Reproductive conflict in the honey bee

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A thesis submitted to the University of Sheffield for the degree of Doctor of Philosophy

Department of Animal and Plant Sciences

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Within an insect society individuals are not equally related, which often leads to reproductive conflicts among them. This thesis investigates reproductive conflict in the honey bee. The main conflict investigated is that over male production. This is an important conflict as workers can lay unfertilised male eggs. In honey bees, this conflict is resolved by worker policing in which workers eat worker-laid eggs. Policing is selectively favoured because of the low average relatedness among workers due to polyandry. A naturally occurring anarchistic colony, in which workers evade normal policing and many workers' sons are reared, was investigated. About half the patrilines produced males, which caused a loss of inclusive fitness for the worker collective, contrary to previously described anarchistic colonies. In hopelessly queenless colonies, worker policing usually stops and the workers rear a last batch of males before the colony dies. Another atypical colony was investigated in which worker policing was not switched off despite being queenless. This caused a maladaptive phenotype in which many workers activated their ovaries and laid eggs that were not reared, leading to the colony producing no males before dying. Variation in worker policing was also investigated in normal queenright colonies. Large between colony variations occurred, and a lower egg policing rate in worker cells, where workers normally do not lay, could reduce the cost of mistaken removal of queen-laid eggs.

Another potential conflict in honey bee societies concerns queen rearing. Workers may increase their inclusive fitness by favouring full-sister queens. Here I show that nepotism was absent in a previously unstudied context, the confinement of queens in their cells by workers. This confirms the general absence of nepotism in queen replacement, which is a largely cooperative process. Last, I investigated the learning abilities of bees towards cuticular hydrocarbons, which are thought to play a role in kin and nestmate recognition. Unsaturated alkenes are shown to be the most likely candidate compounds for recognition in honey bees.

Biographical Sketch

Nicolas Châline was born on the 2nd of March 1973 in Mantes la Jolie, France. His early education took place in Lisieux at the Marie Curie School and the Lycée Gambier. After his Baccalauréat in 1990, he spent three years in Rouen where he did his preparatory classes for the Biology "Grandes Ecoles" at the Lycée Pierre Corneille. He was then admitted to the National Agronomy Institute of Paris-Grignon in 1993, which trains Life engineers, and in the third year he chose to do an MSc in Population Biology, Genetics and Eco-Ethology (DEA) at Tours University. In 1997, he was called for his national service which he did in Martinique as an IT engineer. In August 1998, he moved to Luton, Beds, and then started working at the University of Sheffield in June 1999 in the Department of Animal and Plant Sciences as a research technician in the Sheffield Molecular Genetics Facility. He officially started his PhD on the 1st of October 2001.

I dedicate this thesis to Aude and Ambre, and to my grandfather, Roger Guillamaud, who introduced me to entomology.

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Publications arising from this thesis

The publications arising from this thesis and the corresponding chapters are listed below.

Chapter 4: Châline N, Ratnieks FLW, Raine NE, Badcock NS, Burke T. 2004. Non-lethal sampling of honey bee, *Apis mellifera*, DNA using wing tips. Apidologie, 35, 311-318.

Chapter 5: Châline N, Ratnieks FLW, Burke T. 2002. Anarchy in the UK: Detailed analysis of worker reproduction in a naturally-occurring British anarchistic honeybee, *Apis mellifera*, colony using microsatellite markers. Molecular Ecology, 11, 1795-1803.

Chapter 6: Châline N, Martin SJ, Ratnieks FLW. 2004. Worker policing persists in a hopelessly queenless honey bee colony (*Apis mellifera*). Insectes sociaux, 51, 113-116.

Chapter 8: Châline N, Martin SJ, Ratnieks FLW. In press. Lack of nepotism in honey bee (*Apis mellifera*) interactions between workers and young queens imprisoned in queen cells during swarming. Behavioral Ecology.

Chapter 9: Châline N, Sandoz JC, Martin SJ, Ratnieks FLW, Jones GR. Learning and discrimination of individual cuticular hydrocarbons by honey bees (*Apis mellifera*). (Submitted to Chemical Senses).

Chapter 7: Châline N, Martin SJ, Ratnieks FLW. The influence of cell size and intra and inter colony variability on worker policing in the honeybee, *Apis mellifera*. (In preparation).

Table of Contents

Chapter 1
General introduction

1. Reproductive altruism and Hamilton's inclusive fitness theory

Eusociality is characterised by three traits, which are summarized by Wilson (1971) as: 'Individuals of the same species cooperate in caring for the young; there is a reproductive division of labour, with more or less sterile individuals working on behalf of fecund individuals; and there is an overlap of at least two generations in life stages capable of contributing to colony labour, so that offspring assist parents during some periods of their life'. Reproductive division of labour is the most fundamental characteristic of eusociality and separates it from other forms of sociality, and has puzzled evolutionary biologists ever since Darwin elaborated his theory of natural selection (Darwin, 1859). Darwin saw the presence of sterile workers as a potentially serious problem for his theory as traits of non-reproducing individuals, such as workers, could not be directly transmitted to the next generation.

More generally, the existence of individuals, such as insect workers, who suffer reduced personal reproduction in order to help rear the offspring of another individual appears contrary to natural selection, which normally favours adaptations which increase direct reproduction. The solution of this paradox came with a broader concept of fitness, inclusive fitness (Hamilton 1964a, b), which explained how natural selection could favour altruism (including reproductive altruism) through what John Maynard–Smith (1964) termed 'kin selection' in order to distinguish it from group selection (Wynne-Edwards 1962, 1963). Inclusive fitness extends the direct fitness of an individual by including the fitness gained (or lost) by social actions that affect the reproductive success of relatives. That is, an individual can pass on copies of its own genes both by reproducing directly and by helping a relative to reproduce more, given that relatives have greater genetic similarity than randomly chosen individuals within a population. Kin selection is therefore "the natural selection of genes for social actions via the sharing of these genes between the performer of the action and its relative (kin)" (Bourke and Franks, 1995).

Hamilton showed that altruism is selected for if the product of the increase in reproductive fitness of the receiver of the altruistic act (b) multiplied by the relatedness between the actor and the receiver (r) is greater than the direct reproductive fitness cost incurred by the actor (c). This simple rule (now known as Hamilton's Rule) has led to great advances in our understanding of social behaviour.

Hamilton's Rule shows that the relatedness between interacting individuals is important in the evolution of altruism. However, theoretical developments post-Hamilton have shown that relatedness can also create differences in the reproductive interests of individuals in social groups. Because societies are not generally clonal, kin selection theory predicts that potential conflicts of interest will occur between differently related individuals or groups of individuals (Ratnieks and Reeve 1992, Sundström and Boomsma 2001). These relatedness differences can cause conflict, manipulation and power struggles (Beekman and Ratnieks 2003). A major challenge for evolutionary biologists is to document these potential conflicts and to understand how they can be resolved without loss of social cohesion (Keller, 1999).

Although kin selection theory has been useful in understanding social evolution across the whole range of living organisms, including bacteria, slime moulds, animals and plants, it has probably been most successful and influential in understanding insect societies, particularly the social Hymenoptera (Ratnieks et al. 2001) which in turn have provided some of the best support for the theory itself. In Hymenoptera, males are haploid and develop from unfertilised eggs. This causes the relatedness among relatives to differ from that of diploid organisms. Because males pass their whole genome to their daughters, full sisters (females that have the same mother and father) are related by 0.75, whereas a mother is only related to her daughters by 0.5. This led Hamilton (Hamilton 1964a, b) to suggest that female Hymenoptera would benefit from raising sisters rather than daughters because they are more related to their sisters. The '3/4 relatedness' hypothesis was thought to explain why eusociality has evolved many times independently in the Hymenoptera and few times in diploid organisms (at the time, the only other known eusocial animals were the termites, which are diploid). However, this argument is in fact much weaker than originally put forward because workers are only related to their brothers by 0.25, making the mean relatedness to siblings the same as in diploids, 0.5 (Trivers and Hare 1976, Ratnieks et al. 2001). However, haplodiploidy together with ecological characteristics of the Hymenoptera, particularly the fact that

many solitary species have nest building and maternal care of brood, is still thought to have facilitated the evolution of sociality (reviewed in Bourke and Franks 1995). Although Hamilton was probably incorrect in his 3/4 relatedness explanation for the multiple evolution of sociality in the Hymenoptera, inclusive fitness theory has made many other testable predictions and led to theoretical, conceptual and empirical advances which have transformed our vision of conflict and cooperation in insect societies (Ratnieks et al. 2001).

2. Reproductive conflicts in eusocial Hymenoptera

Hymenopteran societies are characterised by reproductive division of labour among females (queens and workers). Although in many species there are no distinct morphological castes (e.g. Halictid bees, Michener 1974; Polistine wasps, Turillazzi and West-Eberhard 1996; queenless ponerine ants, Peeters 1993), in highly eusocial species these roles or castes are based on morphological differences, in which workers have reduced or even zero ability to reproduce directly. Highly eusocial species typically have one to many morphologically distinct queens (Keller 1993) which are the main reproductive females and from dozens to millions of workers with lesser reproductive potential and which perform the other tasks necessary for their colony to survive. Males usually do not take part in colony life. Mating normally occurs outside the nest with unrelated queens (with the notable exception of the army ants).

Relatedness differences in colonies of social Hymenoptera create a variety of conflicts between colony members. The honey bee (Hymenoptera: *Apis mellifera*) has been a particularly important model organism in studies of social conflict and it is the subject of this thesis. General conflicts within social Hymenoptera will first be presented briefly. Those which are particularly relevant to the honey bee will then be discussed in the following parts of this chapter.

In general, a colony of eusocial Hymenoptera can be considered to consist of three main ‘interest groups’ with their own reproductive interests and power to promote these interests: the queen, workers as individuals and the workers collectively. Conflicts of interest can occur between any or all of these groups. For example, a given worker may have different interests to the worker collective, who may themselves have different interests to the queen. The first conflict of interest between workers and queens to have received the attention of social insect researchers was the conflict over

colony sex-ratio. Workers in hymenopteran societies are more related to their full sisters (0.75) than to their brothers (0.25). This causes the optimal sex investment ratios for the mother queen heading a colony and her daughter workers collectively to differ, with the workers benefiting from a female-biased ratio (Trivers and Hare 1976). Queens can control the sex ratio of the eggs they lay (Ratnieks and Keller 1998) but workers, who care for the brood can also manipulate the final colony sex ratio by preferentially killing male larvae (Trivers and Hare 1976, Sundström et al. 1996, Foster and Ratnieks 2001b) or investing more in queen larvae (Hammond et al. 2002). The end point of this conflict seems to be a partial but incomplete victory for the workers as colonies with singly mated monogynous queens mostly have a sex-ratio that is significantly more female biased than the queen optimum, 1: 1, though not as female biased as 3:1 (Trivers and Hare, 1976). In slave making species, the sex ratio appears to be 1:1, presumably because the workers are not involved in brood rearing, which is carried out by allospecific slaves, thereby allowing the queen to win the conflict (Trivers and Hare 1976, Bourke and Franks 1995).

Trivers and Hare's predictions applied to colonies headed by a single queen mated to a single unrelated male, and with all males reared in the population being queens' sons. However, the kin structure of hymenopteran colonies includes considerable variation both within and among species. Polygyny (the presence of multiple queens) and polyandry (mating of queens to multiple males with resultant multiple paternity of female offspring) are both common (Crozier and Pamilo 1996, Bourke and Franks 1995), even if colonies with a single queen mated to a single male, as assumed by Trivers and Hare (1976) is the most typical kin structure (Boomsma and Ratnieks 1996, Strassmann 2001). Multiple mating by queens can diminish some of the conflicts between queens and workers by bringing their interests closer (Sundström and Boomsma 2001). For example, in sex ratio conflicts, extreme multiple mating brings the optimal sex-ratio of workers closer to 1:1, which is also the optimum of the queen (Bourke and Franks 1995, Crozier and Pamilo 1996) (multiple mating also reduces conflict over male parentage, detailed below in Section 4). Experimental evidence for this comes from populations of *Formica truncorum* and *Formica exsecta* in which colonies headed by singly and multiply mated queens bias the sex ratio accordingly (Sundström 1995, Sundström et al. 1996). However, multiple mating also reduces the mean genetic relatedness among colony members, increasing some areas of potential conflict, such as over the caste fate, queen or worker, of immature females (Wenseleers

and Ratnieks 2004) and introducing additional potential conflicts, such as over the rearing of full-sister versus half-sister queens.

In most species of eusocial Hymenoptera with morphologically distinct queens and workers, the workers have lost the ability to mate but retain functional ovaries and can lay unfertilised eggs that develop into males if reared (Page and Erickson 1988, Visscher 1989). In some species, the workers are fully sterile, with vestigial ovaries (Oster and Wilson 1978), and in some others they lay diploid eggs via thelytoky (*Apis mellifera capensis*, Anderson 1963 and *Cataglyphis cursor*, Cagniant 1980). Haplodiploidy also means that females are more related to their own sons (r = 0.5) than to the sons of full-sisters (full-nephews, r=0.375) or the queen (brothers, r=0.25). This leads to conflict among all females in the colony, both workers and queens, over the production of males. A worker is always more related to her own sons than to any other male that could be reared in the colony, but the average relatedness of workers to worker-produced males changes with the number of fathers (i.e., when the mother queen is mated to more than one male) or mothers (i.e., when the colony is headed by more than one egg-laying queen). These changes in relatedness alter the interests of the workers collectively with regards to the rearing queen-laid versus worker-laid eggs. In species with a queen mated to a single male, workers benefit from rearing other workers' sons over the queen's sons. But when the mother queen is mated to more than two unrelated males, the workers will be less related on average to other workers' sons than to the queen's sons. The workers now benefit from preventing each other from reproducing, instead rearing the queen's sons (Ratnieks 1988, Starr 1984, Woyciechowski and Lomnicki 1987). One of the mechanisms used in this worker policing is eating worker-laid eggs (Ratnieks 1988).

A further prediction of kin selection theory is the differential treatment of differently related nestmate females, in particular immature queens. Consequently, the presence of different matrilines or patrilines within a colony (caused by polygyny or polyandry) makes nepotism (the biasing of altruistic behaviour towards closer kin) a potential strategy for enhancing inclusive fitness (Visscher 1986). An example of when this could occur is during the production of new queens (Visscher 1998).

