic temperatures between the defensive behaviours of the mixed colonies and control groups of pure *A. cerana* and pure *A. mellifera* colonies. Tukey post-hoc multiple comparison tests were used for significant behaviour effects. The core temperatures of the attacking balls formed by mixed colonies and control groups differences were compared using ANOVA procedures. Tukey post-hoc multiple comparison tests were used for significant group effects (Johnson and Wichern, 2002). The means and standard deviations of each variable were calculated. All tests were performed using Statistica$^{©}$ (StatSoft, 2008).

7.3 Results

7.3.1 Defensive behaviours at the entrance

In our observations, *A. cerana* bees, in both control groups and in the two types of mixed colonies, showed shimmering (Fig. 7.1) and heat-balling (Fig. 7.2) behaviours against the wasps. While *A. mellifera* workers did not shimmer, they adopted a characteristic forelegs-stance on the substrate, antennae projected forward, and sometimes wings and mandibles spread, ready to rush toward the flying wasps, and then recruit more *A. mellifera* workers to block the nest entrance (Fig. 7.3). Balling behaviours can also be performed (Fig. 7.4). Guard bees of pure *A. cerana* colonies can recruit about 10 to more than 50 bees to engage in shimmering, while in *A. mellifera* colonies, sometimes more than 100 bees could be seen at the entrance engaged in blocking. This number is not strictly fixed and depends on the specific circumstances, for example, the ambient weather conditions, foraging intensity and other factors.

In the mixed colonies, it can be seen firstly that both species had an active response toward the wasps. *A. cerana* bees shimmered, but *A. mellifera* just used their own shaking behaviour as described above for pure *A. mellifera* colonies. Then, when more *A. mellifera* bees gathered at the entrance, the *A. cerana* workers stopped shimmering and withdraw into the hive, only few of them joined *A. mellifera* for entrance blocking (Fig. 7.5). As for learning flying patterns, both species in the mixed colonies did not change their flying habits.

Fig. 7.1 Shimmering of *A. cerana* guard bees at the entrance

Fig. 7.2 Heat-balling by *A. cerana* bees

Fig. 7.3 Recruiting behaviour of *A. mellifera* guard bees at the entrance

Fig. 7.4 Balling behaviour of *A. mellifera* bees

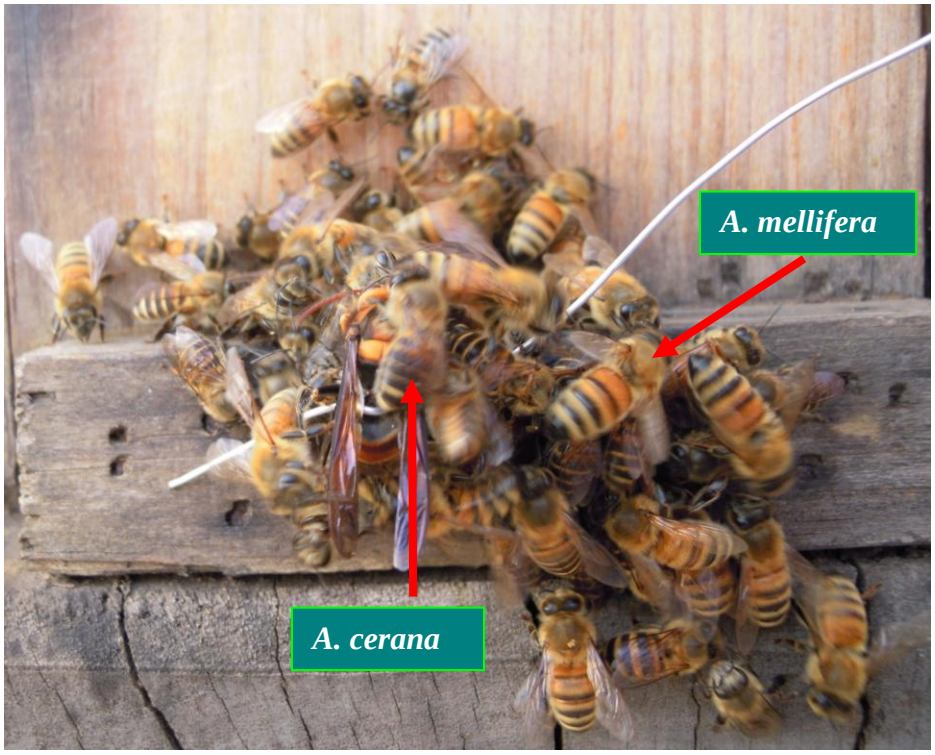


Fig. 7.5 Entrance-blocking of the mixed-species colonies

7.3.2 Heat balling

When the wasps were put closer (about 5 cm) to the hive entrance and were flying, the response of the guard bees in all colonies became more active at the entrance. In pure *A. mellifera* colonies, workers were more likely to start attacking as single individuals, usually only a few (2-4) bees alighted on the wasp to attack it. Most of the bees could be seen either performing their specific defense behaviours or simply blocking at the entrance. A ball was formed only when the wasps were placed on the board at the entrance of the hive. By contrast, in pure *A. cerana* colonies, workers actively attack as a group with dozens of bees shimmering their wings in concert, at first at the entrance, and when the wasps were closer (about 5cm), several bees started flying out and alighting on the wasp and grasping its legs, wings and antenna with their mandibles. Immediately, more than twenty bees flew out simultaneously and engulfed the wasp in less than a second, thus a ball formed. The balls formed by pure *A. cerana* colonies were more tightly packed than those formed by pure *A. mellifera* colonies.

