ความขัดแย้งในการสืบพันธุ์ของผึ้งเอเชีย *Apis florea* และ *Apis cerana*

นางสาวปิยมาศ นานอก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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REPRODUCTIVE CONFLICTS IN THE ASIAN HONEY BEES

Apis florea AND *Apis cerana*

Miss Piyamas Nanork

A Dissertation Submitted in Partial Fulfillment of the Requirements

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ปิยมาศ นานอก : ความขัดแย้งในการสืบพันธุ์ของผึ้งเอเชีย *Apis florea* และ *Apis cerana* (REPRODUCTIVE CONFLICTS IN THE ASIAN HONEY BEES *Apis florea* AND *Apis cerana*) อาจารย์ที่ปรึกษา : ศ. ดร. สิริวัฒน์ วงษ์ศิริ, อาจารย์ที่ปรึกษาร่วม : Assoc. Prof. Dr. Benjamin P. Oldroyd. 93 หน้า. ISBN 974-53-2370-5.

ในรังผึ้งสกุล *Apis* ที่มีนางพญา ผึ้งงานจะมีรังไข่ที่พัฒนาไม่เต็มที่ อย่างไรก็ตามเมื่อรังผึ้งขาดนางพญาแล้ว ผึ้งงานจะเริ่มพัฒนารังไข่และเริ่มวางไข่ที่ไม่ได้รับการปฏิสนธิ เนื่องจากผึ้งนางพญาผสมพันธุ์กับผึ้งตัวผู้หลายตัวจึงทำให้เกิดความแตกต่างในความสัมพันธ์ทางพันธุกรรมระหว่างผึ้งงานที่เกิดมาจากต่างพ่อกัน (ซับแฟมมิลี) ขึ้น ซึ่งคาดว่าจะนำไปสู่ความขัดแย้งในการสืบพันธุ์ คือผึ้งงานจะแข่งขันกันเพื่อผลิตผึ้งตัวผู้ชุดสุดท้ายในรังผึ้งที่ขาดนางพญา วิทยานิพนธ์นี้ได้ศึกษาว่าผึ้งมิ้ม *Apis florea* และผึ้งโพรง *A. cerana* มีการลดความขัดแย้งดังกล่าวอย่างไรเมื่อรังผึ้งอยู่ในสภาพขาดนางพญา

จากการศึกษาพบว่าผึ้งงานในรังผึ้งมิ้มที่ขาดนางพญามีอัตราการพัฒนารังไข่ไม่เท่ากัน แสดงให้เห็นว่าผึ้งงานจากบางซับแฟมมิลี มีความสามารถในการรับรู้การหายไปของเฟอโรโมนจากนางพญาและตัวอ่อนเร็วกว่าผึ้งงานจากซับแฟมมิลีอื่น นอกจากนี้ยังพบว่าในรังผึ้งมิ้มที่ขาดนางพญานั้น มีไข่และดักแด้ตัวผู้ (35.6% และ 22.5% ตามลำดับ) ที่มีกำเนิดมาจากผึ้งงานของรังอื่นอีกด้วย

ในรังผึ้งโพรงพบว่าอัตราการพัฒนารังไข่ในผึ้งงานเพิ่มขึ้นอย่างรวดเร็วหลังจากที่รังอยู่ในสภาพขาดนางพญา ผึ้งงานจากต่างซับแฟมมิลีมีการพัฒนารังไข่ในอัตราที่ใกล้เคียงกันและพบว่ามีไข่และดักแด้ตัวผู้ที่เกิดจากผึ้งงานของรังอื่นอยู่ภายในรังที่ขาดนางพญาเช่นเดียวกันกับที่พบในผึ้งมิ้ม แต่พบไข่และดักแด้ตัวผู้ดังกล่าวในสัดส่วนที่น้อยกว่าที่คาดไว้เมื่อเปรียบเทียบกับจำนวนของผึ้งงานจากรังอื่นที่พบ ซึ่งตรงกันข้ามกับผึ้งมิ้ม ทั้งนี้อาจเนื่องมาจากผึ้งทั้งสองชนิดนี้มีพฤติกรรมการสร้างรังที่แตกต่างกัน และอีกสาเหตุหนึ่งอาจเนื่องมาจากในรังผึ้งโพรงมีอัตราการกินไข่สูง

สุดท้ายได้แสดงให้เห็นว่าผึ้งพันธุ์ *A. mellifera* สามารถจำแนกระหว่างไข่ที่เกิดมาจากผึ้งงานและไข่ที่เกิดมาจากผึ้งนางพญาของผึ้งมิ้มซึ่งมีความสัมพันธ์ทางวิวัฒนาการห่างกันได้ แต่ไม่สามารถจำแนกระหว่างไข่ของนางพญาและผึ้งงานของผึ้งโพรงที่มีความสัมพันธ์ทางวิวัฒนาการใกล้ชิดกันได้ ซึ่งอาจจะเป็นหลักฐานหนึ่งที่แสดงให้เห็นถึงการเปลี่ยนแปลงของระบบการสืบพันธุ์ในผึ้งงานได้

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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In honey bee (genus *Apis*) colonies with a queen, workers usually have inactive ovari
However when colonies become queenless many workers begin to activate their ovaries and l
unfertilized eggs. Differences in relatedness among subfamilies of workers, arising from que
polyandry, is predicted to lead to reproductive conflicts in a queenless colony over which work
should produce the final batch of males. This thesis investigates how these conflicts are played
in terminally queenless colonies of *Apis florea* and *A. cerana*.

The study showed unequal rates of ovary activation among queenless workers of the
dwarf honey bee, *A. florea* colonies, suggesting that some subfamilies have the ability to respon
a lack of queen and brood pheromones earlier than others. Furthermore, this research has shown
queenless *A. florea* colonies are parasitized by eggs of non-natal workers, 35.6% of eggs and 22
of pupae were from non-natal workers.

In colonies of the eastern honey bee, *A. cerana*, the proportion of workers with activ
ovaries rapidly increases a few days after queenlessness. Workers from different subfamilies
uniform rates of ovary activation. Like *A. florea*, eggs and pupae of non-natal workers were f
in queenless *A. cerana* colonies, but the proportion of them was less than expected based on nu
of non-natal workers present. This contrast with *A. florea* may be due to the different n
behaviour of the two species, and the very high rates of worker policing found in *A. cerana*.

Finally it was shown that the western honey bee *A. mellifera* can distinguish queen-la
worker-laid eggs of the distantly related *A. florea*, but cannot distinguish queen-laid and work
eggs of its sister taxa *A. cerana*. This may be evidence for the theoretically predicted 'epis
worker revolution'.

Field of study Biological Sciences Student's signature Piyamas Nanork
Academic year 2005 Advisor's signature
Co-advisor's signature

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Table of Contents

Page

List of Tables

Table Page

List of Figures

Figure Page

CHAPTER I

INTRODUCTION

Honey bees (Genus *Apis*, Order Hymenoptera) are eusocial insects. Three types of individuals or castes are found in a honey bee colony: the queen (a fertile female), workers (subfertile females) and drones (males) (Crozier and Pamilo, 1996; Moritz and Southwick, 1992; Wilson, 1971).

In most species of social Hymenoptera with queen-worker dimorphism, workers cannot mate but retain functional ovaries (Ratnieks and Visscher, 1989). In honey bee colonies that become hopelessly queenless (queen absent in colony), workers eventually begin laying eggs. These worker offspring can be reared and become normal reproductive males (Page and Robinson, 1994; Robinson *et al.*, 1990). In contrast, workers in queen-right colonies (queen present in colony), almost always lack activated ovaries and act to suppress egg production by worker nestmates (Ratnieks and Visscher, 1989). Under normal queen-right conditions the presence of queen substance and pheromones produced by brood inhibit worker ovary development (Free, 1987; Winston and Slessor, 1998; Wongsiri, 1989).

Polyandry, that is, multiple mating by a female with different males, is widespread in highly eusocial Hymenoptera, including honey bees (Cole, 1983; Crozier and Pamilo, 1996; Keller and Reeve, 1994). Kin selection theory (Hamilton, 1964) predicts reproductive conflict among females over the parentage of males, both among workers and between workers and the queen (Barron *et al.*, 2001; Oldroyd and Osborne, 1999; Ratnieks, 1988; Ratnieks and Reeve, 1992).

Worker bees begin to activate their ovaries when their queen is lost and unable to rear a new queen (Miller and Ratnieks, 2001). On relatedness grounds

they should prefer to rear their own male eggs rather than those of the queen or those of their half sisters (Martin *et al.,* 2004; Oldroyd and Osborne, 1999). Here I determine rates of ovary activation in queenless colonies of *A. florea* and *A. cerana*. Furthemore, when honey bee colonies become queenlessness, the colonies will be parasitized by non-natal workers easily. Thus, reproductive parasitism is also examined in queenless colonies of those honey bee species.

The honey bee queen produces a number of pheromones that help maintain colony cohesion and stability (Free, 1987). Queen pheromones are interpreted as honest signals of queen fecundity (Keller and Nonacs, 1993; Seeley, 1985). Signals of this kind, that benefit both sender and receiver, are likely to be conserved over evolutionary time (Keller and Nonacs, 1993). This prediction is tested by cross species policing in 3 honey bee species (*Apis mellifera, A. cerana* and *A. florea*).

Objectives

1. To understand reproductive dominance among workers from different subfamilies in queenless colonies of *Apis florea* and *A. cerana*.
2. To determine whether the queen-produced egg-marking pheromone has persisted over the evolutionary time.
3. To assess the role of reproductive parasitism in queenless honey bee colonies

Anticipated benefit

Honey bee societies provide important model systems for the study of social interactions and the mechanisms by which inter-organismal conflicts are resolved. These principles are now quite well established in the western honey bee *A. mellifera*, but require extension to the Asian species. Each species has its own idiosyncratic mode of reproduction, requiring different methods of regulation of worker reproduction. This project will provide an important and

pioneering extension of our understanding of the control of worker reproduction into Asian species. In particular:

1. The results will provide information on basic biology of honey bee, including reproductive biology and conflicts among queenless workers of two Asian species, *A. florea* and *A. cerana* and contrast this with *A. mellifera*.

2. The study will provide knowledge on evolution and behaviour of honey bees.

3. The cross species policing study will be supported the argument, which will resolve the question as to whether (Monnin and Ratnieks, 2001; Martin *et al*., 2004a) queen pheromones act as queen control or queen signal.

CHAPTER II

LITERATURE REVIEW

2.1 Multiple mating and genetic diversity in social insect colonies

For two reasons, high levels of polyandry are unexpected in the queens of Hymenopteran social insect species. First, multiple mating creates low levels of colony relatedness (Palmer and Oldroyd, 2000; Oldroyd *et al*., 1997, 1998). Low relatedness reduces the kin value of sister queens to the average worker, and creates conflict among workers over which individuals should be reared as queens (Noonan, 1986; Tilley and Oldroyd, 1997; Ratnieks, 1988) Second, polyandry may increase risks to queens while they are on mating flights (such risks include increased of predation, and getting lost) (Keller and Reeve, 1994; Palmer and Oldroyd, 2000; Oldroyd *et al*., 1997, 1998). Despite these apparent disadvantages of polyandry, multiple mating has now been reported from diverse taxa including some eusocial wasps (Foster and Ratnieks, 2001), several ants (Gadau *et al*., 2003; Villesen *et al*., 1999) and most notably, the honey bees (Palmer and Oldroyd 2000). Thus, plausible explanations for the evolution of multiple mating in social insects have been frequently sought. A current view is that polyandry in the social Hymenoptera benefits queens because the higher levels of intracolonial genetic variance increases colony fitness (Boomsma and Ratniekes, 1996; Frank *et al*., 2000b; Oldroyd *et al*., 1998; Palmer and Oldroyd, 2000). Genetic diversity in a colony may:

2.1.1 Increase its capacity to buffer environmental stress (Oldroyd *et al*., 1992a; Palmer and Oldroyd, 2000). Jones *et al*. (2004) found that brood nest temperature in honey bee (*A. mellifera*) colonies with genetically diverse is more stable than in the genetically uniform colonies.

2.1.2 Increase expression of caste or task polymorphism (Oldroyd *et al*., 1992a, 1992b, 1993, 1994a, 1994c; Page *et al*., 1995; Palmer and Oldroyd, 2000). Behavioural polymorphism among subfamilies have been found for a wide variety of critical tasks (Palmer and Oldroyd, 2000). It has been argued that task specialization allows individual bees to focus on particular tasks and become expert in them (Oldroyd *et al*., 1992b; Robinson *et al*., 1994). Recent work suggests that the basis of this polyethisms is variance in the level of a stimulus required to elicit a behaviour, and that this variance is genetically determined (Beshers and Fewell, 2001; Calderone and Page, 1991; Fewell, 2003; Fewell and Bertram, 1999; Page, 1997; Page *et al.*, 1989; review in Palmer and Oldroyd, 2000).

2.1.3 Increase resistance to parasites and pathogens (Schmid-Hempel, 1995; Sherman *et al*., 1988). Multiple mating produces a more diverse range of genotypes, possibly reducing the rate of transmission of disease within a colony (Palmer and Oldroyd, 2000).

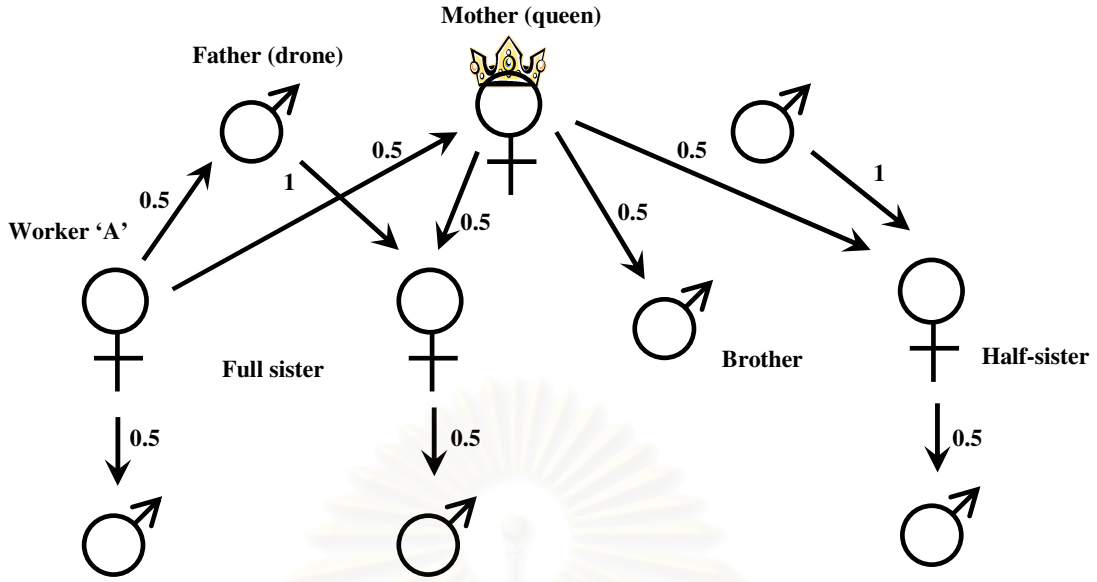
2.1.4 Reduction of genetic costs such as polyandry reducing the risk of mating with sterile males (Frank *et al*., 2000b), or with males carrying the same sex allele as the queen, thereby reducing egg viability (Page, 1980).

In honey bees, extremely high mating frequencies have been widely reported (reviewed in Palmer and Oldroyd, 2000). Estoup *et al*. (1994) used microsatellite analysis to determine number of mating frequency in the western honey bee, *Apis mellifera*, finding 7-20 subfamilies (patrilines). Of the Asian honey bees species, *A. cerana,* the eastern honey bee appears to be the most similar to *A. mellifera*, with a mating frequency ranging from 14-27 (Oldroyd *et al*., 1998). Observed paternity frequency of *A. nigrocincta* and *A. koschevnikovi* ranging from 42-69 (Palmer *et al.*, 2001) and 16-26 (Rinderer *et al.*, 1998), respectively. The mating frequency in the two species of dwarf honey bee, *A. florea* and *A. andreniformis*, are very similar. In *A. florea* 13-19 subfamilies were

found (Palmer and Oldroyd, 2001), while *A. andreniformis* queens mates at least 10-20 times (Oldroyd *et al*., 1997). The giant honey bee, *A. dorsata*, has the highest level of polyandry recorded for any social insect, with the number of subfamilies found per colony ranging from 47-102 (Moritz *et al*., 1995; Oldroyd *et al*., 1996; Wattanachaiyingcharoen *et al*., 2003).

2.2 Colony relatedness and kin structure in honey bee colony

Kin selection theory (Hamilton, 1964) predicts that within polyandrous social insect colonies there is potential for reproductive conflict among females over the parentage of males, both among workers and between workers and the queen (Barron *et al*., 2001; Oldroyd and Osborne, 1999; Ratnieks, 1988; Ratnieks and Reeve, 1992). A worker in species with colony headed by a single queen mated to more than two males, is most related to her own son (r = 0.5), then to the son of full-sister (with whom she shares the same father) (r = 0.375), then to the son of her maternal queen (r = 0.25) and least to the son of a half-sister which only share the mother (r = 0.125) (Figure 2.1) (Barron *et al*., 2001; Oldroyd and Oborne, 1999; Ratnieks and Visscher, 1989). Workers should therefore prefer to rear their own male eggs rather than those of the queen or those of a half sister (Oldroyd and Osborne, 1999).



	Own son	Son of full sister	Queen's son (brother)	Son of half sister
Worker 'A'	0.5	0.375	0.25	0.125

Figure 2.1 A single diploid queen is mated to two haploid males (normally there are many more males). The table gives the relatedness coefficients for the worker A to the possible males that could be produced in her colony (Barron *et al*., 2001).

2.3 Worker reproduction in honey bee

In almost all species of Hymenoptera, fertilized eggs produce females, and unfertilized eggs produce males (Free, 1987; Wilson, 1971). There are three castes in a honey bee colony, the queen (fertile female), workers (subfertile females) and drones (males) (Wilson, 1971). The queen is the only individual that normally lays any eggs (Free, 1987). In the honey bees, *Apis,* and most other social Hymenoptera (Vespinae wasps, *Bombus* and Meliponini bees, all ants except some Ponerinae) workers retain functional ovaries but cannot mate. As a result, any eggs workers lay are unfertilized and can only turn into males. Except

in a few ants, and in *A. mellifera capensis*, unfertilized eggs are diploid via thelytoky and give rise to female offspring (Ratnieks, 2000).

In queen-right honey bee colonies (colonies with a queen), queen pheromones and pheromones from larvae signal to workers that they should refrain from ovary activation and police (destroy) the reproduction of other workers (Barron *et al*., 2001; Miller and Ranieks, 2001). Worker reproduction of queen-right *A. mellifera* colonies is rare; about 1 worker in 10,000 has a fully developed egg in her body (Ratnieks, 1993). These few workers can lay a significant proportion (7%) of the total male eggs in a colony (Visscher, 1996), but only about 1 in 1,000 males reared to adulthood derives from a worker (Visscher, 1989; Ratnieks, 1993).

In contrast, workers in colonies that have lost their queen and have failed to rear a replacement eventually begin laying eggs at high frequency. Ovary development of worker bees and egg laying occurs 1-30 days after loss of the queen, depending on the subspecies (Miller and Ratnieks, 2001) and the species (Oldroyd *et al.*, 2001). Typically, many eggs are laid per cell, and while a few larvae may hatch from these eggs, only one will develop to a male.

The dwarf honey bee, *A. florea* and eastern honey bee, *A. cerana* are broadly distributed throughout Thailand (Ruttner, 1988; Wongsiri *et al*., 1996). As with *A. mellifera,* worker reproduction in *A. florea* colonies, is very rare. Dissections of 800 workers from 4 colonies found no worker with activated ovary, and no worker's sons were found in 564 drones using microsatellite analysis (Halling *et al*., 2001).

Ovary activation is more common among *A. cerana* workers. In colonies with an active queen and brood nest, 1-5% of workers have eggs in their ovaries. However, no worker's sons were detected by microsatellite analysis in a sample

of 652 pupal males from 4 queenright colonies (Oldroyd *et al*., 2001) indicating that worker-laid eggs are actively policed.

2.4 Reproductive competition in queenless workers of *A. mellifera*

After colonies become hopelessly queenless, pheromone signals arising from the queen and brood are lost. In the absence of these signals, workers begin to activate their ovaries and lay eggs that will result in a final brood of drones before the colony perishes (Moritz and Southwick, 1992). In queenless colonies, it is in each individual's interests to contribute as many offspring as possible to the final brood (Page and Robinson, 1994). Therefore, reproductive conflict and reproductive competition among workers is predicted in this situation.

Reproductive conflict in queenless colony of honey bees is suggested based on three lines of evidence (Page and Robinson, 1994).

2.4.1 Occasionally one or a few workers in queenless colonies of European bees produce queen-like pheromones and suppress the egg laying behaviour of their sister nestmates (Robinson *et al*., 1990).