3. The importance of polyandry in social Hymenoptera

Because relatedness is so important, both as a term in Hamilton's rule and in affecting the structure of potential conflicts within societies, the number of queens in a society and the number and relationship of the males they mate with are important to know. Polygyny is frequent in ants (Keller 1991) and also occurs in some bees and wasps. However, polygyny, though important, is outside the scope of this thesis as honey bees, *Apis mellifera*, are typically monogynous (except for the short time when a failing mother queen is being superseded by a daughter queen and the two may coexist, see 5 and chapter 8). I will therefore focus on the occurrence of polyandry in social insects, which reaches its most extreme extent in honey bees (*Apis*) and its effects on the kin structure of the colony.

Polyandry, where a queen mates with more than one male, has been documented in many species of eusocial Hymenoptera using observation, number and volume of stored sperm, and genetic markers including visible markers of body colour, allozymes (for a review, see Crozier and Pamilo 1996, Table 4.1 therein) and DNA markers. For example, analysis of body colour markers and diploid male production had shown decades ago that honey bee queens were multiply mated. The development of DNA markers and in particular microsatellites (highly variable single locus markers characterised by short tandem repeats of nucleotides) now enables the precise assessment of paternity, and other aspects of kin structure, in insect colonies. Using microsatellites, *Apis mellifera* queens have been shown to mate with 7-20 males with a mean of 13.8 (Estoup et al. 1994, Tarpy and Nielsen 2002). Other studies have found that even greater numbers of matings can occur, for example 24 in Neumann et al. (1999). Paternity frequency is also very high in other *Apis* species, with the giant honey bee *Apis dorsata* being the most polyandrous with a mean of 80 mates (Oldroyd et al. 1996, Wattanachaiyingcharoen et al. 2003). Microsatellites also permit the documentation of within-species variation in mating frequency. For example, in *Vespa crabo*, Foster et al. (1999) found that although most queens mate with only one male, double and triple mating also occur. This pattern is widespread in Vespinae and Polistinae wasps, with *Vespula* species showing high levels of polyandry than *Dolichovespula* and *Vespa* (Ratnieks et al. 2001).

In colonies with a multiply mated queen, the paternity contribution of the different males that mate with the queen varies greatly and this must be taken into account when estimating the mean relatedness (Boomsma and Ratnieks 1996). The concept of effective paternity was introduced to allow for the different paternity contributions of males (Starr 1984, Pamilo 1993). Effective paternity (m_e) is defined as the reciprocal of the sum of the squared proportional paternities. It allows to calculate the mean relatedness in colonies (g) though the equation $g=0.25+1/m_e$ (Starr 1984, Pamilo 1993).

Using effective paternity, it is possible to calculate an overall population mating frequency (Foster et al. 2001), which is often lower than 2 even if multiple mating occurs, as in most Vespinae wasps (Foster et al. 1999). In *Apis mellifera*, Estoup et al. (1994) estimated that the mean effective paternity is 12.4, which is slightly lower than the mean number of matings (13.8). Sperm utilization by queens of *Apis mellifera* is fairly constant over time (Estoup et al. 1994), although during the first months of the queen's life there is some variation and sperm admixture improves progressively during this time (Franck et al. 1999). It appears that high effective paternities are actually quite rare in social Hymenoptera. Ants have a mean effective paternity of 1.43, which drops to 1.15 if the leaf cutter ants *Atta* and *Acromyrmex*, effective paternity = 2.14, are excluded. Stingless bees and bumblebees have mean effective paternities of 1.06 and 1.02 respectively. Polistine wasps have a mean effective number of 1.01. Vespinae wasps have a mean effective paternity of 1.12, excluding *Vespula* species which have a mean effective paternity of 3.68 (Strassmann 2001).

Multiple mating presumably has a cost for queens through increased risk of predation and energetic cost (Crozier and Fjerdingstad 2001). It, therefore, seems likely that there should be benefit to multiple mating. Many hypotheses have been advanced in order to explain this (see Table 1.1 modified from Tarpy and Page 2001). Polyandry appears to have evolved secondarily and extreme polyandry occurs only in a few highly eusocial clades such as the leafcutter ants, New and Old World army ants (*Eciton burchelli*, Denny et al. 2004, *Dorylus molestus*, Kronauer et al. 2004), *Apis* bees and *Vespula* wasps. Most importantly for this thesis, high mating frequencies influence the outcome of queen-worker conflicts towards the interest of the queen, especially regarding sex-allocation (Moritz 1985) and male production (Starr 1984,

Woyciechowski and Lomnicki 1987, Ratnieks 1988; see below). It also makes nepotism in queen rearing a possible strategy for workers to increase their inclusive fitness.

Table 1.1. A list of some hypotheses explaining the evolution and maintenance of social insect polyandry in general, and their applicability to honey bees in particular, modified from Tarpy and Page (2001).

Hypothesis	Description	Application to honey bees	References
Direct mechanisms			
1 Convenience	Easier to let multiple males mate than to resist	No, because queens take multiple mating flights	Alcock et al. (1977)
2 Sperm replenishment	Females mate multiply to bolster depleted sperm supply	No, because queens only mate once in their lifetime	Thornill (1976)
3. Sperm limitation	Need for/storage capacity is greater than what one male can provide	No, because males produce enough sperm to fill a queen's spermatheca (Bolten and Harbo 1982, Page and Metcalf 1984)	Cole (1983), Boomsma and Ratnieks (1996), Fjerdingstad and Boomsma (1998) But see Kraus et al. (2004)
4 Nutritive sperm	Males contribute beneficial proteins or compounds in the ejaculate	No, because queens lay eggs over a protracted time span	Lamunyon and Eisner (1994)
5 Fertility-certainty	'Bet-hedging' against mating with only an infertile male	No, because sperm transfer is inefficient (reviewed by Oldroyd et al. 1998)	Walker (1980), Ridley (1988)
Indirect mechanisms			
6 "Good genes"	Increase probability of mating with a genetically superior male	Yes, but no evidence for mate choice by queen bees (Gary 1963, Koeniger et al. 1990)	Thornill and Alcock (1983), Zeh (1997)
7 Sperm competition	Form of passive female choice that allows "best" male to win	Yes, but tested and not supported (Laidlaw and Page 1984, Haberl and Tautz 1998)	Parker (1984), Keller and Reeve (1995)
8 Effective population size	Increases the N_e of the population	No, because polygyny is more effective than polyandry to increase N_e (Crozier and Page 1985)	Wilson (1963)
9 Genetic variation			
a Task diversity	Increase fitness due to more diverse worker force	Yes	Oster and Wilson (1978), Robinson and Page (1989)
	More efficient exploitation of foraging environment	Yes	Oldroyd et al. (1991), Oldroyd et al. (1992), Oldroyd et al. (1993)
	Rare tasks	Yes, but only at extreme mating numbers	Robinson and Page (1988), Fuchs and Moritz (1999)
b Environmental tolerance	Allows for a buffer against environmental changes	Yes	Calderone et al. (1989), Page et al. (1995)
c Disease tolerance	Reduces the transmission of parasites and pathogens among colony members	Yes	Hamilton (1987), Sherman et al. (1988), Schmid-Hempel (1998), Palmer and Oldroyd (2003), Tarpy (2003)
d Genetic benefits	Reduces the optimal sex-ratio of the workers to that of the queen	Yes[1]	Moritz (1985), Woyciechowski and Lomnicki (1987), Pamilo (1991), Queller (1993), Ratnieks and Boomsma (1995), Sundstrom and Ratnieks (1998)
	Reduces the genetic load caused by the sex locus	Yes	Page (1980), Page and Metcalf (1982), Crozier and Page (1985), Ratnieks (1990), Pamilo et al. (1994), Crozier and Pamilo (1996)

1Difficult to distinguish cause from effect; polyandry may have evolved in honey bees for other reasons, and the approximate 1:1 sex ratio (Page and Metcalf 1984) is a consequence of the resultant change in genetic relatedness among female nestmates

4. Worker reproduction and worker policing

Worker reproduction is common in the eusocial Hymenoptera. This may lead to conflicts between the queen and the workers over the production of the males (Woyciechowski and Lomnicki 1987). The ability of workers to lay eggs varies (Monnin et al. 2003). In species where queen and worker roles are not based on morphological differentiation, workers can mate and lay both male and female eggs. This situation is common in Stenogastrinae and Polistinae wasps, and in Halictidae and Anthophoridae bees. In queenless Ponerinae ants workers are morphologically different from queens, having evolutionarily lost their wings, but retain full reproductive potential (Wilson 1971, Michener 1974, Monnin and Ratnieks 2001). In many species, workers cannot mate but retain functional ovaries and are able to produce unfertilised males that develop into males (as mentioned above). This is the case in eusocial Apidae bees (honey bees, Apinae, bumble bees, Bombinae, most stingless bees, Meliponinae), Vespinae wasps and most ants (Barron et al. 2001). Full sterility of workers that have vestigial ovaries is very rare although it well known in some ant genera (Oster and Wilson 1978) and has recently been discovered in stingless bees (Boleli et al. 1999)

Worker reproduction is at its most apparent when the queen heading the colony dies without being replaced and the colony becomes queenless (Robinson et al. 1990), a situation commonly observed in honey bees and most other species of eusocial Hymenoptera. In *Apis mellifera*, many workers activate their ovaries after the queen's death (Page and Erickson 1988, Robinson et al. 1990) and they start laying eggs within three weeks of becoming queenless if the queen is not replaced (they become hopelessly queenless), managing to rear a last batch of male brood before the colony dwindles due to the non-replacement of the worker force (Miller and Ratnieks 2001). In queenless colonies, there is a subfamily variation in the tendency for workers to activate their ovaries, lay eggs and have to have their eggs reared into adults (Robinson et al. 1990, Page and Robinson 1994, Martin et al. 2004a). Racial variation also occurs, with subspecies like *Apis mellifera capensis* colonies starting male production within only 6 days (Ruttner and Hesse 1979). In other honey bee species, like *Apis cerana*, the onset of worker reproduction in queenless colony is also fast, and within 5 days between 40-50% of the workers can have full sized eggs in their ovaries (Oldroyd et al. 2001).

Successful worker reproduction in queenright colonies is less common. There may be direct mechanisms by which the queen can control worker reproduction, such as by eating any worker-laid eggs ('queen policing', Ratnieks 1988). In honeybees, it is the presence of the queen and brood in the colony that inhibits the activation of ovaries in most workers. This is achieved via brood and queen pheromones (Arnold et al. 1994). Some authors postulate that rather than manipulating workers against their interests, the queen, queen-laid eggs (Endler et al. 2004) and brood pheromones give workers an honest signal of the queen fecundity (Keller and Nonacs 1993). The knowledge that the colony is queenright induces workers to refrain from activating their ovaries. This is known as self-policing (Ratnieks 1988). In other species where the number of individuals is lower, direct aggression towards laying workers and oophagy by the queen can be observed, as in the queenless ants *Diacamma* sp. and *Dinoponera quadriceps* (Gobin et al. 1999, Kikuta and Tsuji 1999, Liebig et al. 1999, Monnin and Ratnieks 2001) or in *Dolichovespula* wasps (Foster et al. 2001).

The relatedness of a worker to other worker's sons is influenced by the mating frequency of queens. In monandrous species, there is only one patriline inside the colony and the mean relatedness of a worker to its nephews is 0.375 (the relatedness to its brothers is 0.25) (Ratnieks 1988). Therefore, in monandrous colonies, workers would benefit on relatedness grounds from rearing other workers' sons in preference to the queen's sons. In contrast, in polyandrous species the presence of more than one patriline decreases the mean relatedness of workers to other worker-laid males, with relatedness to nephews becoming less than 0.25 (the relatedness to brothers) when the effective mating frequency exceeds two (Ratnieks 1988). In this situation, workers benefit from preventing each other from reproducing, instead rearing only the queen's sons. This is called 'worker policing' and may occur through a variety of mechanisms such as by workers eating each other's eggs or by aggressing workers with active ovaries (Ratnieks 1988).

In queenright *A. mellifera* colonies, worker reproduction is rare. Only c. 0.01% - 0.1% of the workers are laying eggs at any time (Ratnieks 1993). Worker policing through aggression of workers with activated ovaries may occur, as studies of introduced workers with active ovaries have shown workers to be able to detect ovary development (Visscher and Dukas 1995, Dampney et al. 2002). Although workers with active ovaries can lay a significant proportion of the haploid male eggs in honeybee

colonies (7%, Visscher 1996) only around 0.1% of the adult males in the colonies are derived from worker-laid eggs (Visscher 1989). This is because workers recognise and eat (police) worker-laid eggs (Ratnieks and Visscher 1989). Worker policing is effective because workers easily discriminate between queen-laid and worker-laid eggs, probably because the queen marks her eggs with a pheromonal signal (Ratnieks 1995, Appendix 1). There is also strong evidence of worker policing in other *Apis* species. In *Apis florea*, Halling et al. (2001) found no significant ovary activation in a sample of 800 workers, no worker produced males in 4 colonies and worker-laid eggs introduced to queenright colonies are removed twice as fast as queen-laid eggs. In *A. cerana*, although some workers have active ovaries, no worker-laid male could be detected in a sample of 652 pupae (Oldroyd et al. 2001). In *A. dorsata*, lack of worker reproduction also suggests effective worker policing (Wattanachaiyingcharoen et al. 2002).

