

# Chapter 4

## Intraspecific and Interspecific Comb-Building

**Abstract** *A. florea* was tested to determine whether they would salvage wax from their own deserted natal combs in preference to other conspecific combs and from heterospecific facsimiles of other species. Preferences for natal comb were significantly greater than for non-natal combs, no wax being collected from heterospecific combs. Behavioural variations for wax choice were also assessed using *A. capensis*, *A. florea*, *A. cerana* and *A. dorsata* waxes, Japan wax, candelilla wax, bayberry wax and ozokerite, which were tested in *A. m. capensis*, *A. florea* and *A. cerana* colonies. *A. m. capensis* accepted only the beeswaxes. *A. cerana* and *A. florea* accepted the *A. cerana*, *A. florea* and *A. dorsata* waxes, but rejected *A. m. capensis* and the other waxes. Comb-building in *A. cerana* and *A. mellifera* mixed-species colonies was examined with foundation made from the waxes of these species and then given to colonies having either an *A. cerana* or an *A. mellifera* queen. The colonies did not discriminate between the waxes and comb-building was the combined efforts of both species.

### 4.1 Introduction

Aside from competition during foraging, nest site selection, and robbing, few other interspecific interactions among honeybee species have been investigated in any depth. But, among these, the activities of the Cape honeybee, *A. m. capensis*, have become notorious as exemplars of intraspecific parasitism (Moritz et al. 2011); and, likewise, interspecific social parasitism is widespread in *A. cerana* (Nanork et al. 2006), and *A. florea* (Chapman et al. 2009). There have also been studies of reciprocal transfers of *A. cerana* workers with *A. koschevnikovi* (Koeniger et al. 1996), *A. cerana* with *A. nuluensis* (de Guzman et al. 1996), heterospecific queen rearing with *A. cerana* and *A. mellifera* (Oschmann 1965; Ruttner and Maul 1983; Potichot et al. 1993), interspecific ovarial activation (Hepburn 1994; Tan et al. 2009), communication (Su et al. 2008; Tan et al. 2008), thermoregulation in *A. cerana* and *A. mellifera* mixed-species colonies (Yang et al. 2010a, b, c), and

defense behaviour (Tan et al. 2010). Such heterospecific studies are at a nascent stage; but the complexity of the behaviours observed produce data that reveal activities which have survived speciation in honeybees. In this chapter we discuss a new dimension to such studies relating to the intraspecific and interspecific utilization of different waxes and of comb-building in mixed-species colonies of honeybees.

The nature of speciation has been analysed and reconstructed in quite considerable detail, particularly with emphasis on reproductive isolation (Wilson 1971; Bush 1975; Via 2001). However, many aspects of physiology and behaviour remain shared among species after speciation, but have been little touched upon, and their study illuminates the extent to which some important features have been conserved. For example, it was shown recently that *A. cerana* and *A. mellifera* mixed-species colonies are quite viable (Tan et al. 2006) and, indeed, each can interpret the waggle dances of the other (Su et al. 2008; Tan et al. 2008). Turning to beeswax, intraspecific comparisons of the beeswaxes among the races of *A. mellifera* show that they can only be distinguished after careful calculation of the peak-elution patterns of selected compounds (Brand-Garnys and Sprenger 1988; Fröhlich et al. 2000a), which obviously indicates that speciation within *A. mellifera* is an on-going process. However, there are notable species-specific differences in beeswaxes among species (Aichholz and Lorbeer 1999). Although the waxes vary, they all share a complex mixture of homologous neutral lipids in common (cf. Chap. 16).

## 4.2 Intraspecific Comb Wax Salvage

The secretion of wax and construction of combs represents a large metabolic investment by honeybees, so that desertion of the nest, for whatever reason, constitutes an energetically hefty expenditure (Hepburn et al. 1984; Pirk et al. 2011). Nonetheless, nest desertion by absconding or migrating colonies is a common feature of tropical honeybees in Africa (Hepburn and Radloff 1998) and Asia (Oldroyd and Wongsiri 2006; Hepburn 2011). Despite the possible cost effectiveness of cannibalising wax from a deserted nest and reusing it in the construction of a new one (Pirk et al. 2011), this behaviour is thus far only known for three species of honeybees, *A. andreniformis* (Duangphakdee, pers. obs.; Wongvilas, pers. obs.), *A. florea* (Akranakul 1977; Dutton and Free 1979; Wongsiri et al. 1997; Hepburn et al. 2009, 2010), and *A. m. capensis* (Hepburn and Radloff 1998).

Inasmuch as *A. florea* will accept heterospecific beeswaxes inserted into their nests (Hepburn et al. 2009), Hepburn et al. (2010) conducted experiments on absconding *A. florea* colonies to determine whether these bees would preferentially salvage wax from their own, original natal comb over that of other conspecific combs; and, whether they would salvage wax from crown comb facsimiles of *A. florea* combs fashioned from the combs of *A. cerana*, *A. dorsata*, and

*A. mellifera*. Because *A. florea* colonies also tend to nest near one another (Rinderer et al. 2002; Wattanachaiyingcharoen et al. 2008), this demographic characteristic invites competition for accessible, free-standing, empty combs, the wax of which is a valuable and metabolically expensive resource (Hepburn et al. 1984; Pirk et al. 2011).