In the mixed colonies, *A. mellifera* bees were more likely than *A. mellifera* to depart from the entrance and alight on the wasp to initiate an attack by biting the legs, wings

and antenna or stinging. But their stinging behaviour was not very successful and in our experiment only one sting was found left on the body of the wasp (Fig. 7.6). As long as *A. mellifera* bees launched the attack, *A. cerana* join them immediately, thus a mixed ball formed within a minute (Fig. 7.7).

When the balls were firmly established (about just 1 min for all colonies), they were moved several meters away from the entrance to measure the temperatures of the bees (Table 7.1). Interestingly, although the workers from mixed colonies formed mixed balls, *A. mellifera* workers were mainly at the outer edge of the ball while *A. cerana* workers were in the core (Fig. 7.7).

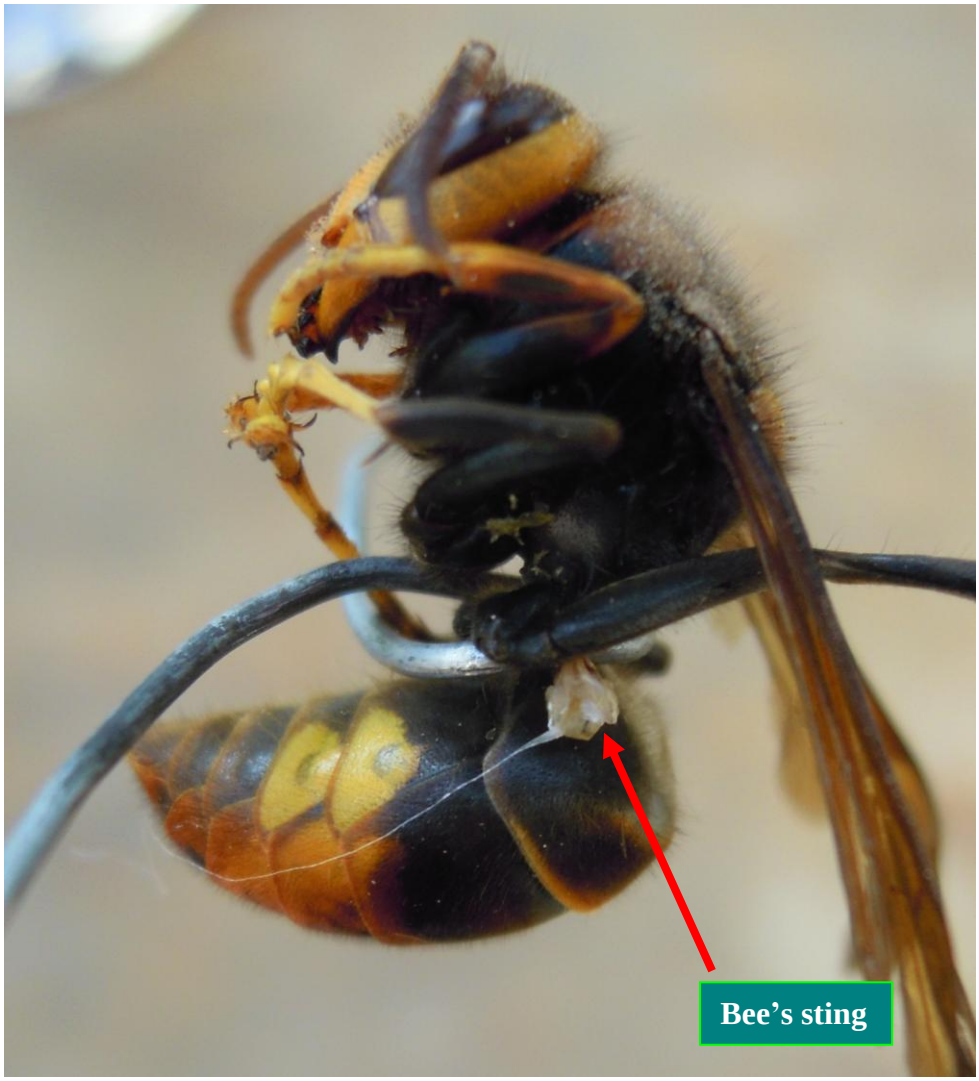


Fig. 7.6 A bee sting left on the body of a wasp

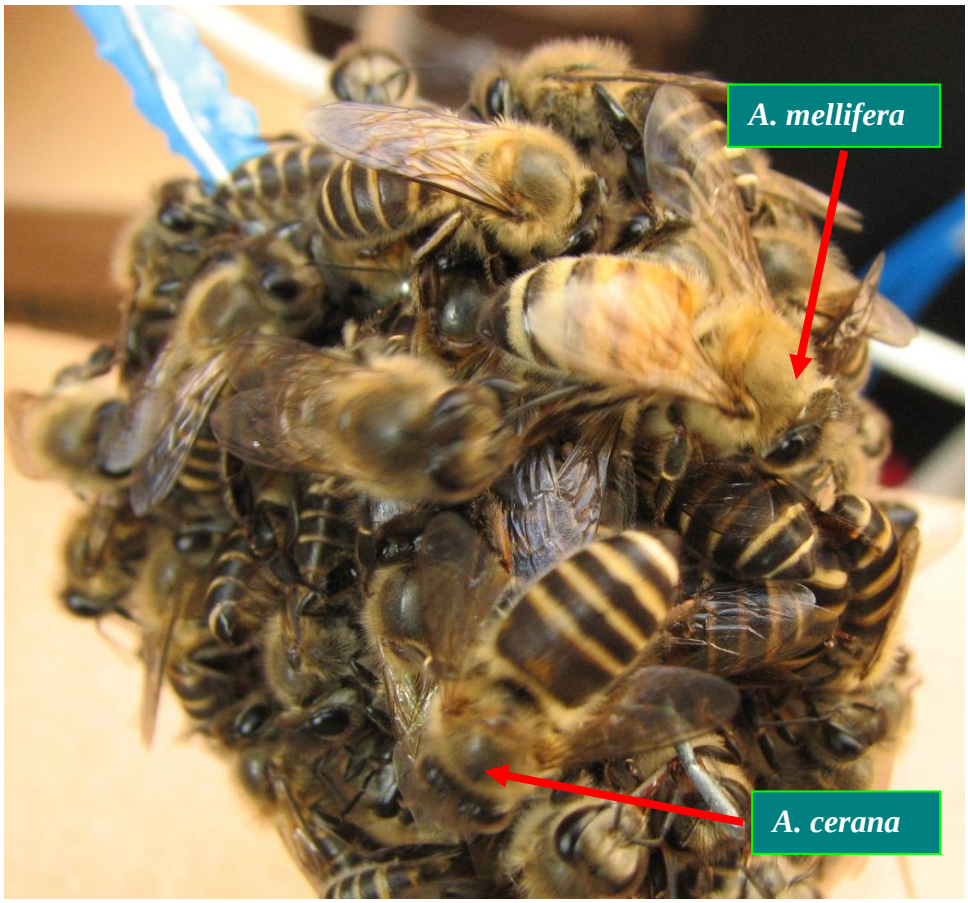


Fig. 7.7 Wasp heat-balling of the mixed-species colonies

7.3.3 Thoracic temperature changes

The guard bees' body temperature changes from normal conditions to that causing alert by the wasps and then actual attacks of the balls were measured (Table 7.1). *A. cerana* workers in all colonies showed the same tendency when faced different background: when faced by wasps continuously harassing them, the guard bees