Worker policing has also been well documented in the Vespinae wasps. In *Dolichovespula saxonica*, worker policing appears to be facultative and depends on the queen mating frequency (Foster and Ratnieks 2000). In other *Dolichovespula* species, reproductive conflicts have not been resolved, with worker reproduction and ovary activation being common, although an intermediate proportion of adult worker's sons (3-21%) suggests that the queens still maintain some reproductive power and that worker policing occurs (Foster et al. 2001c, Wenseleers et al. unpublished data). In *Vespula vulgaris,* worker policing occurs even though there is no relatedness benefits for policing as workers are equally related to worker's and queen's sons because paternity is close to 2 (1.9, Foster and Ratnieks 2001a). This suggests that worker policing in *V. vulgaris* has been selected for other reasons than relatedness, such as colony-level benefits of reducing reproductive conflict (Keller 1999, Ratnieks 1988) or causing a female biased sex allocation ratio (Foster and Ratnieks 2001b) . Other *Vespula* species with high mating frequencies also show absence of worker reproduction, as expected (Foster and Ratnieks 2001c). In general, the Vespinae wasps fit the predicted pattern of relatedness benefit well with *Vespula spp.* showing policing and *Dolichovespula spp.* showing extensive worker reproduction at low paternity. In contrast, the hornet *Vespa crabo* is exceptional. In worker reproduction and policing the situation is similar to *V. vulgaris* with no workers' sons reared in queenright colonies, few workers with active ovaries, and worker policing. However, paternity frequency is only 1.1 (Foster and Ratnieks 2001c, Foster et al. 2002), similar to that of *Dolichovespula* species with extensive worker reproduction. Relatedness and kinship

are therefore insufficient alone to explain the evolution of worker policing in the Vespinae wasps and colony level costs of worker reproduction can provide an additional benefit to worker policing, and can select for worker policing at paternities below two (Ratnieks 1988). Species where overt queen-worker conflict occurs have small colony sizes whereas *Vespula* colonies, where worker policing occurs, have larger colonies with thousands of workers in the reproductive phase of the annual life cycle. So it is likely that the resolution of reproductive conflicts which are costly to the colonies, for example because of adult male killing related to sex-ratio conflicts, played an important role in the evolutionary success of some species (Foster and Ratnieks 2001c). Absence of worker reproduction and the occurrence of worker policing by egg eating have also been documented in ant species with low paternity frequencies or in the clonal ant *Platythyrea punctata*, which again suggests a high cost of worker reproduction (Hartmann et al. 2004). Worker policing by egg eating also occurs in *Diacamma* sp. (Kikuta and Tsuji 1999) and has recently been discovered in two other ant species, the facultative polygynous *Pachycondyla inversa* (D'Ettore et al. 2004) and *Camponotus floridanus* (Endler et al. 2004).

4.1 Anarchy

Although the rearing of workers' sons is normally rare in queenright *Apis mellifera* colonies, it is common in the rare "anarchistic" colonies. In anarchistic colonies, a large proportion of the males reared are workers' sons even though the queen is present. Anarchistic colonies are very rare, approximately one colony per 1000-10,000 (Barron et al. 2001), but anarchy can be easily detected during routine inspection of beekeeper-managed hives because they have male brood above the queen excluder, a part of the nest the queen cannot enter because of her larger body size and which can normally only be used by the colony to store honey (see chapter 3). In those colonies, the frequency of workers with active ovaries, although low (<1%; Montague and Oldroyd 1998, Oldroyd and Osborne 1999), is still far greater than normal colonies (0.01%, Ratnieks 1993). The kin structure of two naturally-occurring anarchistic colonies from Australia has been described (Oldroyd et al. 1994, Montague and Oldroyd 1998). In both, the workers were the offspring of a single queen mated to many males, as is normal, and one patriline of workers produced most of the workers' sons. The anarchistic trait is genetically determined (Oldroyd and Osborne 1999) and Oldroyd and Osborne (1999) have been able to select lines that express the anarchistic

behaviour to a much greater extent. However, these colonies are weak because of the inbreeding induced by the selection process and they soon fail because workers lay eggs in worker cells, thereby preventing the colony from sustaining its work force which can arise only from queen-laid eggs reared in worker cells. Up to 80% of the brood reared in an anarchist colony can be worker's sons (Barron et al. 2001).

Anarchistic workers in queenright colonies are more likely to activate their ovaries than workers in normal colonies and, furthermore, the eggs they lay are less likely to be policed by other workers. These characteristics indicate a lower sensitivity to the pheromonal signals that normally inhibits worker reproduction in queenright colonies, possibly involving changes in the production and/or perception of pheromones (Barron et al. 2001). The anarchistic syndrome has an influence on the activation of ovaries, but the phenotype of the colony that contains the anarchistic workers also plays a part. Anarchistic workers fostered in wild-type colonies and non-anarchistic workers fostered in anarchistic colonies had, respectively, a lower and higher likelihood of developing active ovaries (Barron and Oldroyd 2001, Oldroyd et al. 1999). Anarchistic workers are able to evade worker policing by laying eggs that are more acceptable to their fellow workers (Oldroyd and Ratnieks 2000). In selected anarchistic colonies, their eggs are policed at a slower rate than wild-type worker-laid eggs in wild-type colonies during the first 24 h. Mechanistically, this may be either by counterfeiting the queen-produced egg-marking pheromone, which allows discrimination between worker and queen-laid eggs in normal colonies or by masking the true nature of their eggs (Appendix 1.2). It is possible that anarchistic colonies as a whole also have a less efficient system of worker policing, as shown by a study of the selected anarchistic lines (Oldroyd and Ratnieks 2000). Anarchy does not seem to be an epiphenomenon of queenlessness because anarchistic workers do not have any advantage in producing males in queenless colonies (Montague and Oldroyd 1998). The evolution of anarchistic behaviour is a very interesting example of the reproductive conflict that takes place between individuals at the colony level. Anarchistic workers who evade worker policing gain at the expense of other workers because while they are more related to their sons than to the queen's sons other workers are not, therefore causing a tragedy of the commons (Hardin 1968, Wenseleers and Ratnieks 2004). At the colony level, anarchy is likely to be costly because anarchistic workers "work" less, reducing colony efficiency and output, or because of an excessive production of males, which are expensive to produce (Dampney et al. 2004, Barron et al. 2001). These costs are likely to keep

anarchy rare. An additional reason for the rarity of anarchistic colonies may be the need of two mechanisms for anarchistic workers to successfully produce males: the ability to activate their ovaries and the ability to lay eggs that are not policed. These characteristics are probably under different independent genetic determinism, which are rarely associated in the same individual and can be costly for both workers and colonies if present alone (Barron et al. 2001). It is also interesting to see that the presence of many anarchistic patrilines in a colony will decrease the mean relatedness of workers to males (chapter 5), and that worker policing will then be selected for because only the anarchistic genes gain fitness in this situation. This situation is a perfect example of a selfish genetic unit that distorts the production of male gametes by the colony such that they carry the anarchistic trait (Barron et al. 2001).

5. Queen replacement and nepotism

Evidence for discrimination in the rearing of young queens, often referred to as nepotism (the biasing of altruistic behaviour towards closer kin), in social insects is weak and controversial. Only one study, in the polygynous ant *Formica fusca*, has shown a convincing biasing in reproductive investment by workers in more closely related offspring of the queen (Hannonen and Sundström 2003). In polyandrous colonies, one potential opportunity for a worker to increase its inclusive fitness occurs during queen production by favouring full sister queens over half sister queens. In the honey bee, queen rearing occurs in three situations during the colony cycle: when new queens are produced before swarming; when new queens are produced when an old failing queen is superseded; and when a queen dies (emergency queen rearing). If workers can favour full-sister queens belonging to their own patriline and the take-over of the colony by a full sister, they will increase their inclusive fitness because full-sisters are related to them by 0.75, while half sisters are only related to them by 0.25 (Visscher 1986). In order to do this, workers must have a way of assessing relatedness of other members of the colony. A possible mechanism is the use of cuticular hydrocarbons. Arnold et al. (1996) showed that there are significant differences between the cuticular hydrocarbons extracted by solvent from workers from different patrilines when they are kept separately. However, these differences may fade in a colony environment and only some of the patrilines then still show characteristic odours (Arnold et al. 2000). Evidence of kin recognition and kin selection in the honey bee in

the context of queen rearing is controversial. Some studies have showed weak effects of kinship on queen rearing (Page and Erickson 1984, Visscher 1986, Page and Erickson 1986, Noonan 1986). However, these early studies have been criticised for using colonies with few patrilines and for the use of body colour markers such as the recessive gene *cordovan*, which could have influenced the outcome of the experiments (Breed et al. 1994). Another problem is that it is difficult to discriminate between true kin recognition and a genetically determined greater likelihood (coined "royalty alleles" by Osborne and Oldroyd 1999) of certain patrilines to be reared as queens possibly due to having a more attractive brood pheromone or a higher intensity of begging (Breed et al. 1994). The use of DNA microsatellite markers has allowed experiments on naturally mated queens. Tilley and Oldroyd (1997) and Châline et al. (2003) (Appendix 2.1) showed differences between the proportion of queens of each patriline reared and the proportion of those patrilines in the workers. These differences are not always present and it is still not easily possible to distinguish between true kin recognition and the aforementioned "larval attractiveness" hypothesis. In order to investigate this question, Osborne and Oldroyd (1999) investigated the possible existence of "royalty alleles" by giving queenless colonies worker brood from their own and other colonies from which to rear emergency queens to determine whether the same patrilines of worker larvae were consistently selected to rear into queens in different queenless colonies. There results showed no such differences and they concluded that nepotism is the more likely cause of these subfamily differences.

Most research has focused on possible nepotism in interactions between workers and larval queens. More recently, research has investigated the effect of relatedness on interactions between workers and adult queens who have emerged from their cells following the departure of the prime swarm (reviewed in Tarpy et al. 2004). Newly-emerged adult queens within a colony compete with each other to head a new daughter colony. A newly emerged queen has two potential mechanisms for enhancing the probability that she survives to head a colony: First by killing other virgin queens to head the established nest or leave with half of the colony workforce to found a new nest (known as a secondary swarm or afterswarm). When queens attempt to eliminate each other, the process is characterized by numerous queen-queen interactions, including "duels" between emerged adult queens and "assassinations" in which a pupal queen in her cell is killed by an adult queen free in the colony. Vibratory signals made by adult

queens (piping) and queen-worker interactions (vibration signals, aggressive behaviour, feeding) are thought to influence the outcome of the queen selection process.

Workers could play an important role in the queen selection process, for example by preventing or permitting duels and assassinations, or by forming secondary swarms, motivated either by nepotism or "quality control", removing low quality queens regardless of nepotism (Tarpy et al. 2004). Tarpy and Fletcher (1998) found that queens that were sisters of the workers had an advantage in winning duels over unrelated queens. However, Gilley (2003) found that in colonies with naturally-mated queens aggressive behaviour by workers was not more directed towards emerged half-sister queens. Queen quality had little influence on worker-queen interactions and survival (Gilley et al. 2003, Schneider and DeGrandi-Hoffman 2003, Tarpy et al. 2000). These behaviours are mainly aggressive and the absence of nepotism in these is understandable as colony workers would risk harming the queens and failing to have an available replacement. When colonies produce afterswarms, adult queens often remain in their queen cells for up to one week (Bruinsma et al. 1981, Fletcher 1978, Grooters 1987) before exiting into the colony. During this time workers prevent the queens from exiting their special queen cells while feeding them through temporary openings. They also protect the queens by aggressively preventing access by queens who have already left their cells (Gilley, 2001). Workers may well express nepotism during this period, but this has not been studied yet. In chapter 8, I studied the influence of kinship on the interactions between workers and queens which have not exited their cells.

Evidence for nepotism in queen rearing exists, but in most cases the tendency to bias the patriline of new queens is weak (Tarpy et al. 2004). It seems that the likelihood to express nepotism is also genetically determined as there is great between-colony and even between-patriline variation, which is further influenced by the fact that patrilines have different likeliness to perform queen care (Visscher 1998). Ratnieks and Reeve (1991) also showed, using a model, that a mixed ESS of nepotists and non-nepotists was quite logical. Possible reasons for only weak kin selection include the limitations of the discrimination ability of bees due to a paucity of cues or ambiguity of cues present in larvae, or that kin selection is costly to the colony (if for example workers kill queen larvae from other patrilines) (Ratnieks and Reeve 1991, Visscher 1998).

6. Chemical communication and the role of hydrocarbons

Chemical communication is the main channel through which colony integration is achieved in honey bees (Breed 1998). It is used to inform colony members about division of labour, the reproductive status of the queen and other colony members, the origin of eggs, nest origin (nestmate recognition) and kin. Although specific pheromones secreted by various glands are of primary importance in chemical communication (like for example the honey bee queen mandibular pheromone), cuticular hydrocarbons are also thought to play an important role (Lenoir et al. 1999). One reason for this is that studies have shown them to show enough variation to be used as useful information sources. In a recent study, however, Dani et al. (2004a) have shown that although cuticular hydrocarbons have sufficient variation to distinguish between related and unrelated nestmates (different matrilines, *Polistes dominulus* and *Vespa crabo*), they may not be sufficient to separate differently related nestmates (half vs. full-sisters, *Vespa crabo*). Behavioural evidence for their role has recently become increasingly available. This includes foraging regulation (*Pogonomirmex barbatus,* Greene and Gordon 2003), reproductive status (*Dinoponera quadriceps,* Peeters et al.1999; *Diacamma ceylonense,* Cuvillier-Hot et al. 2002; *Myrmecia gulosa,* Dietemann et al. 2003), egg policing (*Camponotus floridanus,* Endler et al. 2004, but see Martin et al. 2004d, Appendix 1.3 for the situation in honey bees), nestmate recognition (*Polistes*, Dani et al. 2001, Panek and Gamboa 2000; *Vespa crabo,* Ruther et al. 2002; *Cataglyphis niger,* Lahav et al 1999), kin recognition (*Formica truncorum*, Boomsma et al. 2003) and social parasitism (*Polistes sulcifer,* Sledge et al. 2001; *Polyergus rufescens,* D'Ettore et al. 2002).

In the honey bee, although cuticular hydrocarbons differences could be used to separate between nestmates and non-nestmates, between different degrees of kinship within a colony (Arnold et al. 2000) and egg origin (Martin et al. 2004d, Appendix 1.3), behavioural evidence is more controversial. Supplementation experiments (i.e. adding cuticular hydrocarbons to focal workers) have shown some effects of some hydrocarbons, especially alkenes on nestmate recognition (Breed 1997, 1998, Dani et al., in press). However, Fröhlich et al. (2000, 2001) have shown that bees do not seem to be able to discriminate between comb wax and cuticular waxes using the hydrocarbon fraction. Furthermore, Breed et al. (2004) now postulate that fatty acids rather than cuticular hydrocarbons may play the main role in nestmate recognition in

honey bees. The question however remains open and new tests have to be designed to confirm or refute the role of hydrocarbons. The critical problem is to be able to link correlation studies which details difference, olfactory ability of bees, and behavioural evidence. In chapter 9, I have used an established learning bioassay, the conditioning of the proboscis extension reflex (Bitterman et al. 1983) to investigate differences in learning ability of cuticular hydrocarbons by worker bees in order to try to pick out candidate compounds for recognition in the honey bee.