2.4.2 Workers behave agonistically toward each other on the basis of egg-laying status and genetic relatedness (Evers and Seeley, 1986; Visscher and Dukas 1995) but see Dampney *et al*. (2002).

2.4.3 At least some members of all subfamilies of queenless colonies engage in some egg laying; however, egg-laying activity is not equal among subfamilies (Robinson *et al*., 1990).

Robinson *et al*. (1990) reported that there are subfamily differences in drone production in queenless *A. mellifera* colonies, but these biases are not always explained by subfamily differences in oviposition behaviour. They

conducted two experiments to determine whether worker reproduction in queenless honey bee colonies is influenced by genetic structure of the colonies. In the first, Robinson *et al*. (1990) demonstrated subfamilial differences in worker egg-laying behaviour. In the second, they showed that the parentage of worker-derived drones differed from that which would be expected if eggs of all subfamilies had similar viability. They suggested that this may be because of genetic variance for egg production, differential survival of eggs and larvae among patrilines, or both.

Page and Robinson (1994) found that subfamily biases in drone brood production within a colony changed significantly with brood stage. Temporal changes in the subfamily composition of brood suggest that workers selectively cannibalize eggs and larvae. Immature individuals of some subfamilies were more likely to be cannibalized than those of other subfamilies.

Martin *et al*. (2004) found reproductive competition in queenless workers of *A. mellifera* among subfamilies. Subfamilies vary in the speed of ovary activation after queen-loss, and in the survival of eggs to the larval stage. They suggested that workers of some subfamilies lay eggs that are more acceptable to queenless workers than those laid by workers of other subfamilies.

A. florea and *A. cerana* differ from *A. mellifera* in that workers activate their ovaries after only a few days of queenlessness, suggesting that patterns of reproductive conflicts may differ in these species and potentially be more severe than in *A. mellifera*. This is especially so in *A. cerana*, where 1-5 % of workers have activated ovaries even in queenright colonies, and 15% and 40% have activated ovaries after 4 and 6 days of queen-loss, respectively (Oldroyd *et al*., 2001). Therefore, because workers are reproductively active in queenright colonies of *A. cerana*, actual conflict among workers over which individuals should be permitted to be reproductively active is expected. This expectation will be tested in this project.

In addition to reproductive competition for the production of drone offspring among queenless workers, four studies have investigated patrilinial differences in reproductive success during emergency queen rearing Tilley and Oldroyd (1997) found that in long-established queenless colonies with repeated introductions of young brood, some patrilines were preferentially reared as queens. Additional studies have shown that subfamily proportions vary between emergency queens and workers but that the pattern is not consistent among colonies and that this may be due to weak nepotism (Châline *et al*., 2003; Osborne and Oldroyd, 1999). On the other hand, Frank *et al*. (2002) failed to find any patriline differences between worker and queen brood.

2.5 Social parasitism in honey bees

A relationship between two species in which the parasite benefits in many ways from brood care or other socially managed resources at the expense of the host society is known as "social parasitism" (Schmid-Hempel, 1998). Social parasitic tactics can occur within a species (Roubik, 1989). Social parasitism have been found in all major groups of social insects; ants, bees, termites (Wilson, 1971) and wasps (Akre *et al*., 1976).

Lopez-Vaamonde *et al*. (2004) found intraspecific social parasitism by male-producing reproductive workers in eusocial bumble bee (*Bombus terrestris*). They showed that bumble bee workers enter unrelated colonies and lay eggs that produce adult male offspring. The socially parasitic workers reproduce earlier and are more reproductive and aggressive than resident workers.

A well-known example of social parasitism in honey bees (genus *Apis*) is the Cape honey bee's (*A. mellifera capensis*) parasitism of *A. m. scutellata* (Beekman *et al*., 2000; Calis *et al*., 2002; Greeff, 1996; Moritz *et al*., 1999; Nuemann and Hepburn, 2001; Nuemann and Moritz, 2002; Oldroyd, 2002).

Workers of *A. m. capensis* can produce diploid eggs parthenogenetically in a process called thelytoky (Greeff, 1996; Nuemann and Hepburn 2001; Nuemann and Moritz, 2002). This allows rare *A. m. capensis* workers to parasitize colonies of *A. m. scutellata*. *A. m. capensis* workers enter the host colony and, because they do not react to signals from the brood or queen, activate their ovaries and lay eggs. Offspring of these parasitizing workers are also able to lay eggs, and thus the population of parasitizing workers rapidly increases. In time, the number of social parasitic workers exceeds the number of host workers, and the colony will dwindle and die. Cape honey bee workers have a unique series of traits that reflect important physiological and genetic pre-adaptations for intra specific social parasitism: high fecundity, longevity, high and fast pheromonal development and thelytoky (Nuemann and Hepburn, 2001).

Another well-known of social parasitic tactics in honey bees is so-called robbing behaviour, where workers of one colony steal the honey stores of another colony (Moritz and Southwick, 1992). Not only workers but also sexual reproductives of foreign colony can enter a host colony and benefit from its resources at the expense of the host (Nuemann and Moritz, 2002). Nuemann *et al*. (2000) reported that male sexuals (drones) of the honey bee drift among colonies, which might constitute a social parasitic tactic. Rinderer *et al*. (1985) found that Africanized drone honey bees (*A. mellifera*) migrate into European honey bee colonies and gain mating advantage for Africanized bees because it both inhibits European drone production and enhances Africanized drone production.

2.6 Worker policing in the honey bee

Worker policing is any behaviour of workers that prevents other workers from producing sons, perhaps by destroying (eating) worker-laid eggs or by aggression toward reproductive workers (Ratnieks and Visscher, 1989). Worker policing can evolve due to the relatedness asymmetries that arise from polyandry

(see Figure 2.1 above). Worker policing is not predicted in colonies of bumble bees and stingless bees in which queens mate once, because in these species workers are more related to their male offspring (r = 0.5), then male offspring of other workers (r = 0.375) and least related to male offspring of the queen (r = 0.25) (Ratnieks, 1988). Thus some worker reproduction is predicted in these species and is widespread in bumble bees (Bourke and Ratnieks, 2001).

Worker policing has now been demonstrated in three *Apis* species, *A. mellifera* (Ratnieks and Visscher, 1989), *A. florea* (Halling *et al*., 2001), and *A. cerana* (Oldroyd *et al*., 2001) and there is strong circumstantial evidence that it also occurs in *A. dorsata* (Wattanachaiyingcharoen *et al*., 2002). Worker policing is also present in some polyandrous wasps (Foster and Ratnieks, 2000) and ants (Gobin *et al*., 1999; Monnin and Ratnieks, 2001).

Ratnieks (1993) found that both worker egg-laying and worker policing occurs in queenright colonies of *A. mellifera*. To reduce the biological costs of policing, including those of search time and of errors of identification of eggs, worker policing appears to be focused on drone cells where workers are more likely to lay eggs, rather than on worker cells which are more likely to contain queen-laid eggs (Halling and Oldroyd, 2003).

Worker policing is absent or rare in the Cape honey bee (*A. m. capensis*) because of thelytokous parthenogenesis. As a result, workers are as related to worker-laid eggs as they are to queen-laid eggs (Beekman *et al*. 2002; Moritz *et al*., 1999). In anarchistic honey bee colonies in which the majority of drones are the offspring of workers rather than the queen (Oldroyd *et al*. 1994b), eggs laid by anarchistic workers have low removal rates (Oldroyd and Ratnieks, 2000). The low rate of worker policing in anarchistic colonies is presumably because eggs laid by workers were marked by mimicking queen substance (Beekman *et al*. 2004; Martin *et al*., 2004a; Ratnieks, 2000).

Halling *et al*. (2001) showed that in *A. florea*, worker-laid eggs are removed by workers approximately twice as fast as queen-laid eggs, indicating that a mechanism of worker policing in *A. florea* is oophagy of worker-laid eggs. They also found that all males produced were sons of queen, not workers. No workers with activated ovaries were found in 800 workers from 4 colonies. These results suggest that worker policing is an effective component of the mechanisms that maintain worker sterility in this species.

A. cerana police worker-laid eggs in the same way that *A. florea* and *A. mellifera* do, but are perhaps slightly more tolerant of worker-laid eggs than the other species. In queen-right colonies of *A. cerana,* where 1-5% of workers have activated ovaries, worker-laid eggs are actively policed, because no male derived from a worker was detected in a sample of 652 males (Oldroyd *et al*., 2001).

2.7 Queen-produced egg-marking pheromone

A pheromone is a chemical, secreted from the exocrine gland of an animal, that elicits a behavioural or physiological response by another animal of the same species and so acts as a chemical message. It is secreted as a liquid and transmitted as a liquid or gas (Free, 1987). Pheromonal communication is fundamental in social insects for regulating a multitude of intracolonial activities (Katzav-Gozansky *et al*., 1997). The queen honey bee produces a number of pheromones which jointly attract workers to her. The presence of queen pheromones (and those of her brood) maintains colony cohesion (Free, 1987). Pheromones from the mandibular glands of honey bee queen (queen mandibular pheromone, QMP) are probably important in eliciting retinue behaviour (Free, 1987). Queen honey bees also signal workers about their presence by secretions from their tergite glands, the median oviduct, the sting gland, the Dufour's gland and the Koschevnikov gland (Free, 1987; Katzav-Gozansky *et al*., 1997).

Because worker policing behaviour is well developed in honey bees (Oldroyd *et al*., 2001; Oldroyd and Osborne, 1999; Halling *et al*., 2001; Ratnieks, 1988; Ratnieks and Visscher, 1989), workers must have a well-developed mechanism to discriminate worker-laid and queen-laid eggs. The most likely mechanism is a queen-produced egg-marking pheromone (Oldroyd *et al*., 2002). Eggs laid by workers are assumed to lack this putative pheromone and are removed by police workers (Ratnieks and Visscher, 1989). Originally it was thought that this pheromone is produced by the Dufour's gland (Ratnieks, 1995), but recent work casts doubt on this view, and the source of the pheromone is as yet unknown (Katzav-Gozansky *et al*., 2001; 2002a, 2002b; Martin *et al*., 2002b; Sole *et al*., 2002). Queen-produced egg-marking pheromones may involve substances from other sources, as was specifically noted by Ratnieks (1995) (Oldroyd *et al*., 2002).

Traditionally the role of queen's pheromone has been interpreted as a mechanism by which the queen controls worker reproduction (e.g. Wilson, 1971). However, Seeley (1985) and Keller and Nonacs (1993) both argued that queen pheromones are much better interpreted, within a framework of kin selection, as honest signals of queen fecundity. That is, the queen benefits from her signals as she obtains a reproductive monopoly over the nest. The workers benefit from the signal because in the presence of the queen and brood they increase their inclusive fitness by refraining from reproduction (Keller and Nonacs, 1993). Signals of this kind, that benefit both sender and receiver are likely to be conserved over evolutionary time because any change to the signal is unlikely to be at a selective advantage. In contrast, if the queen's pheromones are used to chemically suppress worker ovaries, then one would expect rapid evolutionary change of the signal. This is because mutations that allow a worker to escape the queen's suppression of worker fertility would be at a strong selective advantage in workers. Likewise mutations in queens that counter the worker's fertility mutations would be highly selected in queens. This should lead to rapid evolution of the queen pheromones across the time.

If the queen-produced egg-marking pheromone is an honest signal of queen fecundity, then it should be conserved across evolutionary time and be active in all species of *Apis.* Theory suggests that a queen-produced egg-marking signal (pheromone) would be selective favoured, because both queen (sender) and police workers (receivers) of the signal would benefit (Seeley, 1985). Ratnieks (2000) suggests that the sender and receivers of the signal do not benefit equally, but the important thing is that both parties do benefit. The egg marking signal would help police workers kill workers' sons (nephew) but keep the queens' sons (brothers).

2.8 The evolution of the honey bee (*Apis*)

Nine species of *Apis* are currently recognized: *A. dorsata, A. laboriosa, A. florea, A. andreniformis, A. mellifera, A.cerana, A. nuluensis, A. koschevnikovi,* and *A. nigrocincta* (Koeniger and Koeniger, 2000; Oldroyd and Wongsiri 2006). Several lines of evidence have been used to clarify the evolutionary history of these species and infer taxonomic groupings. These include fossil evidence, morphology, behaviour, and DNA sequence data (Alexander, 1991; Engel, 1998; Garnery *et al*., 1991; Oldroyd *et al*., 1998; Ruttner, 1988).

The consensus of these studies (Alexander, 1991; Arias and Shepard, 2005; Cameron *et al*., 1992; Engel *et al*., 1997; Ganery *et al*., 1991; Lockhart *et al*., 1994; Oldroyd *et al*., 1998; Oldroyd and Wongsiri, 2006) is the phenogram shown in Figure 2.2. The group of *A. cerana-A. nigrocincta-A. nuluensis* separated after the divergence of *A. koschevnikovi* followed by *A. mellifera*, then the group of giant honey bees *A. dorsata-A. laboriosa* followed by the *A. florea-A. andreniformis* group. However the branch lengths and species divergence times of the honey bee species is decidedly unclear.

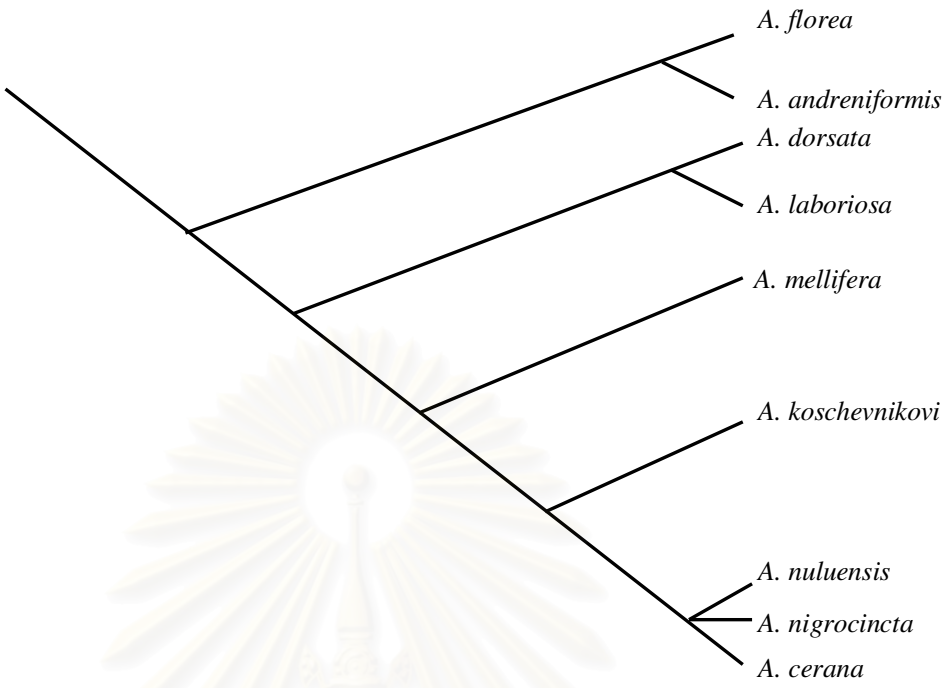


Figure 2.2 Phylogeny for the genus *Apis* (Oldroyd and Wongsiri, 2006).

2.9 The red dwarf honey bee, *Apis florea*

The red dwarf honey bee, *A. florea*, is native to south Asia (Figure 2.3) (Ruttner, 1988). Colonies usually build a single, exposed comb in the stratum of dense bushes and small trees (Figure 2.4) (Free, 1981; Ruttner, 1988; Wongsiri *et al*., 1996), but occasionally on high trees, and very rarely on buildings (Lekprayoon and Wongsiri, 1989).

A. florea is found in Pakistan, India, Sri Lanka, Thailand, Indochina, Malaysia, parts of Indonesia, Palawan (Ruttner, 1988; Wongsiri *et al*., 1996) and Iran (Tirgari, 1971). *A. florea* is also presented in Sudan, probably the result of human-assisted introduction (Lord and Nagi, 1987; Mogga and Ruttner, 1988). In southeast Asia it is usual to find a nest of *A. florea* in almost any village (Akaratanakul, 1976). Ruttner (1988) suggested that *A. florea* evolved in tropical Asia as cold-temperature climate in the Plieistocene, meant that open-nesting honey bees could not have lived in Europe. Because of their ecological

requirements, they must have disappeared from Europe and only survived in tropical south Asia, sharing the fate of all subtropical plants and animals of Europe.

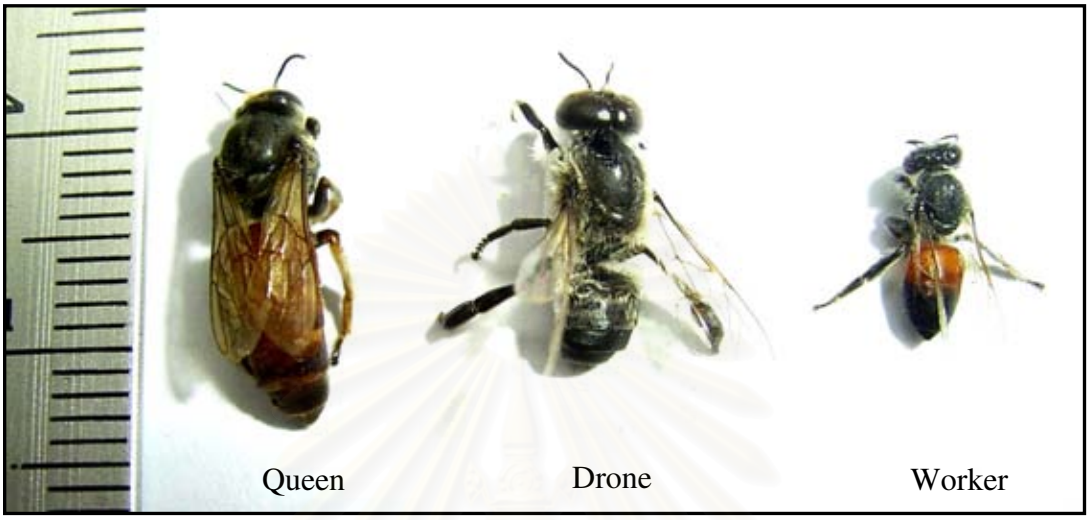


Figure 2.3 Three castes of *A. florea*; queen, drone and worker.

Figure 2.4 Nest of *A. florea*.

2.10 Eastern honey bee, *Apis cerana*

Apis cerana, a native Asian honey bee species which is closely related to the western honey bee *A. mellifera* (Figure 2.5) is found throughout Asia from eastern Indonesia, west to Iran and north to Japan (Ruttner, 1988). They construct their nest in cavities, and their nests comprise multiple parallel combs (Figure 2.6) (Koeniger and Koeniger, 2000; Otis, 1991; Ruttner, 1988).

The reproductive biology of *A. cerana* workers is different from other *Apis* species. Whereas workers with activated ovaries are extremely rare in queenright colonies of *A. florea* and *A. mellifera* (Halling *et al.* 2001; Ratnieks, 1993), queen-right workers of *A. cerana* often activate their ovaries (see above; Oldroyd *et al.*, 2001). *A. cerana* workers begin laying eggs in 2-3 days after queen loss (Blanford, 1923), much more quickly than workers of *A. mellifera* (Oldroyd *et al.*, 2001).

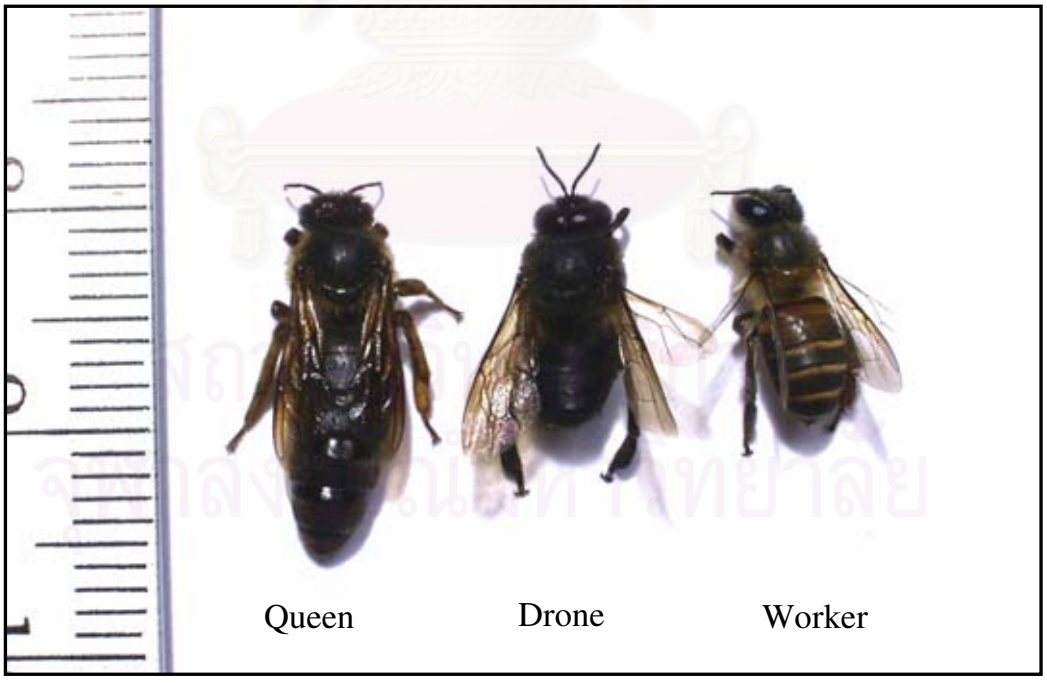


Figure 2.5 Queen, drone and worker of *A. cerana*.