Hepburn et al. (2011) studied *A. florea* colonies that occur naturally in a small wood of about 3.65 ha, at King Mongkut's University of Technology, Chom Bueng, Thailand. The occurrence of these non-experimental colonies constitutes possible intercolonial competition for wax salvage among *A. florea* colonies. Over two seasons, *A. florea* colonies were collected, three or four at a time, moved at dusk, and each nest was suspended under its own open-sided bamboo shelter at the edge of a copse. The following day, about a thousand workers from each colony on the crowns of the combs were marked with a dot of craft paint, one unique colour per colony. At dusk the same day, the brood comb extending below the crown was cut away and removed to induce absconding. The comb crown is strictly a honey store, which prior to absconding, is virtually emptied of honey to provide fuel for the ensuing absconding flight (Hepburn et al. 2011). The next day, the colonies were continuously observed until they absconded and settled in a new tree, after which compass directions and the distances flown were measured for each colony.

In the first experiment, as soon as a colony absconded, two additional empty *A. florea* comb crowns were placed adjacent to the original crown (about 10 cm apart) of the recently absconded nest. The relative positions of the three crowns were assigned using a different set of random numbers for each colony and set of comb crowns. Within an hour of absconding and settling elsewhere, colonies issued foragers which returned to their original nest sites to scavenge wax. One hour after absconding, the three experimental crowns at each shelter were checked and the numbers of colony-specific colour-marked bees on the combs were counted; this was repeated three times at 30 min intervals. Then the positions of the combs relative to one another were changed again on the basis of random numbers, and the numbers of marked bees arriving at each comb were again counted.

Foragers from six colonies which had absconded returned to their natal nests to salvage wax. When these wax-salvage foragers reached the shelter and encountered three adjacent but different *A. florea* comb crowns, including their own original natal one, their preferences for the combs from which they salvaged wax differed significantly. Some of the colour-marked foragers reconnoitred all three combs but only landed on and recovered wax from their own original natal combs. Foragers from one-third of the colonies collected wax from all three combs. In two wax-scavenging episodes, foragers retrieved more wax from the non-natal combs than their own natal ones (Table 4.1). It is worth noting that some unmarked *A. florea* foragers also salvaged wax from these combs, but because these individuals could not be linked to a specific colony source, such bees were not counted. Some of these bees could have been unmarked bees from the natal colony that absconded; however, among them were bees whose departing flight paths were different from the compass directions in which the test colonies had flown

**Table 4.1** Preferences of six, wax-salvaging *A. florea* colonies from natal and non-natal combs. Numerical values represent the sum of wax-scavenging events (Hepburn et al. 2010)

Combs					
Colony	Natal	Non-natal	G-value	df	<i>P</i> -value
1	106	0	232.9	1	<0.0001
2	112	0	246.1	1	<0.0001
3	95	0	208.7	1	<0.0001
4	6	19	1.0	1	0.3102
5	2	0	4.4	1	0.0361
6	13	62	9.7	1	0.0019
Total	334	81	702.8	6	<0.0001
Heterogeneity G			312.9	5	<0.0001
Mean $\pm$ SD	56.7 $\pm$ 53.7	13.5 $\pm$ 24.9			

after absconding. These bees were designated as ‘free-lance’ wax-scavengers from other colonies in the vicinity.

The results from this experiment demonstrate that *A. florea* wax-salvaging foragers from different colonies differed significantly as to whether they would cannibalize wax from non-natal *A. florea* combs; some did, others not. Given that the hydrocarbons of comb waxes vary among colonies of the same species and that *A. cerana* and *A. mellifera* worker bees can discriminate intraspecifically between combs of different colonies (Breed et al. 1988, 1995; Sasaki et al. 2000; Wilde et al. 2001), Hepburn et al. (2010) interpreted this data to indicate that (1) *A. florea* has just as a refined level of discriminatory ability as do the other two species; and, (2) that the observed differences in wax-salvage behaviour probably reflect genetic differences for this trait in *A. florea*. When two *A. florea* non-natal comb crowns were placed with the natal one, many returning marked foragers indiscriminately cannibalized wax from all three combs. At the same time, there were other foragers, the so-called ‘free-lance’ wax scavengers derived from other nests in the vicinity that retrieved comb crown wax. It appears that a deserted *A. florea* nest is a resource worth securing by any colony of this species in the surrounds.

### 4.3 Interspecific Wax Salvage

On completion of the above tests with three *A. florea* comb crowns, a second experiment was conducted; but this time *A. florea* comb crowns were tested against facsimiles made from *A. cerana*, *A. dorsata*, and *A. mellifera* combs. Test specimens of *A. cerana* and *A. mellifera* combs were prepared from frames of drawn combs by cutting away about 3 cm of drawn comb and adhering this to the top bars of whole frames. *A. dorsata* combs were cut into 3 cm strips which were wax-melted onto bare frame top bars. Thus, all combs were about 3 cm high and 12 cm wide, very similar to an *A. florea* crown when the top bars of the test combs

**Table 4.2** Preferences of five *A. florea* wax-salvaging colonies for conspecific *A. florea* as well as *A. dorsata*, *A. cerana* and *A. mellifera* crowns. Numerical values represent the sum of wax-scavenging events (Hepburn et al. 2010)

Crown combs				
Colony	<i>A. florea</i>	<i>A. dorsata</i>	<i>A. cerana</i>	<i>A. mellifera</i>
1	7	0	0	0
2	62	0	0	0
3	22	0	0	0
4	89	0	0	0
5	56	0	0	0
Total	236	0	0	0
Mean $\pm$ SD	47.2 $\pm$ 32.8	0	0	0

were inverted to simulate the *A. florea* crown combs. The positions of the four wax crowns were again assigned randomly. Procedurally, this experiment was exactly like the first experiment. In both experiments none of the comb specimens had been used for brood rearing, were about of the same light colour, and therefore, probably of the same age, and were collected in the same area.