7. Conclusion

Social insects are an ideal model to test the predictions of kin selection theory, and recent developments have led to a number of predictions and tests relating to conflict and conflict resolution within social insect colonies (Ratnieks et al. 2001). The existence of a high level of polyandry in the honey bee makes it an excellent species for studying conflict in social systems, in particular conflict over male production (leading to worker policing) and conflict over queen rearing (nepotism). These conflicts are the primary focus of this thesis. Because both conflicts involve chemical communication at a mechanistic level, through the recognition of the maternal origin of male eggs and the identity of young queens as full-sisters or half-sisters, an additional focus of this thesis has been the ability of worker bees to learn various cuticular hydrocarbons, which may be involved in nestmate recognition.

Chapter 2
How the thesis evolved

1. How it all started

I first met Francis Ratnieks at the National Honey Show in 1998, where he gave a talk about his research on honey bees and worker policing, just after my national service ended. I already had a keen interest in honey bees and in the question of conflict and cooperation in social insect colonies. This interest had been kindled during my undergraduate degree at the National Institute of Agronomy of Paris-Grignon, with the lectures of Pierre-Henry Gouyon and Jean-Pierre Henry. My Master's degree at University of Tours and the Laboratory of Invertebrate Comparative Neurobiology (LNCI, Bures-sur-Yvette) investigated whether nepotism occurred in honey bee colonies during emergency queen rearing.

During a visit to Sheffield University in spring 1999, I was able to formulate a research plan for a potential PhD. The aim was to focus on studying several aspects of reproductive conflict in the honeybee, *A. mellifera*, specifically worker policing in male production and nepotism in queen rearing. The research would use DNA microsatellites to assess paternity and maternity in colonies in order to study the role of colony structure, kinship and potential division of labour in these conflicts.

As funding was not available immediately, I spent two years employed as a research technician in Professor Terry Burke's Molecular Ecology Laboratory (Sheffield Molecular Genetics Facility). During these two years, I became familiar with the use of microsatellites to carry out paternity analyses in birds and was a co-author in the following three publications:

Birkhead TR, Châline N, Biggins JD, Burke T, Pizzari T (2004) Non-transitivity of paternity in a bird. *Evolution*, **58**, 416-420.

Davies NB, Butchart SHM, Burke T, Châline N, Stewart IRK (2003) Reed warblers guard against cuckoos and cuckoldry. *Animal Behaviour*, **65**, 285-295.

Hatchwell BJ, Ross DJ, Châline N, Fowlie MK, Burke T (2002) Parentage in the cooperative breeding system of long-tailed tits *Aegithalos caudatus*. *Animal Behaviour*, **64**, 55-63.

Although I officially started my PhD in October 2001, I was able to work on my PhD project beforehand for a month in the summers of 2000 and 2001. The work conducted fell in five categories, only four of which are included in the thesis (sections 2, 4, 5 and part of section 6 below) as I was not the principal investigator in the other one (3). The work included in the thesis therefore represents two-thirds of my workload during the three years of my PhD.

2. Worker policing and worker reproduction

Francis then introduced me to the mutant Anarchistic colonies discovered in Australia by Dr. Ben Oldroyd, who was also visiting Sheffield on a sabbatical at the time. Francis obtained an anarchistic colony of honey bees from a beekeeper in Widnes, Lancashire, and this led to the first study of this thesis (Chapter 5) which was to use DNA microsatellites to unravel the colony's kin structure and determine the maternal origin of the males that were reared at three different levels: workers' sons versus queen's sons, sons of workers of different patrilines, and sons of different individual workers. The genetic analysis used 17 microsatellite loci and led to perhaps the most detailed study of worker reproduction ever carried out in a colony of insects. The queen in this "British Anarchist" did not survive the winter thereby preventing any further studies.

The next year, 2001, during routine work studying worker policing, which involved making queenless colonies to obtain worker-laid eggs (chapter 3, section 7), one of the queenless colonies behaved abnormally, in that worker-laid eggs were never observed in the cells (as is typical in a "hopelessly queenless" colony) even one month after dequeening. This led to a study to determine what was taking place: Were the workers not laying eggs at all, or were they laying eggs which were policed (i.e., eaten by other workers)? The results showed that the latter explanation was correct, and led to us describing a novel genetic variant for worker reproduction in the honey bee (chapter 6). That is, colonies which do not switch off their policing even when queenless.

I also investigated in more details the factors affecting worker policing by repeating a series of policing trials (chapter 3, section 7) on three discriminator colonies, with special attention to the rate of egg-removal in drone cells and worker cells, but also the removal rate of queen-laid and worker-laid eggs and the inter-colony

variation. Because workers preferentially lay eggs in drone cells, I hypothesised that police worker should be more discriminating against worker-laid eggs in drone cells than in worker cells. A similar study conducted in Sydney at the same time prevented me from submitting the results straightaway, but by pooling the results with another experiment, conducted in South Africa by my lab colleague Dr. Stephen Martin, I was able to get a very good picture of how worker policing is indeed dependent on cell size, but also of colony and that the more effective the policing, the more queen-laid eggs are erroneously eaten (chapter 7).

3. Egg-marking chemistry involved in worker policing and worker reproduction

During my PhD, I was involved in the search for the hypothesised queen-produced egg-marking pheromone which allows workers to distinguish queen-laid eggs from worker-laid eggs. These experiments, in collaboration with Dr. Stephen Martin, the principal investigator, and Dr. Graeme Jones of Keele University lasted all through the thesis and the principal results were that esters (Martin et al. 2002b, Appendix 1.1) and hydrocarbons (Martin et al. 2004d, Appendix 1.3), which are both secreted by the Dufour's gland, are not the true egg-marking pheromone of queen-laid eggs. However, anarchistic workers appear to increase the acceptability of their eggs by secreting the usually queen specific esters, thus probably scrambling egg recognition (Martin et al. 2004c, Appendix 1.2). The use of various solvents on both worker-laid eggs and queen-laid eggs and additional experiments showed that the egg marking pheromone was indeed produced by the queens and was very robust (Martin et al. 2004b, Appendix 1.4). This also showed that the pheromone was unlikely to be a non-polar compound and the latest advance was the development of an extraction technique enabling the transfer of the signal from queen-laid eggs to worker-laid eggs, the nature of which is under investigation.

4. Nepotism in queen rearing

My interest in nepotism, dating back from my MSc, was renewed by recent review papers and a series of experiments investigating aggressive behaviour of workers towards newly-emerged queens (Tarpy and Fletcher 1998, Gilley 2001, Gilley 2003, see Chapter 1 and 8). I decided to study the occurrence of nepotism in a context

and stage in the young queen's life which up to then had never been studied. This is when a young queen has emerged from her pupa but is still confined to her cell, and is fed by workers through a small slit in the tip of the cell. The workers may also prevent the queen from leaving her cell. This behaviour is very common and occurs after the primary swarm has departed with the mother queen. Because young adult queens may stay in their cells for as long as a week this seemed like a context in which nepotism could occur. This study required the development of an apparatus which prevented young queens from exiting their cells while still allowing observation of normal interactions between workers and the imprisoned queens. DNA microsatellites allowed me to determine kinship among interacting workers and queens. The sample size obtained was the largest so far in any study of nepotism in naturally mated colonies (chapter 8).

The increased familiarity of the literature that I gained during my PhD combined with the extensive training in writing also allowed me to write and submit two papers on nepotism using the data from my MSc thesis, one of which is published (Châline et al. 2003, Appendix 2.1), while the other is currently under review (Châline and Arnold, Appendix 2.2).

5. Discrimination of colony odours

Recognition underlies the ability of workers to manipulate brood rearing, whether in policing eggs or in queen-rearing nepotism, and the ability to recognise nestmates. But in the honey bee, contrary to a growing number of social insects, the nature of the chemical cues used by workers in recognition is still largely unclear. There is evidence that cuticular hydrocarbons are involved in nestmate (Dani et al., submitted) and kin (Arnold et al. 2000) discrimination. However, the specific compounds involved are still unknown. In my final field season, 2003, I used the Proboscis extension reflex (PER) bioassay to investigate differences in learning and discrimination abilities of workers towards long chain alkenes and alkenes, the main hydrocarbons present on worker cuticles (Chapter 9). I found large differences; especially that alkanes and heavier alkenes are not perceived well while the other alkenes are. This suggests that alkenes are more likely to be involved in recognition and that perception of the compounds is greatly influenced by their chemical structures.

6. Beekeeping and Apis Biodiversity in Europe (the BABE project)

During my PhD, I was also involved in the BABE project, an EU-funded research network across 6 laboratories. The first aim of this project for Sheffield was to develop a non-lethal DNA sampling method which could be used on queens to be included in breeding programmes. This forms chapter 4 of this thesis. In addition, I was also involved in another BABE project which investigated queen mating distances and mating isolation of the Hope Valley and Edale in the Peak district as part of a broader project to conserve the British black bee, *Apis mellifera mellifera*, the native honey subspecies of northern Europe (including France). The results of this study have been submitted (Jensen et al., Appendix 3). Following the development of the non-lethal sampling technique, an ambitious project was developed by A. Perez-Sato and F. Ratnieks in order to carry out intra-colony selection for hygienic behaviour in a black bee population. I have assisted in this project and also helped design and set-up another project which will investigate the mating success of normal size and small size honey bee males produced in worker cells.

Chapter 3
General methods

1. Study organism

The western honey bee, *Apis mellifera*, belongs to the Apini, one of the four tribes of corbiculate bees of the Apidae family which also include the social Meliponini and Bombini and the non-social Euglossini. It arose in central Asia (around the Himalayas, Ruttner 1988) and spread west into Western Asia, Africa and Europe. It is one of the seven species in the *Apis* genus, with all the other species native to Asia. Because of the great extent and ecological diversity of its natural range and the presence of many barriers to gene flow, such as the Alps or the Mediterranean, it has evolved into 24 distinct subspecies (Ruttner 1988). In Europe, 13 of them occur around the Mediterranean with four (*mellifera*, *ligustica*, *carnica* and *caucasica)* in Western Europe (other island races also evolved like *sicula* in Sicily and *cecropia* in Crete) (Ruttner 1988). These four western European races are the main ones exported worldwide from Europe for general beekeeping purposes. The honey bee is now a cosmopolitan species which is the most economically important insect for both pollination and hive products, mainly honey but also beeswax, pollen, propolis, bee brood, royal jelly and venom. In the UK, the value of honey bee pollination has been estimated to £172 millions a year (Carreck and Williams 1998).

The native subspecies of honey bee in the UK and northwest Europe is the black bee, *Apis mellifera mellifera* (Cooper 1986). However because extensive introduction of non-native subspecies like *ligustica* (the Italian bee) and *carnica* (the Carnolian bee) have occurred since 1859, especially after the Isle of Wight disease wiped out most of British colonies (c. 1915), the black bee is no longer the predominant subspecies in Britain. The Buckfast bee, which was selected by hybridising bees from numerous origins by Brother Adam, is also popular among British beekeepers. At the Laboratory of Apiculture and Social Insects (LASI), and with the collaboration of the Bee Improvement and Bee Breeders Association (BIBBA), an effort has been made to conserve the native black bee. The laboratory apiary therefore mainly hosts bees of the

mellifera subspecies. However, because there is no reproductive isolation between the lab and other potential sources of male bees in Sheffield, hybridisation with non-native subspecies occurs.

Figure 3.1. Picture of a worker (Left) and queen marked with an Opalithplättchen numbered tag (Right). (Photos N. Châline)

Honey bees, *Apis mellifera*, usually live in colonies of up to 60 000 individuals, headed by a single queen (Figure 3.1). As described in chapter 1, the queen produces most of the brood in the colonies. The queens mate early in their life with a large number of males (average 14 males, Tarpy and Nielsen 2002) during a number of mating flights which occur between the second and third week of their adult life (after emergence).

2. The bee hive

The honey bee has been kept in hives by beekeepers for over four thousands years (Crane 1992). Modern beekeeping began in 1851, when, in the USA, the Reverend L.L. Langstroth invented the modern movable frame hive (Crane 1992, Figure 3.2). This revolutionary design is still used today by most beekeepers across the world. It allows the removal of frames, making colony inspection and management much easier and more effective. For example, the queen can be relocated, the brood checked for diseases, and frames of brood or honey can be moved between hives. Another important development was the queen excluder in the early 1800s which allow the queen to be confined to a part of the colony. Because honey bees do not move brood from cell to cell, the queen excluder, which contains openings large enough for a

worker to go through but too small for the queen, prevents her access to the honey chamber, keeping it free of brood and making it easier to harvest honey (Figure 3.2).

Figure 3.2. Standard Langstroth hive with lid removed. The hive is in two deep boxes separated by a queen excluder. The queen is confined to the lower box. (Photo A. Perez-Sato)

Figure 3.3. a. Worker emerging from its brood cell. Capped brood cells and cells filled with honey can be seen (Photo N. Châline) **b.** Frame with naturally built emergency queen cells. (Photo A. Perez-Sato)

The movable frames are double sided combs consisting of hexagonal cells made of wax secreted by intersegmental wax glands of the workers on the ventral surface of the abdomen. These cells are used both for brood rearing and food storage (honey and pollen) (Figure 3.3a). The queen lays a single egg in each cell located in the brood chamber. The eggs hatch after three days and the larvae are fed progressively within the

cells by nurse bees. When a larva is fully fed but before it pupates, its cell is sealed with a thin layer of wax. Drones are reared in the same way but in larger hexagonal cells ("drone cells") which can also be used for food storage. The queen has control over whether an egg will be fertilized or not and will change fertilisation according to the cell size (Ratnieks and Keller 1998). Queens are reared in very different acorn-shaped 'queen cells', which are oriented downwards and are normally built at the bottom of the wax combs (Figure 3.3b).