A. B.

Figure 2.6 Nests of *A. cerana.*

2.11 Identifying subfamilies in honey bee colonies using microsatellites

Microsatellites are short tandem repeated sequence motifs consisting of repeat units of 2-6 bp in length. They are highly abundant in eukaryotic genomes, and also occur in prokaryotes but at lower frequencies. Microsatellite arrays are highly variable in length due to the change in the number of copies of the repeated sequence resulting from errors during replication of the DNA. Different microsatellites are defined for the purposes of assay and study by the unique sequences flanking them. Microsatellites generally occur in noncoding regions of genome. They are inherited in typically Mendelian fission, that is each diploid individual has two copies (alleles) of the microsatellite one inherited from its mother and the other from the father. As there is a high degree of variability of the sizes of microsatellite alleles, it is not uncommon for an individual to have two different alleles at a locus and, as the sizes of the alleles can be easily measured, it is possible to determine which of the alleles was inherited from the mother, which from the father (Avise, 1994; Scholotterer, 1998).

Microsatellites are a powerful genetic marker for detecting genetic variation and estimating relatedness (Crozier and Pamilo, 1996). Microsatellite

analysis has been used to answer diverse questions in honey bee biology including population-level genetic diversity (e.g Deowanish *et al.*, 1996; Estoup *et al.*, 1995; Franck *et al.*, 1998, 2000a, 2001; Rowe *et al.*, 1997; Sittipraneed *et al.*, 2001; Viard *et al.*, 1998), relatedness in aggregations of colonies (McNally and Schneider, 1996; Oldroyd *et al.*, 1995, 1997) and for determining the parentage of individual bees (e.g. Halling *et al.*, 2001;Oldroyd *et al.*, 1996, 1998, 2001; Palmer and Oldroyd, 2001; Palmer *et al.*, 2001; Wattanachaiyingcharoen *et al.*, 2002, 2003).

CHAPTER III

REPRODUCTIVE PARASITISM IN QUEENLESS *Apis florea* COLONIES

(Note: a version of this chapter appeared in Nature *437: 829)*

Abstract

The red dwarf honey bee, *Apis florea*, constructs a single exposed comb (Wongsiri *et al.*, 1996). In queenless colonies, workers activate their ovaries and lay eggs that are reared. Egg rearing necessitates cessation of worker policing rendering them vulnerable to parasitism by the eggs of workers from other colonies. The result was shown that queenless *A. florea* colonies are parasitized by non-natal workers. In queenright nests about 2% of workers are non-natal. This proportion rises to 4.5% after dequeening. Furthermore, 35.6% of eggs and 22.5% of pupae derived from non-natal workers. This suggested that an important reproductive tactic *A. florea* workers is to seek out and parasitize queenless nests with their eggs.

Introduction

The red dwarf honey bee (*Apis florea*) is a small honey bee species that nests in the open by building a single comb attached to a twig (Wongsiri *et al.*, 1996). Few if any workers have activated ovaries and the queen's reproductive monopoly is enforced by worker policing (Halling *et al.*, 2001; Ratnieks and Visscher, 1989). Functional worker sterility and policing in colonies with a queen is thought to maximize worker's inclusive fitness due to reduction of conflict among individual workers over which should be reproductively active (Ratnieks, 1988). However in queenless colonies, workers can only maximize their reproductive success by personal reproduction, necessitating the breakdown of worker policing (Miller and Ratnieks, 2001).

In this study I determined the maternity of males produced in queenless colonies of *A. florea*. Most unexpectedly, I show that a significant proportion of the males produced by queenless colonies are sons of parasitic non-natal workers.

Materials and methods

Experimental colonies and sample collection

Four wild *A. florea* nests with drone comb were collected (one in 2003 and three in 2004), and transported them to the grounds of Chulalongkorn University in Bangkok where they were tied in convenient locations on low tree branches at least 5 m from any other nest. The grounds host many wild colonies. After taking a sample of workers from each colony (n = 100), I removed the queens from the translocated colonies and removed any queen cells that subsequently developed. Adult workers were sampled after one week, and again after four weeks. As worker-produced eggs, larvae and pupae appeared in the drone combs, I collected samples, but this was not possible for colonies 3 and 4,

as these colonies absconded before larvae were reared. All samples were kept at -20°C.

To obtain eggs laid by workers, drone combs of colonies 1 and 2 were cut away and taken to the laboratory. After taking egg samples, I furnished each comb with a loop of light wire so that I could hang the drone combs back in their natural position (underneath the colony's comb, Figure 3.1) to let workers rear their brood. I used the experimental colonies with drone comb because worker bees prefer drone cells to lay their eggs (Halling and Oldroyd 2003). I decided to use their own drone comb rather than provide combs from other colonies because odors associated with comb are important in honey bee nestmate recognition systems (Breed *et al*., 1998).

Adult workers of *A. florea* were dissected according to Dade (1977) to determine the level of ovary activation. Ovaries were classified as ovarioles not discernable (inactive), ovarioles visible (inactive), small eggs present (<50% of full size, activated) and eggs >50% full size (activated) (Oldroyd *et al.*, 2001). I then used microsatellite loci to determine the parentage of the dissected workers and of the worker-produced males (Halling *et al*., 2001; Palmer and Oldroyd, 2001).

A.

B.

Figure 3.1 A normal queenright *A. florea* (A.) and a queenless *A. florea* colony with an experimental drone comb attached (B.).

DNA Extraction

Egg samples

An egg was ground in a 0.6 ml microcentrifuge tube using a pipette tip with 5 µl of distilled water, 50 µl of boiling Chelex® 100 (5% w/v in $TE_{0.1}$, see Walsh *et al*., 1991) was then added and mixed with the egg fragments by vortexing for 3 sec. Samples were boiled for 15 min and centrifuged at 1,200 rpm for 15 min. The supernatant was then transferred to a new microcentrifuge tube and stored in 4°C.

Worker, larval and pupal samples

DNA extraction from hind leg of adult workers, from whole larvae and from match-head sized portion of pupae. Worker legs, larval and pupal tissue were chopped in a 1.5 ml microcentrifuge tube and 500 µl of boiling Chelex® 100 (5% w/v in $TE_{0.1}$) solution was then added. Samples were boiled for 15 min., centrifuged at 1,200 rpm for 15 min. Supernatants were transferred to a new microcentrifuge tube and diluted 1:2 with distilled water. The same process was performed on whole larvae using 200 µl Chelex. Extracts were diluted 1:1 with distilled water.

Microsatellite analysis

Six microsatellite loci A8, A76, A88, A107, B124 and Ap249 (Table 3.1) were used to determine the subfamily of workers and the origin of worker-produced males. Diluted DNA (1 µl) was used in 5 µl PCR reactions (1X PCR buffer, 1.5 mM $MgCl_2$, 0.625 mM of each dNTP, 0.4 mM of forward and reverse primer (reverse primer Hex labeled) and 0.25 units of *Taq* polymerase (BioLabs);see also Table 3.1). PCR products were electrophoresed on 5% urea/polyacrylamide on an automated DNA fregment analyzer (Corbett Research, Sydney, Australia) at 1,400 V and 38º C. Lengths of microsatellite alleles were determined in base pairs using the software package OneDscan (Scananalytics, Fairfax, Vt., USA).

Once the worker bees had been scored, maternal alleles were identified: either the same allele was present in all workers (when the queen is homozygous at the locus), or two alleles were each present in half of them indicating that the queen was heterozygous for these two alleles (Estoup *et al*., 1994). The paternity of a worker and the number of subfamilies present was then determined. For each colony, the queen's genotype was inferred from the genotypes of 300 workers. To determine the origin of a worker-laid male I assessed whether a male carried

an allele that could have been produced by a worker of any patriline of the colony at any locus. Males that did not carry a 'natal allele' were classified as offspring of non-natal workers.

Table 3.1 Primer sequences and PCR conditions for six microsatellite loci used to detect paternity in *A. florea*.

Locus	Primer sequences	$MgCl_2$ conc. (mM)	Annealing temp. (°C)	Number of cycle	References
A8	5'CGAAGGTAAGGTAAATGGAAC	1.5	55	35	Estoup *et al.*,
	5'GGCGGTTAAAGTTCTGG				1994
A76	5'GCCAATACTCTCGAACAATCG	1.5	55	35	Estoup *et al.*,
	5'GTCCAATTCACATGTCGACATC				1994
A88	5'CGAATTAACCGATTTGTCG	1.5	55	35	Estoup *et al.*,
	5'GATCGCAATTATTGAAGGAG				1994
A107	5'CCGTGGGAGGTTTATTGTCG	1.5	55	35	Estoup *et al.*,
	5'GGTTCGTAACGGATGACACC				1994
B124	5'GCAACAGGTCGGGTTAGAG	1.5	55	35	Estoup *et al.*,
	5'CAGGATAGGGTAGGTAAGCAG				1994
Ap249	5'CGCGCGACGACGAAATGT	1.5	57	9	Solignac *et*
	5'CAGTCCTTTGATTCGCGCTACC		55	9	*al.*, 2003
			52	9	
			49	15	

The number of alleles and average heterozygosities among workers (H) were high (Table 3.2).

Table 3.2 Number of alleles and average heterozygosities among 600 *A. florea* workers.

	Locus					
	A8	A76	A88	A107	B124	Ap249
Number of alleles	3	3	2	3	3	8
H	0.547	0.717	0.302	0.686	0.445	0.796

Statistical analysis

To compare the proportions across two classes (e.g. the proportion of non-natal and natal workers with and without active ovaries), 2x2 contingency tables were constructed followed by *G* tests with one degree of freedom. All tests were pooled across the available colonies.

Results

Before queen removal, the number of non-natal workers was low (averaging 2.0 %, Table 3.3) and none of these workers had activated ovaries. After dequeening, the proportion of non-natal workers rose significantly ($P = 0.008$) to 4.5%. Furthermore, the results suggesting that parasitic workers actively seek out queenless colonies because significantly ($P < 0.001$) more non-natal workers (42.6%) had activated ovaries than natal ones (17.7%). Moreover, non-natal workers had significantly ($P < 0.001$) higher reproductive success than natal workers: 3.2% of workers in colonies 1 and 2 were non-natal, but these laid 35.6% of the eggs and 22.5% of the pupae.

Table 3.3 Reproductive parasitism of queenless dwarf bee colonies

	Colony				
Before dequeening	1	2	3	4	Average
Number of non-natal workers	1	5	0	2	
Number of natal workers	99	95	96	94	
% Non-natal workers	1	5	0	2.08	2.0
After dequeening					
Number of non-natal workers	0	13	8	14	
Number of natal workers	200	187	184	178	
% Non-natal workers	0	6.5	4.2	7.3	4.5
% Non-natals with activated ovaries	-	15.4	62.5	50.0	42.6
% Natals with activated ovaries	28.0	27.8	4.9	10.1	17.7
% Offspring derived from non-natal workers (n)					
Eggs	44.4 (115)	26.7 (120)	-	-	35.6
Larvae	38.5 (143)	25.6 (125)	-	-	32.6
Pupae	30.0 (100)	14.9 (94)	-	-	22.5

Error rate for misclassification of sons of non-natal workers

A son of a non-natal worker can be erroneously classified as the son of a natal worker if he fortuitously carries a 'natal allele' at all loci. This probability is $\prod_i p_i$, where p_i is the frequency of the male's allele at the i^{th} locus. Because I did not have population-wide allele frequencies, the array of alleles in the workers sampled were used to obtain an approximation. Based on this, the average and approximate probability of a non-natal-derived male having the same genotype as a genotype-matched resident-derived male is 0.031, ± S.E. 0.003, n = 222 males for colony 1 and 0.029 ± 0.003, n = 261 for colony 2.

Error rate for misclassification of sons of natal workers

A son of a natal worker can be erroneously classified as the son of a non-natal if his mother's patriline was not sampled among the workers. The more rare a patriline is, the greater the possibility that it will not be sampled. The probability of not sampling a patriline of proportion k is $(1-k)^n$, where n is the number of workers sampled (Foster *et al*., 1999). 288-300 workers were sampled per colony. This sample size means that the probability of non-detection of a rare worker patriline of proportion 1% due to not sampling it, is low ($P = 0.05$).

The possibility of non-detection of a subfamily because two fathering males shared the same genotype is not relevant to the detection of the sons of non-natal males.

Discussion

The study has shown that an important reproductive tactic of *A. florea* workers is to actively seek out and parasitize queenless nests with their eggs due to worker policing being switched off in queenless colonies (Miller and Ratnieks, 2001). This parasitic behaviour is apparently absent from *A. mellifera* where offspring of non-natal workers are rare or absent in queenless nests (Martin *et al*., 2004). This study probably explains the common phenomenon of queenless dwarf bee colonies absconding their nest. During my study, 41 colonies of *A. florea* were used for the experiment. Most of them absconded in a week or so, 4 colonies of them stayed for 4 weeks after dequeening, only 2 of them reared male offspring. The absconding of queenless colonies also occurs in the sibling species the black dwarf honey bee, *A. andreniformis*. Colonies of *A. andreniformis* abscond more often and faster than *A. florea* (personal observations). Kin selection theory would predict that the queenless colonies should stay to put and rear their own males. But it seems likely that such workers seek to join other nests, and because they are unrelated to them, parasitize them with their eggs.

Their ability to do this may be facilitated because, in contrast to *A. mellifera*, *A. florea* nests in the open habitats. This nesting habit makes the species more vulnerable to parasitism than cavity nesting species. It is also possible that workers from queenright *A. florea* colonies may parasitize queenless nests as found in *A. m. capensis* (Neumann and Hepburn, 2001), preferring the chance of personal reproduction in a queenless nest to contributing to the reproductive output of their own colony.

CHAPTER IV

UNEQUAL RATES OF OVARY ACTIVATION AMONG WORKER SUBFAMILIES IN QUEENLESS *Apis florea* COLONIES

Abstract

In honey bees (*Apis*) workers cannot mate, but retain functional ovaries. When colonies have lost their queen, workers begin to activate their ovaries and lay eggs which eventually result in a final batch of males before the colony finally perishes. Because the honey bee queen mates with numerous drones, her colony comprises multiple subfamilies. This genetic diversity leads to the possibility of reproductive conflicts among subfamilies over which workers should lay eggs to produce the males in queenless colonies.

Over a two year period I collected 41 wild dwarf honey bee (*Apis florea*) colonies. Queens of these colonies were removed. I collected approximately 100 adult workers before queen removal. Thirty nine colonies absconded within a week or so but two remained *in situ.* 100 workers were sampled after 4 days, 1 week, 2 weeks, 3 weeks and 4 weeks of queenlessness. The samples were dissected to determine rate of ovary activation. Microsatellite loci were then used to determine subfamily of dissected workers. As with *A. mellifera,* I found reproductive competition among queenless workers of *A. florea*. In both colonies, some subfamilies have high proportion of workers with activated ovaries.

Introduction

Honey bee colonies have been characterized as “superorganisms” in which reproduction is channeled exclusively through the queen, and selection on workers is restricted to maximizing their efficiency as parts of coordinated whole (Seeley, 1989). In queen-right honey bee colonies, ovaries of workers are normally inactive (Barron *et al*., 2001; Miller and Ratnieks, 2001; Ratnieks, 1995). However, if a colony becomes hopelessly queenless, workers that have not yet begun foraging begin to activate their ovaries and lay eggs producing a final batch of males before the colony perishes. This behaviour is an adaptive response to queenlessness, for without parthenogenetic reproduction by workers, the reproductive fitness of their colony is zero.

Kin selection theory (Hamilton, 1964) predicts reproductive competition among queenless laying workers, because differences in relatedness among subfamilies results in differences in inclusive fitness associated with raising males of different origins (Oldroyd and Wongsiri, 2006; Robinson *et al*., 1990). Thus, competition is predicted among subfamilies over the relative share of the drones that the queenless colony is able to rear. As predicted, strongly unequal reproductive success has been observed in queenless colonies of *A. mellifera* (Martin *et al*., 2004; Robinson *et al*., 1990). Differential reproductive success probably arises from workers preferentially rearing or cannibalizing eggs to which they are differentially related and different rates of ovary activation among workers (Martin *et al*., 2004, Robinson *et al*., 1990).

Although workers with activated ovaries are very rare in queen-right *A. florea* colonies (Halling *et al*., 2001), workers activate their ovaries after only in a few days of queenlessness (Nanork *et al*., 2005, see chapter 3). A colony of this species comprises 13-19 subfamilies (Palmer and Oldroyd, 2001), and so reproductive competition among workers from different subfamilies is predicted as in *A. mellifera* (Martin *et al*., 2004). Furthermore, Nanork *et al*. (2005, see

Chapter 3) demonstrated that queenless *A. florea* colonies are parasitized by eggs laid by workers from other nests. Egg production in queenless nests is extraordinarily prolific, with many tens of eggs laid per cell (Akratanakul, 1977; Oldroyd and Wongsiri, 2006; Ruttner, 1988). Thus the patterns of reproductive competition are expected to be different in *A. florea* from *A. mellifera.*

Here I examine patterns of reproductive competition among subfamilies within colonies of *A. florea*. This chapter extends the analysis presented in Chapter 3 which described the reproductive parasitism in queenless *A. florea.*

Materials and methods

Experimental colonies and sample collection

Forty one colonies of wild *A. florea* were obtained from Samutsongkram Province, Thailand and were moved to the grounds of Chulalongkorn University, Bangkok, Thailand. The experiments were conducted during December 2003 - October 2004. The experimental colonies were tied in convenient location on branches of small trees, at least 5 m apart from each others. For each relocated nest I collected approximately 100 adult workers and then removed the queen to induce ovary activation in worker bees. Any queen cells that subsequently developed were removed. One hundred workers were collected again after 4 days, 1 week, 2 weeks, 3 weeks and 4 weeks of queenlessness. Samples were kept at –20º C.

Adult workers were dissected according to Dade (1977) to determine level of ovary activation. Ovaries were classified into 4 categories as in Oldroyd *et al*., (2001, see also Chapter 3). Microsatellite loci were used to determine the parentage of the dissected workers (Nanork *et al*., 2005, Chapter 3).

DNA extraction and microsatellite analysis

A hind leg of a worker bee was ground in a 1.5 ml microcentrifuge tube. DNA was extracted using 500 µl of boiling Chelex® 100 solution (5% w/v in $TE_{0.1}$). Samples were boiled for 15 min, centrifuge at 1,200 rpm for 15 min, then diluted the supernatants 1:2 with sterile distilled water and stored in 4º C.

Six microsatellite primers (A8, A76, A88, A107, B124 and Ap249, Table 3.1 in Chapter 3) were used to determine the genotypes of *A. florea* workers (Halling *et al*., 2001; Palmer and Oldroyd, 2001). PCR conditions and PCR products electrophoresis are as in Chapter 3.

The queen's genotype of each colony was inferred from the genotypes of 282-299 workers. The coefficient of relatedness in each colony, *g*, among female offspring of a queen as,

$$g = 0.25 + 0.5\sum_{i=1}^{k} p_i^{\,2} \quad , \qquad (1)$$

(Boomsma and Ratnieks, 1996), where

$\sum_{i=1}^{k} p_i^{\,2}$ is calculated as

$$(N\sum_{i=1}^{k} y_i^{\,2} - 1)/N - 1,$$

k is the number of subfamilies observed, y_i is the observed proportion of the i^{th} subfamily, and *N* is the number of workers scored. The effective mating frequency (Boomsma and Ratnieks, 1996; Crozier and Pamilo, 1996), *m*, was computed from

$$m = 1/\sum_{i=1}^{k} p_i^{\,2} \, , \qquad (2)$$

(Starr, 1984).

Statistical analysis

A contingency table for each colony was constructed that compare subfamilial representation among dissected adult workers using *G* test (Zar, 1996).

Where a large number of cells in the contingency table have expected value <5, the *G* test can produce levels of significance that deviate from the actual. Therefore a sampling modification of Fisher's exact test (Lewontin and Felsenstein, 1965) was also performed using the program Monte Carlo RxC (W. Engels, University of Wisconsin).

Results

Over a two-year period of study, 41 colonies of *A. florea* were relocated. Thirty nine colonies were absconded within a week or so after queen removal, only two colonies remained long enough for me to obtain samples of eggs larvae and pupae, and adult workers over a protracted period. Dissection of workers to investigate rate of ovary activation in both colonies showed that some workers activated their ovaries within 4 days of queen removal. The proportion of workers with activated ovaries increased across time (Figure 4.1).