In this experiment, wax-salvaging by *A. florea* foragers from the experimental crowns of *A. florea*, *A. cerana*, *A. dorsata* and *A. mellifera* was observed. In separate trials of five different colonies, the number of paint-marked *A. florea* bees that salvaged wax from *A. florea* crowns was  $47.2 \pm 32.8$ , while paint-marked *A. florea* foragers did not retrieve waxes from the crowns of *A. cerana*, *A. dorsata* or *A. mellifera* (Table 4.2). The five colonies differed significantly in the numbers of wax-salvaging bees on *A. florea* crowns compared to the other species combs. Some unmarked *A. florea* foragers salvaged wax from the *A. cerana* crown, and approached but did not salvage wax from the *A. dorsata* and *A. mellifera* crowns. Because these bees were unmarked, they were excluded because of the possibility that such individual bees were not from the test colonies.

In this experiment the results were significant in that paint-marked *A. florea* foragers did not salvage wax from *A. cerana*, *A. dorsata*, or *A. mellifera* crowns. This indicates an unequivocal sensory capacity of *A. florea* foragers to distinguish between *A. florea* and non-*A. florea* waxes. However, the fact that unmarked *A. florea* foragers also salvaged wax from *A. cerana* comb suggests a greater behavioural plasticity than indicated just by the experimental colonies.

When the data of these experiments are juxtaposed to similar ones of hetero-specific wax utilization within combs (Hepburn et al. 2009), some context-specific anomalies appear. In an experiment with heterospecific waxes, small squares of beeswax foundation fashioned from comb waxes of *A. florea*, *A. cerana*, *A. dorsata* and *A. mellifera* colonies were inserted in ‘windows’ cut in the middle of *A. florea* combs. All the *A. florea* colonies unequivocally accepted the wax inserts of *A. cerana*, *A. dorsata* and *A. florea* and built on them, but rejected the *A. mellifera* wax inserts. However, in this experiment, paint-marked *A. florea* foragers did not salvage wax from the combs of *A. cerana*, *A. dorsata* or *A. mellifera*. This contrast obviously

indicates that sensory discrimination of waxes by *A. florea* is exercised in the field but not in the nest; a context in which it should be unnecessary in the absence of heterospecific nest parasitism by other honeybee species.

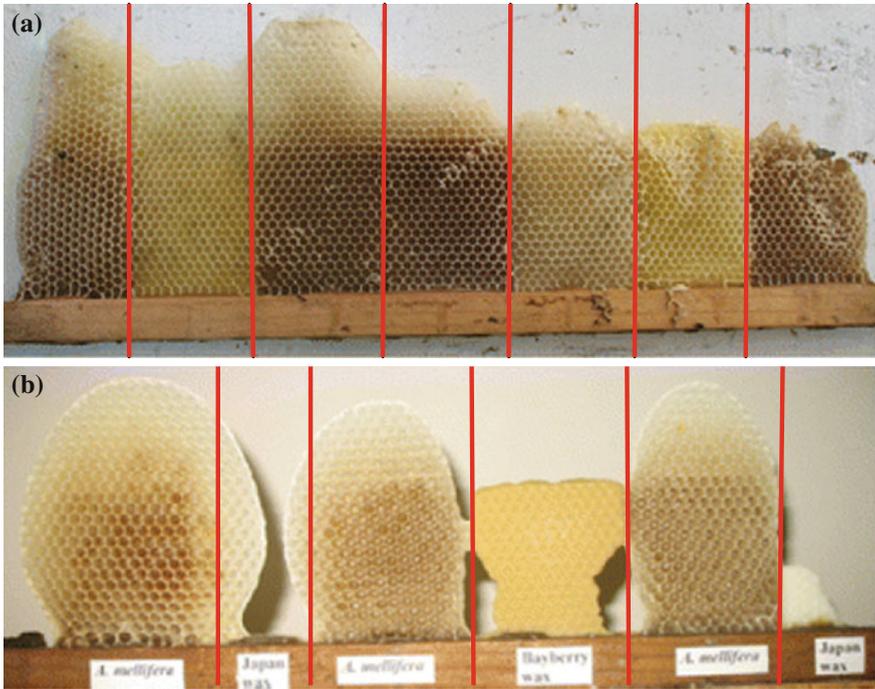
#### 4.4 Interspecific Wax Discrimination

Because *A. mellifera* can distinguish olfactory differences between combs of different colonies (Fröhlich et al. 2000b) and different ages of the same species (Breed et al. 1998), questions arise as to what extent is there flexibility for wax choice among honeybee species? Do they discriminate among waxes that they might naturally encounter (as in the Southeast Asian species), compared with waxes foreign to them? Finally, what are the Euclidean distances based on chemical composition of beeswaxes of different sister-groups, and are these similarities and differences related to wax choice in different species of honeybees?

Although *A. andreniformis* (Duangpakdee, pers. obs.; Wongvilas, pers. obs.), *A. florea* (Akratanakul 1977; Hepburn et al. 2009, 2010) and *A. m. capensis* (Hepburn and Radloff 1998), are known to scavenge wax conspecifically from abandoned combs, there have not yet been any reports of heterospecific salvage. To assess behavioural flexibility for wax choice using several beeswaxes, plant and mineral waxes as the test materials, Hepburn et al. (2009) used the Cape honeybee, *A. m. capensis*, colonies in South Africa, and *A. cerana* and *A. florea* colonies in Thailand. *A. m. capensis*, *A. florea*, *A. cerana* and *A. dorsata* beeswaxes, three plant waxes (Japan wax—ex: *Toxicodendron*, candelilla wax—ex: *Euphorbia*, and bayberry wax—ex: *Myrica*) and ozokerite, a mineral wax, were moulded into small sheets of wax foundation (inserted in the grooves of normal frame top bars), or, were cut into small squares and inserted in ‘windows’ cut from the host combs.