3. Queen rearing

Queen rearing is carried out by beekeepers. Commercial breeders may rear thousands of queen cells for shipping to other beekeepers to breed from or to requeen their colonies. . Queen rearing depends on the fact that female larvae are totipotent, and specifically that young larvae in worker cells can develop into queens if given royal jelly and reared in queen cells. It is based on the artificial grafting of larvae into modified cells made of plastic or wax. Queen rearing was used in chapter 4 and 8. In both cases the same technique, described below, was used.

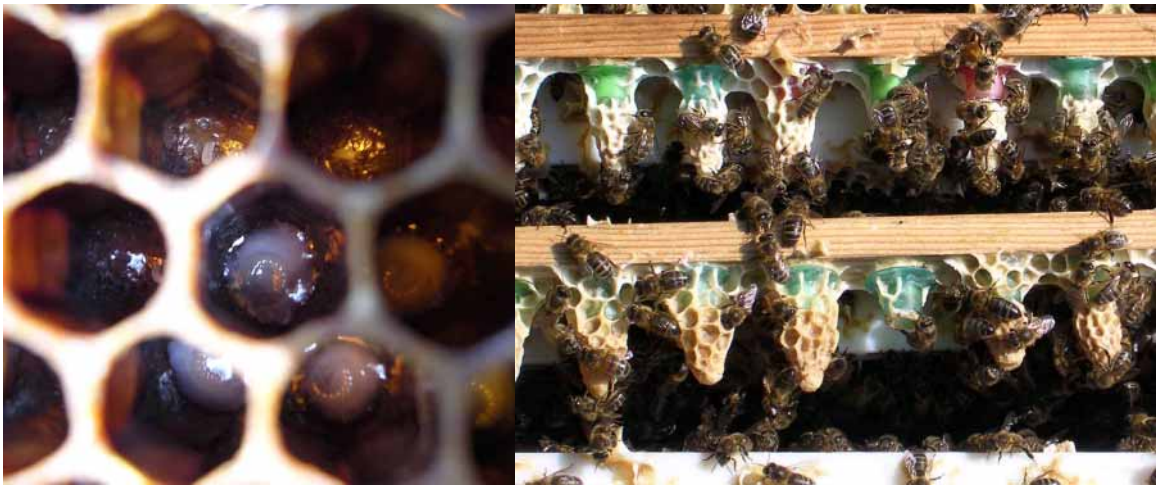


Figure 3.4. a. Frame containing young larvae for grafting. **b.** Finished grafted queen cells. The plastic cups used for the grafting can be seen. The queen cell is finished by the bees from the starter-finisher colony with wax. (Photos A. Perez-Sato)

A frame from the selected mother queen colony with very young larvae is chosen and transferred to the laboratory (Figure 3.4a). There, using a standard grafting tool, larvae of the 1st or second instar are transferred to plastic queen cell cups (JZ-BZ) primed with a drop of water (known as dry grafting as opposed to grafting with a drop of royal jelly). The cups are attached to wooden bars fixed to a modified frame (Figure

3.4b) and then transferred to a queenright starter-finisher colony for rearing (Laidlaw and Page 1997). The queenright starter-finisher is a populous colony housed in two deep frames standard Langstroth hive boxes separated by a queen excluder. It is fed sucrose syrup *ad libitum* in order to stimulate queen rearing. This type of colonies will rear up to 50 queens despite the presence of the queen which is isolated away from the queen cells by the queen excluder so preventing her from destroying the queen cells. After ten days, one day prior to the queen emergence from their cells, they are transferred from the colony into individual cages and into an incubator at 34C until emergence. In chapter 8, the colonies used for rearing were the colonies where the larvae originated so guaranteeing that the queens were reared by sister workers. After emergence, queens can be used for various purposes such as the chemical analysis of the different glands associated with the reproductive system.

4. Queen mating nucleus hives

For breeding or experimental purposes, virgin queens obtained through queen rearing may have to be mated before shipping or use in an experiment. The usual procedure in order to do that is to use small sized hives, each known as a "mating nucleus". This is because it is more economical in terms of bees and equipment and often more successful than in full-sized established colonies. Mating nuclei used at the laboratory were polystyrene Warnholz mating nuclei. These nuclei require only about 500 workers and contain four mini-frames and a feeder compartment for syrup or sugar candy (Figure 3.5). A sealed queen cell or virgin queen in a cage is introduced in each nucleus.

This method was used to produce mated queens destined for chemical analysis (see Appendix 1) and also to test whether the non-lethal DNA sampling method developed in chapter 4 (clipping the wing tips) allowed normal mating.

5. Instrumental insemination

Artificial insemination (AI) is an ideal way to identity and number of males with which a queen mates. Natural mating in the honey bee occurs in mid air and is impossible to control, expect by using an isolated valley or island without honey bees. But even here all that can be controlled is the source of the males, not the number or

actual identity of those that mate a queen. For research purposes, it allows control not only of the number of mates and their origin, but also more elaborate experimental designs like the insemination of several queens with the mixed sperm of the same males. Detailed books describing the procedure are available (Laidlaw and Page 1997, Moritz 1987).

The possibility of using AI during the PhD was discussed with Prof Ratnieks, especially for the nepotism project in chapter 8. Some earlier studies of nepotism, for example, used queens mated to three males by AI. However, DNA microsatellites are sufficiently polymorphic to allow research to use naturally mated queens with many patrilines. Consequently, we decided that it was better to carry out the nepotism study in chapter 8 using naturally mated queens with a normal complement of patrilines. However, I had still the opportunity to follow a training course and to practise the technique in the lab.

Figure 3.5. Nicolas Châline inspecting mating nuclei in the Pisgah garden mating apiary. Nuclei are arranged by four, each facing a different direction and painted a different colour to reduce drifting. (Photo A. Hart)

6. Worker and queen dissections

Dissections of workers and queens were used throughout the PhD and had several purposes. First, the dissection of worker abdomens was used to quantify ovary activity (Chapter 5, Appendix 1). Second, dissections of workers and queens were used to collect various pheromone producing glands like the Dufour's gland (Appendix 1). Finally, dissection of the spermathecae of queens can be used to ensure that they are mated (Chapter 4).

The first method used to dissect bees is to fix a freshly killed or frozen bee with a pin in a Petri dish which is then part filled with pure water. Cuts are made laterally along both sides of the abdomen from the sixth to the second tergite. Another dorsal lateral cut through the top of the abdomen allows a flap of exoskeleton to be folded back and held by another pin. The lower part of the gut is then separated from the honey crop by cutting through the proventriculum. The lower gut is then pulled back and fixed with a pin. Because the distal tips of the ovaries sometimes encircles the gut, the tracheal system around the gut system often has to be cut to allow its removal without damaging the ovaries. After this procedure, the ovaries can be seen ventrally and assessed for ovary activation. The glands associated with the sting are also then visible and can be sampled. The second method that can be used to dissect gland is to just tear the sting and the last segment of the abdomen from the bee. The gland associated with the sting and the reproductive system and the ovaries stay attached to the sting and can be dissected. When dissecting a queen, another organ which can be easily seen and removed is the spermatheca. The spermatheca is covered by a tracheal net. Once this net has been removed by gentle manipulation in the fingers, the spermatheca itself can be inspected. If it has no sperm inside it is transparent and the queen is unmated. If creamy white, she has been successfully inseminated.

Ovary activation in workers can be quantified in various ways. For studies of worker reproduction, it is most useful to determine the proportions of workers whose ovaries are substantially or partially active by using three categories, depending on the size of the largest oocyte present in the two ovaries. The categories are: low activation: no oocytes or largest oocyte < 0.8mm, with the ovaries typically threadlike; medium: largest oocyte between 0.8 and 1.2mm; and high: largest >1.2mm, the ovaries are usually quite distended (Miller and Ratnieks 2001). Eggs in cells are approximately 1.3-1.8mm long (Ratnieks 1993, Winston 1987) so the high category represents workers

who are currently capable of laying eggs. Figure 3.6 show the ovaries of a worker with high activation.

Figure 3.6. Activated worker ovaries (Photo N. Châline)

7. Worker policing bioassays

The study of worker policing by egg eating requires a robust bioassay which can be compared across colonies, treatments and experiments. The bioassay used in this thesis was developed by Ratnieks and Visscher (1989). The first requirement is obtaining queen-laid and worker-laid eggs. Queen-laid eggs are obtained easily from queenright colonies. If eggs of a particular age or sex are required, the queen can also be caged in a one-frame queen excluder containing an empty frame of worker or drone cells or given frames at particular times to lay on. Worker-laid eggs are obtained from hopelessly queenless colonies. The usual procedure to obtain such a colony is to transfer around five frames of brood from the brood box of a populous queenright colony into the honey chamber, above the queen excluder. After ten days, all the brood in these frames will be capped and the top box can be removed, taken to another apiary and set up as a hive. This colony is unable to rear a replacement queen because there are no young larvae in worker cells from which to rear emergency queens. After three weeks, the workers have activated ovaries and have started to lay unfertilised male eggs. If the colony is given a frame of drone sized cells they will preferentially lay in these, which facilitates the harvesting of eggs as the cells are larger. These queenless colonies can be reinforced, if need be, by transferring additional frames containing sealed worker cells

from another colony to provide a continuing source of young workers without allowing queen rearing.

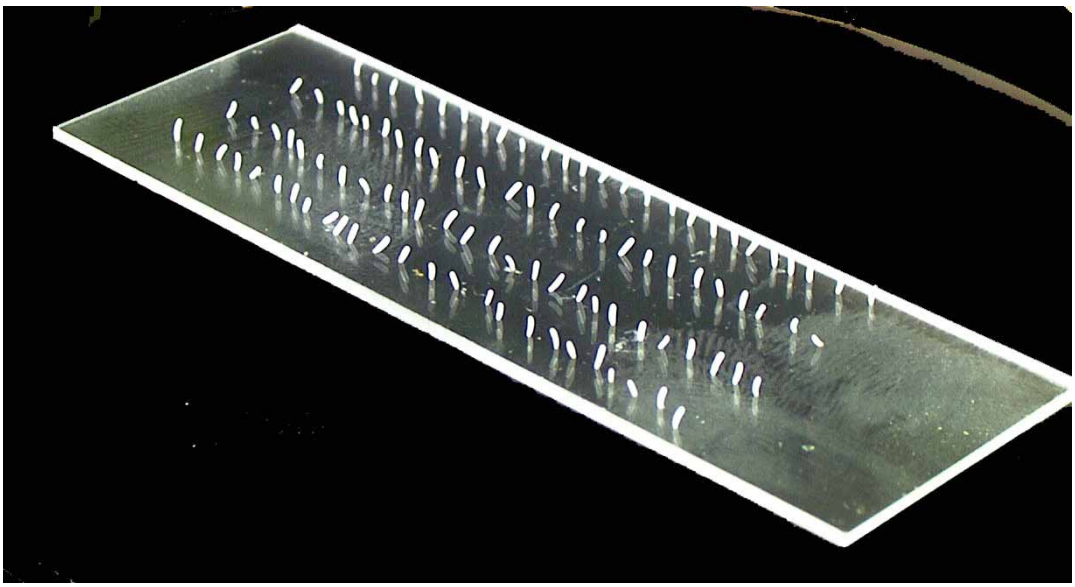


Figure 3.7. Rows of eggs transferred to a microscope slide. (Photo A. Gregson)

In some cases the policing trials are performed in particular test colonies which are of interest for their policing phenotype (Chapter 6). Normally, however, any queenright colony can be used as a discriminator colony in which to study the rates of removal of different classes of eggs. Discriminator colonies are populous queenright colonies housed in two standard Langstroth deep boxes (Figure 3.2) separated by a queen excluder. The queen is confined to the bottom box. An empty frame of drone comb (the test frame) is inserted into the top box 24 hours at least prior to the experiments to acquire the colony odour. Alternately, by using comb foundation (a sheet of bees wax, commercially available, stamped in a hexagonal pattern in the size of either drone or worker cells) new frames can be constructed, including frames that are half worker cells and half drone cells (i.e., two half sheet of foundation are used) (Chapter 7). Two frames of young brood (ideally with many eggs and larvae) are then taken from the lower box and placed one on each side of the test comb. These brood frames have to be replaced regularly by new frames of young brood. The idea behind doing this is that the test frame stays within the brood chamber. As the discriminators hives are usually very populous and tend to swarm, the old frames of brood placed around the test frame are sometimes removed and given to another colony. Any spaces in the hive where frames have been removed are filled with an empty frame.

Before a trial, frames containing newly-laid queen-laid and worker-laid eggs are collected from the source colonies. Workers do not discriminate between unfertilised (normally male) and fertilised (normally female) queen-laid eggs (Oldroyd and Ratnieks 2000). Hence, queen-laid eggs can be collected from either drone or worker cells. Worker-laid eggs and queen-laid eggs usually do not originate from the same colony. This could add an additional effect in the policing trials. However, queen-laid eggs from different colonies are not treated differently, nor are worker-laid eggs of a normal phenotype (Ratnieks and Visscher 1989). Eggs are then removed from their cells using special forceps and transferred to a microscope slide (Figure 3.7) before being transferred into the test comb in adjacent lines of cells. While on the glass slide they can also be treated, if necessary, with various chemicals or gland extracts (Appendix 1) or they can just be transferred to the test frame from the discriminator colony. Twenty eggs per treatment are typically used (Figure 3.8). The test frame is then returned to the discriminator colony. Egg removal rate is determined by removing the frame at intervals and counting the number of eggs remaining. The usual intervals used are after 1, 2 and 20 hours (next day), but this can vary according to the experiment purposes.

Figure 3.8. Worker-laid and queen-laid eggs transferred to a drone frame (test frame). (Photo F. Ratnieks)

Chapter 4

Non-lethal sampling of honey bee, *Apis mellifera*, DNA using wing tips

1. Abstract

DNA sampling of insects frequently relies upon lethal or invasive methods. Because insect colonies contain numerous workers it is often possible to destructively sample workers for genetic analysis. However, this is not possible if queens or workers must remain alive after sampling. Neither is it possible to remove an entire leg, wing or other appendage as this will often hinder normal behaviour. This study investigates the possibility of genotyping queen honey bees *Apis mellifera* using DNA extracted from wing tips so that flight and other activities are unaffected. Our results show that wing tip samples (*c.* 1.3mm^2) provide good quality DNA which gives reliable genotypes when PCR amplified (94.3% success rate). Wing tip DNA sampling will permit a variety of novel research approaches, including genotyping of queens at emergence in breeding programs where certain patrilines or genotypes are preferred, and genotyping workers and queens which must behave normally following sampling.