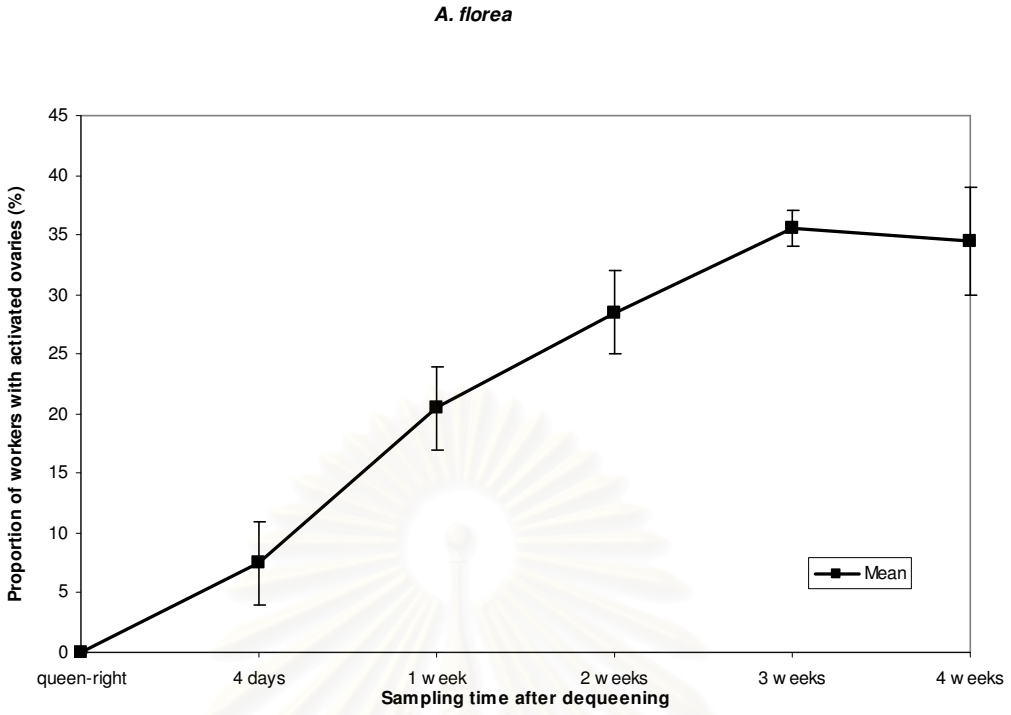


Figure 4.1 Average proportion (%) of *A. florea* workers with activated ovaries in 2 colonies across 4 weeks of queen removal (including non-natal workers). The *bars* indicate SEs of the mean.

Microsatellite analysis based on 299 and 282 workers from colonies 1 and 2 respectively indicated that there were 20 subfamilies in colony 1 and 24 subfamilies in colony 2 (Table 4.1).

Table 4.1 Observed and effective mating frequency and coefficient of relatedness for 2 colonies of *A. florea*.

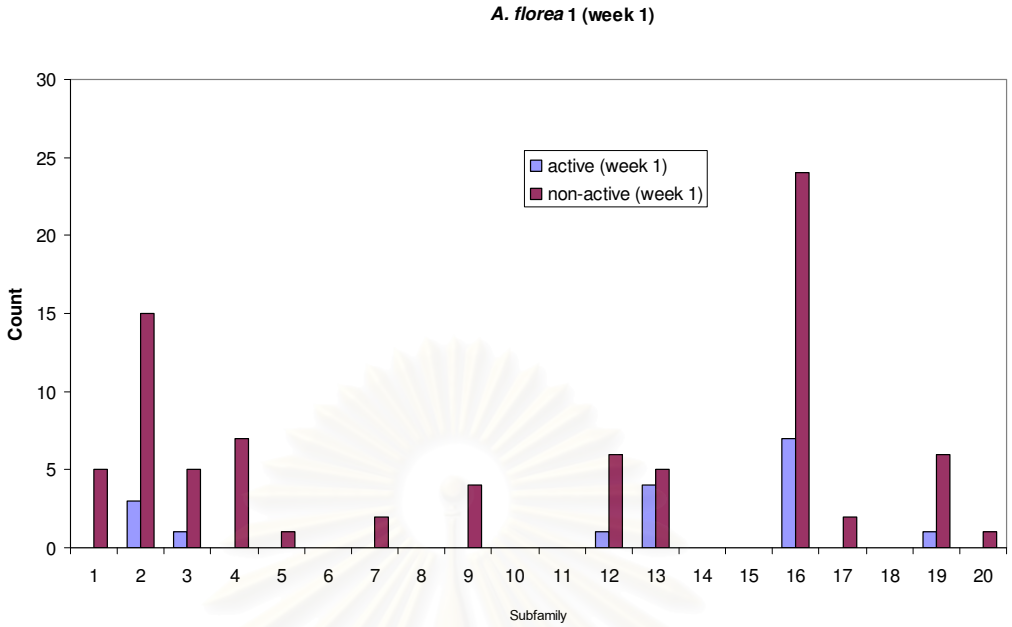
colony	Observed mating frequency	Effective mataing frequency	Coefficient of relatedness
1	20	9.44	0.3
2	24	15.54	0.28
Mean (± S.E.)	22 ± 2.0	12.5 ± 3.05	0.29 ± 0.01

In both colonies, one week after dequeening there was no significant difference in the proportion of workers with activated ovaries among different subfamilies (Table 4.2, Figures 4.2, 4.3). However, after 4 weeks some subfamilies had a much higher proportion of workers with activated ovaries than others (Table 4.2, Figures 4.2, 4.3). This indicates that, like *A. mellifera*, some subfamilies are more likely to activate their ovaries than others in queenless colonies.

Table 4.2 Contingency table analyses of the proportions of adult workers with activated ovaries among different subfamilies in *A. florea* colonies. P_{Fisher} is the *P* value from a Monte Carlo approximation of the Fisher's exact test and *P* is the probability associated with *G* test.

Colony	Sampling time	*G*	*df*	*P*	P_{Fisher}
1	Week 1	12.548	12	0.4	0.778
	Week 4	33.92	13	0.001	0.002
	Totals	32.258	15	0.006	0.012
	Heterogeneity	14.254	10	0.161	
2	Week 1	20.446	18	0.308	0.491
	Week 4	37.694	19	0.006	0.015
	Totals	58.14	21	0.112	0.163
	Heterogeneity	20.074	16	0.023	

A.



B.

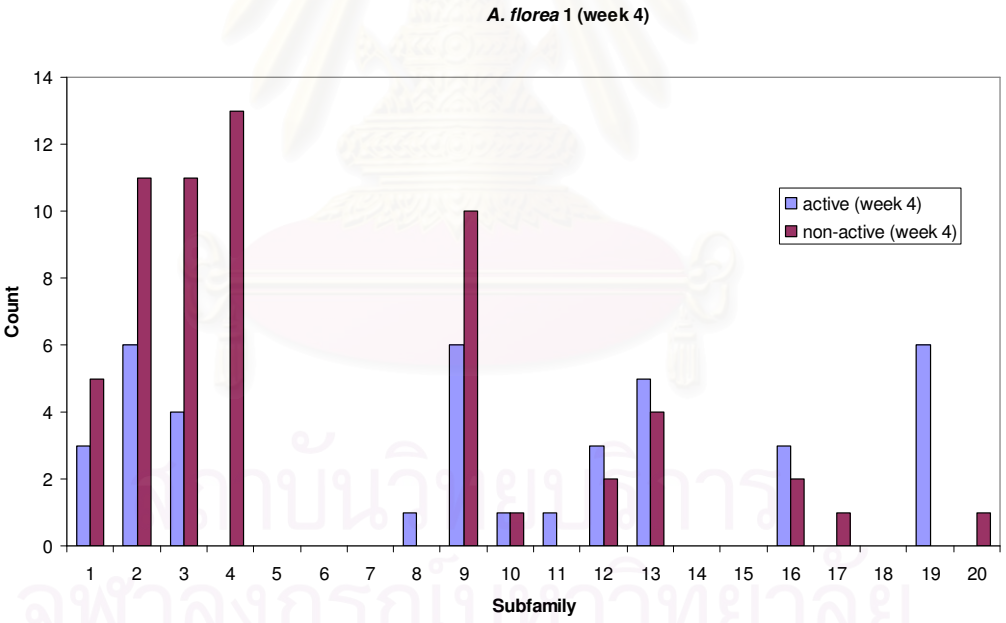
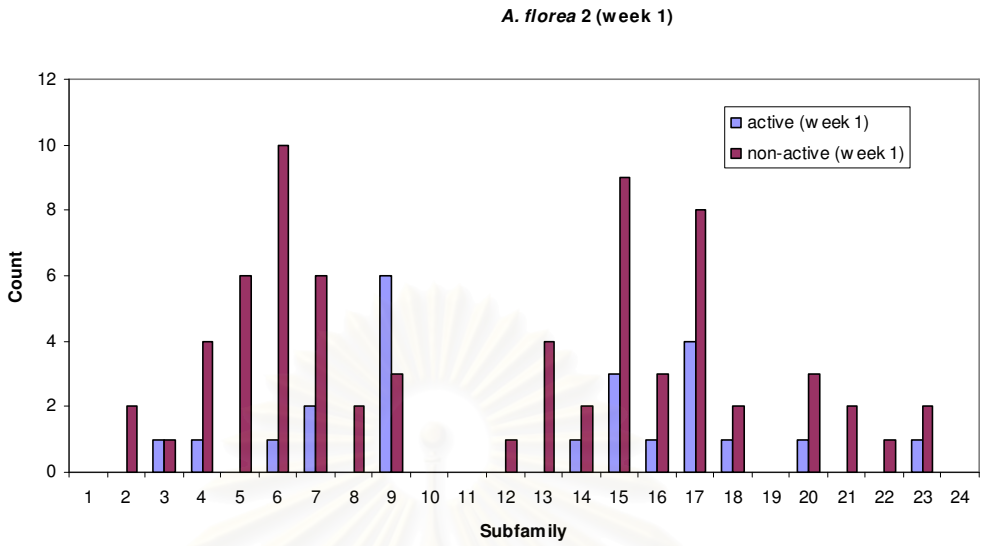


Figure 4.2 Proportion of workers of *A. florea* (colony 1) with and without activated ovaries among different subfamilies after 1 week (A) 4 weeks (B) of dequeening.

A.



B.

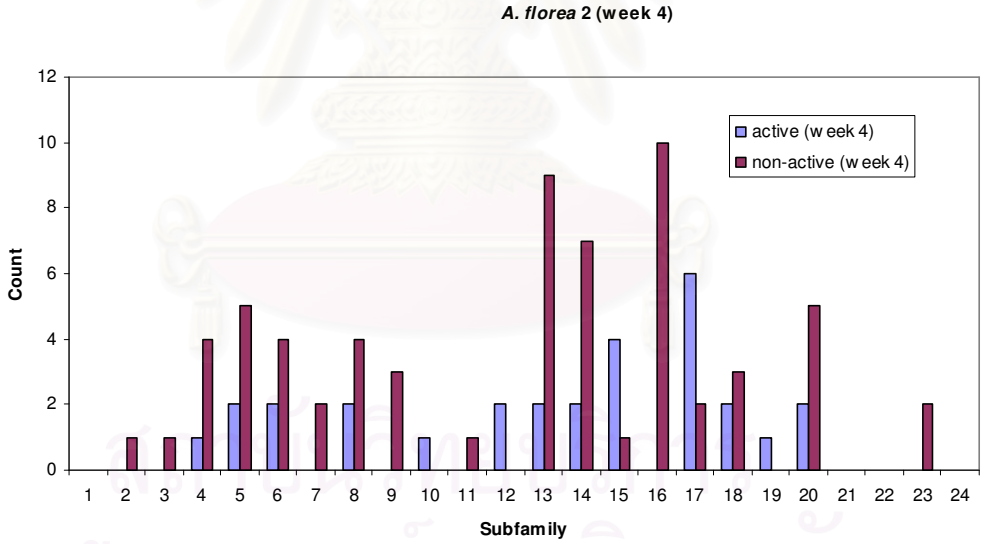


Figure 4.3 Proportion of workers of *A. florea* (colony 2) with and without activated ovaries among different subfamilies after 1 week (A) 4 weeks (B) of dequeening.

Discussion

These results provide evidence for the predicted reproductive competition among subfamilies in queenless *A. florea* colonies. The results show that, as with queenless *A. mellifera* colonies (Martin *et al*., 2004), reproductive competition occurs among queenless workers in *A. florea*. Workers of some subfamilies have significantly higher proportion of individuals with activated ovaries than others. It seems likely that certain subfamilies have the ability to respond to the lack of queen and brood pheromones earlier than others. In colony 2, the dominant subfamily changed over time significantly (P = 0.023) while the dominant subfamily in colony 1 did not change over time (P = 0.161). The proportion of workers with activated ovaries in both *A. florea* colonies increased across 4 weeks of queenlessness (Figure 4.1), suggesting that it may be a useful tactic by *A. florea* workers to delay ovary activation until egg oophagy rates have declined, when they may be able to maximize their reproductive success.

When *A. florea* colonies become queenless, the colonies are easy to parasitize because worker policing breaks down (Miller and Ratnieks, 2001). I found reproductive parasitism in the colonies (Chapter 3). Male eggs of workers from other colonies were found. This suggested that the natal workers should have tactic to compete with non-natal workers to maximize their reproductive success. Reproductive competition among queenless workers and, among natal and non-natal workers could also occur in the egg stage. Workers may be able to discriminate between eggs laid by their half-sister and super-sisters and only destroy the former (Martin *et al*., 2004). Competition may also be expressed in larval rearing by workers to which they are differentially related (Robinson *et al*., 1990).

The number of workers per colony (282-299 bees) used to determine mating frequency in this study was higher than the earlier ones (92-159 bees,

Palmer and Oldroyd, 2001). However, the levels of polyandry found in these two colonies are similar to those reported by Palmer and Oldroyd (2001) (Table 4.1).

CHAPTER V

REPRODUCTIVE CONFLICT IN QUEENLESS *Apis cerana* COLONIES

Abstract

Apis cerana is unusual among honey bee species in that 1-5% of workers in queenright colonies have activated ovaries. I investigated the rate at which workers activate their ovaries after dequeening in three colonies. Four days after dequeening, 39.33% of workers had activated ovaries and 47.66% did so after 7 days. The very high proportion of workers with activated ovaries apparently coincides with the very high rate of worker policing that appears within 3 days of queenlessness. Unlike *A. florea* and *A. mellifera*, workers from different subfamilies show approximately uniform rates of ovary activation. Finally, I demonstrate that reproductive parasitism occurs in queenless *A. cerana* colonies.

Introduction

In colonies of the western honey bee, *Apis mellifera* (Jay, 1970; Ratnieks, 1993), the red dwarf honey bee, *A. florea* (Halling *et al*., 2001) and the giant honey bee, *A. dorsata* (Wattanachaiyingcharoen *et al*., 2002) with a queen, few if any workers have activated ovaries. However, if a colony loses its queen and is unable to rear a new one, the workers of these species eventually begin to lay eggs, curtail worker policing, and produce a last batch of reproductive males before the colony finally perishes due to a lack of replacement workers (Barron *et al*., 2001; Miller and Ratnieks, 2001; Ranieks, 1995; Visscher, 1989, 1996). Because reproduction by queenless workers represents an opportunity for them to increase their personal fitness at a time when the reproductive success of the group is severely compromised, adaptations that enhance a workers' reproductive success when queenless are expected (Oldroyd *et al*., 2001), and this probably explains unequal rates of ovary activation and reproductive success among worker subfamilies in queenless *A. mellifera* (Martin *et al*., 2004; Page and Erickson, 1988; Page and Robinson, 1994; Robinson *et al*., 1990) and *A. florea* (Nanork *et al*., 2005; see Chapter 4).

In stark contrast to all other investigated honey bee species, *A. cerana* workers are often reproductively active in queen-right colonies (Bai and Reddy, 1975; Blanford, 1923; Oldroyd *et al*., 2001), with 1-5% of workers having activated ovaries. The presence of full-sized eggs in the ovarioles of queen-right workers implies that some workers lay eggs in queen-right colonies. Nevertheless, no worker's sons were detected in a sample of 652 pupal males sampled from 4 queenright colonies indicating that policing is efficient in queenright colonies despite high rates of worker oviposition (Oldroyd *et al*., 2001).

A. cerana workers activate their ovaries extremely quickly after queen loss, such that 15% of randomly sampled workers have activated ovaries 4 days

after dequeening, and after 6 days, 40% (Oldroyd *et al*., 2001). Queenright *A cerana* police worker-laid eggs in the same way that *A. florea* and *A. mellifera* do, but are perhaps slightly more tolerant of worker-laid eggs than the other species. Sakagami and Akahira (1958) suggested that *A. cerana* workers often do not rear replacement queens following queen loss, and rapidly develop their ovaries instead. But Wongsiri and Deowanish (1995) found that emergency queen cells of *A. cerana* present after a few days of queen-lost.

In all honey bee species investigated genetically, colonies contain a small number of non-natal workers. In *A. dorsata* about 1.2% of workers are non-natal (Paar *et al*., 2002), in *A. florea* about 2% (Nanork *et al*., 2005) and usually non-natal was not found in *A. mellifera* colonies (Martin *et al.,* 2004), except in *A. m. capensis* (up to 5% of non-natals were found) (Neumann *et al*., 2000, 2001). Non-natal workers have the potential to adversely affect their host colony either by stealing honey (Downs and Ratnieks, 1999, 2000) spreading diseases or parasites (Cook, 1987; Fries and Camazine, 2001) or parasitizing the nest with their eggs (Neumann and Hepburn, 2001; Nanork *et al*., 2005). *A. cerana* generally nest in a cavity as do *A. mellifera*. In addition to protection from the weather, the cavity with its heavily guarded entrance hole is the first line of defence against predators and conspecific parasites. In contrast, *A. florea* nests in the open and Nanork *et al*. (2005) suggested that it is this nesting habit makes the species more vulnerable to parasitism than cavity nesting species.

Here I investigate reproductive conflicts in queenless *A. cerana.* I predict that because the species is cavity nesting, and because of the extremely high policing rates in queenless colonies (Oldroyd *et al*., 2001), reproductive parasitism should be absent in *A. cerana* as it apparently is in arrhenotokous subspecies of *A. mellifera* (Martin *et al*., 2004). Reproductive parasitism occurs in the thelytokous subspecies *A. m. capensis* (Neumann and Hepburn., 2001). I also investigate whether there is intra-colony conflict, with workers of some subfamilies achieving higher rates of ovary activation than others as occurs in

A. mellifera (Martin *et al*., 2004, Page and Robinson, 1994) and *A. florea* (see Chapter 4). Finally, I provide new and more accurate estimates of the level of polyandry in *A. cerana.*

Materials and methods

Experimental colonies and sample collection

Three colonies of *A. cerana* were transferred from Chumporn Province to Chulalongkorn University, Bangkok in May 2004. To minimize passive drifting of workers among colonies, the experimental colonies were placed at least 2 m apart from other colonies, and the entrances of the colonies were orientated in different directions with respect to the other colonies (Pfeiffer and Crailsheim, 1998). Approximately 100 adult workers were collected before removal of the queen. The queen cells that subsequently developed were also removed. After 4 days, and at 1, 2, 3, and 4 weeks after queen removal, I sampled 100 workers from each colony. I also collected worker-produced eggs, larvae and pupae that appeared in drone combs, storing them at -20° C for later analysis.

Adult workers of *A. cerana* were dissected according to Dade (1977) to determine their level of ovary activation. Ovary activation was classified into 4 categories as in Oldroyd *et al*. (2001; see Chapter 3). Microsatellite loci were used to determine the parentage of the dissected workers (Oldroyd *et al*., 1998) and that of the eggs and pupae.

DNA extraction

Egg samples

An egg was ground in a 0.6 ml microcentrifuge tube using a pippett tip with 5 µl of distilled water. 50 µl of boiling Chelex® 100 solution (5% w/v in $TE_{0.1}$) was added and mixed with the egg fragments by vortexing for 3 sec.

Samples were then boiled for 15 min and centrifuged at 1,200 rpm for 15 min. The supernatant was transferred to a new microcentrifuge tube and store in 4°C.

Worker and pupa samples

DNA was extracted from the hind legs of adult bees and from match-head sized portion of pupae using 500 µl of boiling Chelex® 100 solution (5% w/v in $TE_{0.1}$). Samples were boiled for 15 min, centrifuge at 1,200 rpm for 15 min, then diluted the supernatants 1:2 with sterile distilled water and stored in 4º C.

Microsatellite analysis

Five microsatellite primers (A14, A76, A107, B124 and Ap43, Table 5.1) were used to determine the genotypes of *A. cerana* workers, eggs and pupae (Oldroyd *et al*., 1998). PCR conditions are given in Table 5.1 (see also Chapter 3). PCR products were electrophoresed on 5% urea/polyacrylamide on an automated DNA fragment analyzer (Corbett Research, Sydney, Australia) at 1,400 V and 38° C. The length of microsatellite alleles was determined using the software package OneDscan (Scanalytics, Fairfax, Vt., USA).