at the entrance raise their thoracic temperatures about 5ºC and shimmered, and temperature further increased another 5-6ºC when attacking the wasp in a ball. Their thoracic temperatures reached about 35ºC, which is about 11ºC higher than that of normal guarding bees. By contrast guard bees in all colonies of *A. mellifera* did not increase their body temperatures significantly, but when they attacked wasps by balling, their temperatures increased about 7ºC from normal conditions.

Table 7.1 Means (±S.D.) of thoracic temperatures of guard bees under different defense/attack behaviours (ºC). (*N* = 3 colonies per colony type, 20 bees per colony)

Colony type	Workers	Normal guarding	Defensing	Attacking
pure *A.cerana*	*A.cerana*	24.3±1.1[a]	29.8±1.6[b]	35.2±5.6[c]
pure *A.mellifera*	*A.mellifera*	24.9±1.2[a]	24.8±1.3[a]	31.9±4.0[b]
mixed(*A.cerana* queen)	*A.cerana*	24.4±3.4[a]	29.4±4.1[b]	35.9±4.2[c]
	A.mellifera	24.6±2.2[a]	24.1±2.0[a]	32.9±3.0[b]
mixed(*A.mellifera* queen)	*A.cerana*	24.7±2.3[a]	28.6±4.3[b]	34.1±5.2[c]
	A.mellifera	24.9±1.4[a]	24.3±2.6[a]	30.7±5.4[b]

[a]Means within one row followed by the same letter are not significantly different (Tukey multiple comparisons: $P > 0.05$)

The core temperatures of the balls were measured, the mixed balls formed by the two species did not differ significantly from that of pure colonies (ANOVA, $F_{3,32} = 0.465$, $P = 0.709$; Table 7.2).