Photographs were taken to document the results once the combs were drawn. All four kinds of beeswax were accepted by colonies of *A. m. capensis* and cells were constructed on them (Fig. 4.1a). (As an aside, the honeyguides that damaged the combs shown in Fig. 4.1 are specialist feeders on beeswax and have the enzymic capacity to digest it (Downs et al. 2002; Diamond and Place 2008). The Asian orange-rumped honeyguide also consumes beeswax (Cronin and Sherman 1976; Underwood 1992), but no physiological studies have been reported on its digestive capacity). Both the Japan and bayberry waxes were gnawed away and removed by the bees, while candelilla and ozokerite waxes remained untouched (Fig. 4.1b; Table 4.3). The *A. cerana* colonies accepted the strips of *A. cerana*, *A. florea* and *A. dorsata* wax and extended their combs on these; however, they either gnawed or avoided the wax of *A. m. capensis* as well as all plant and mineral waxes (Table 4.3).

After 1 week the *A. florea* colonies had repaired the wax inserts in their combs. However, they did not refashion the larger cell base to *florea*-size, but constructed new cell walls much thicker than normal so that cell diameter was a close approximation to normal size. Over subsequent weeks, all the *A. florea* colonies had accepted



**Fig. 4.1** **a** Comb construction by *A. m. capensis* on foundation sheets made from (left to right) the waxes of *A. m. capensis*, *A. cerana*, *A. m. capensis*, *A. florea*, *A. dorsata*, *A. cerana*, and *A. m. capensis*; **b** Comb construction by *A. m. capensis* on foundation sheets made from (left to right) the waxes of *A. m. capensis*, Japan wax, *A. m. capensis*, bayberry, *A. m. capensis* and Japan wax. The obvious damage to the combs in (a) was inflicted by the Lesser Honeyguide, *Indicator minor*, feeding on them (Hepburn et al. 2009)

**Table 4.3** Reactions of *A. m. capensis*, *A. cerana* and *A. florea* honeybees to thin sheets of different beeswaxes, plant and mineral waxes (Hepburn et al. 2009)

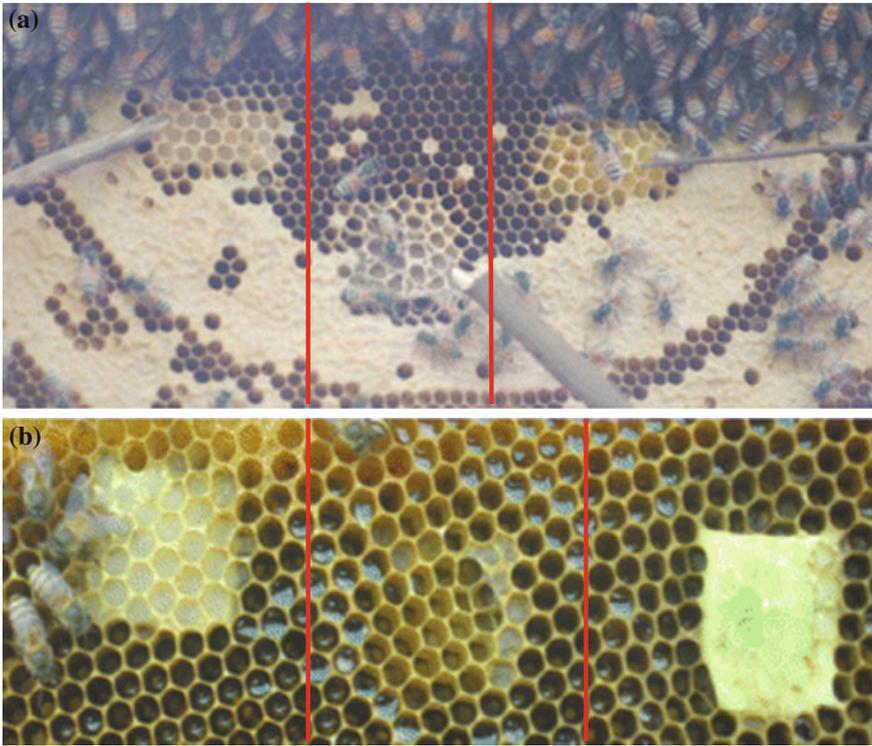
Waxes		Host colonies		
		<i>A. m. capensis</i>	<i>A. cerana</i>	<i>A. florea</i>
Beeswax	<i>A. cerana</i>	Accepted builds	Accepted builds	Accepted builds
	<i>A. florea</i>	Accepted builds	Accepted builds	Accepted builds
	<i>A. dorsata</i>	Accepted builds	Accepted builds	Accepted builds
	<i>A. m. capensis</i>	Accepted builds	Gnawed/untouched	Gnawed
Plant wax	Bayberry	Gnawed	Untouched	Not tested
	Japan wax	Gnawed	Untouched	Not tested
	Candelilla	Untouched	Untouched	Not tested
Mineral wax	Oxokerite	Untouched	Untouched	Not tested

the *A. cerana* and *A. dorsata* foundation wax inserts (Fig. 4.2a). One colony accepted the *A. mellifera* wax, but the other two simply gnawed away at the wax. *A. cerana* colonies readily and equally accepted the waxes of only three species (*A. cerana*, *A. dorsata* and *A. florea*), and partially or completely rejected that of *A. m. capensis* (Table 4.3, Fig. 4.2b). Based on a parsimonious cluster analysis (cf. Chap. 16, Fig. 16.3), the giant honeybee group (*A. dorsata* and *A. laboriosa*) is clearly segregated from the other species, as are the dwarf species (*A. andreniformis* and *A. florea*), while *A. mellifera* is placed close to its sister-group, *A. cerana*.