2. Introduction

Non-lethal sampling for DNA fingerprinting is becoming increasingly important for conservation, behavioural and population studies (Gerken et al. 1998, Lushai et al. 2000, Starks and Peters 2002). It is also important in selection and breeding programs. For small animals such as insects, one methodological challenge is to develop tissue sampling methods that do not affect individual survivorship while still providing adequate quality DNA for genetic analysis (Gerken et al. 1998). A study on damselflies (Fincke and Hadrys 2001) has shown that removal of one tibia provides sufficient tissue for DNA extraction but does not kill the insect. Haemolymph from larval and adult scorpionflies is another non-lethal tissue source (Gerken et al. 1998, Kurtz and Sauer 1999), although the subsequent effect on adult survivorship was not recorded. Studies

on butterflies successfully used 2 mm^2 of wing edge (Rose et al. 1994) and 3 mm^2 of wing tip (Lushai et al. 2000) to extract usable DNA without killing the insects.

In social insects, the survival of sampled individuals is not always important. In species with large numbers of workers, individuals can be sacrificed to provide the samples needed for many types of genetic analyses, such as for determining kinship and relatedness among progeny (e.g. honey bees: Chapter 5, wasps: Foster et al. 2001, ants: Bourke et al. 1997). However, lethal sampling can be problematic when small colonies are studied, and is unsuitable for genotyping queens destined to head colonies or workers whose subsequent behaviour must be studied (Starks and Peters 2002). In addition, extensive sampling from a population can alter the subsequent population structure (Starks and Peters 2002). Non-lethal sampling of an entire leg has been used in *Polistes* wasps (Starks and Peters 2002) but it had significant effects on the behaviour and survivorship of sampled workers. The removal of one to three tarsi for marking purposes has also been used in *Leptothorax acervorum* (Bourke 1991, 1993) without apparently harming the queens or hindering their behaviour.

In honey bees, *Apis mellifera*, non-lethal sampling would be valuable in several types of studies such as behavioural studies of workers in relation to genotype or patriline, and studies of queens. Non-lethal sampling of queens would also permit novel breeding programs, such as selecting among newly-emerged queens reared from a single mother colony according to patriline or genotype. Any potential tissue sampling method should not interfere with the queen's ability to mate (unless instrumental insemination is used) or to carry out colony duties.

The purpose of this study was to determine whether small pieces of wing tip could be used for the extraction of DNA suitable for genotyping queen bees with polymerase chain reaction (PCR) amplification. Our results show that small areas of wing tip (*c.* 1.3 mm^2) taken from newly-emerged queens provided good quality DNA in 95% of cases. The genotypes scored from wing tips were the same as those from large tissue samples (whole wings or antennae).

3. Materials and Methods

Two DNA extraction experiments were carried out using worker and queen honey bees (*Apis mellifera*) taken from colonies kept at the Laboratory of Apiculture and Social Insects, University of Sheffield. The first experiment used workers to determine the suitability of two methods (freezing at -20°C and ethanol at room temperature) for storing two tissue samples (wings, tarsi) for later DNA analysis. The second experiment determined whether wing tips of newly-emerged queens could provide sufficient DNA for PCR amplification and analysis at 4 commonly used nuclear microsatellite loci. These wing tip genotypes were compared with those obtained from extractions of whole forewing and antenna samples.

3.1. Worker samples

A frame of capped worker brood was incubated overnight at 34°C. The following day 30 newly-emerged workers were marked using numbered tags (Opalithplättchen). The tarsi from one middle and one hind leg were removed with fine forceps, and one forewing was clipped 3mm from the thorax. The forewing was then cut in half across the length, yielding a proximal and a distal sample. Each half (proximal or distal) was randomly allocated to one of the two storage methods: (1) in 1 ml of 95% ethanol at room temperature and (2) dry (without buffer) at -20°C. Tarsi were similarly allocated to these two storage methods. Subsequently, workers were kept in a cage at 34°C with syrup, pollen and water *ad libitum*. After ten days, ten workers were killed by freezing and used to collect a second set of the same appendages which were then stored in the same ways. In addition, the heads were collected and frozen at -20°C as a control. DNA extractions and genetic analyses were made one month after the final samples were taken.

3.2. Queen samples

The queens used were reared during the spring and summer of 2002 following standard queen rearing methods (larval grafting into artificial queen cells in a two-storey queenright starter-finisher colony which was fed sucrose syrup; Chapter 3, Laidlaw and Page 1997). Larvae from five different mother colonies were used during

the season. Eight days after grafting larvae into queen cups the sealed queen cells containing pupae were removed from the hive and incubated at 34°C until emergence. Newly-emerged queens were marked with numbered tags (Opalithplättchen) and kept in individual cages in a "queen bank" colony unless they were introduced into mating nucleus colonies (see below).

A first set of 12 queens were introduced into small queenless colonies (queen mating nucleus colonies) 2-14 days after emergence. The purpose of this first set of queens was to see if it was possible to genotype mated queens from their clipped wings, because clipping wings of mated queens is a common beekeeping practise to reduce swarming (Laidlaw and Page 1997). These colonies were regularly inspected for egg-laying. Three days after extensive egg-laying was observed, the queen was removed from her colony, and her right fore and hindwing were clipped 3 mm from the thorax and frozen at -20°C. These queens were subsequently caged and returned to the queen bank colony for later use and stored at -20°C after death. In September, any queens still alive were killed and stored at -20°C. Seven of the queens were confirmed to have mated successfully. Two did not mate. Three laid eggs but we were unable to determine if they were mated or not after dissection (Chapter 3) because their spermathecae were damaged when they were collected.

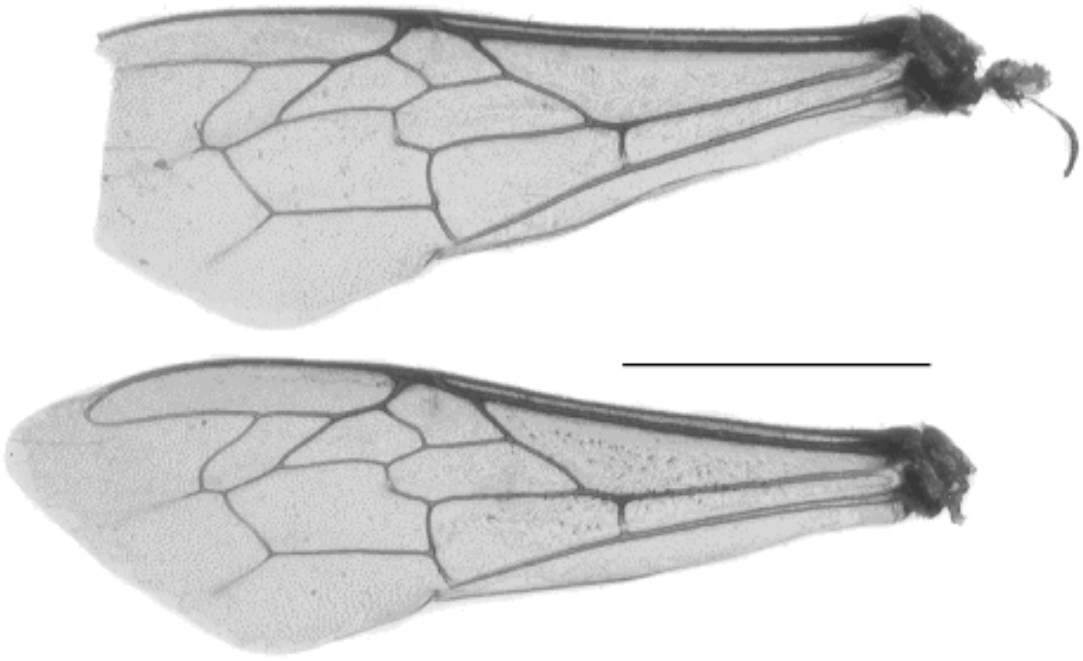


Figure 4.1. Forewings of two queens, one complete and the other clipped for DNA extraction from the wing tip (scale bar = 3.4 mm).

A second set of 35 queens were reared and at emergence a small piece of the tip of each forewing was cut off using fine scissors and stored at -20°C (Figure 4.1). We estimated the area removed from each wing at 1.3 mm^2 by approximating the wing tip as a triangle with dimensions given by graticule measurements made under a binocular microscope. The removed area was around 7.5% of each forewing surface. Seven of these queens were introduced to mating nuclei, five of them mated successfully, whilst two became drone layers. These seven queens were collected from the mating nuclei and kept in the queen bank colony until they died. Following emergence, the remaining 28 queens were kept in individual cages in the queen bank until death, after which they were stored at -20°C. The purpose of keeping the queens in the bank colony was to determine if it was possible to obtain DNA extractions from wings of older bees. Because wings are mainly dried cuticle through which a few veins circulate haemolymph (Snodgrass 1956) and the epidermis cells degenerate after emergence (Richards and Davies 1977), it is possible that wings will become unsuitable with time for DNA extractions.

For all genetic analyses, both antennae of each dead queen were collected and stored at -20°C as control samples. The remaining part of one forewing of the queens in the second set of queens was also clipped 3mm from the thorax at death and stored at -20°C.

3.3. DNA extractions

DNA was extracted from worker heads using high-salt extractions (Bruford et al. 1998, Miller et al. 1988). Heads were added to 250 µL of proteinasing solution (0.2 mg/ml proteinase K, 50 mM Tris, 120 mM NaCl, 1% SDS, 20 mM EDTA, pH 8.0) and crushed thoroughly. The remaining cuticle of the head was then removed and the solution digested (with constant agitation) at 55°C for 3 h. An equal volume of 4M ammonium acetate was then added and the solution was vortexed and left at room temperature for 15 min. The sample was centrifuged at 8000*g* for 10 min and the supernatant decanted into an autoclaved labelled eppendorf tube. To precipitate the DNA from the supernatant, two volumes of 100% ethanol were added and the sample was centrifuged at 8000*g* for 10 min. The supernatant was decanted and the pellet was rinsed in 1 ml of 70% ethanol and air-dried for 30 min. DNA samples were dissolved overnight in 250 ml 10 mM Tris, 0.1 mM EDTA.

All the other tissue samples (tarsus, antenna, full forewing and wing tip) were extracted using chelex®100 extraction (Walsh et al. 1991). The samples were placed in liquid nitrogen for 5 min and then crushed thoroughly with a disposable pestle. Different amounts of 5% chelex®100 solution were added according to the nature of the sample: 200 µl was added to antenna and tarsus samples, 100 µl was added to full forewing samples and 50 µl was added to wing tip samples. The samples were then incubated at 56°C for 2 hours with constant agitation, vortexed for 10 s, boiled at 100°C for 15 min and vortexed for another 10 s. Following 3 min of centrifugation at 8000g, 20 µl of the supernatant was pipetted into 200 µl microtitre plates. All the extractions were used neat for PCR reactions. All the steps following the incubation period were repeated if more DNA samples were needed.

3.4. Microsatellite analysis

Polymerase chain reaction (PCR) amplifications were used to amplify 4 microsatellite markers: A76, A107, A113 and B124 (Table 4.1). A76, A107 and A113 were previously isolated from *Apis mellifera* (Estoup et al. 1994, 1995) and B124 was isolated from *Bombus terrestris* (Estoup et al. 1994). PCRs were performed with a Hybaid thermal cycler in a 10.5 µl volume containing 1.5 µl of DNA sample, 1.0 µM of each primer, 0.2 mM of each dNTP, 1.5 or 2.0 mM $MgCl_2$, and 0.05 units of *Taq* DNA polymerase (Thermoprime plus, Advanced Biotechnologies), in the manufacturer's buffer at a final concentration of 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0 and 0.01% (w/v) Tween. The reaction profile for each locus was 94°C for 1 min, followed by 39 cycles of 94°C for 30 s, annealing temperature (Table 4.1) for 30 s, and 72°C for 30 s, followed by a last elongation stage of 5 min at 72°C. The forward primer of each marker was 5' end-labelled with a fluorescent phosphoramidite (NED, 6-FAM or HEX). The PCR products were visualised on an Applied Biosystems (ABI) 377 DNA sequencer using an internal size-standard (ROX). Because of the size and dye differences between the PCR products for the 4 loci we were able to multiplex them in a single set of markers after diluting them with different amounts of ddH_2O (Table 4.1). The gels were analysed using ABI Genescan software (version 3.1) and Genotyper DNA fragment analysis software (version 2.5).

Table 4.1. The 4 DNA microsatellite markers used.

Locus	Fluorescent label	T_a (°C)	Dilution (µl)	$MgCl_2$	Number of alleles	Size-range (bp)	Heterozygosity
A107	Hex	58	0	1.0	15	160-186	0.946
A113	6-Fam	58	30	1.2	6	202-234	0.875
A76	Ned	58	10	1.2	18	208-315	0.875
B124	Hex	54	10	1.5	13	207-251	0.786

T_a annealing temperature.
$MgCl_2$ published concentrations of $MgCl_2$ for PCR reactions.
Numbers of alleles and heterozygosities, calculated with CERVUS (Marshall *et al.* 1998) and based only on the 6 colonies of honey bees used in the experiments (n = 46 queens and 10 workers).

The annealing temperatures used and the 1.5 mM concentration of $MgCl_2$ were obtained previously by optimisation on phenol extracted samples and were used in the first reactions. The worker samples were only amplified once at these conditions. However, the presence of chelating agents in the DNA samples might cause amplification problems and for the queen samples, any individuals with failed reactions after the first PCRs were redone at two $MgCl_2$ concentrations, 1.5 and 2.0 mM. All PCR reactions were performed using both negative (water) and positive controls (DNA extracted from worker heads using classic phenol technique and of known genotypes).

Because we amplified several samples from the same individuals and performed more than one successful PCR amplification on some samples we could check the reliability of the genotypes obtained from the wing tip samples and that using very little tissue for the extraction did not cause allelic dropout during the amplifications, as sometimes occurs (Taberlet et al. 1999).

3.5. Statistical analyses

A generalised linear model with binomial error structure was used to test whether the amplification efficiencies of the 4 microsatellite loci were significantly different for the various sampling and storage regimes. For this purpose the individual samples were scored as 1 if they successfully amplified at the 4 loci and as 0 if at least one of them did not. When multiple pair-wise comparisons were done, we used the Bonferroni correction to adjust the level of significance.