Table 7.2 Means±S.D. of core temperatures of the balls (ºC). (*N* = 3 colonies per colony type, 3 days per colony)

Colony type	Core temperature (ºC)
pure *A. cerana*	45.4±1.0
pure *A. mellifera*	45.5±1.2
mixed(*A. cerana* queen)	45.9±0.9
mixed(*A. mellifera* queen)	45.6±1.0

7.4 Discussion

In observations on the defensive behaviours of the mixed colonies, we found that the two species defend their colonies with their species-specific patterns: *A. mellifera* workers did not learn to shimmer their wings like *A. cerana*, nor change their flying patterns to avoid predation by the wasps. On the other hand, *A. cerana* in the mixed colonies did not learn the defensive behaviour of *A. mellifera* bees either. When *A.*

mellifera bees recruit their nestmates to defend their colonies, more and more *A. mellifera* bees gather at the entrance to block it, and *A. cerana* worker then stopped shimmering and withdrew into the hives. Thus we suggest that the defense behaviours are distinctly determined by their genetics, and have changed following speciation.

Different honeybee species have different risk assessments, resulting in different thresholds for response (Gordon, 1996; Jones et al., 2004; Jones and Oldroyd, 2007). *A. cerana* is generally reported as being mild, tolerant and timid in defense behaviour (Ruttner, 1988); however, they show a number of behavioural patterns which prove to be very effective against traditional enemies. One of the most striking traits is group defense. For example, if attacked by powerful enemies such as wasps and hornets, they do not counter attack, as *A. mellifera* bees do (Schneider and Kloft, 1971). In our observations, we found in all mixed colonies that when a wasp was placed close to the entrance, *A. mellifera* workers initiated the attack first, though by single individuals, and then *A. cerana* bees joined and formed a mixed ball. Interestingly, in the mixed balls *A. mellifera* bees were at the outer edge of the ball while *A. cerana* bees formed a tight inner core. This is obviously determined by their specific genetics (Gordon, 1996).

Although heat-balling wasps as such is well documented (Ono et al., 1987; Tan et al., 2005), the behavioural sequence of attracting additional recruits to the guard bee cohort, increased numbers of wing-shimmering guard bees that raise thoracic temperature prior to striking a wasp have not been previously measured for either *A. cerana* or *A. mellifera*. Un-alerted guard bees of both *A. cerana* and *A. mellifera* have relatively low thoracic temperatures, about 24^{o}C, but when hawking wasps approach them, unlike *A. mellifera*, the *A. cerana* guard bees are immediately alerted and begin body shaking and wing shimmering. Likewise, thoracic temperature rapidly increases about 5^{o}C and those guard bees with the higher thoracic temperatures more readily attack wasps than those at lower temperature. The wing shimmering behaviour is directly associated with increasing the guard bee cohort and may be mediated by the simultaneous release of a pheromone. Because shimmering guard bees increase their surface temperatures during wing-shimmering, this would facilitate the dispersal of any recruiting pheromones (Stabentheiner et al., 2002). Likewise, during fanning *A. cerana* face away from the nest entrance (Sakagami, 1960), and this would direct any pheromonal plume backwards into the nest. However, it has been reported that *A. cerana* does not expose its Nasanov gland during shimmering (Koeniger et al., 1996e). Wing-shimmering is also interpreted

as an anti-predator visual pattern disruption mechanism, similar to that of *A. nuluensis* (Koeniger et al., 1996e).

In contrast, *A. mellifera* guard bees do not exhibit these behavioural responses to hawking wasps and there is no rapid elevation of thoracic temperature. This apparent inability to rapidly detect wasps and to respond defensively accounts for the three-fold greater wasp presence at colonies of *A. mellifera* than *A. cerana* and for an eight-fold greater hawking-take of the former over the latter in autumn (Ken Tan et al., 2007). *A. cerana* may also withdraw into its nest which *A. mellifera* does not do. *A. cerana* guard bees use wing-shimmering as a visual pattern disruption mechanism, similar to *A. nuluensis* (Koeniger et al., 1996e), another trait absent from the behavioural repertoire of *A. mellifera.*

The venom of *A. cerana* is identical with that of *A. mellifera* in the amino acid sequence of the melittin, its main component, and alarm substance: isopentyl acetate was found in workers of *A. cerana*, but in much lower quantities than in *A. mellifera* (about 1 ug/bee vs. about 2 ug/bee) (Seeley, 1985). In the mixed colonies, we found that *A. mellifera* initiates attacks first, while *A. cerana* bees follow to form mixed balls. While *A. mellifera* bees did not show any signs of being recruited by *A. cerana* to form a mixed ball, this might be caused by both differences in alarm pheromones and different defense habits.

In any event, *V. velutina* preferentially hawk *A. mellifera* foragers when both *A. mellifera* and *A. cerana* occur in the same apiary (Ken Tan et al., 2007). Our observations suggest a reciprocal co-evolution in the prey/predator relationship between *V. velutina* and *A. cerana* both of which are endemic to and sympatric in southeast Asia (Li, 1993; Tan et al., 2005) while *A. mellifera* was introduced from Europe, where there is no widespread wasp predation. The fact that the behavioural sequences described here for *A. cerana* also occur in *A. nuluensis* (Koeniger at al., 1996e) and *A. dorsata* (Kastberger et al., 1998; Kastberger and Stachl, 2003) suggests a general soft co-evolution between a cache of predators and honeybees in Southeast Asia.