Given the ubiquitous nature and abundance of surface waxes throughout the plant kingdom (Kolattukudy 1976), it is perhaps surprising that elaborate glands for wax synthesis and secretion evolved in honeybees in the first place (Hepburn et al. 1991). This is particularly curious as many other apoid bees utilize various plant exudates for building their nests (Roubik 1992). Nevertheless, all *Apis* species have such glands, but their product of secretion, beeswax, has also changed with speciation, there being only 13 out of 82 chromatographic elution peaks shared in common across all species (Aichholz and Lorbeer 1999, cf. Chap. 16). The plant and mineral waxes were uniformly rejected, possibly because they lack some or all of the 13 shared compounds present in all beeswaxes and/or are actually repellent for other reasons (Sackin 1998). The plant waxes often contain terpenoid compounds, which are known honeybee repellents (Hamilton 1995). Given that the alkanes, monoesters and diesters, hydroxymonoesters, hydroxydiesters are shared in common within all beeswaxes, these compounds could be interpreted as the ‘essence’ of beeswax, which may be necessary and sufficient to induce bees to build comb.

The rejection of the *A. m. capensis* wax by *A. cerana* and *A. florea* is difficult to account for, but could possibly be due to the presence of a series of saturated fatty acids, C<sub>22</sub>–C<sub>36</sub>, all of which are absent from *A. cerana*, *A. florea* and largely from *A. dorsata* wax. Indeed, using the proboscis extension reflex technique, Fröhlich et al. (2000b) showed that *A. mellifera* workers can recall and distinguish the fatty acids and hydrocarbons of wax. It is also pertinent to mention cell size in relation to the foundation strips of wax given to the bees in this experiment. The *A. m. capensis* cell size used in making the moulds was about 4.8 mm in width; however, the cells of *A. florea* are 2.9 mm and *A. cerana* 4.3 mm. Inasmuch as both *A. florea* and *A. cerana* readily accepted the *A. cerana*, *A. dorsata* and *A. florea* wax foundation made to the *A. m. capensis* cell size (4.8 mm), then rejected the *A. m. capensis* wax, cannot be attributed to differences in cell size.

Considering the Euclidean distances of the beeswaxes, the dwarf and giant honeybees are distinct groups, but *A. mellifera* is only slightly skewed away from *A. cerana*. It is somewhat curious that both *A. cerana* and *A. florea* accepted the *A. dorsata* wax, which, based on chemical cladistics for wax, is the most distant from both. Perhaps this would appear to be a small discrepancy in light of the currently prevailing phylogenies for *Apis* based on nesting sites (Lindauer 1956), morphometrics (Alexander 1991), DNA sequences (Arias and Sheppard 2005) and behaviour (Raffiudin and Crozier 2007). In any event, the close proximity of the



**Fig. 4.2** **a** Comb construction by *A. florea* on foundation 'windows' made from (left to right) the waxes of *A. dorsata*, *A. m. capensis* and *A. cerana*; **b** Comb construction by *A. cerana* on foundation 'windows' made from (left to right) the waxes of *A. florea*, *A. m. capensis* and *A. dorsata* (Hepburn et al. 2009)

beeswax cluster groups to those based on DNA and morphometrics suggests that the wax glands were a highly conserved feature during honeybee evolution.

Previous studies on comb-building in *A. mellifera* have shown that some very simple building rules (Darchen 1954 et seq.; Hepburn and Whiffler 1991) which, coupled to the physico-chemical properties of beeswax as a building material (Pirk et al. 2004; Buchwald et al. 2006), can parsimoniously explain several aspects of comb-building behaviour. Indeed, regulation of behaviour through self-organisation (Bonabeau et al. 1997; Boomsma and Franks 2006; Detrain and Deneubourg 2006), specifically in honeybee societies, can be used to interpret behaviours including comb construction (Belic et al. 1986; Hepburn 1998), the arrangement of food-storing and brood-rearing in the combs (Camazine et al. 1990; Camazine 1991), and the regulation of food collection behaviour (Jenkins et al. 1992).

## 4.5 Comb-Building in Mixed-Species Colonies

Mixed-species colonies of honeybees offer us a valuable opportunity to investigate the relationships within and between the two species and provide us with a new perspective to examine the theories of self-organisation in honeybees and investigate the evolution of behaviour. Division of labour in mixed-species colonies remained an intriguing issue, which was not previously considered until quite recently with experiments by Yang et al. (2010c). They examined the comb-construction behaviour of mixed-species colonies of *A. cerana* and *A. mellifera* to answer several questions: (1) Will mixed-species colonies accept each other's waxes? (2) Will colonies of pure *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 during comb-building? (4) Do *A. cerana* 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 emerge in the newly constructed combs? And (6) once constructed, how are these cells used in the economy of the nest?

### 4.5.1 Organisation of Mixed-Species Colonies and Wax Foundation

Yang et al. (2010c) established mixed-species colonies of both *A. cerana* and *A. mellifera* workers: three colonies were headed by *A. cerana* queens, and reciprocally, three colonies were headed by *A. mellifera* queens. Frames of sealed brood about to emerge as young adults of each species were placed in the colonies of the other species (Tan et al. 2006). Observations were made on the wax-building behaviour when the newly emerged workers of the two species were about 10–18 days old, the peak age of wax secretion (Rösch 1927; Hepburn et al. 1984; Seeley 1995). Pure *A. cerana* and *A. mellifera* colonies with the same age cohort of workers were also selected as control colonies, which were equalised for size, number of combs, adult bees, nectar and pollen stores and brood. In these experiments, beeswax was extracted from both *A. cerana* and *A. mellifera* combs and used to make small sheets of beeswax foundation of the two worker cell sizes: *A. cerana*, about 4.75 mm in diameter (Ruttner 1988), and *A. mellifera*, 5.35 mm in diameter (Winston 1987), using silicon rubber moulds (Hepburn et al. 2009). Both *A. cerana* and *A. mellifera* cell-size foundation was introduced into pure *A. cerana* and pure *A. mellifera* colonies. The experiments on cell size and wax discrimination, and comb-building cooperation were conducted with colonies of *A. cerana* and *A. mellifera* at an apiary at the King Mongkut's University of Technology, Chom Bueng, Thailand. The four types of beeswax foundation sheets (two wax types and two cell sizes) were fixed on the top bars of frames, and their

relative positions in the hives determined by random number assignment; they were then inserted into the centre of the hives.