The queen's genotype of each colony was inferred from the genotypes of 289-293 workers. The coefficient of relatedness in each colony, *g*, among female offspring of a queen as,

$$g = 0.25 + 0.5\sum_{i=1}^{k} p_i^{\,2} \quad , \qquad (1)$$

(Boomsma and Ratnieks, 1996), where

$\sum_{i=1}^{k} p_i^{\,2}$ is calculated as

$$(N\sum_{i=1}^{k} y_i^{\,2} - 1) / N - 1,$$

k is the number of subfamilies observed, y_i is the observed proportion of the i^{th} subfamily, and N is the number of workers scored. The effective mating frequency (Boomsma and Ratnieks, 1996; Crozier and Pamilo, 1996), m, was computed from

$$m = 1/\sum_{i=1}^{k} p_i^{\,2}, \qquad (2)$$

(Starr, 1984).

To determine the origin of a worker-laid male I assessed whether a male carried an allele that could have been produced by a worker of any patriline of the colony at any locus. Males that did not carry a 'natal allele' were classified as offspring of non-natal workers.

Table 5.1 Primer sequences and PCR conditions for five microsatellite loci used to detect paternity in *A. cerana*.

Locus	Primer sequences	$MgCl_2$ conc. (mM)	Annealing temp. (°C)	Number of cycles	References
A14	5'GTGTCGGAATCGACGTAACC 5'GTCGATTACCGATCGTGACG	1.5	55	35	Estoup *et al.,* 1994
A76	5'GCCAATACTCTCGAACAATCG 5'GTCCAATTCACATGTCGACATC	1.5	55	35	Estoup *et al.,* 1994
A107	5'CCGTGGGAGGTTTATTGTCG 5'GGTTCGTAACGGATGACACC	1.5	55	35	Estoup *et al.,* 1994
B124	5'GCAACAGGTCGGGTTAGAG 5'CAGGATAGGGTAGGTAAGCAG	1.5	55	35	Estoup *et al.,* 1994
Ap43	5'GGCGTGCACAGCTTATTCC 5'CGAAGGTGGTTTCAGGCC	1.5	60	35	Solignac *et al.*, 2003

Statistical analysis

A contingency table was constructed for each colony that compared subfamilial representation among dissected adult workers. Where workers had a haplotype that could potentially belong to two or more subfamilies, those subfamilies were pooled for all analyses.

Where a large number of cells in a contingency table have expected values <5, the *G* test can produce levels of significance that deviate from the actual. Therefore a sampling modification of Fisher's exact test (Lewontin and Felsenstein, 1965) was also performed using the program Monte Carlo RxC (W. Engels, University of Wisconsin).

To compare the proportions across two classes (e.g. the proportion of non-natal and natal workers with and without active ovaries), 2x2 contingency tables were constructed followed by *G* tests with one degree of freedom. All tests were pooled across the available colonies.

Results

Few workers had activated ovaries before queen removal in colonies 1 and 2, but no worker with activated ovaries was found in colony 3 (Figure 5.1). The proportion of workers with activated ovaries increased significantly ($P<0.001$) in all colonies within 4 days of queenlessness (Fig 5.1).

The number of alleles and average heterozygosities among workers (H) was high (Table 5.2)

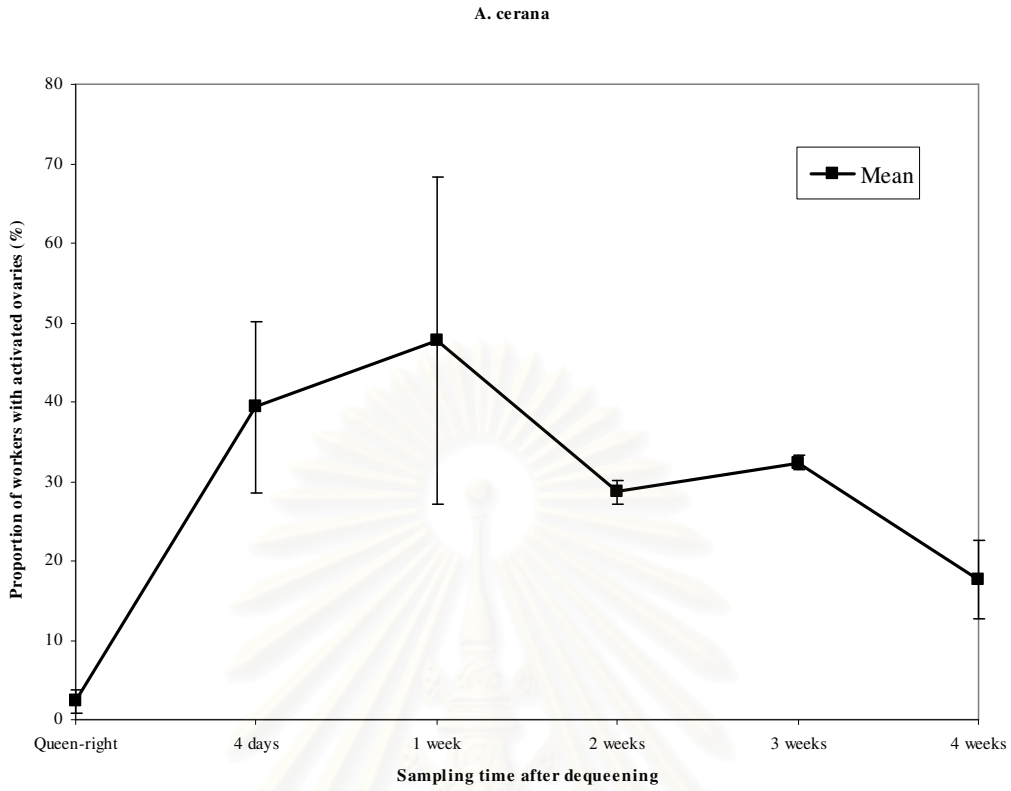


Figure 5.1 Average proportion (%) of *A. cerana* workers with activated ovaries in 3 colonies across 4 weeks of queen removal (including non-natal workers). The *bars* indicate SEs of the mean.

Table 5.2 Number of alleles and average heterozygosities among *A. cerana* workers.

	Locus				
	Ap43	A107	A76	B124	A14
Number of alleles	19	7	5	5	4
H	0.815	0.541	0.341	0.575	0.524

Based on 300 workers of each colonies, microsatellite analysis of 5 loci showed that there were 40 subfamilies in colony 1 and 39 subfamilies in colony 2 and 35 subfamilies in colony 3 (Table 5.3).

Table 5.3 Observed and effective mating frequency and coefficient of relatedness for 3 colonies of *A. cerana*.

Colony	Observed mating frequency	Effective mating frequency	Coefficient of relatedness
1	40	9.43	0.3
2	39	21.45	0.27
3	35	14.76	0.28
Mean (± S.E.)	38 ± 1.53	15.21 ± 3.48	0.28 ± 0.009

There was no significant difference in the proportion of workers with activated ovaries among different subfamilies before and after dequeening in colonies 2 and 3. In colony 1, the proportion of workers with activated ovaries before and after 4 days of queenlessness was not significantly different among subfamilies. However, after 3 weeks of queen removal, workers of some subfamilies had a significantly higher proportion of workers with activated ovaries than others (Table 5.4).

Table 5.4 Contingency table analyses of the proportions of adult workers with activated ovaries among different subfamilies in *A. cerana* colonies. P_{Fisher} is the *P* value from a Monte Carlo approximation of the Fisher's exact test and *P* is the probability associated with *G* test.

Colony	Sampling time	*G*	*df*	*P*	P_{Fisher}
1	Day 0	3.415	28	1	1
	Day 4	35.027	21	0.028	0.131
	Week 3	41.946	24	0.013	0.007
	Totals	48.478	39	0.142	0.499
	Heterogeneity	31.91	34	0.57	
2	Day 0	10.098	26	0.998	0.988
	Day 4	33.442	24	0.095	0.252
	Week 3	24.706	23	0.366	0.819
	Totals	56.438	37	0.027	0.181
	Heterogeneity	11.808	36	0.99	
3	Day 4	28.35	22	0.164	0.106
	Week 3	34.162	26	0.131	0.343
	Totals	45.438	33	0.073	0.13
	Heterogeneity	17.074	15	0.314	

The proportion of non-natal workers present in the three colonies before queen removal averaged 4.3% (Table 5.5). The proportion of non-natal workers with activated ovaries (7.7%) was not significantly different ($P = 0.29$) from natal workers (2.1%). After dequeening the proportion of non-natal workers decreased significantly ($P = 0.033$) to 1.8%. There was no significant difference ($P = 0.19$) between the proportion of non-natal workers with activated ovaries (37.5%) and natal ones (26.3%). Non-natal workers had significantly ($P = 0.028$) higher reproductive success than natal workers: 2.7% of workers in colonies 1, 2 and 3 were non-natal, but these laid 5.2% of the eggs and 5.5% of the pupae.

Table 5.5 Reproductive parasitism of queenless *A. cerana* colonies

	Colony			
Before dequeening	1	2	3	Average
Number of non-natal workers	3	4	6	
Number of natal workers	97	96	93	
% of non-natal workers	3	4	6.06	4.3
% Non-natals with activated ovaries	33.3	0	0	7.7
% Natals with activated ovaries	1.03	5.21	0	2.1
After dequeening				
Number of non-natal workers	8	3	0	
Number of natal workers	192	197	200	
% of non-natal workers	4	1.5	0	1.8
% Non-natals with activated ovaries	75	66.7	-	37.5
% Natals with activated ovaries	41.7	42.1	25.5	26.3
Offspring derived from non-natal workers (n)				
Eggs	4.51 (133)	7.9 (126)	3.15 (127)	5.2
Pupae	3.19 (94)	11.48 (61)	3.75 (80)	5.5

Error rate for misclassification of sons of non-natal workers

The probability that a son of a non-natal worker could be erroneously classified as the son of a natal worker if he fortuitously carried a 'natal allele' at all loci was calculated according to Nanork *et al*., 2005 (see Chapter 3). The average and approximate probability of a non-natal-derived male having the same genotype as a genotype-matched natal-derived male is 0.021 ± S.E. 0.002, n = 218 males for colony 1, 0.047 ± 0.005, n = 170 for colony 2 and 0.023 ± 0.003, n = 199 for colony 3.

Error rate for misclassification of sons of natal workers

The probability of non-detection of a rare worker patriline of proportion 1% due to not sampling it was calculated according to Foster *et al*. (1999) base on 299-300 workers, is low ($P = 0.05$).

The possibility of non-detection of a subfamily because two fathering males shared the same genotype is not relevant to the detection of the sons of non-natal males.

Discussion

The reproductive behaviour of *A. cerana* workers is qualitatively different to that of its sibling species, *A. mellifera* and the more distantly-related, *A. florea*. In queenright *A. cerana* colonies about 5% of workers have activated ovaries but they are not reproductively successful due to efficient policing. Ovary activation rises rapidly after dequeening, peaking with about 50% of workers having active ovaries after one week, before falling again (this study, Oldroyd *et al*., 2001). Thus ovary activation peaks while there is still strong worker policing activity (Oldroyd *et al*., 2001). Rates of ovary activation in queenless workers are similar and uniformly high among subfamilies, and there is a low level of reproductive parasitism.

After a honey bee colony becomes queenless, and with no brood from which to raise another, it's only possibility for reproduction rests with the few drones that it may raise before it finally dies. The rearing of this last batch of drones requires that the colony ceases worker policing behaviour – the removal of worker laid eggs (Miller and Ratnieks, 2001). The cessation of worker policing renders the colony vulnerable to parasitism by workers from other nests, and also increases the possibility that individual workers and subfamilies will come into conflict over which eggs the colony will rear. Thus a colony (and

ultimately natural selection) must trade off the level of policing against the possibility of parasitism, and individual workers must trade off their personal reproductive success against that of other workers and the nest as a whole. If the colony does not curtail policing behaviour then the reproductive success of the workers is zero. If it does curtail policing the colony may be parasitized. If a worker allows other workers to reproduce it may reduce its own chances for reproduction, but if no workers reproduce colony-level fitness is zero. Furthermore, there is likely to be an interaction between the optimal phenotype when queenless and the optimal phenotype when queenright. Thus there is likely to be a reproductive premium for rapid ovary activation when queenless, but large numbers of reproductive or nearly reproductive workers in queenright nests may reduce colony-level fitness.

It appears that there are subtle differences among the honey bee species in how the conflicting stresses inherent in the reproductive biology of workers are resolved. In arrhenotokous *A. mellifera*, some subfamilies have greater reproductive success than others, but few if any of the males reared are the sons of non-natal workers (Martin *et al*., 2004). This implies that colonies switch off worker policing behaviour (Miller and Ratnieks, 2001) but are still able to defend themselves against reproductive parasites, either by removing non-natal workers or, less likely, by selectively removing the eggs of non-natals. Subfamilies differ in their reproductive success, presumably because of differing genetically-based thresholds required for ovary activation (Châline *et al*., 2002; Montague and Oldroyd, 1998).

In *A. florea*, subfamilies differ strongly in the rates at which they activate their ovaries, suggesting that as with *A. mellifera*, there is differential reproductive success among queenless nestmates. Furthermore, queenless *A. florea* nests are extremely vulnerable to parasitism from workers from other nests (Nanork *et al*., 2005, see chapter 4). As with *A. florea* (Nanork *et al*., 2005; see Chapter 3), queenless *A. cerana* colonies are parasitized by eggs laid by workers

from other colonies. In queen-right colonies the proportion of non-natal workers had activated ovaries was not significantly different to natal ones.

After dequeening, the proportion of non-natal workers decreased significantly. The proportion of non-natal workers that had activated ovaries was not significantly different from that of the natals. This suggests that queenless *A. cerana* workers are better able to defend themselves against non-natal parasitic workers than can *A. florea*, and this may be because *A. cerana* are cavity nesting. However, non-natal workers had higher reproductive success than natal workers. This suggests that some non-natal eggs evade worker policing and are accepted. The proportion of non-natal eggs and pupae detected was less than expected based on the number of non-natal workers present. This may be because of rate of worker policing is still high in the first few days of queenlessness (Oldroyd *et al*., 2001).

I speculate that queen-right *A. cerana* are tolerant of drifted workers, for they may contribute to the welfare of the nest. Any eggs that they lay are removed by police workers, and so drifted workers are potentially more of a benefit than a cost. However, when a colony is queenless, drifted workers become potential parasites, and I suggest that they are actively removed and their eggs are policed in favour of natal eggs.

The reason why rates of ovary activation are so high in queenright *A. cerana* colonies (relative to all other species studied) remains a mystery. Potentially this is an adaptation to frequent queenlessness (Oldroyd *et al*., 2001), though why *A. cerana* is more likely to become queenless than other species is unclear. Neumann and Hepburn (2001) suggested that parasitizing *A. m. capensis* is more likely to come from queenright nests than from queenless ones, and this remains an important area for investigation.

My results demonstrate that the paternity frequency of workers in *A. cerana* is 35-40. The frequency is higher to that observed by Oldroyd *et al*. (1998), 14-27. This may be the sample size of each colony (289-293) is higher than Oldroyd *et al*. (1998), they used 55-101 bees from each colony. Thus, some rare patrilines were detected in this study, and these may have been undetected in the earlier study.

CHAPTER VI

PRESERVATION AND LOSS OF THE HONEY BEE (*Apis*) EGG MARKING SIGNAL ACROSS EVOLUTIONARY TIME

Abstract

Worker policing is any behaviour in which workers act to reduce reproduction in nestmate workers, and in honey bees, *Apis*, policing occurs mainly via the selective removal of worker-laid eggs. Workers can distinguish eggs laid by queen and eggs laid by workers almost certainly because queen-laid eggs are marked by a pheromonal mark. When the policing is efficient, workers are predicted to evolve self restraint or 'acquiescence' and cease laying eggs because it is unlikely to lead to success reproduction. In *Apis cerana*, workers have high rates of ovary activation but have low rates of worker reproductive success. This indicates that the evolution of 'acquiescence' is incomplete in *A. cerana*, whereas complete or nearly complete acquiescence has evolved in all other *Apis* species. An 'episode of revolution' (Wenseleers *et al*., 2004b) in worker policing may be occurring in *A. cerana*, associated with a change in the signal used by workers to distinguish queen-laid and worker-laid eggs. I tested this prediction by studying cross-species policing in 3 species: *A. mellifera*, *A. cerana* and *A. florea* and found that although *A. mellifera* can readily distinguish queen and worker laid eggs of *A. florea* (implying that the egg recognition signal has been preserved to some extent in these two evolutionary lineages), *A. mellifera* is unable to distinguish queen and worker laid eggs of its sister taxa *A. cerana*. This supports the hypothesis that there has been a recent evolutionary change in the queen's egg marking signal in the *A. cerana* lineage.

Introduction

In honey bee (*Apis*) colonies that have lost their queen and have failed to rear a replacement, workers eventually begin laying eggs, which can give rise to fully functional males (Beekman and Oldroyd, 2005; Winston, 1987). In contrast, workers in queen-right (queen present) colonies almost always lack activated ovaries (Ratnieks, 1995) and act to suppress egg production by worker nestmates by recognizing and eating any eggs laid by workers (Ratnieks, 1988; Ratnieks and Visscher, 1989). This 'worker policing' behaviour is thought to have evolved due to the relatedness coefficients that arise from polyandry (Barron *et al*., 2001; Ratnieks, 1988; Wenseleers *et al*., 2004a, 2005, see Chapter 2).

Honey bee workers can readily distinguish queen-laid (QL) eggs, from worker laid (WL) eggs. The nature of the egg recognition signal that workers use to distinguish QL and WL eggs is as yet unknown (Barron *et al*., 2001; Katzav-Gozansky *et al*., 1997, 2001; Martin *et al*., 2002b), but is unlikely to be a physical signal (Beekman and Oldroyd, 2005; Katzav-Gozansky *et al*., 2003) and most likely to be a pheromone placed on eggs by queens (Beekman *et al.*, 2004; Oldroyd *et al*., 2002; Ratnieks, 1995). Eggs laid by workers lack this queen-specific pheromone and are removed (Ratnieks, 1989). It was originally suggested that the putative egg-marking pheromone is produced by the queen's Dufour's gland (Ratnieks, 1995), but recent work casts doubt on this view, and the source of the queen's egg marking signal remains unknown (Beekman *et al.*, 2004; Katzav-Gozansky *et al*.,2001, 2002a, 2002b, 2003; Martin *et al*., 2002b; Sole *et al*., 2002) .

Worker policing based on oophagy of WL eggs has now been demonstrated in three honey bee species: *A. mellifera* (Ratnieks and Visscher, 1989), *A. florea* (Halling *et al*., 2001), and *A. cerana* (Oldroyd *et al*., 2001), and there is strong circumstantial evidence that it also occurs in *A. dorsata* (Wattanachaiyingcharoen *et al*., 2002). Comparative analysis suggests that

polyandry and worker policing evolved before the radiation of the genus *Apis* (Oldroyd *et al*., 1996; Oldroyd and Wongsiri, 2006), and is therefore very ancient. Worker policing via oophagy has also evolved independently in a wide variety of social insects including Vespidae wasps (Foster and Ratnieks, 2000, 2001, 2002) and three ant species (Endler *et al*., 2004; D'Ettore *et al*., 2004; Kikuta and Tsuji, 1999).

When worker policing is efficient, and the production of eggs by workers is unlikely to lead to successful reproduction, workers are predicted to evolve mechanisms of self restraint or 'acquiescence' (Wenseleers *et al*., 2004a). That is, efficient worker policing removes the incentive for individual workers to attempt selfish reproduction. The Asian hive bee *A. cerana* is unusual among honey bees in that 3-5% of workers have fully active ovaries even in queenright colonies (Bai and Reddy, 1975; Oldroyd *et al*., 2001; see Chapter 4). Nonetheless *A. cerana* workers police worker-laid eggs, and few if any male offspring arise from worker-laid eggs (Oldroyd *et al*., 2001). High rates of ovary activation in association with low rates of worker reproductive success in *A. cerana* indicate that the evolution of 'acquiescence' is incomplete in *A. cerana*. As complete or nearly complete acquiescence has evolved in all other species of *Apis* studied thus far, and is found in the basal *A. florea* (Halling *et al*., 2001) there may have been an evolutionarily recent perturbation or 'episode of revolution' (Wenseleers *et al*., 2004b) in the worker policing system of *A. cerana*, possibly associated with the signals used to distinguish QL and WL eggs.