CHAPTER 8

General Discussion

Honeybees are a well-established model for the study of social organization of insects and the evolution of sociability. Compared with other social insects such as ants, of which there are hundreds or thousands or even more species (Holldobler and Wilson, 1990), there are only nine species of honeybees, but they play an extremely important role in ecology by pollinating many plants. This might be one of the reasons why they are so attractive to biologists and ecologists. Another even more important reason is that they have a social organization in which some aspects are very similar to human society. And their social organization and division of labour are so effective that they can give inspiration to us on how to make our society run more effectively.

A. cerana and *A. mellifera* live in cavities or hives, regulate their hive temperatures in a stable narrow range of 32-36ºC and, they have an extremely effective form of social communication, and work force allocation. Accordingly, these two species have been raised by humans to make commercial bee products and for pollination of green house plants.

In this thesis, the mixed-species colonies of two species, *A. cerana* and *A. mellifera* were organized to assess the behavioural relationships between them. We tested their common social characters such as queen-workers' "honest" or "suppress" pheromone regulation, waggle dance dialects, self-organization in task allocation and group cooperation.

The mixed-species colonies provide us some very valuable information about the behaviours and organization developing in the course of evolution. It might bring the two species back to the point upon which they are about to diverge from each other in terms of behaviour. Thus the results could be of significance for the study of the regulation of evolution in social insects.

8.1 Queen retinues

The general method to set up mixed-species colonies is reciprocal brood exchange. Thus it is very important to test if the introduced individuals can get along well with the host members: the queen and the workers, because the queen pheromones of the honeybee colonies play the most important role in the stability of the honeybee colonies. So it is of significance to investigate if the two species would exhibit the typical retinue behaviours to the queen and then transmit its pheromones.

The results show that workers of the two species could get along well with each other and no fighting behaviour occurred. This is due to the fact that the nest-mate recognition system of honeybees is primarily organized after the workers become adults and young workers acquire the colony specific odour when they begin outside tasks.

In our observations, we found that although three pheromonal components (9-ODA, 9-HDA and 10-HDA) of the queens of both species were significantly different, the workers did not show any obvious avoidance behaviour towards either of the hetero-specific queens. Both species were attracted by the queens, engaged in retinue behaviour, licked the queens and showed normal grooming and feeding behaviour. These results suggest that the retinue response was not related to a specific queen pheromone or colony environment, and this is consistent with the results of other investigations (Pankiw et al., 1994; Hoover et al., 2005). This non-specific queen retinue behaviour in the mixed-species colonies indicates that the queen pheromones can be transmitted among the workers of the two species without any obstacles, irrespective of possible "suppressive agents" (Fletcher and Ross, 1985) or "honest signals" (Peeters et al., 1999; Strauss et al., 2008). Thus we argue that the basic queen signalling mechanism is conserved and queen pheromones and retinue formation preceded speciation in *Apis* because workers of both species respond to heterospecific queens. However, there is a pheromonal nuance because *A. cerana* workers responded less to *A. mellifera* queens and there are significant differences in the proportions of 9-ODA, 10HDA, 9HDA and in the ratio of 9-ODA/(9-ODA+10-HDA+10-HDAA) that could have led to differences in retinue responses. The queen pheromones appear to be quantitatively different between queens and could be 'interpreted' as different pheromonal "dialects". This would be a parsimonious explanation for the differences in

the attractiveness of queens for *A. cerana* workers, but begs the question for the *A. mellifera* workers. Nonetheless, this leaves unanswered questions such as 1) What does it mean if retinues of similar proportions are measured in the two species while the queens of one of these species produces more pheromone?, and 2) Why do *A. mellifera* queens attract fewer workers in mixed-species colonies compared to pure colonies? We conclude that retinue behaviour itself as well as the pheromones of the queens that induce this behaviour are both ancestral, conserved traits that preceded speciation in apine bees.

8.2 Ovarian activation

The reproduction division of labour in honeybees is the most striking character of their social lives in which the queen monopolizes egg-laying while workers do not have active ovaries, thus can not reproduce normal offspring. This system operates very well when sufficient queens' pheromones are present, but if the pheromones are removed workers might activate their ovaries and the reproduction of the colony would collapse. Although the workers could attend the hetero-specific queens allowing the pheromones to spread to both species, we have found the queens' pheromones of the two species are different. Thus how the workers behave under hetero-specific queens needs to be investigated. The fact that all queenright colonies, except for one comparison, showed significantly less ovarian activation in workers than the queenless counterparts in both *A. cerana* and *A. mellifera* demonstrates that the queens of the two species have pheromonal equivalence in the conspecific inhibition of worker ovarian activation.

Even the comparison of queenright mixed-species colonies headed by an *A. cerana* queen with its queenless counterpart, although not significantly different in the extent of the ovarian activation, showed a significant difference in the rate of activation, supporting the idea that queen presence affects workers of both species. However, in none of the queenright colonies is the inhibitory effect complete as indicated by the proportion of workers with activated ovaries. This partial ovarian activation is nonetheless sufficient to preclude reproductive competition by workers as none of the bees reached the laying worker stage.