Video-recordings were made of comb-building activity for the test and control colonies at 10 s intervals three times a day and every day for the replicates (Table 4.4). On replaying the video clips, detailed information was obtained 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 the festoon bees 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 comb building was complete. When the foundation sheets had been extended beyond their original lengths by the addition of several cm of new wax, the combs were removed from the hives and replaced with new top bars with the same four kinds of foundation (Yang et al. 2010c).

### ***4.5.2 Cell-Size and Wax Discrimination***

Pure *A. cerana* colonies ignored all beeswax foundation and began building new combs either from the top bar, or from the lower edges of the foundation sheets (Fig. 4.3a). By contrast, the pure *A. mellifera* colonies accepted both the *A. cerana* and *A. mellifera* foundation sheets and built cells on both cell sizes (Fig. 4.3b). In the two types of mixed-species colonies, all four types of foundation were accepted (Fig. 4.3c, d); workers of both species were seen building cells on the foundation (Fig. 4.4, Table 4.5). None of these mixed-species colonies showed any preference to a particular type of foundation with respect to wax type or cell size.

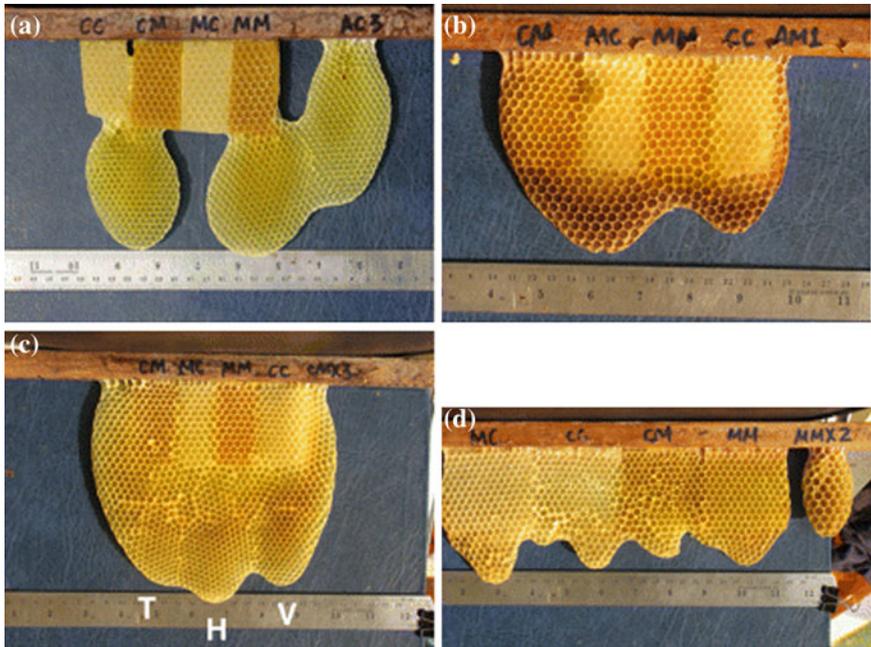
### ***4.5.3 Cell-Size Modification of Foundation Sheets***

All the *A. mellifera* cell-size sheets of foundation were built to their original size without any modification (Table 4.5); but the *A. cerana* cell-size foundation sheets were modified in all colonies, except for the pure *A. cerana* colonies. Some of these cells were squeezed to make space for enlarging neighbouring cells. The percentages of combs that had modified cells in the test and control groups are shown in Table 4.5. In *A. mellifera* queen-headed mixed-species colonies, all the *A. cerana* foundation sheets were modified, and in the pure *A. mellifera* colonies, nearly all were modified which was significantly different to the *A. cerana* queen-headed mixed-species colonies and pure *A. cerana* colonies (Yang et al. 2010c).

**Table 4.4** Numbers (mean  $\pm$  S.D.) of worker bees engaged in comb-building on four types of foundation (Yang et al. 2010c)

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

*N* is the number of pure colonies and *n* is the number of repetitions



**Fig. 4.3** Comb built by an *A. mellifera* queen-headed, mixed-species colony. 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. AC3, an example comb built by a pure *A. cerana* colony (Colony 3); AM1; an example comb built by a pure *A. mellifera* colony (Colony 1); CMX3, an example comb built by an *A. cerana* queen-headed, mixed-species colony (Colony 3); MMX2, an example comb built by an *A. mellifera* queen-headed, mixed-species colony (Colony 2) (Yang et al. 2010c)

#### 4.5.4 Freely-Built Combs

On completion of the comb-building trials using different species, waxes and cell sizes on the artificial foundation sheets, the workers from the four types of colonies were observed starting to build new combs at several sites (Table 4.6). Pure *A. mellifera* colonies and *A. mellifera* queen-headed mixed-species colonies had significantly more festoons at new comb-building sites than pure *A. cerana* and *A. cerana* queen-headed colonies (Table 4.6). In *A. cerana* queen-headed mixed-species colonies, workers of both species were seen working together in festoons, although significantly more *A. mellifera* workers ( $57.9 \pm 6.2\%$ ) were involved than *A. cerana* workers ( $42.1 \pm 6.2\%$ ). Similarly, in the *A. mellifera* queen-headed mixed-species colonies, significantly more *A. mellifera* workers ( $67.5 \pm 4.8\%$ )