3.6. Behavioural analysis

Twenty forager bees were collected at the entrance of an observation hive and anaesthetised by chilling at 4°C for ten minutes. They were then marked with a dot of white paint and their wing tips clipped in the same way as the newly-emerged queens (i.e., removal of 1.3 mm^2). They were released 20 m from their original colonies to determine if they could fly back to their colony.

4. Results

4.1. Worker samples

There was no significant effect of type of tissue, storage method or age of bee on the mean amplification success of the 4 loci for the worker DNA samples (Table 4.2, n=86, P>0.2) although the B124 and A113 loci amplified for fewer individuals than the other two loci, A76 and A107. It was therefore decided to use the simpler method, freezing, as the storage method for the subsequent queen samples.

Table 4.2. Experiment 1. Number of unsuccessful PCR amplifications for each sampling method for the worker samples at the 4 loci that were tested.

	Locus					
Tissue sample (sample size)	A107	A113	A76	B124	Total	Mean %
Head (n=10)	0	1	0	4	5	12.50
Tarsus ethanol (n=9)	1	1	2	1	5	13.89
Tarsus ethanol ten days (n=9)	0	0	0	1	1	2.78
Tarsus frozen (n=10)	0	0	0	1	1	2.50
Tarsus frozen ten days (n=10)	0	1	1	0	2	5.00
Half wing ethanol (n=8)	1	2	0	2	5	15.62
Half wing ethanol ten days (n=10)	0	1	0	1	2	5.00
Half wing frozen (n=10)	1	2	1	1	5	12.50
Half wing frozen ten days (n=10)	0	0	1	0	1	2.50
Total	3	8	5	11	27	
Mean %	3.48	9.30	5.81	12.79	7.85	

4.2. Queen samples

We analysed 44 antenna samples, 46 full forewing samples and 35 wing tip samples. The 46 queens were aged between 2 and 142 days (mean ± s.e.: 34.45 ± 5.31) before the final sampling (antennae for the first set, full forewing and antennae for the second). After the first PCRs, the tissue type had a significant effect on the

amplification success (Table 4.3, n=125, P<0.001). The pair-wise comparisons of the 3 sampling methods showed that only the wing tip and the antennae samples were significantly different from each other (n=79, P<0.001). Locus A76 amplified for more samples than all the others (3.2% of failures vs. 12.8% for A107 and A113 and 22.4% for B124). We amplified all individuals with missing genotypes again at all loci with two different magnesium concentrations: 1.5 mM and 2.0mM. Locus B124 amplified better at 2.0 mM $MgCl_2$ which is 0.5 above the recommended concentration with the published sequence (Table 4.1), and could be necessary because of the presence of a chelating agent in the DNA samples. 1.5 mM $MgCl_2$ was already above the recommended concentration for the other markers (Table 4.1).

Table 4.3. Experiment 2. Number of unsuccessful PCR amplifications in the two sets performed on the queen samples (first/cumulated results after second amplification). The second amplifications was done on all the unsuccessful samples from the first PCR at two different MgCl2 concentrations.

	Locus				
Tissue sample	A107	A113	A76	B124	Mean %
Antennae (n=44)	2/0	2/0	0/0	2/0	3.4/0
Wing tips (n=35)	11/1	13/2	4/1	14/2	30.0/4.3
Full wing (n=46)	3/0	1/0	0/0	12/0	8.7/0
Mean %	12.8/0.8	12.8/1.6	3.2/0.8	22.4/1.6	

Following the second round of amplifications, all samples could be scored at all loci except for two of the wing tip samples, giving an overall amplification failure of 4.3% for wing tips (n = 140 genotypes), 0% for full wings (n =184) and 0% for antennae (n =176). One of the wing tip samples did not give any product with any of the markers used. The other unsuccessful wing tip sample gave a product only for two loci (A107 and A76; Table 4.3). For all other amplifications, the genotypes were identical and consistent for the different samples of the same individual and different PCR amplifications of the same samples. In addition, the five different colony origins of the queens could be identified using their genotypes.

Sufficient DNA was extracted from the wing tip samples to perform at least 20 PCR amplification reactions using wing tips and at least 50 with DNA extracted from whole wings.

4.3. Behavioural analysis

All the wing tip clipped workers released 20m from their observation hives were seen flying back home and on subsequent days some of them were seen leaving the hive on foraging trips and on the combs inside. It was not possible to record all the bees as only two combs of the nine frame observation hive were observable.

The mating success of the wing tip clipped queens introduced to mating nuclei (5/7) and of unclipped queens (7/9) was not significantly different (Fisher's exact test, P=0.3) suggesting no adverse effect of wing clipping. Poor weather conditions and robbing of the mating nucleus colonies by other colonies may have caused the failure of some of the queens to mate.

5. Discussion

Our results clearly show that it is possible to extract DNA from wings and wing tips using standard and simple techniques and that the quality of the DNA is good enough to perform PCR amplifications. The wing tip samples proved to be harder to amplify but the success rate of 33/35 (94.3%) at the four loci was still very good. If minimal impact on survival or behaviour is desired then this is clearly the preferred method.

The removal of legs or tarsi in honey bees for DNA sampling, even if suitable for genetic analyses (Starks and Peters 2002), is probably not the best option as they are essential for conducting many colony activities. In addition, queen and worker tarsi produce important pheromones (Lensky and Slabezki 1981, Winston, 1987) and queens with missing tarsi are superseded more frequently (Woyke 1988). Queens with missing legs, which sometime occur naturally, appear less able to move around in the colony and lay eggs more slowly (FLWR, personal observation). Although queens need their wings for making mating flights (and workers obviously use their wings for foraging, defence, removal of corpses from the nest, etc.) both queens and workers frequently have worn wing tips, showing that they can fly despite losing part of their wings. A study on bumblebee wing wear (Hedenström et al. 2001) showed that a 10% reduction of the wing surface did not significantly affect forager survivorship. Clipping full wings of mated queens in colonies is a common beekeeping practice which does not affect the

queen's ability to carry out her in-nest duties, but prevents them from swarming (honey bee queens never remate once egg-laying has begun; Laidlaw and Page 1997).

Because it is more difficult to amplify DNA from wing tip samples, it is recommended that PCR conditions be optimised for all the markers using control samples. Although each PCR product that was obtained could be scored reliably, we still recommend performing two amplifications of each locus to ensure maximal scoring accuracy (Taberlet et al. 1999). For the whole wing extractions, no amplification problems occurred and even wings of older queens (up to 142 days) gave good quality DNA.

Whole wing and wing tip sampling of queens can be a useful method in honey bee breeding and conservation programs. Honey bees are economically important for their honey production and as major pollinators of crops and wild plants (e.g. Carreck and Williams 1998, Roubik 2002). Typically, breeding programs select for desirable traits such as low defensiveness and high disease resistance (Spivak 1996, Spivak and Reuter 1998) or can attempt to conserve local races (Cooper 1986). Being able to select queens before allowing them to mate naturally or before instrumental insemination has the potential to speed up the selection process and reduce the amount of work involved. Sometimes the presence of only one or a few patrilines with the desired trait is sufficient to make the whole colony express a desirable phenotype such as hygienic behaviour (e.g. Trump et al. 1967), a phenomenon known as behavioural dominance (Craig 1980). The standard breeding approach of randomly selecting queens from colonies with a desirable phenotype can then be a relatively inefficient way of artificial selection. By using the wing tip sampling methods, however, a breeder could specifically select queens from the preferred patrilines if these have been determined by behavioural studies of workers. Such within colony selection can increase the response to selection in breeding programs (Wenseleers and Ratnieks, unpublished). In addition, bee genetics and behavioural studies are now well developed and quantitative trait loci responsible for certain behaviour like foraging (Hunt et al. 1995), defence (Hunt et al. 1998) and hygienic behaviour (Lapidge et al. 2002) are starting to be identified so that newly-emerged queens could also be selected based on specific genes or markers. Non-destructive tissue sampling could also be used for other purposes. For example, in behavioural studies, the patrilines of particular workers could be determined before they are studied, which could give better designed experiments (e.g., equal sample sizes per

patriline). Or in parentage studies, the queens could be non-destructively sampled to increase confidence in parentage assignments.

Chapter 5

Anarchy in the UK: Detailed genetic analysis of worker reproduction in a naturally-occurring British anarchistic honeybee, *Apis mellifera*, colony using DNA microsatellites.

1. Abstract

Anarchistic behaviour is a very rare phenotype of honeybee colonies. In an anarchistic colony, many workers' sons are reared in the presence of the queen. Anarchy has previously been described only in two Australian colonies. Here we report on a first detailed genetic analysis of a British anarchistic colony. Male pupae were present in great abundance above the queen excluder, which was clearly indicative of extensive worker reproduction and the hallmark of anarchy. Seventeen microsatellite loci were used to analyse these male pupae, allowing us to address whether all the males were indeed workers' sons, and how many worker patrilines and individual workers produced them. In the sample, 95 of 96 of the males were definitely workers' sons. Given that c. 1% of workers' sons were genetically non-distinguishable from queen's sons, this suggests that workers do not move any queen-laid eggs between the part of the colony where the queen is present to the area above the queen excluder which the queen cannot enter. The colony had 16 patrilines, with an effective number of patrilines of 9.85. The 75 males that could be assigned with certainty to a patriline came from 7 patrilines, with an effective number of 4.21. They were the offspring of at least 19 workers. This is in contrast to the two previously studied Australian naturally-occurring anarchist colonies, where most of the workers' sons were offspring of one patriline. The high number of patrilines producing males leads to a low mean relatedness between laying workers and males of the colony. The importance of studying such colonies in the understanding of worker policing and its evolution is discussed.

2. Introduction

Insect societies show great diversity in their mating systems (Boomsma and Ratnieks 1996, Strassmann 2001) and in the way reproduction is shared among colony members (Bourke and Franks 1995, Crozier and Pamilo 1996, Foster and Ratnieks 2001c, Foster et al. 2001). Documenting this variation among species and colonies is crucial in understanding reproductive conflicts because queen mating frequency greatly affects colony kin structure and the relatedness among female offspring (Pamilo et al. 1997). This in turn may influence reproductive conflicts among colony members, e.g. over the optimal sex ratio (Trivers and Hare 1976) or male parentage (Ratnieks 1988).

Honeybees, *Apis mellifera*, typically have a single queen who is the main reproductive individual within the colony. The workers cannot mate but retain functional ovaries and can lay unfertilised eggs which develop into males (Winston 1987, Page and Erickson 1988, Visscher 1989, Seeley 1995). However, the reproductive output of workers in most queenright colonies is negligible (Visscher 1989, Ratnieks 1993, Visscher 1996). Several mechanisms are responsible for this. At a proximate level, few workers have active ovaries (Ratnieks 1993), and the presence of both the queen (Butler and Fairey 1963) and brood (Arnold et al. 1994) inhibits worker ovary activation. In queenless colonies, this inhibition disappears and 5 to 24% of workers activate their ovaries (Miller and Ratnieks 2001). In addition, most worker-laid eggs are eaten (policed) by other workers (Ratnieks and Visscher 1989, Ratnieks 1993, Visscher 1996, Barron et al. 2001). Worker policing is favoured in honeybees on relatedness grounds because *Apis* queens typically mate with multiple males (Estoup et al. 1994, Oldroyd et al. 1997, Palmer and Oldroyd 2000). As a result, honeybee workers are on average more related to the queen's sons than to their sister workers' sons, and they benefit by worker policing as this causes the rearing of queen's sons rather than the less related workers' sons (Ratnieks 1988, Barron et al. 2001).

Although worker policing normally ensures that few worker sons are reared in queenright *Apis mellifera* colonies, many males are worker-derived in "anarchistic colonies". Anarchistic colonies are very rare, approximately one colony per 1,000-10,000 (Barron et al. 2001). Although there is no overt difference in the appearance of workers' and queen's sons within a colony, anarchistic colonies can be easily detected in managed hives when a queen excluder is used to confine the queen to the lower hive boxes. The co-occurrence of male brood above the excluder and brood of both sexes,

and the queen, below the excluder strongly suggests anarchy. The kin structure of two naturally-occurring anarchistic colonies from Australia has been described (Oldroyd et al. 1994, Montague and Oldroyd 1998). In both colonies, the workers were the offspring of a single queen mated to many males, as is typical, but only one patriline of workers produced the majority of the workers' sons (98% in one, Oldroyd et al. 1994, and 84-92% in the other, Montague and Oldroyd 1998).

Here we provide a detailed genetic analysis of a naturally-occurring anarchistic colony of *Apis mellifera* from Britain. We used 17 polymorphic DNA microsatellite loci to distinguish between workers' sons and queen's sons, and between the offspring of different worker patrilines and even individual workers. In contrast to the two Australian anarchistic colonies our results show that at least 8 of the 16 worker patrilines produced males. Our results also show that many individual workers produced these males. In addition, since the queen was not the mother of any of the males reared above the queen excluder, our data show that queen eggs or larvae were not transferred from below the queen excluder.

3. Material and methods

In April 1999, a novice beekeeper from Widnes, Lancashire, England reported a honeybee colony, *Apis mellifera*, with brood above the queen excluder to an Internet newsgroup on beekeeping for advice as to what was going on. One of us (FR) visited the beekeeper and confirmed that it was a queenright colony with brood of both sexes below the excluder but only male brood (many eggs, and hundreds of larvae and pupae) above the excluder. The queen had been marked with a paint dot, which indicated that she had been reared before 1999 and, therefore, that the colony had not been queenless at any time in the previous few months. If the colony had been temporarily queenless and had recently been requeened, worker reproduction could have been caused by the absence of the queen (Winston 1987, Page and Erickson 1988, Miller and Ratnieks 2001). The colony bore all the hallmarks of anarchy. This was only the second naturally-occurring anarchistic colony that FR had seen in 18 years of beekeeping during which he has inspected more than 1000 colonies with queen excluders. The beekeeper donated the colony for research and it was transported to the laboratory apiary.