Signals like the queen's egg marking signal, benefit both sender and receiver. The sender (the queen) benefits from the signal because it reinforces her reproductive monopoly. The receivers (police workers) benefit because the signal allows them to rear more related QL eggs than less related WL eggs. Signals such as these, that benefit both parties, are likely to be conserved over evolutionary time because changes to the signal are unlikely to be at a selective advantage. However, instances of selfish reproductive behaviour by workers are

known. Foremost of these are so-called 'anarchistic' strains of *A. mellifera* in which workers activate their ovaries and lay eggs (Châline *et al*., 2002; Montague and Oldroyd, 1998; Oldroyd *et al*., 1994b) which have intermediate acceptance between QL and WL eggs of wild type strains (Oldroyd and Ratnieks, 2000). Such workers lay eggs that carry a signal that mimics that produced by queens (Martin *et al*., 2004a). An inability of police workers to accurately distinguish QL and WL eggs should lead to rapid evolution of the queen's egg marking signal, because such changes benefit both queens and police workers. I tested this prediction by studying cross-species policing in 3 species: *A. mellifera*, *A. cerana*, and *A. florea*. The occurrence of an 'episode of revolution' in *A. cerana*, characterized by worker reproduction, would be supported if the egg marking signal in this species can not be recognized by *A. mellifera* police workers, whereas the queen's egg marking signal of more distantly related *A. florea* can be recognised. Here I show that this prediction is supported, suggesting that frequent worker reproduction in *A. cerana* has apparently led to rapid evolution of the queen's egg marking signal, and that an 'episode of revolution (Wenseleers *et al.*, 2004b) is currently occurring in *A. cerana*.

Materials and methods

Sources of eggs

A. florea

I transferred six wild-caught colonies of *A. florea* from Samutsongkram province to the campus of Chulalongkorn University, Bangkok, Thailand. After moving the colonies I found that three had become queenless and these were used as a source of worker-laid (WL) eggs. To facilitate harvesting of WL eggs, I suspended a section of *A. florea*-built drone comb beneath the nests (Halling *et al*., 2001). These comb sections were always laid in, and could be readily transferred to the laboratory in order to harvest the eggs under a low-powered dissecting microscope. In queenright colonies, the presence of the queen was

confirmed before eggs were collected. QL eggs were obtained from any of three queenright colonies by cutting a small section of comb from the nest.

A. cerana

The two colonies of *A. cerana* used in this study were wild-caught from Samutsongkram province. These colonies were transferred into single-storey box hives and were left at the natal sites in Samutsongkram for two weeks to allow the colonies to establish their nests in the artificial hives. Then I moved the colonies to Chulalongkorn University in March 2004. One colony lost its queen during the transfer, and was maintained as the queenless colony. The queenless colony built drone combs which provided WL eggs. The other queen-right colony provided the QL eggs for these experiments.

Due to the lack of male QL eggs (i.e. unfertilized eggs laid by a queen in drone cells) at the time of the experiments, I was compelled to use female (i.e. fertilized eggs harvested from worker cells) QL eggs. However, this is most unlikely to have affected the results. First, Oldroyd and Ratnieks (2000) tested the survival rate of *A. mellifera* female and male QL eggs in policing assays, and found that they are not different, presumably because both types of eggs are laid by the queen and are presumably marked by the same way (Halling *et al*., 2001). Second, I was able to confirm that the removal rates of female and male QL eggs of *A. florea* are no different in *A. mellifera* discriminator colonies (see below). Unfortunately, I was unable to test the relative survival of male and female *A. cerana* QL eggs because my queenright source colony never produced any.

Policing assays

With minor modifications required by the biological material available, I performed standard policing bioassays (Oldroyd and Ratnieks, 2000; Ratnieks and Visscher, 1989) using a total of four *A. mellifera* discriminator colonies. The

discriminator colonies comprised 6-7 frames of bees and brood. Environmental conditions for honey bees were good at the time of the assays and brood nests were expanding.

To perform an assay I identified three rows of drone cells in an *A. mellifera*-built drone comb with colored drafting pins. I then transferred one row of 20 QL and one row of 20 WL eggs into the test rows using modified forceps (Ratnieks and Visscher, 1989; Taber, 1961). One row was left blank to control for any queen or worker oviposition by the discriminator colony during the assay. After loading the comb, I sandwiched it between two brood combs of the discriminator colony. Because the discriminator colonies available were relatively small, I was unable to isolate the queens heading the discriminator colonies from the test combs, as is usual practice (Oldroyd and Ratnieks, 2000; Ratnieks and Visscher, 1989). However, because my assays were so brief, oviposition is most unlikely. Moreover, if oviposition did occur within the test rows it would have been random with respect to treatment.

A. florea

I used four queen-right *A. mellifera* discriminator colonies to assay the removal rates of *A. florea* eggs from different sources. Five trials were conducted in each of three discriminator colonies and seven trials in another colony. A total of 440 QL and 440 WL *A. florea* eggs were transferred to discriminator colonies. Assays were conducted over a period of six days. I inspected the test combs after 10, 20, 30, 40 minutes and 1 hour, recorded the number of remaining WL and QL eggs and checked for the presence of any eggs in the control row.

To test whether male and female *A. florea* eggs have different survival rates I used two queen-right *A. mellifera* discriminator colonies to assay the survival of female and male eggs laid by two *A. florea* queens. Two trials were conducted in one discriminator colony, and three trials in another colony. Rows

of 20 female and male eggs used in each trial. The test combs were inspected after 10, 20 and 30 minutes.

A. cerana

Three queen-right *A. mellifera* discriminator colonies were used for the assays. Five trials were conducted in each of two discriminator colonies and three trials in one colony. A total of 260 QL and 260 WL eggs were transferred to discriminator colonies. Egg survival was then determined after 10, 20, 30 minutes, 1, 1.5 and 2 hours. Assays were conducted over a period of three days.

Statistical analysis

I analysed the data using a Cox regression survival analysis (Collett, 1994) as implemented in SPSS. I compared the survival of eggs laid by queens and laying workers using exact failures (i.e. a transferred egg was removed at min 10, 20, 30, 40 or 60 for *A. florea* assays and at min 10, 20, 30, 60, 90 and 120 for *A. cerana* assays) and right censoring (for those eggs still remaining at min 60 for *A. florea* assays and at 120 min for *A. cerana* assays). The null hypothesis in a Cox regression model assumes that the hazard rate (i.e. probability of egg removal) at any given time for an individual egg in one group is proportional to the hazard at that time for a similar egg in the other group. The model I constructed included egg source, trial and discriminator as variables. Graphically, I present the mean proportion of surviving eggs at each time, as these are more informative than the hazard function plots.

Results

A. florea

Both QL and WL eggs were rapidly removed by *A. mellifera* workers, but QL eggs survived significantly longer than WL eggs (P = 0.003) (Table 6.1,

Figure 6.1). Discriminator colonies did not differ significantly in their treatment of eggs (P = 0.082); all discriminators removed WL eggs more quickly than QL eggs.

Cox regression analysis indicated that the survival of male and female *A. florea* eggs in *A. mellifera* discriminator colonies is not significantly different (P = 0.886).

Table 6.1 Likelihood ratios comparing the survival of *A. florea* eggs of different sources (QL and WL; n = 440 for each egg source) in *A. mellifera* discriminator colonies. The survival function is modelled without ('Null') and with egg source, trial and discriminator colony ('Overall') as factors. The procedure then tests the effect of adding 'Source of eggs', 'Trial' or 'Discriminator'.

Term	-2 log likelihood	χ^2	*df*	*P*
Null	11005.145			
Overall	10987.897	17.296	3	0.001
Source of eggs		8.536	1	0.003
Trial		5.146	1	0.023
Discriminator		3.031	1	0.082

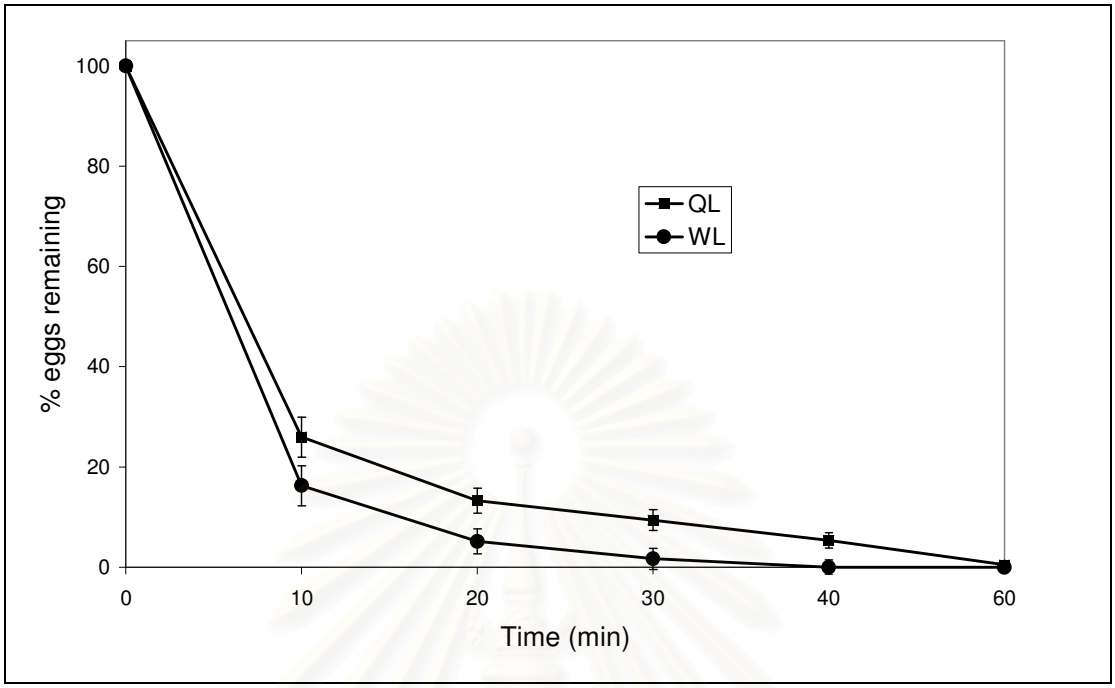


Figure 6.1 Survival of worker-laid (WL) and queen-laid (QL) eggs of *A. florea* in queenright *A. mellifera* discriminator colonies. *Bars* indicate standard errors of the means.

A. cerana

Cox regression analysis showed that the survival of QL and WL eggs in *A. mellifera* discriminator colonies is not significantly different (P = 0.416 Table 6.2, Figure 6.2). Discriminator colonies differed significantly in their treatment of eggs (P = 0.023), but the rate of eggs removal in both QL and WL eggs was the same in all discriminator colonies.

Table 6.2 Likelihood ratios comparing the survival of *A. cerana* eggs of different sources (QL and WL; n = 260 for each egg source) in *A. mellifera* discriminator colonies. The survival function is modelled without ('Null') and with egg source, trial and discriminator colony ('Overall') as factors. The procedure then tests the effect of adding 'Source of eggs', 'Trial' or 'Discriminator'.

Term	-2 log likelihood	χ^2	*df*	*P*
Null	5599.720			
Overall	5568.938	31.511	3	< 0.001
Source of eggs		0.662	1	0.416
Trial		21.298	1	< 0.001
Discriminator		5.164	1	0.023

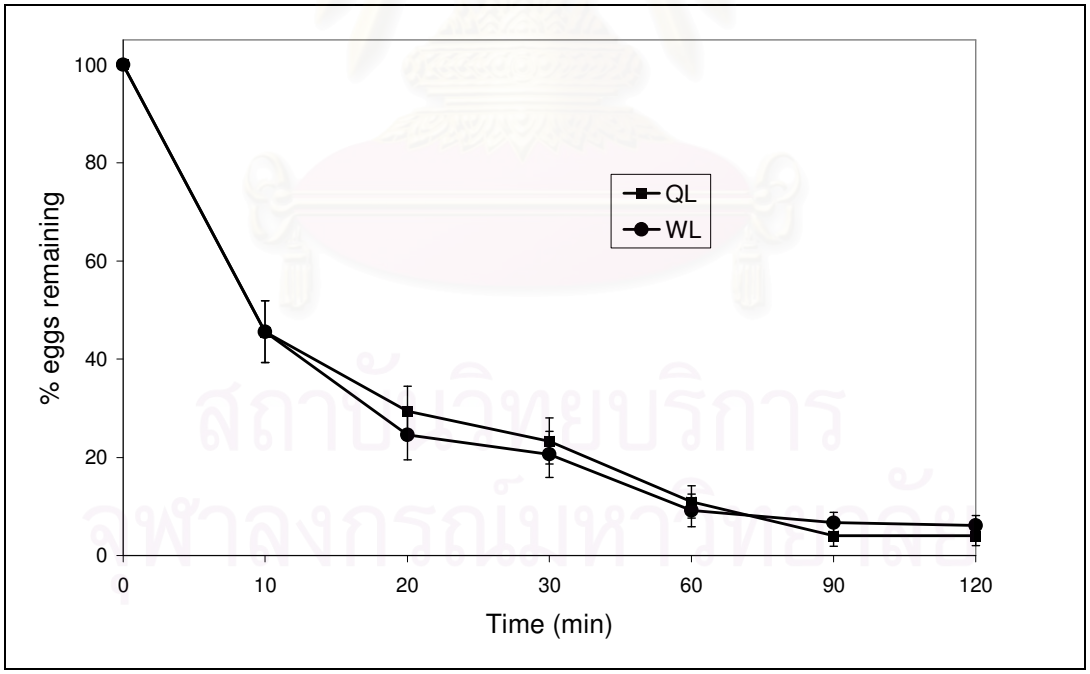


Figure 6.2 Survival of worker-laid (WL) and queen-laid (QL) eggs of *A. cerana* in queenright *A. mellifera* discriminator colonies. The *bars* indicate SEs of the mean.

Discussion

The study shows that although *A. cerana* eggs are far more acceptable than *A. florea* eggs overall, *A. mellifera* police workers can distinguish QL and WL eggs produced by *A. florea*, but are unable to distinguish QL and WL eggs of its sister taxa *A. cerana*. As *A. mellifera* and *A. cerana* probably diverged 1-2 million years ago whereas *A. florea* diverged from the *A. mellifera-A. cerana* clade 6-10 million years ago (Oldroyd and Wongsiri, 2006). On phylogenetic grounds, therefore, I would expect that it would be more likely that *A. mellifera* workers could distinguish QL and WL eggs of *A. cerana* than they could distinguish QL and WL eggs of *A. florea.* Conversely if an 'episode of revolution' (Wenseleers *et al*., 2004b) is occurring in *A. cerana*, as manifest by relatively high rates of worker ovary activation (Bai and Reddy, 1975; Oldroyd *et al*., 2001), I would predict rapid changes in the signals placed on eggs by *A. cerana* queens and workers. These changes have apparently resulted in an inability of *A. mellifera* to distinguish QL and WL *A. cerana* eggs, even though they retain some ability to distinguish *A. florea* eggs.

In queenright *A. cerana* colonies, 1-5% of workers have eggs in their ovarioles, and rapidly activate their ovaries after dequeening (Oldroyd *et al*., 2001). Although worker-laid males are rare in *A. cerana*, indicating that worker reproduction is curtailed by policing in *A. cerana*, the rate at which WL eggs are removed by police workers is much lower (Oldroyd *et al*., 2001) than it is in *A. mellifera* (Beekman and Oldroyd, 2003, 2005; Beekman *et al*., 2004; Halling and Oldroyd, 2003; Oldroyd and Ratnieks, 2000; Ratnieks and Visscher, 1989) or *A. florea* (Halling *et al*., 2001). I speculate that an episode of worker rebellion is currently occurring in *A. cerana*. Presumably, *A. cerana* workers evolved the ability to mimic the queen's egg marking pheromone. The existence of reproductively successful workers and reduced policing efficiency reduces the incentive for self-restraint by workers (Wenseleers *et al*., 2004a, 2004b, 2005). Hence quite high levels of ovary activation are observed in *A. cerana.*

I recognize that these experiments provide only circumstantial evidence that an 'episode of revolution' is currently occurring in *A. cerana*. Confirmation of my hypothesis will require identification of the queen's egg marking signal, potentially a cuticular hydrocarbon (Beekman *et al.*, 2004), and dissection of its constituents in several species of honey bee, and a demonstration that the *A. cerana* compound is very different from the other species. The elucidation of the honey bee queen's egg marking pheromone has proved extremely elusive (Katzav-Gozansky *et al*., 2001, 2002, 2003; Martin *et al*. 2002b, 2004a, 2004b; Oldroyd *et al*. 2002), and so such an analysis is not currently possible. In the meantime, these cross-species policing data provide tantalizing support for the existence of an 'episode of revolution' in *A. cerana*

CHAPTER VII

CONCLUSIONS

7.1 Rates of ovary activation in queenless colonies of *A. florea* and *A. cerana*

Kin selection theory provides a framework for exploring the costs and benefits of individual-level and colony-level reproduction in honey bee colonies. Workers are always more related to their own sons ($r = 0.5$) than the sons of the queen ($r = 0.25$) or the sons of their half sisters ($r = 0.125$) (Barron *et al*., 2001; Crozier and Pamilo, 1996; Pamilo, 1994). Thus all workers would prefer to raise their own sons while preventing other workers from producing theirs. Resolution of the inherent conflict in honey bee societies over which individuals will produce the male eggs is not straightforward: no single strategy is better than all others under all circumstances (Barron *et al*., 2001). Each honey bee species has evolved its own idiosyncratic methods of resolving conflict over worker reproduction, and these are reflected in the varying reproductive status of workers, both when queenless and queenright. In the western honey bee, *A. mellifera* and the red dwarf honey bee, *A. florea*, few if any workers have activated ovaries (Barron *et al*., 2001; Halling *et al*., 2001; Ratnieks, 1993; Visscher, 1989; Chapter 3). Workers with activated ovaries are more common in the eastern honey bee, *A. cerana*, where 1-5% of workers have eggs in their ovaries (Oldroyd *et al*., 2001, see also Chapter 5). The reasons why natural selection tips the balance in favor of personal reproduction in *A. cerana* are unclear, but potentially it is because *A. cerana* colonies frequently lose their queen (Ruttner, 1988) and those workers that already have active or partially activated ovaries will be advantaged.

After a colony losses its queen and cannot rear a replacement, workers begin to activate their ovaries and lay unfertilized eggs which will develop into males (Barron *et al*., 2001; Ratnieks, 1993). Because honey bees are

polyandrous, kin selection theory (Hamilton, 1964) predicts that the balance of costs and benefits over which individuals should lay eggs shifts away from the collective (the queen's eggs) towards the individual: the colony must rear worker-laid eggs, for there are no other eggs available. But because not all eggs are equally valuable to all workers, a queenless nest is much more conflict-ridden than a queenright one.

This study has shown that workers of both *A. florea* and *A. cerana* activate their ovaries after only a few days of queenlessness. Reproductive competition was found among worker patrilines in queenless *A. florea* colonies, with workers of some subfamilies having much higher rates of ovary activation than others. Similar phenomena have been observed in *A. mellifera* (Martin *et al*. 2004). This suggests that certain subfamilies have greater ability to respond to a lack of queen and brood pheromones earlier than others (Martin *et al*., 2004). In contrast, there was little difference in the proportions of workers with activated ovaries among patrilines in queenless *A. cerana* colonies, suggesting that there is no genetic variance for worker reproduction.

7.2 Reproductive parasitism in queenless colonies of *A. florea* and *A. cerana*

When a honey bee colony becomes hopelessly queenless and must switch off worker policing in order to lay eggs, it becomes vulnerable to social parasitism: workers from non-natal nests may either actively or passively join the queenless nest and lay eggs there. The possibility of social parasitism generates another level of potential conflict within honey bee nests. All workers are collectively and individually disadvantaged by social parasitism, and yet there primary defence against it, worker policing, must be switched off if the colony is to have any chance of reproduction. I have shown that when colonies of *A. florea* and *A. cerana* become hopelessly queenless, they are parasitized by eggs of workers from other colonies. This study has shown that the proportion of non-natal eggs and pupae reared by queenless *A. florea* is very high and parasitism

may be facilitated the species' open nests (Nanork *et al*., 2005, see Chapter 3). In queenless *A. cerana* colonies, a much smaller proportion of eggs and pupae are of non-natal origin. This suggests that cavity nesting and high rates of worker policing in queenless *A. cerana* colonies may reduce the ability of non-natal workers to parasitize queenless colonies.

The reproductive life cycle of parasitic workers of *A. florea* and *A. cerana* may occur as in Figure 7.1 (adapted from Neumann and Hepburn, 2001 and Neumann and Moritz, 2002). Transmission of workers to new hosts can occur via individual worker intrusion (drifting, step 1 and dispersing, step 2) and /or via colony absconding and merger (step 3). Parasitic workers successfully invade queenless colonies, and lay eggs (step 4). The cessation of worker policing in queenless colonies (step 5) allows the eggs of non-natal workers to be reared (step 7) and become reproductive males (step 8) which may possibly mate with a queen and produce workers. The grey box and dotted lines symbolizes the possible pathway of parasitic workers in *A. cerana* colonies.