That significantly fewer workers underwent ovarian activation in the mixed queenless colonies is most parsimoniously explained as the effect of worker-worker pheromonal inhibition. Perhaps a much greater inhibition was derived from the presence of *A. cerana* workers in the same colonies as well as from other *A. mellifera* workers.

In contrast to this, *A. cerana* workers of queenright mixed-species colonies (34.6%) showed significantly greater ovarian activation than their workers in queenright colonies (14.2%). However, because queenless worker bees can also inhibit ovarian activation in other workers, comparisons among them in queenless, mixed-species colonies allows an estimation of the separate effects of queen-worker and worker-worker inhibition. There was no significant difference in the extent of ovarian activation between *A. mellifera* workers of queenright mixed-species colonies (21.7%) and their respective conspecific queenright colonies (11.7%).

These interpretations of ovarian activation are consistent with the results of the [9-ODA / (9-ODA + 10-HDA + 10-HDAA)] ratios. *A. cerana* queens have more strongly queen-biased signals than *A. mellifera* queens, results consistent with other published data (cf. Keeling et al., 2001 for *A. cerana*; Crewe and Velthuis, 1980 for *A. mellifera*). Thus it is reasonable to conclude that this interspecific bias of queen signals largely accounts for the greater rate and extent of ovarian activation in *A. cerana* workers in mixed-species colonies headed by *A. mellifera* queens.

One could speculate that the stronger queen biased signal of the *A. cerana* is the result of a higher degree of social parasitism in natural populations of *A. cerana.* Indeed, the strong queen signal is comparable to queens of the African subspecies *A. m. capensis* (cf. Wossler, 2002) in which workers can reproduce despite the presence of a reproducing queen (Neumann and Hepburn, 2002; Wossler, 2002). Another similarity is that workers of *A. m. capensis* are less affected by the queens' pheromones of other *A. mellifera* subspecies, as were the *A. cerana* workers in the mixed-species colonies headed by the *A. mellifera* queen. Mandibular gland pheromones are likely to have played a central role in the evolution of social parasitism in honeybees (Dietemann et al., 2007). The importance of these pheromones is based on their multiple functions in determining reproductive status and allowing individuals to prevent reproduction by their nestmates (Velthuis et al., 1990; Simon et al., 2005).

8.3 Interspecific communication

The fact that insects have a symbolic "language" has amazed all biologists. In all animals, as long as they are highly related, (for example the same species), individuals could communicate with each other for some specific goal such as mating, but the dance language of the honeybees is obviously operating at a higher level, because much information has been encoded and cannot only be used for food foraging, but also for scouting nesting sites. Although waggle dances could provide a number of recruitment stimuli, it remains unknown which the bees use; and, indeed, those features of the dance that assist followers to stay with the dancers need not be the same as those that carry the direction and distance signal (Dyer, 2002). Although there are internal differences in the waggle dances of *A. cerana* and *A. mellifera* foragers, the basic structure of the waggle dance is the same in both (Lindauer, 1956). For the successful interpretation of the waggle dance of any group of honeybees, it is an a priori requirement that there must be a dancer with information to transmit. Such a dancer needs an audience to which it can deliver its information and members attending such dances must acquire and act on that information. Foragers of *A. cerana* and *A. mellifera* fulfil these conditions when each performed waggle dances and successfully recruited foragers of the other species together in a mixed-species colony.

Thus, it is demonstrable that both species can acquire and act on information provided by each other's waggle dances in mixed-species colonies of *A. cerana* and *A. mellifera.* Inasmuch as the round dances change to waggle dances at different distances, target distance should be overshot in the one and undershot in the other. However, the same percentage of *A. cerana* and *A. mellifera* recruits both undershot and overshot the target, under both conspecific and heterospecific dance conditions. Towne and Gould (1988) showed that the spatial precision of the dance in *A. mellifera* is neither so accurate that they usually find areas which have already been depleted nor so inaccurate that they usually fail to find the advertised resources altogether. Moreover, the bees' distance errors decrease greatly with increasing distance to the target. It is just this pre-speciation flexibility in precision that allows about 40% *A. cerana* and *A. mellifera* recruits to accurately home into a target on the first time out.

8.4 Comb construction

Comb building is an important behaviour in honeybees' lives, for almost all individuals use their wax gland to perform the building tasks at the proper age. Comb building behaviour is an extremely amazing model of group cooperation in the honeybees, for the bees are working in a dark cavity or hive where there is no central source of information. When construction begins, the workers cling together in elongated chains or festoons, forming a dense cluster which facilitates an equable temperature for wax secretion and manipulation (Hepburn, 1986; Winston, 1987). Numerous comb building workers, with active wax glands, engage in the task of comb construction. But, instead of building a single comb together, several festoons begin at independent sites and begin building several cells, hence combs, simultaneously and only later connect them with some irregular transitional cells (Hepburn, 1986; Winston, 1987; Hepburn and Whiffler, 1991). In this case, the parallelism rule of comb construction can be achieved indirectly at the finishing stage of comb building, with many irregular cells and seam connections between several branches started from separate sites (Hepburn and Whiffler, 1991). Our videos and pictures of the mixed-species colonies showed that the two species have almost the same building behaviours. Likewise, the irregularly built cells verified that both species engaged in the task, and indicate that the regulation of building behaviour is also preserved after their speciation. Interestingly, the *A. cerana* workers, as a colony did not accept any type of beeswax foundation, but as individuals, they can be stimulated by *A. mellifera* workers to engage in comb building. We confirm that in the mixed-species colonies, these two species did in fact cooperate in comb building, even though irregular cells arise through their joint efforts. We can also infer that, although the comb building workers are poorly-informed and lack a central controller (Pratt, 2004), it is really a task which can only be finished by some smaller groups in which individuals closely cooperate to achieve progress.