**Fig. 4.4** Comb-building by a mixed-species, wax-building chain of *A. cerana* and *A. mellifera* workers (Yang et al. 2010c)



**Table 4.5** Percentages of *A. cerana* cell size foundation with modifications (Yang et al. 2010c)

Colony type	<i>A. cerana</i> cell-size foundations		<i>A. mellifera</i> cell-size foundations	
	Number	Percentage with modified signs (%)	Number	Percentage with modified signs (%)
Pure <i>Apis cerana</i> ( $N = 3$ , $n = 12$ replicates)	24	0	24	0
Pure <i>Apis mellifera</i> ( $N = 3$ , $n = 12$ replicates)	24	83.3	24	0
<i>A. cerana</i> queen-headed mixed colony ( $N = 3$ , $n = 14$ replicates)	28	10.7	28	0
<i>A. mellifera</i> queen-headed mixed colony ( $N = 3$ , $n = 10$ replicates)	20	100	20	0

$N$  is the number of pure colonies and  $n$  is the number of repetitions

than *A. cerana* workers ( $32.5 \pm 4.8\%$ ) were engaged in comb-building in the festoons (Table 4.6). 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 (Table 4.6).

As for irregular cells on the new combs, pure *A. cerana* and *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

**Table 4.6** Characteristics of freely-built combs, mean ± SD (Yang et al. 2010c)

Parameter	<i>A. cerana</i> queen-headed mixed colonies (N = 3, n = 14 replicates)	<i>A. mellifera</i> queen-headed mixed colonies (N = 3, n = 10 replicates)	Pure <i>A. cerana</i> colonies (N = 3, n = 12 replicates)	Pure <i>A. mellifera</i> colonies (N = 3, n = 12 replicates)
Number of festoons	2.3 <sup>b</sup> ± 0.5	4.2 <sup>a</sup> ± 1.4	1.9 <sup>b</sup> ± 0.9	3.9 <sup>a</sup> ± 1.1
Number of <i>A. cerana</i> workers on the festoons	61.4 ± 13.4	36.8 ± 10.7	108.0 ± 29.1	–
Number of <i>A. mellifera</i> workers on the festoons	84.6 v 16.1	75.6 ± 16.3	–	90.3 ± 25
Total number of two species of workers on the festoons	146.1 <sup>a</sup> ± 22.0	112.4 <sup>b</sup> ± 24.5	1-8.0 <sup>b</sup> ± 29.1	90.3 <sup>b</sup> ± 25.0
Percentage of irregular cells (%)	9.1 <sup>a</sup> ± 3.6	10.8 <sup>a</sup> ± 4.7	0.8 <sup>b</sup> ± 0.5	2.7 <sup>b</sup> ± 1.7
Patterns of newly built combs:	V + H: 29 % V + H + T: 22 % V + T: 21	V + H: 60 % V: 40 %	V: 75 % V + H: 17 % T: 8 %	V: 83 % V + H: 17 %
V = vertical	V: 14 %; T: 7 %			
H = horizontal	V + H + R: 7 %			
T = tilted				
R = rosette				
Cell size of newly built combs (mm)	5.41 <sup>b</sup> ± 0.27	5.93 <sup>a</sup> ± 0.61	4.38 <sup>c</sup> ± 0.06	5.74 <sup>a, b</sup> ± 0.61

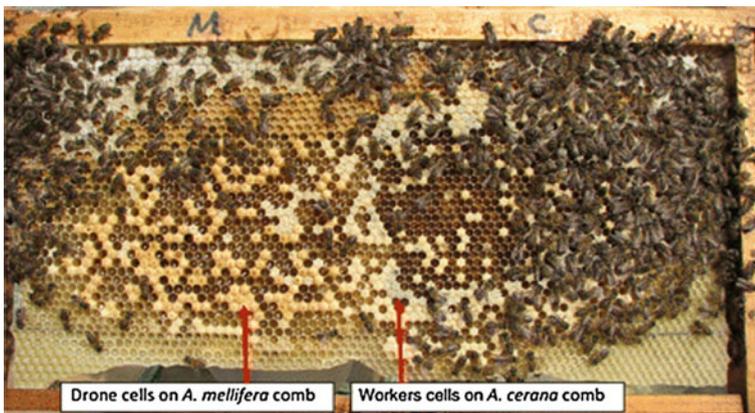
Means within one row followed by the same letter are not significantly different (Tukey multiple comparisons:  $p > 0.05$ ). N is the number of pure colonies and n is the number of repetitions (Yang et al. 2010c)

(Table 4.6). 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 mixed-species colonies, pure *A. cerana* and *A. mellifera* colonies; different festoons on one comb built patterns different to those built on other combs (Table 4.6). *A. mellifera* queen-headed, mixed-species colonies built new combs mainly in vertical and horizontal patterns (Fig. 4.3d); in pure *A. cerana* and *A. mellifera* colonies, the patterns of cell orientation were more homogeneous and mainly vertical (Fig. 4.3a, b; Table 4.6).