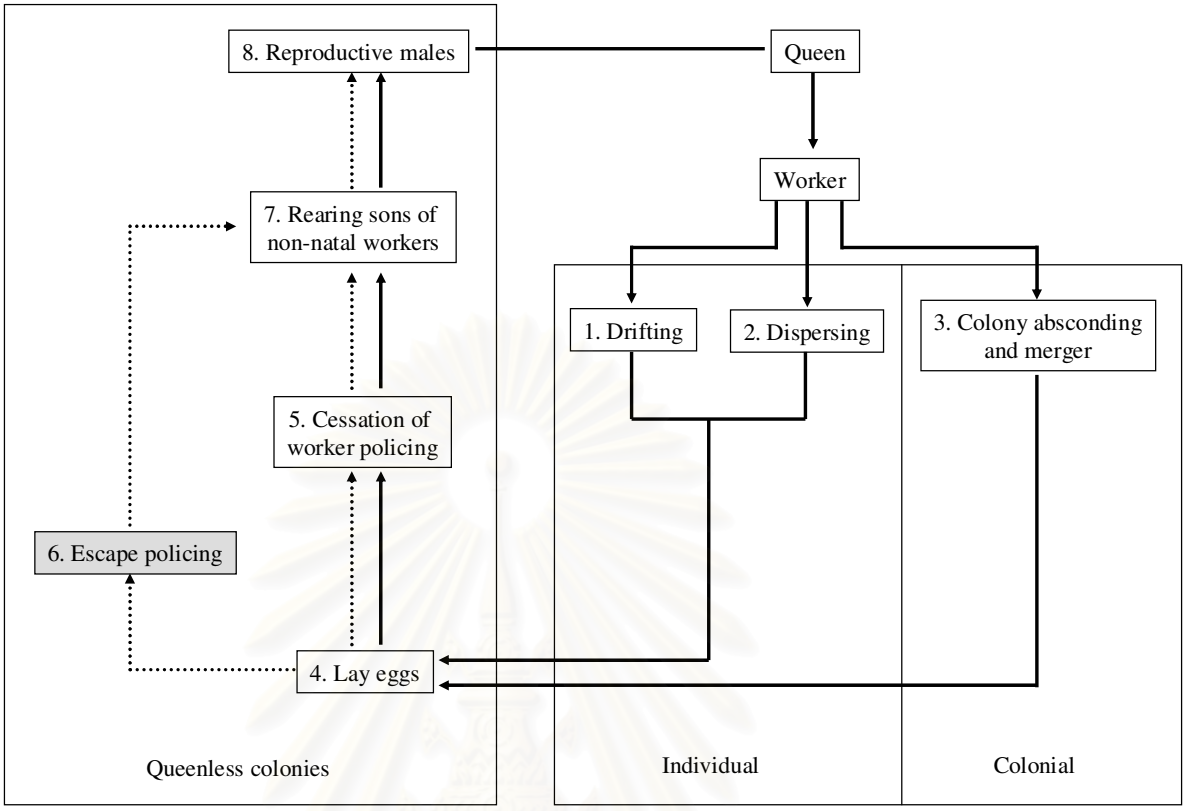


Figure 7.1 Reproductive cycle of social parasitic workers in *A. florea* and *A. cerana* colonies.

7.3 Preservation and loss of the honey bee (*Apis*) egg marking signal across evolutionary time

This study has provided evidence that rapid evolutionary change may have occurred in the signals placed on eggs by *A. cerana* queens and workers. When offered QL and WL eggs of *A. cerana* in a standard policing assay (Oldroyd and Ratnieks, 2000; Ratnieks and Visscher, 1989) *A. mellifera* workers cannot distinguish them, whereas they retain some ability to distinguish *A. florea* eggs. This observation supports the theoretical prediction that 'episodes of revolution' (Wenseleers *et al*., 2004b) may occasionally occur in eusocial insect colonies, that are characterized by rapid evolutionary change in the queen's egg marking signal. Such a revolution may be currently occurring in *A. cerana*,

presumably because workers have evolved the ability to mimic the queen's egg marking pheromone.

7.4 Suggestions for further work

7.4.1 In the Cape honey bee, *A. m. capensis*, it has been proposed that parasitic workers enter host colonies by passive "drifting" (Greeff, 1997), resulting from slight orientation errors of young workers and sometimes of foragers (Free, 1958). However, long-range drifting (dispersal) of parasitic workers could also occur (Neumann *et al*., 2001). Furthermore, Neumann *et al*. (2001) found that the queenstate (*i.e*. queenless or queenright) of mother and host play a role on drifting and dispersal of workers and on the hosting of these workers in *A. m. capensis*, *A. m. scutellata* and their natural hybrids. They found that parasitic *A. m. capensis* is more likely to come from queenright nests than from queenless ones.

The origin of parasitic workers in colonies of *A. florea* and *A. cerana* is still unclear. Do the parasitic workers arise from queenright or queenless colonies? This could be tested by experiments as similar to Neumann *et al*. (2001). Nine colonies of queenright and queenless of *A. florea* and *A. cerana* would be used, 3 circles of 3 queenless and 3 queenright colonies of each species would be set up. The colonies within each circle would be spaced 1 m apart, the circles are 40 m apart. Workers would be paint-marked according to colony and reintroduce into their queenless or queenright mother colonies and recapture after 10 days to investigate the origin of parasitic workers. This is an important study because it may reveal the origins of policing behaviour. If Asian species are constantly invaded by parasitizing workers from queenright nests, then their evolved response is most likely to be policing of the parasitizing eggs. But if the parasites are rare, and mainly arise from dispersing queenless nests, then a role of social parasites if the evolution of policing is less likely.

7.4.2 Parasitic workers and reproductive parasitism are not found in arrhenotokous *A. mellifera* colonies (Martin *et al*., 2004). This suggests that *A. mellifera* has effective defense against reproductive parasitism in at least the first month of queenlessness. Rates of worker policing decline after the colonies lose the queen (Miller and Ratnieks, 2001) and make them vulnerable to be being parasitized. The optimal time to invade *A. mellifera* host colonies may be after one month of queenlessness. This prediction could be tested by collecting adult, workers eggs, larvae and pupae, and performing a worker policing test (Oldroyd and Ratnieks, 2000) every week after queen loss until the colonies perish and followed by determination of parentage using microsatellite analysis. Such a study would show if social parasitism increases after worker policing ceases.

7.4.3 This study has shown that in *A. cerana* colonies before queen removal, non-natal workers found accounted for 4.35% of workers and 7.7% of these had activated ovaries (see Chapter 5). This suggests that non-natal workers have higher rates of ovary activation than natals (2.1%). This samples size is too small to definitively conclude that non-natals have higher rates of ovary activation than natals. In a future study approximately 1,000 workers will be collected from each of 3 *A. cerana* colonies. These samples will be dissected to determine rates of ovary activation, the genotypes of each worker will be then analyzed using microsatellite loci provide an accurate assessment of rates of ovary activation in both natal and non-natal workers.

7.4.4 In queenright *A. cerana* colonies there is effective worker policing, but the rate appears to increase after queen loss (Oldroyd *et al*., 2001). This may explain why the proportion of eggs laid by non-natal workers in queenless *A. cerana* colonies is lower than that observed in *A. florea* (see Chapter 3, 5 and Nanork *et al*., 2005). As I proposed above, this may suggest that worker policing first arose as a defense from social parasitism in *A. cerana*. I will examine rates of worker policing in queenless *A. florea* colonies. I postulate that unlike *A. cerana*, policing behaviour is rapidly switched off in *A. florea*, leaving them

more vulnerable to parasitism. The experiment will be conducted using 1 queenright and 4 queenless *A. florea* colonies. Standard policing assays (Oldroyd and Ratnieks, 2000) will be performed using natal and non-natal worker-laid eggs, and queen-laid eggs will be used as a control.

7.4.5 In polyandrous honey bees, workers can potentially increase their inclusive fitness by rearing full-sister queens. If the mother queen dies suddenly, workers feed a few larvae in worker cells with royal jelly and rear them into queens (Châline *et al*., 2003). Four studies on patriline differences in emergency queen rearing were conducted using microsatellites. Some patrilines were preferentially reared as queens but the pattern is not consistent among colonies (Châline *et al*., 2003; Osborne and Oldroyd, 1999; Tilley and Oldroyd, 1997). On the other hand, Frank *et al*. (2002) failed to find any patriline differences between worker and queen brood. I will collect emergency queen larvae of *A. cerana* and *A. florea* and determine differences among their patrilines using microsatellite loci.

7.4.6 In queenless colonies, multiple eggs laid by workers are often found in any one brood cell. What is the origin of the eggs in any one cell? Does an individual worker guard a cell and prevent other workers from laying in it? Does she personally provision her own larvae? I will determine the maternity of worker laid eggs in particular cells using microsatellites. If individual workers do exclusively use one cell, then this would constitue an extra-ordinary reversion to near solitary behaviour by workers in queenless nests.

7.5 Conclusions

This thesis has revealed much the reproductive conflicts in terminally queenless colonies of *A. florea* and *A. cerana*. Workers of different subfamilies activate their ovaries at different rates and their eggs have differential survival. The study has revealed that each honey bee species evolved its own strategies to

resolve conflict over worker reproduction. Moreover, the study has shown the important reproductive tactic of workers to contributing to their own reproductive output by parasitizing the queenless colonies. Finally, an‘episode of worker revolution’ is suggested to currently occurring in *A. cerana* because workers have ability to mimic the queen’s egg marking pheromone and the high levels of ovary activation was found in the colonies. However, more studies as suggested above are needed to fill up the gaps of how the reproductive conflict in colonies of *A. florea* and *A. cerana* are resolved.

References

Akaratanakul, P. 1976. Honey bees in Thailand. *American Bee Journal*. 116(3): 120-121, 124, 126.

Akaratanakul, P. 1977. *The natural history of the dwarf honeybee, Apis florea F. in Thailand*. Ph.D. thesis, Cornell University, Ithaca, New York.

Akre, R.D., Garnett, W.B., McDonald, J.F., Green, A. and Landolt, P. 1976. Behavior and colony development of *Vespula pensylvanica* and *V. atropolisa* (Hymenoptera: Vespidae). *J. of Kans. Entomol. Soc.* 49: 63-84.

Alexander, B.A. 1991. Phylogenetic analysis of the genus *Apis* (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am*. 84: 137-149.

Arias, M.C. and Sheppard, W.S. 2005. Phylogenetic relationships of honey bees (Hymenoptera:Apinae:Apini) inferred from nuclear and mitochondrial DNA sequence data. *Mol. Phyl. Evol.* 37:25-35

Avise, J.C. 1994. *Molecular Markers, Natural History and Evolution*. New York : Chapman & Hall.

Bai, A.R.K. and Reddy, C.C. 1975. Ovary development and egg laying in *Apis cerana indica* workers. *J. Apicult. Res.* 14:149-152.

Barron, A.B., Oldroyd, B.P. and Ratnieks, F.L.W. 2001. Worker reproduction in honey-bees (*Apis*) and the anarchistic syndrome: a review. *Behav. Ecol. Sociobiol*. 50: 199-208.

Beekman, M., Calis, J.N.M. and Boot, W.J. 2000. Parasitic honeybees get royal treatment. *Nature*. 404: 723.

Beekman, M., Good, G., Allsopp, M.H., Radloff, S., Pirk, C.W.W. and Ratnieks, F.L.W. 2002. A non-policing honey bee colony (*Apis mellifera capensis*). *Naturwissenschaften*. 89: 479-482.

Beekman, M., Martin, C.G. and Oldroyd, B.P. 2004. Similar policing rates of eggs laid by virgin and mated honey-bee queens. *Naturwissenschaften.* 91:598-601.

Beekman, M., and Oldroyd, B.P. 2003. Different policing rates of eggs laid by queenright and queenless anarchistic honey bee workers (*Apis mellifera* L.). *Behav. Ecol. Sociobiol*. 54: 480-484.

Beekman, M. and Oldroyd, B.P. 2005. Honey bee workers use cues other than egg viability for policing. *Biology Letters*. 1(2): 129-132.

Beshers, S.N., Fewell, J.H. 2001. Models of division of labor in social insects. *Ann. Rev. Ent*. 46:413-440.

Blanford, E.J. 1923. Chinese bees as we find them. *Bee World*. 5: 104-106.

Boomsma, J.J. and Ratnieks, F.L.W. 1996. Paternity in eusocial Hymenoptera. *Phil. Trans. R. Soc. Lond. B*. 351: 947-975.

Bourke A.F.G. and Ratnieks, F.L.W. 2001. Kin-selected conflict in the bumble bee *Bombus terrestris* (Hymenoptera: Apidae). *Proc. R. Soc. London. B.* 268: 347-355.

Breed, M.D., Leger, E.A., Pearce, A.N. and Wang, Y.J. 1998. Comb wax effects on the ontogeny of honey bee nestmate recognition. *Animal Behaviour*. 55: 13-20.

Calderone, N.W. and Page, R.E. 1991. Evolutionary genetics of division of labor in colonies of the honey bee (*Apis mellifera*). *Am. Nat*. 138:69-92.

Calis, J.N.M., Boot, W.J., Allsopp, M.H. and Beekman, M. 2002. Getting more than a fair share: nutrition of worker larvae related to social parasitism in the Cape honey bee *Apis mellifera capensis*. *Apidologie*. 33: 193-202.

Cameron, S.A., Derr, J.N., Austin, A.D., Wooley, J.B. and Wharton, R.A. 1992. The application of nucleotide sequence data to phylogeny of the Hymenoptera: a review. *J. Hymenoptera Res.* 1: 63-79.

Châline, N., Arnold, G., Papin, C. and Ratnieks. F.L.W. 2003. Patriline differences in emergency queen rearing in the honey bee *Apis mellifera*. *Insectes Soc*. 50: 234-236.

Châline, N., Ratnieks, F.L.W. and Burke, T. 2002. Anarchy in the UK: detailed genetic analysis of worker reproduction in a naturally-occurring British anarchistic honeybee, *Apis mellifera*, colony using DNA microsatellites. *Mol. Ecol*. 11: 1795-1803.

Cole, B.J. 1983. Multiple mating and the evolution of social behavior in the Hymenoptera. *Behav. Ecol. Sociobiol*. 12: 191-201.

Collett, D. 1994. *Modelling Survival Data in MedicalRresearch*. Chapman & Hall, London.

Cook,V.A. 1987. The spread of *Varroa jacobsoni* and *Tropilaelaps clareae*. *Bee World*. 68:163-164.

Crozier, R.H. and Pamilo, P. 1996. *Evolution of Social Insect Colonies: Sex Allocation and Kin Selection*. Oxford University Press, Oxford.

Dade, H.A. 1977. *Anatomy and Dissection of the Honeybee*. International Bee Research Association, London.

Dampney J.R., Barron A.B. and Oldroyd B.P. 2002. Policing of adult honey bees with activated ovaries is error prone. *Insectes Soc*. 49:270-274.

Deowanish, S., Nakamura, J., Matsuka, M. and Kimura, K. 1996. MtDNA variation among subspecies of *Apis cerana* using restriction fragment length polymorphism. *Apidologie*. 27: 407-413.

D' Ettore, P., Heinze, J. and Ratnieks, F.L.W. 2004. Worker policing by egg-eating in the ponerine ant *Pachycondyla inversa*. *Proc. R. Soc. London.B.* 271: 1427-1434.

Downs, S.G. and Ratnieks, F.L.W. 1999. Recognition of conspecifics by honeybee guards uses acquired in the adult stage. *Animal Behaviour*. 58: 643-648.

Downs, S.G. and Ratnieks, F.L.W. 2000. Adaptive shifts in honeybee (*Apis mellifera* L.) guarding behavior support predictions of the acceptance threshold model. *Behav. Ecol.* 11: 326-333.

Endler, A., Liebig, J., Schmitt, T., Parker, J.E., Jones, G.R., Schreier, P. and Holldobler, B. 2004. Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. *PNAS*. 101; 2945-2950.

Engel, M.S. 1998. Fossil honey bees and evolution in the genus *Apis* (Hymenoptera: Apidae). *Apidologie*. 29: 265–281.

Engel, M.S. and Schultz, T.R. 1997. Phylogeny and behavior in honey bees (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* 90: 43-53.

Estoup, A.; Garnery, L.; Solignac, M.; and Cornuet, J. M. 1995. Microsatellite variation in honey bee (*Apis mellifera* L.) population: Hierarchical genetic structure and test of infinite allele and stepwise mutation models. *Genetics*. 140: 679-695.

Estoup, A, Solignac, M. and Cornuet, J.-M. 1994. Precise assessment of the number of patrilines and of genetic relatedness in honeybee colonies. *Proc. R. Soc. Lond. B*. 258: 1-7.

Ever, C.A. and Seeley, T.D. 1986. Kin discrimination and aggression in honey bee colonies with laying workers. *Animal Behaviour.* 34: 924-944.

Fewell, J.H. 2003. Social insect networks. *Science*. 301:1867-1870.

Fewell, J.H. and Bertram, S.M. 1999. Division of labor in a dynamic environment: response by honeybees (*Apis mellifera*) to graded changes in colony pollen stores. *Behav. Ecol. Sociobiol*. 46:171-179.

Foster, K. R., Seppa, P., Ratnieks, F. L. W. and Thoren, P. A. 1999. Low paternity in the hornet *Vespa crabro* indicates that multiple mating by queens is derived in vespine wasps. *Behav. Ecol. Socio*. 46, 252-257.

Foster, K.R. and Ratnieks, F.L.W. 2000. Facultative worker policing in a wasp. *Nature*. 407: 692-693.

Foster, K.R. and Ratnieks, F.L.W. 2001. Paternity, reproduction and conflict in vespine wasps: a model system for testing kin selection predictions. *Behav. Ecol. Sociobiol.* 50: 1-8.

Foster, K.R. Gulliver, J. and Ratnieks, F.L.W. 2002. Worker policing in the European hornet *Vespa crabro*. *Insectes Soc.* 49: 41-44.

Franck, P., Garnery, L., Solignac, M. and Cornuet, J.M. 1998. The origin of west European subspecies of honeybees (*Apis mellifera*) : new insights from microsatellite and mitochondrail DNA. *Evolution* 52(4) : 1119-1134.

Franck, P., Garnery, L., Solignac, M. and Cornuet, J.M. 2000a. Molecular confirmation of a fourth lineage in honeybees from the Near East. *Apidologie*. 31 : 167-180.

Frank, P., Koeniger, N., Lahner, G., Crewe, R.M., and Solignac, M. 2000b. Evolution of extreme polyandry: an estimate of mating frequency in two

African honeybee subspecies, *Apis mellifera monticola* and *A. m. scutellata*. *Insectes Soc*. 47: 364-370.

Franck, P., Ganery, L., Loiseau, A., Oldroyd, B.P., Hepburn, H.R., Solignac, M. and Cornuet, J.-M. 2001. Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. *Heredity*. 86: 420-430.

Frank, P., Solignac, M., Vautrin, Cornuet, J.M., Koeniger, G. and Koeniger, N. 2002. Sperm competition and last-male precedence in the honeybee. *Animal Behaviour.* 64:503-509.

Free, J.B. 1958. The drifting of honeybees. *J. Agric. Sci.* 51: 294-306.

Free, J.B. 1981. Biology and behavior of the honey bee, *Apis florea*, and possibilities for beekeeping. *Bee World*. 62(2): 46-59.

Free, J.B. 1987. *Pheromones of Social Bees*. Chapman & Hall, London.

Fries, I. and Camazine, S. 2001. Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie*. 32:199-214.

Gadau, J., Strehl, C.-P., Oettler, J. and Hölldobler, B. 2003. Determinants of intracolonial relatedness in *Pogonomyrmex rugosus* (Hymenoptera; Formicidae): mating frequency and brood raids. *Mol. Ecol*. 12: 1931-1938.

Garnery, L., Vautrin, D., Cornuet, J.M., and Solignac, M. 1991. Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie*. 22: 87-92.

Gobin, B., Billen, J. and Peeters, C. 1999. Policing behaviour towards virgin eggs layers in a polygynous ponerine ant. *Animal Behaviour.* 58: 1-6.

Greeff, J.M. 1996. Effects of thelytokous worker reproduction on kin-selection and conflict in the Cape honeybee, *Apis mellifera capensis*. *Phil. Trans. R. Soc. Lond. B*. 351: 617-625.

Greeff, J.M. 1997. The Cape honeybee and her way north: an evolutionary perspective. *S. Afr. J. Sci.* 93. 306-308.

Halling, L.A. and Oldroyd, B.P. 2003. Do policing honeybee (*Apis mellifera*) workers target eggs in drone comb? *Insectes Soc*. 50: 59-61.

Halling, L., Oldroyd, B.P., Wattanachaiyingcharoen, W., Barron, A.B., Nanork, P. and Wongsiri, S. 2001. Worker policing in the bee *Apis florea*. *Behav. Ecol. Sociobiol*. 49: 509-513.

Hamilton, W.D. 1964. The genetical evolution of social behaviour I. *J. Theor. Biol*. 7: 1-16.

Jay, S.C. 1970. The effect of various combinations of immature queen and worker bees on the ovary development of worker honeybees in colonies with and without queens. *Can J. Zool*. 48: 169-173.

Jones, J.C., Myerscough, M.R., Graham, S. and Oldroyd, B.P. 2004. Honey bee nest thermoregulation: diversity promotes stability. *Science*. 305: 402-404.