8.5 Thermoregulation

Thermoregulation of nests is the most marked trait of the cavity nesting honeybees. They are able to maintain a stable high temperature about 33-36ºC (unbelievably close

to the body temperatures of mammals!). For this reason they are regarded as having reached the highest evolutionary stage of insects. Due to different habits of the two species for ventilation at the hive entrance, this behaviour gives us a good opportunity to investigate the mechanism of task allocation in thermoregulation.

When the two species of workers were fanning together at the entrance, they maintained their species-specific postures, their heads facing in opposite directions as was observed by Atwal and Dhaliwal (1969). However, it was strange (but reasonable) that in both types of mixed-species colonies, *A. cerana* workers were the main force in ventilating the hive, while only few *A. mellifera* workers fanned. This probably explains why both types of mixed-species colonies have thermoregulation efficiency similar to pure *A. cerana* colonies. But why does *A. mellifera* seem more "defective?" We have thus tested the altruism theory. At first sight, altruism would reduce fitness of the concerned individual and strengthen its opponent's fitness, but this theory is not compatible with individuals of social insets, which live together with hundreds to thousands of nestmates. At the colony level, some new ideas have been introduced such as the famous Kin selection theory (Hamilton, 1963) and the Prisoner's Dilemma of game theory (Chase, 1980) in order to interpret evolutionary history in social insects. Hamilton's kin selection theory suggests that social animals only aid related animals to gain group genetic benefits, while the Prisoner's Dilemma game mainly deals with balance between cooperation and defection. Because two individuals can cooperate or defect, the payoff to a player is in terms of the effect on its fitness (survival and fecundity). No matter what the other does, the selfish choice of defection yields a higher payoff than cooperation. But if both defect, both do worse than if both had cooperated. In this thesis, we found that *A. cerana* workers are more sensitive to temperature changes and initiated ventilation fanning earlier than *A. mellifera* workers. On the other hand the *A. mellifera* workers abandoned fanning when they detected that the fanning task was been done by others. Thus *A. cerana* workers could be defined as more "cooperative" while *A. mellifera* workers as more "defective". The idea of "diversity promotes stability" does not easily apply to mixed-species colonies of honeybees, because the workers from different species adopt different techniques in ventilating the hive at the entrance. If both of them fan using their own unique postures, it really reduces the effectiveness of the fanning effort rather than strengthening it.

Task allocation in honeybees is very complicated probably because there is in fact no central information source available and no "controller" bees engaged in task allocation.

It has been suggested that each individual has to make its own decisions which could be produced as a self-organization mechanism (Page and Mitchell, 1998; Bonabeau et al., 1997). This mechanism is sufficiently effective that each work force is properly "arranged". Thus, if one task is being done by enough nestmates, newly recruited individuals might stop to perform other tasks, and this might explain the case of *A. mellifera* workers' performances in our mixed-species colonies. Thus we conclude that *A. mellifera* workers' behaviour is more adaptive for stability for the mixed-species colonies, rather than apparently "defectiveness".

8.6 Coordination in defense

In order to test how well the mixed-species colonies could exist in natural conditions, where the predators are always hunting around by the entrances, we tested the cooperation in colonies defense against the wasps as an aim at investigate the defense strategy in relation to evolution. The two species normally have different responses to the wasp's predating due to their different distributions and evolutionary backgrounds. So the defensive behaviour against wasps of the mixed-species colonies also gives us a good chance to investigate mutual cooperation. We found that the two species defend with their species-specific patterns: *A. mellifera* workers did not learn to shimmer their wings like *A. cerana*, nor change their flying patterns to avoid predation by the wasps. On the other hand, *A. cerana* in the mixed-species colonies did not learn the defensive behaviour of *A. mellifera* bees either. When *A. mellifera* bees recruit their nestmates to defend their colonies, more and more *A. mellifera* bees gather at the entrance to block it, and *A. cerana* workers then stopped shimmering and withdrew into the hives. Thus we suggest that the defense behaviours are distinctly determined by their genetics, and have changed following speciation.

Different honeybee species have different risk assessments, resulting in different thresholds for response (Gordon, 1996; Jones et al., 2004). *A. cerana* is generally reported as being mild, tolerant and timid in defense behaviour (Ruttner, 1988); however, they show a number of behavioural patterns which prove to be very effective against traditional enemies, for example, if attacked by wasps and hornets, they do not counter attack, as *A. mellifera* bees do (Schneider and Kloft, 1971). In our observations,

we found in all mixed-species colonies that when a wasp was placed close to the entrance, *A. mellifera* workers initiated the attack first, though by single individuals, and then *A. cerana* bees joined and formed a mixed ball. Interestingly in the mixed balls *A. mellifera* bees were at the outer edge of the ball while *A. cerana* bees formed a tight inner core. This is obviously determined by their specific genetics (Gordon, 1996).