The different mixed-species colonies built significantly different sized cells (Table 4.6). 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*

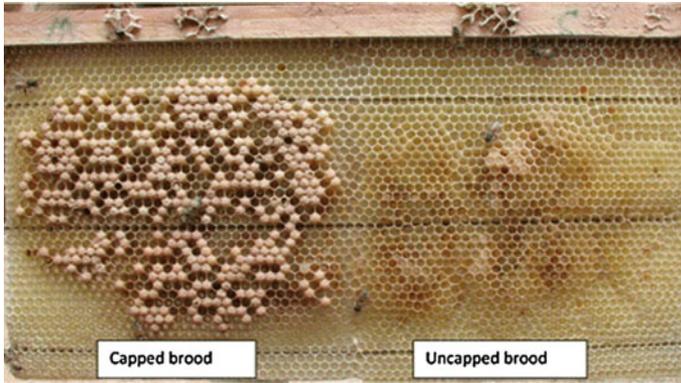


**Fig. 4.5** Utilisation of combs built on two types of cell size foundation in pure *A. cerana* colonies; *A. mellifera* size cells (*left*) were used for storing food, while the *A. cerana* size cells (*right*) were used for brood rearing (Yang et al. 2010c)



**Fig. 4.6** Utilisation of combs built on two types of cell size foundation in pure *A. cerana* colonies; *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 (Yang et al. 2010c)

queen-headed mixed-species colonies were similar to *A. mellifera* drone cells (European type, 6.0–6.3 mm), whereas in the *A. cerana* queen-headed mixed-species colonies, the cells had a diameter of  $5.41 \pm 0.27$  mm, which is like the normal *A. mellifera* worker size cells. The pure *A. cerana* colonies built cells  $4.38 \pm 0.06$  mm in size, which is the normal *A. cerana* worker size cell.



**Fig. 4.7** Utilisation of combs built on two types of cell size foundation in pure *A. mellifera* colonies, the brood cells on the *A. mellifera* cells (*left*) are already capped but the larvae on the *A. cerana* cell size foundation (*right*) still need about three 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 (Yang et al. 2010c)

#### 4.5.5 Utilisation of the Newly Built Combs

In their experiments, Yang et al. (2010c) inserted both *A. cerana* cell size (4.75 mm in diameter) and *A. mellifera* cell-size (5.35 mm diameter) foundation strips into pure *A. cerana* 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; while 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 the *A. mellifera* colonies in the subsequent use of these cells. The pure *A. cerana* colonies used the *A. mellifera* size cells either for food storage (Fig. 4.5) or drone brood rearing, while the *A. cerana* size cells were normally used for rearing worker brood (Fig. 4.6). In pure *A. mellifera* colonies, queens mainly laid eggs in both *A. mellifera* and *A. cerana* size cells, but they all showed a preference for *A. mellifera* size cells and laid eggs in these cells first and more regularly (Fig. 4.7).

#### 4.5.6 General Comb-Building

It is common knowledge that cavity-dwelling honeybees build multiple, parallel combs and that this parallelism is recognised as a building rule (Darchen 1954; Hepburn 1986; Hepburn and Muller 1988). Comb-building bees work 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 that facilitates an equable temperature for wax secretion and manipulation (Hepburn 1986). Numerous comb-building workers with active wax glands engage in the task of comb construction. But, instead starting to build at a single site, several festoons begin at independent sites, constructing starting strips of cells several cells (hence combs) simultaneously, and only later do they connect these using irregular transitional cells (Hepburn 1986; 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 new combs started at separate sites (Hepburn and Whiffler 1991).

#### 4.5.7 Comb-Building in Mixed-Species Colonies

*A. cerana* and *A. mellifera* workers cooperate heterospecifically in the same festoons in comb-building (Yang et al. 2010c); but it is somewhat strange that in the pure *A. cerana* colonies, none of the four types of foundation (foundation made from *A. mellifera* wax in both *A. mellifera* and *A. cerana* worker size cells and foundation made from *A. cerana* wax both in *A. cerana* and *A. mellifera* worker size cells) were accepted, although two of the four foundations were embossed with normal *A. cerana* size cell. In sharp contrast to this, in the pure *A. mellifera* colonies, workers were seen building cells on both types of wax foundation and of both cell sizes. These results indicate that *A. mellifera* workers are more tolerant of cell size factors in wax foundation. 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 species of 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 mixed-species colonies (Table 4.6). This certainly suggests that comb-building workers *A. mellifera* can somehow stimulate *A. cerana* workers to start building comb. 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 festoons, so it is not surprising that the cell sizes were similar to normal *A. mellifera* worker-sized cells.

It is interesting to note that in an *A. cerana* queen-headed mixed-species 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 in festoons, the two species cannot really perform the comb-building tasks

harmoniously. The fact that the combs in the pure *A. mellifera* colonies and *A. mellifera* queen-headed colonies mixed-species were built to normal *A. mellifera* drone-sized cells may be related to the season in which in the experiment was conducted.

In conclusion, *A. cerana* workers as colonies did not accept any type of beeswax foundation, but as individuals were stimulated by *A. mellifera* workers to engage in comb-building. So, the results are consistent with the idea that honeybee comb-building behaviour is an example of self-organisation. It was also confirmed that in the mixed-species colonies, these two closely related honeybee species did in fact cooperate in comb-building, even though irregular cells arose through their joint efforts. It can also be inferred that, although the comb-building workers are poorly informed and lack a central controller (Pratt 2004), comb-building is really a task that can only be finished by smaller groups, in which individuals cooperate closely to achieve progress. This might explain, in part, why *A. mellifera* workers do not dominate the comb-building effort.

The results presented here, based on mixed-species colonies, reinforce the conclusion that this experimental method is extremely useful for testing underlying mechanisms that evoke or suppress certain behaviours. Such an experimental context has been successfully used to elucidate disruption of social networks as in ovarian activation (Tan et al. 2009), stimulation of social networks, dance language (Tan et al. 2008) and retinue behaviour towards queens (Yang et al. 2010a). The results from the comb-building experiments provide additional evidence for the value of mixed-species colonies as experimental probes for investigating pre- and post-speciation behaviour in honeybees.

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