Katzav-Gonzansky, T., Soroker, V., Francke, W. and Hefetz, A. 2003. Honeybee egg-laying workers mimic a queen signal. *Insectes Soc*. 50: 20-23.

Katzav-Gozansky, T., Soroker, V. and Hefetz, A. 2002a. Evolution of worker sterility in honey bees: egg-laying workers express queen-like secretions in Dufour's gland. *Behav. Ecol. Sociobiol*. 51: 588-589.

Katzav-Gozansky, T., Soroker, V., Hefetz, A. 2002b. Honeybees Dufour's gland - idiosyncrasy of a new queen signal. *Apidologie*. 33:525-537.

Katzav-Gozansky, T., Soroker, V., Hefetz, A., Cojocaru, M., Erdmann, D.H. and Franke, W. 1997. Plasticity of caste-specific Dufour's gland secretion in the honey bee. *Naturwissenschaften*. 84: 238-241.

Katzav-Gozansky, T., Soroker, V., Ibarra, F., Francke, W. and Hefetz, A. 2001. Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal? *Behav. Ecol. Sociobiol*. 51: 76-86.

Keller, L. and Nonacs, P. 1993. The role of queen pheromones in social insects: queen control or queen signal. *Animal Behaviour*. 45: 787-794.

Keller, L. and Reeve, H.K. 1994. Genetic variability, queen number, and polyandry in social Hymenoptera. *Evolution*. 48: 694-704.

Kikuta, N. and Tsuji, K. 1999. Queen and worker policing in the monogynous and monandrous ant, *Diacamma* sp. *Behav. Ecol. Sociobiol.* 46: 180-189.

Koeniger, N., and Koeniger, G. 2000. Reproductive isolation among species of the genus *Apis*. *Apidologie*. 31: 313-339.

Lekprayoon, C. and Wongsiri, S. 1989. Queen production with *Apis florea* in Thailand. *Proceedings of the first Asian-Pacific conference of Entomology.* Chiang Mai.

Lewontin, R.C., Felsenstein, J. 1965. The robustness of homogeneity tests in 2x2 tables. *Biometrics*. 21: 19-33.

Lockhart, P.J., Steel, M.A., Hendy, M.D. and Penny, D. Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* 11: 605-612.

Lopez-Vaamonde, C., Konning, J.W., Brown, R.M., Jordan, W.C. and Bourke, F.G. 2004. Social parasitism by male-producing reproductive workers in a eusocial insect. *Nature*. 430: 557-560.

Lord, W.G. and Nagi, S.K. 1987. *Apis florea* discovered in Africa. *Bee World*. 68: 39-40.

Martin, C.; Oldroyd, B.P.; and Beekman, M. 2004. Differntial reproductive success among subfamilies in queenless honey bee colonies (*Apis mellifera* L.) *Behav. Ecol. Sociobiol*. 56: 42-49.

Martin, S.J., Beekman, M., Wossler, T.C. and Ratnieks, F.L.W. 2002a. Parasitic Cape honeybee workers, *Apis mellifera capensis*, evade policing. *Nature*. 415: 163-165.

Martin, S.J., Châline, N., Oldroyd, B.P., Jones, G.R. and Ratnieks, F.L.W. 2004a. Egg marking pheromones of anarchistic worker honeybees (*Apis mellifera*). *Behav. Ecol*. 15:839-844

Martin, S.J., Jones, G.R., Châline, N. and Ratnieks, F.L.W. 2004b. Role of hydrocarbons in egg recognition in the honeybee. *Phys. Ent*. 29:395-399.

Martin, S.J., Jones, G.R., Ratnieks, F.L.W., Middleton, H. and Châline, N. 2002b. Reassessing the role of the honeybee (*Apis mellifera*) Dufour's gland in egg marking. *Naturwissenschaften*. 89: 528-532.

McNally, L. C. and Schneider, S. S. 1996. Spatial distribution and nesting biology of colonies in the African honey bee *Apis mellifera scutellata* (Hymenoptera: Apidae) in Botswana, Africa. *Environmental Entomology*. 25 (3): 643-652.

Miller, D.G. and Ratnieks, F.L.W. 2001. The timing of worker reproduction and breakdown of policing behaviour in queenless honey bee (*Apis mellifera* L.) societies. *Insectes Soc*. 48: 178-184.

Mogga, J. and Ruttner, F. (1988). *Apis florea* in Africa: source of the founder population. *Bee World*. 69(3): 100-103.

Monnin, T. and Ratnieks, F.L.W. 2001. Policing in queenless ponerine ants. *Behav. Ecol. Sociobiol*. 50: 97-108.

Montague, C.E. and Oldroyd, B.P. 1998. The evolution of worker sterility in honey bees: an investigation into a behavioral mutant causing failure of worker policing. *Evolution*. 52: 1408-1415.

Moritz, R.F.A., Kryger, P. and Allsopp, M.H. 1999. Lack of worker policing in the Cape honeybee (*Apis mellifera capensis*). *Behaviour*. 136: 1079-1092.

Moritz, R.F.A., Kryger, P., Koeniger, N. Estoup, A., and Tingek, S. 1995. High degree of polyandry in *Apis dorsata* queens detected by DNA microsatellite variability. *Behav. Ecol. Sociobiol.* 37: 357-363.

Moritz, R.F.A. and Southwick, E.E. 1992. *Bees as Superorganisms: An Evolutionary Reality.* Springer-Verlag. Berlin.

Nanork, P., Parr, J., Chapman, N.C., Wongsiri, S. and Oldroyd, B.P. 2005. Asian honeybees parasitize the future dead. *Nature*. 437: 829.

Neumann, P. and Hepburn, R. 2001. Behavioural basis for social parasitism of cape honeybees (*Apis mallifera capensis*). *Apidologie*. 33: 165-192.

Nuemann, P. and Moritz, R.F.A. 2002. The Cape honeybee phenomenon: the sympatric evolution of the social parasite in real time? *Behav. Ecol. Sociobiol.* 52:271-281.

Neumann, P., Moritz, R.F.A. and Mautz, D. 2000. Colony evaluation is not affected by drifting of drone and worker honeybees (*Apis mellifera* L.) at a performance testing apiary. *Apidologie*. 31: 67-79.

Neumann, P., Radloff, S.E., Moritz, R.F.A., Hepburn, H.R. and Reece, S.L. 2001. Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid host colonies. *Behav. Ecol*. 12(4); 419-428.

Noonan, C. 1986. Recognition of queen larvae by worker honeybees (*Apis mellifera*). *Ethology*. 73: 295-306.

Oldroyd, B.P. 2002. The Cape honey bee: an example of a social cancer. *Trends Ecol. Evol*. 17(6): 249-251.

Oldroyd, B.P., Clifton, M.J., Parker, K., Wongsiri, S., Rinderer, T.E. and Crozier, R.H. 1998. Evolution of mating behavior in the genus *Apis* and an estimate of mating frequency in *A. cerana* (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am*. 91: 700-709.

Oldroyd, B.P., Clifton, M.J., Wongsiri, S., Rinderer, T.E., Sylvester, H.A. and Crozier, R.H. 1997. Polyandry in the genus *Apis*, particularly *Apis andreniformis*. *Behav. Ecol. Sociobiol*. 40: 17-26.

Oldroyd, B.P., Halling, L.A., Good, G., Wattanachaiyingcharoen, W., Barron, A.B., Nanork, P., Wongsiri, S. and Ratnieks, F.L.W. 2001. Worker policing and worker reproduction in *Apis cerana*. *Behav. Ecol. Sociobiol.* 50: 371-377.

Oldroyd, B.P. and Osborne, K.E. 1999. The evolution of worker sterility in honeybees: the genetic basis of failure of worker policing. *Proc. R. Soc. Lond. B*. 266: 1335-1339.

Oldroyd, B.P. and Ratnieks, F.L.W. 2000. Evolution of worker sterility in honey bees: how anarchistic workers evade worker policing by laying eggs that have low removal rates. *Behav. Ecol. Sociobiol*. 47: 268-273.

Oldroyd, B.P., Ratnieks, F.L.W. and Wossler, T.C. 2002. Egg-marking pheromones in honey-bees *Apis mellifera*. *Behav. Ecol. Sociobiol*. 51: 590-591.

Oldroyd, B.P., Rinderer, T.E. and Buco, S.M. 1992a. Intracolonial foraging specialism by honey bees (*Apis mellifera*) (Hymenoptera: Apidae). *Behav. Ecol. Sociobiol*. 30: 291-295.

Oldroyd, B.P., Rinderer, T.E., Buco, S.M. and Beaman, L.D. 1993. Genetic variance in honey bees for preferred foraging distance. *Anim. Behav*. 45: 323-332.

Oldroyd, B.P., Rinderer, T.E., Harbo, J.R. and Buco, S.M. 1992b. Effects of intracolonial genetic diversity on honey bee (Hymenoptera: Apidae) colony performance. *Ann. Entomol. Soc. Am*. 85: 335-343.

Oldroyd, B.P., Rinderer, T.E., Schwenke, J.R. and Buco, S.M. 1994a. Subfamily recognition and task specialization in honey bees (*Apis mellifera* L.) (Hymonoptera: Apidae). *Behav. Ecol. Sociobiol*. 34: 169-173.

Oldroyd, B.P., Smolenski, A.J., Cornuet, J.-M. and Crozier, R.H. 1994b. Anarchy in the beehive. *Nature*. 371: 749.

Oldroyd, B.P., Smolenski, A.J., Cornuet, J.-M., Wongsiri, S., Estoup, A., Rinderer, T.E., et al. 1995. Levels of polyandry and intracolonial genetic relationships in *Apis florea*. *Behav. Ecol. Sociobiol*. 37: 329-335.

Oldroyd, B.P., Smolenski, A.J., Cornuet, J.-M., Wongsiri, S., Estoup, A., Rinderer, T.E., et al. Crozier, R.H. 1996. Levels of polyandry and intracolonial genetic relationships in *Apis dorsata* (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am*. 89: 276-283.

Oldroyd, B.P., Sylvester, H.A., Wongsiri, S. and Rinderer, T.E. 1994c. Task specialization in a wild bee, *Apis florea* (Hymenoptera: Apidae), revealed by RFLP banding. *Behav. Ecol. Sociobiol*. 34: 25-30.

Oldroyd, B.P. and Wongsiri, S. 2006. *Asian honey bee: biology, conservation, and human interaction*. Harvard University Press. inpress.

Osborne, K.E. and Oldroyd, B.P. 1999. Possible causes of reproductive dominance during emergency queen rearing by honeybees. *Animal Behaviour*. 58: 267-272.

Otis, G. W. 1991. A review of the diversity of species within *Apis*. In Smith, D. R. (ed.), *Diversity in the Genus Apis*, pp. 29-49. Westview Press. Oxford.

Page, R.E. 1980. The evolution of multiple mating behavior by honey bee queens (*Apis mellifera* L.). *Genetics*. 96: 263-273.

Page, R.E. 1997. The evolution of insect societies. *Endevour*. 21:114-120.

Page, R.E. and Erickson, E.H. 1988. Reproduction by worker honey bees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* 23: 117-126.

Page, R.E. and Robinson, G.E. 1994. Reproductive competition in queenless honey bee colonies (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* 35: 99-107.

Page, R.E., Robinson, G.E., Calderone, N.E. and Rothenbuhler, W.C. 1989. Genetic structure, division of labor, and the evolution of insect societies. In: Breed, M.D., Page, R.E. (eds) *The Genetics of Social Evolution*. Westview Press, Boulder, pp 15-30.

Page, R.E., Robinson, G.E., Fondrk, M.K., and Nasr, M.E. 1995. Effects of worker genotypic diversity on honey bee colony development and behavior (*Apis mellifera*). *Behav. Ecol. Sociobiol.* 36: 387-396.

Palmer, K.A. and Oldroyd, B.P. 2000. Evolution of multiple mating in the genus *Apis*. *Apidologie*. 31: 235-248.

Palmer, K.A. and Oldroyd, B.P. 2001. Mating frequency in *Apis florea* revised (Hymenoptera, Apidae). *Insectes Soc*. 48: 40-43.

Palmer, K.; Oldroyd, B. P.; Franck, P.; and Hadisoesilo, S. 2001. Very high paternity frequency in *Apis nigrocinta*. *Insectes Soc*. 48: 327-332.

Pamilo, P., Sundström, L., Fortelius, W., and Rosengren, R. 1994. Diploid males and colony level selection in Formica ants. *Ethol. Ecol. Evol*. 6:221–235.

Parr, J., Oldroyd, B.P., Huettinger, E. and Kastberger, G. 2002. Drifting of workers in nest aggregations of the giant honeybee *Apis dorsata*. *Apidologie*. 33: 553-561.

Pfeiffer, K.J. and Crailsheim, K. 1998. Drifting of honeybees *Insectes Soc*. 45: 151-167.

Ratnieks, F.L.W. 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. *Am. Nat*. 132: 217-236.

Ratnieks, F.L.W. 1993. Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. *Behav. Ecol. Sociobiol*. 32: 191-198.

Ratnieks, F.L.W. 1995. Evidence for a queen-produced egg-marking pheromone and its use in worker policing in the honeybee. *J. Apic. Res*. 34: 31-37.

Ranieks, F.LW. 2000. Worker policing in the honey bee: basic facts and ideas. *Ins. Soc. Life*. 3: 3-10.

Ratnieks, F.L.W. and Reeve, H.K. 1992. Conflict in single-queen hymenopteran societies: the structure of conflict and processes that reduce conflict in advanced eusocial species. *J. Theor. Biol*. 158: 33-65.

Ratnieks, F.L.W. and Visscher, P.K. 1989. Worker policing in the honeybee. *Nature*. 342: 796-797.

Rinderer, T.E., Hellmich, R.L., Danka, R.G. and Collins, A.M. 1985. Male reproductive parasitism: a factor in the Africanization of European honey-bee populations. *Science*. 228: 1119-1121.

Rinderer, T.E., Stelzer, J.A., Oldroyd, B.P. and Tingek, S. 1998. Levels of polyandry and intracolonial genetic relationships in *Apis koschevnikovi. J. Apic. Res*. 37: 281-287.

Robinson, G.E., Page, R.E. and Arensen, N. 1994. Genotypic differences in brood rearing in honey bee colonies: context specific? *Behav. Ecol. Sociobiol.* 34: 125-137.

Robinson, G.E., Page, R.E. and Fondrk, M.K. 1990. Intracolonial behavioral variation in worker oviposition, oophagy, and larval care in queenless honey bee colonies. *Behav. Ecol. Sociobiol*. 26: 315-323.

Rowe, D. J.; Rinderer, T. E.; Stelzer, J. A.; Oldroyd, B. P.; and Crozier, R. H. 1997. Seven polymorphic microsatellite loci in honeybees. *Insectes Soc*. 44: 85-93.

Rubik, D.W. 1989. *Ecology and natural history of tropical bees.* Cambridge University Press. Cambridge.

Ruttner, F. 1988. *Biogeography and Taxonomy of Honeybees*. Springer-Verlag, Berlin.

Sakagami, S.F. and Akahira, Y. 1958. Comparative of ovarian size and number of ovarioles between workers of Japanese in European honey bees. *Kontyo*. 26: 103-107.

Schlotterer, C. 1998. Microsatellites. In Hoelzel, A. R. (ed.), *Molecular Genatic Analysis of Populations: A practical Approch*, pp. 237-262. Oxford University Press. New York.

Schmid-Hempel P. 1995. Parasites and social insects. *Apidoligie*. 26: 255-271.

Schmid-Hempel, P. 1998. *Parasites in social insects*. Princeton University Press. N.J.

Seeley, T.D. 1985. *Honeybee Ecology*. Princeton University Press, Princeton, N.J.

Seeley, T.D. 1989. The honey bee colony colony as a superorganism. *Am. Sci.* 77: 546-553.

Sherman, P.W., Seeley, T.D. and Reeve, H.K. 1988. Parasites, pathogens and polyandry in social hymenoptera. *Am. Nat*. 131: 602-610.

Sittipraneed, S.; Laoaroon, S.; Klinbunga, S.; and Wongsiri, S. 2001. Genetic differentiation of honey bee (*Apis cerana*) in Thailand: evidence from microsatellite polymorphism. *J. Api. Res.* 40(1): 9-16.

Sole, C.L., Kryger, P., Hefetz, A., Katzav-Gonzansky, T. and Crewe, R.M. 2002. Mimicry of queen Dufour's gland secretions by workers of *Apis mellifera scutellata* and *A. m. capensis*. *Naturwissenschaften*. 89: 561-564.

Solignac, M., Vautrin, D., Loiseau, A. Mougel, F., Baudry, E. et al. 2003. Five hundred and fifty microsatellite markers for the study of the honeybee (*Apis mellifera* L.) genome. *Molecular Ecology Notes*. 3: 307-311.

Starr, C.K. 1984. Sperm competition, kinship and sociality in the aculeate Hymenoptera. In: Smith, R.L. (ed.) *Sperm competition and the evolution of animal mating systems*. Academic Press, Orlando, F.L., pp 427-464.

Tabler, S. 1961. Forceps designed for transferring honey bee eggs. *J. Econ. Entomol.* 54: 247-250.

Tilley, C.A. and Oldroyd, B.P. 1997. Unequal subfamily proportions among honey bee queen and worker brood. *Animal Behaviour*. 54: 1483-1490.

Tirgari, S. 1971. On the biology and manipulation of *Apis* (*Macrapis*) *florea* F. in Iran. *Proc. Int. Apic. Congr.* 23: 330-332.

Villesen, P., Gertsch, P.J., Frydenberg, J., Mueller, U.G. and Boomsma, J.J. 1999. Evolutionary transition from single to multiple mating in fungus-growing ants. *Mol. Ecol*. 8: 1819-1825.

Viard, F.; Franck, P.; Dubois, M. P.; Estoup, A.; and Jarne, P. 1998. Variation of microsatellite size homoplasy across electromorphs, loci, and populations in three vertebrate species. *J. of Molecular Evolution*. 47: 42-51.

Visscher, P.K. 1989. A quantitative study of worker reproduction in honey bee colonies. *Behav. Ecol. Sociobiol*. 25: 247-254.

Visscher, P.K. 1996. Reproductive conflict in honey bees: a stalemate of worker egg-laying and policing. *Behav. Ecol. Sociobiol*. 39: 237-244.

Visscher, P.K. and Dukas, R. 1995. Honey bees recognize development of nestmates' ovaries. *Animal Behaviour*. 49:542-544.

Walsh, P.S., Metzger, D.A. and Higuchi, R. 1991. Chelex ® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*. 10: 507.

Wattanachaiyingcharoen, W., Oldroyd, B.P., Good, G., Halling, L., Ratnieks, F.L.W. and Wongsiri, S. 2002. Lack of worker reproduction in the giant honey bee *Apis dorsata* Fabricius. *Insectes Soc*. 49: 80-85.

Wattanachaiyingcharoen, W., Oldroyd, B.P., Wongsiri, S., Palmer, K., and Parr, J. 2003. A scientific note on the mating frequency of *Apis dorsata*. *Apidologie*. 34: 85-86.

Wenseleers, T., Hart, A.G. and Ratnieks, F.L.W. 2004a. When resistance is useless: and the evolution of reproductive acquiescence in insect societies. *The American Naturalist*. 164(6): E154-E167.

Wenseleers, T., Hart, A.G., Ratnieks, F.L.W., Ribeiro, M.F., Alves, D.A. and Imperatriz-Fonseca, V.-L. 2005. Working-class royalty: bees beat the caste system. *Biology Letters*. 1: 125-128.

Wenseleers, T., Helanterä, H., Hart, A.G. and Ratnieks, F.L.W. 2004b. Worker reproduction and policing in insect societies: an EES analysis. *J. Evol. Biol.* 17: 1035-1047.

Wilson, E.O. 1971. *The Insect Societies*. Harvard University Press, Cambridge, Mass.

Winston, M.L. 1987. *The Biology of the Honey Bee*. Harvard University Press, Cambridge, Mass.

Winston, M.L. and Slessor, K.N. 1998. Honey bee primer pheromones and colony organization: gaps in our knowledge. *Apidologie*. 29: 81-95.

Wongsiri, S. 1989. *Biology of honey bee* (in Thai). Ton-or Press. Bangkok. pp. 77-86.

Wongsiri, S. 1995. Queen production of *Apis cerana*. In: Kevan, P.G. (ed) *The Asiatic hive bee: Apiculture, biology and role in sustainable development in tropical and subtropical Asia*. Enviroquest, Ltd. Ontario, pp.91-106.

Wongsiri, S., Lekprayoon, C., Thapa, R., Therakhupt, K., Rinderer, T.E., Sylvester, H.A., Oldroyd, B.P. and Booncham, U. 1996. Comparative biology of *Apis andreniformis* and *Apis florea* in Thailand. *Bee World.* 77(4): 23-35.

Zar, J.H. 1996. *Biostatistical Analysis*. Prentice-Hall, Upper Sadle River, N.J.

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