Although heat-balling wasps as such is well documented (Ono et al., 1987; Tan et al., 2005), the behavioural sequence of attracting additional recruits to the guard bee cohort, increased numbers of wing-shimmering guard bees that raise thoracic temperature prior to striking a wasp have not been previously measured for either *A. cerana* or *A. mellifera*. Un-alerted guard bees of both *A. cerana* and *A. mellifera* have relatively low thoracic temperatures, about 24^{o}C, but when hawking wasps approach them, the *A. cerana* guard bees are immediately alerted and begin body shaking and wing shimmering. Likewise, thoracic temperature rapidly increases about 5^{o}C and those guard bees with the higher thoracic temperatures more readily attack wasps than those at lower temperature. The wing shimmering behaviour is directly associated with increasing the guard bee cohort and may be mediated by the simultaneous release of a pheromone. Because shimmering guard bees increase their surface temperatures during wing-shimmering, this would facilitate the dispersal of any recruiting pheromones (Stabentheiner et al., 2002). Likewise, during fanning *A. cerana* face away from the nest entrance (Sakagami, 1960), and this would direct any pheromonal plume backwards into the nest. However, it has been reported that *A. cerana* does not expose its Nasanov gland during shimmering (Koeniger et al., 1996e). Wing-shimmering is also interpreted as an anti-predator visual pattern disruption mechanism, similar to that of *A. nuluensis* (Koeniger et al., 1996e).

In contrast, *A. mellifera* guard bees do not exhibit these behavioural responses to hawking wasps and there is no rapid elevation of thoracic temperature. This apparent inability to rapidly detect wasps and to respond defensively accounts for the three-fold greater wasp presence at colonies of *A. mellifera* than *A. cerana* and for an eight-fold greater hawking-take of the former over the latter in autumn (Ken Tan et al., 2007). *A. cerana* may also withdraw into its nest which *A. mellifera* does not do. *A. cerana* guard bees use wing-shimmering as a visual pattern disruption mechanism, similar to *A. nuluensis* (Koeniger et al., 1996e), another trait absent from the behavioural repertoire of *A. mellifera.*

The venom of *A. cerana* is identical with that of *A. mellifera* in the amino acid sequence of the melittin, its main component, and alarm substance: isopentyl acetate was found in workers bees from *A. cerana* bees, but much lower in quantities than in *A. mellifera* (about 1 ug/bee vs. about 2 ug/bee) (Seeley, 1985), in the mixed-species colonies, We found that *A. mellifera* initiate attacks first, while *A. cerana* bees follow to form mixed balls. While *A. mellifera* bees did not show any signals to be recruited by *A. cerana* bees to form a mixed ball, this might be caused by both the factors alarm pheromones difference and different defense habits.

8.7 General conclusion

Though few in number, the species in the genus of *Apis* have various habits, for example some species, especially in the tropical areas, build nests in the open air on twigs or rocks, whilst others have evolved a cavity living habit. It remains a controversial topic whether or not the cavity species are derived from the open nesting species. Many scientists believe that open nesting species are more primitive and gradually gained more adaptive environment independence ability to living in cavities thus spreading into wider living areas (Alexander, 1991). But Koeniger (1976b) have a different view. They argue that the open nesting behaviour is the result of an adaptation to the hot climate of the tropical areas, thus implying that cavity nesting is a more primitive character. In this thesis, the study of the behaviours of the mixed-species colonies gave us a new perspective to investigate the main cavity living characters, such as communication and cooperation in comb construction in dark hives, thermoregulation of the hives, nest defense at the entrances, which collectively may be helpful for us to understand these behaviours during evolution and speciation.

In this thesis, I try to test the mixed-species colonies to determine if their behaviours could be interpreted by some of the well-known theories: non-cooperative game theory, self organization theory, repress or honest signal, altruism (the evolutionary of altruism), etc. Indeed these theories do not account for some of the behaviours. For example, workers from both species take part in the comb building task through a self organization discipline, while *A. mellifera* tend to skip the thermoregulation task to reduce conflict, and the queens' pheromones are more like an honest signal for the

workers because workers of both species form the typical retinue behaviour and do not show significant ovarian activations. However, it is not easy to tell if in one mixed colony there was more competition or less cooperation, or vice versa, in fact these two concepts were introduced from human society, and may well be anthropomorphic. It would be wiser to become a member of their society and we might have the chance to reveal the real organization of honeybees.

It is quite strange to conclude that it seems that these theories are very suitable to describe all the behaviour of the mixed-species colonies, but maybe in fact none of them is accurate. Thus I feel that this study about mixed honeybee colonies for my thesis, like a poem, is never finished, only abandoned, and the work will surely be carried on.

CHAPTER 9

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