The colony had a healthy egg-laying queen, brood of both sexes, and approximately 30,000 workers. New male brood continued to be observed above and below the queen excluder during the spring. On 26th May 1999 frames of brood were taken from below and above the queen excluder and kept in a freezer. Samples of worker and male pupae were taken from the frame below the excluder and male pupae were taken from the frame above the excluder. Pupae rather than adult bees were collected to exclude bees that drifted from adjacent colonies, which can represent as many as 89% of the adult drones and 14% of the adult workers (Neumann et al. 2000). By sampling workers and males at the same time, the workers in the sample were younger than those that laid the eggs that gave rise to the sample of male pupae. However, because sperm use by *Apis mellifera* queens becomes consistent a few months after mating (Estoup et al. 1994, Franck et al. 1999) and because the queen was at least 8 months old, the patriline proportions in the colony at the sampling time should be comparable to the proportions at the time the male eggs were laid.

DNA from the heads of 214 pupae (n = 94 workers, n = 96 males from above the excluder, n = 24 males from below the excluder) was extracted using phenol (Bruford et al. 1998). Polymerase chain reaction (PCR) amplifications were used to amplify 17 microsatellite markers (Table 5.1) previously developed for *Apis mellifera* and *Bombus terrestris* (Estoup et al. 1994, Estoup et al. 1995, Baudry et al. 1998). PCRs and PCR visualisation were conducted as in Chapter 3.

Many markers had to be analysed to obtain a clear picture of the colony kin structure. For example, when trying to determine the mother of a male there are several possibilities (e.g., the queen and workers of different patrilines) and these potential mothers all have many genes identical by descent because they are related. Males are haploid and each male inherits one or the other of his mother's two alleles at each locus. Because workers are all daughters of the queen, a worker's son inherits a queen-derived allele at a locus with a probability of 0.5. When this happens, that locus is uninformative in assigning the male to a particular patriline, and it also makes the male indistinguishable from a queen's son. This causes a large proportion of the workers' sons to be indistinguishable from queen's sons when only a small number of marker loci are used. Even when a male inherits a paternal allele it may not be possible to assign the male as a worker's son, if the paternal allele is the same as one or both of the queen's alleles at that locus. When assigning worker's sons to their maternal patriline, the fact that the males fathering the different patrilines can share alleles with the queen

and between each other makes the assignment of maternity to different patrilines more difficult.

Table 5.1. The 17 DNA Microsatellite markers used. Markers were isolated from *Apis mellifera* except B124 which was isolated from *Bombus terrestris*.

Marker set	Locus	Fluorescent label	T_a (°C)	Number of alleles	Size-range (bp)	Heterozygosity
1	A107[1]	Hex	60	7	165-186	0.742
	A113[2]	6-Fam	60	4	203-227	0.667
	A24[2]	Ned	55	4	96-106	0.095
	A35[1]	Hex	57	4	114-125	0.624
	A43[1]	6-Fam	55	3	126-139	0.326
	A76[1]	Ned	58	8	230-308	0.947
	A88[2]	Ned	55	2	143-151	0.447
	B124[1]	Hex	55	7	218-242	0.691
2	A14[1]	6-Fam	58	8	219-255	0.946
	A28[1]	Ned	58	2	131-137	0.731
	A29[1]	6-Fam	54	8	134-163	0.737
	A7[1]	Hex	58	4	110-132	0.558
3	Ap14[3]	Ned	62	4	134-148	0.839
	Ap16[3]	Hex	52	2	143-157	0.042
	Ap19[3]	6-Fam	56	7	134-146	0.916
	Ap33[3]	Hex	54	9	226-253	0.958
	Ap37[3]	6-Fam	56	3	188-193	0.589

Multiplexing and labelling with one of three fluorescent dyes allowed us to run the 17 loci in 3 marker sets on an ABI 377 automated DNA sequencer.
T_a annealing temperature.
The markers used were published by [1]Estoup et al. (1994), [2]Estoup et al. (1995) and [3]Baudry et al. (1998).
Numbers of alleles and heterozygosities, calculated with CERVUS (Marshall et al. 1998) are given based on this colony alone (n=94 workers).

4. Results

4.1. Kinship of worker offspring

The microsatellite markers used were highly polymorphic with 2-9 different alleles per locus (mean 5.11) detected across all the males and workers analysed.

Heterozygosities were calculated using CERVUS (Marshall et al. 1998) and ranged from 0.042 to 0.958 (Table 5.1).

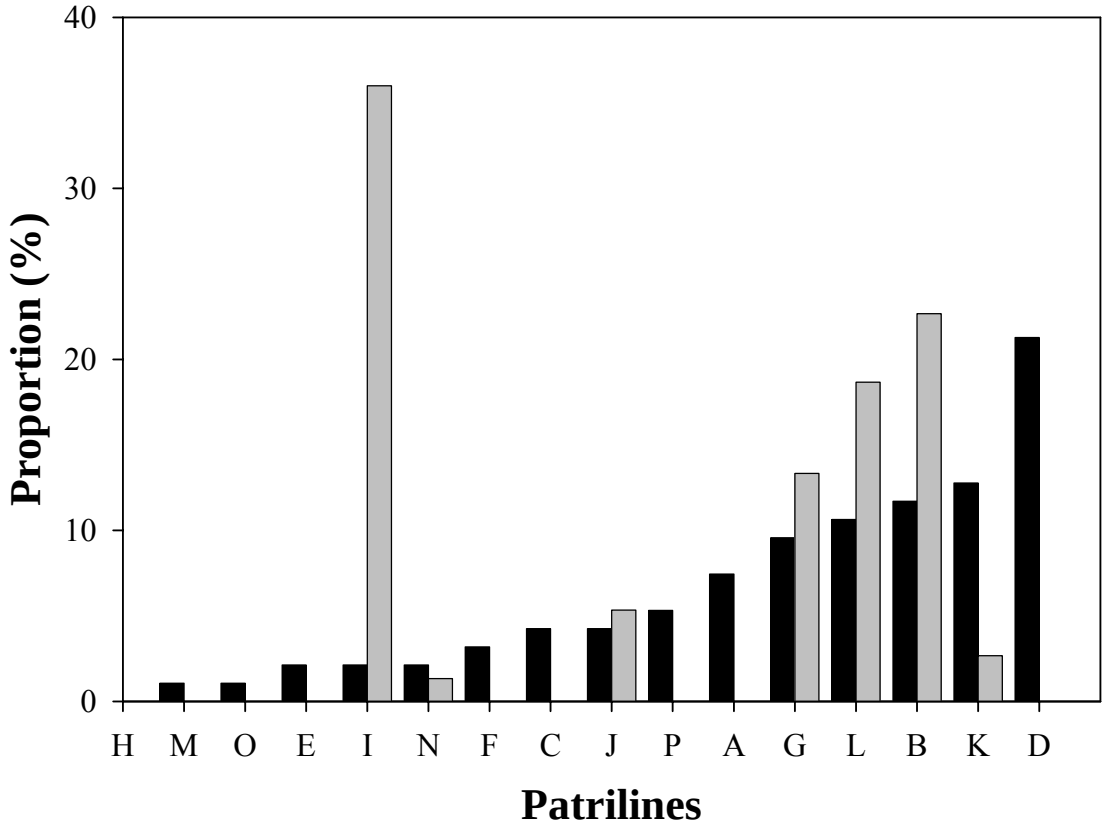
We inferred the genotype of the queen from the workers' genotypes. If the queen is heterozygous at a locus then the workers will have one of two maternal alleles with approximately equal frequency. If the queen is homozygous then all the workers will carry the same maternal allele. The genotype of each worker's father was then determined by subtraction and the total number of fathers and their relative paternity determined.

In total, 16 patrilines (named A-P) were found in the 94 workers. The large number of loci used and their high variability means that it is unlikely that we failed to find any fathers due to genetic non-detection. However, because of the large number of fathers it is possible that unsampled rare patrilines were present. By analysing 94 workers, any male who contributed to 3% or more of the offspring has a greater than 95% probability of being represented in the sample (Boomsma and Ratnieks 1996). No undetected patrilines appeared in the workers' sons, which further suggests that we sampled all patrilines. Because the workers were not equally frequent among patrilines (Figure 5.1) the effective mating frequency (Me) is 9.05 (Starr 1984, Boomsma and Ratnieks 1996), and 9.85 if corrected for sample size (Pamilo 1993). This corresponds to a mean relatedness among workers of 0.30 (Pamilo 1993).

When genotyping individual bees, there is a risk of mistyping the individuals and of mutations. This is mostly problematic for patrilines represented by a single or two workers or patrilines for which the implied paternal genotype only differs at one or two loci from that of another father. When this occurred, new PCRs were performed on the individuals and run on new gels to check for correct typing. This leaves the possibility of mutations. In our sample, most paternal genotypes differed at more than three loci. However, four patriline pairs differed at less than three loci. Patriline J and P differ at one locus but are represented by 4 and 5 workers respectively. Patriline O, represented by a single worker, differs from patriline K at two loci, and it is unlikely that two mutations would occur at the same time. Patriline N, represented by two workers, differs from patriline E at one locus but it is unlikely that the same mutation occurred twice in the two N individuals. Patriline H, represented by a single worker only differs from patriline D at one locus. There is a chance that this is due to a mutation. We chose to include this worker from patriline H as belonging to a distinct patriline in the results. However, combining D and H into one patriline would not

change the results in a significant way as the mean relatedness among workers would become 0.307 as opposed to 0.305 with 16 patrilines. Given the high mating frequency of honeybee queens and non-equal sperm use, it is highly likely to find patrilines represented only by one worker in a sample of 94.

Figure 5.1. Patriline distribution of workers (n = 94, black bars) and workers' sons (n = 75 assigned to specific patrilines, grey bars) from above the queen excluder. Assigned males from below the excluder are not included, as they constitute another independent sample.



4.2. Males from above the queen excluder

4.2.1. Workers' sons or Queen's sons?

The detection of workers' sons is made difficult by the fact that, at any locus, the son of a worker inherits his mother's paternal allele only half the time. In addition, the father of the egg-laying worker may also share an allele with the queen at a given locus, which leads to this locus being uninformative for the whole patriline. The probability, p, of being able to detect a worker's son of a given patriline is

$$p = 1 - 0.5^{l}$$

where l is the number of informative loci for this patriline, that is the number of loci where the father's allele is different from both the queen's alleles. These probabilities ranged from 93.75% (patriline A) to more than 99.9% (patrilines B, C, L, N) (Table 5.2). A mean detection probability can be calculated (Foster and Ratnieks 2001a) as

$$p = \sum_{i=1}^{n} p_i(1-0.5^{li})$$

where n is the number of patrilines, p_i is the proportional representation of the ith patriline and l_i is the number of informative loci analysed for the ith patriline. This probability was 99.2 % in the study colony. Of the 96 males from above the queen excluder, 95 were positively identifiable as workers' sons because each carried at least one paternal marker. The remaining male could not be assigned. However, with a detection probability of 99.2 % the probability that at least one out of 96 workers' sons will have no paternal allele is high ($1 - 0.992^{96} = 0.537$; Figure 5.2). It is, therefore, fully consistent with the genetic detection probabilities that the non-assigned male is also a worker's son.

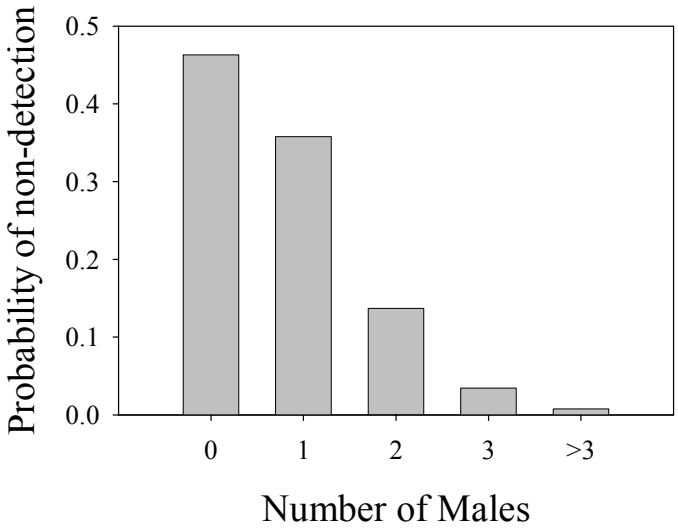


Figure 5.2. Probability of not detecting worker's sons in a sample of 96 males calculated from a binomial distribution with n=96 and p=0.992.

Table 5.2 Genotypes of the queen and father of each worker patriline (A-P) detected using 17 microsatellite markers (n=94 workers).

	A107	A113	A24	A35	A43	A76	A88	B124	A14	A28	A29	A7	Ap14	Ap16	Ap19	Ap33	Ap37	Number of workers	Numbers of alleles different from the queen	Probability of detection	Mean relatedness of workers to workers' sons	Mean relatedness of workers to all males
Queen	174 188	215 221	96 96	114 119	126 126	230 288	143 151	222 237	225 235	137 137	141 155	110 132	134 142	143 143	134 142	238 242	193 193					
A	165[i]	227[i]	96	119	126	230	143	218[i]	235	137	155	132	134	143	134	250[i]	193	7	4	0.9375	0.125	0.193
B	168[i]	221	96	123[i]	126	232[i]	143	222	230[i]	131[i]	163[i]	110	140[i]	143	140[i]	253[i]	189[i]	11	10	0.9990	0.182	0.219
C	171[i]	203[i]	96	123[i]	126	244[i]	143	242[i]	230[i]	131[i]	143[i]	110	140[i]	143	134	245[i]	189[i]	4	11	0.9995	0.125	0.193
D	172[i]	203[i]	96	119	138[i]	244[i]	143	222	230[i]	131[i]	141	110	134	143	138[i]	250[i]	193	20	8	0.9961	0.125	0.193
E	186[i]	221	104[i]	125[i]	126	234[i]	143	222	221[i]	137	139[i]	110	134 142	157[i]	146[i]	247[i]	188[i]	2	10	0.9990	0.125	0.193
F	174	221	106[i]	119	126	234[i]	143 151	239[i]	230[i]	137	134[i]	110 132	134 142	143	144[i]	226[i]	189[i]	3	8	0.9961	0.125	0.193
G	174	215	96	114	126	238[i]	143	222	237[i]	131[i]	139[i]	110	140[i]	143	140[i]	236[i]	188[i]	9	8	0.9961	0.158	0.208
H	174	203[i]	96	119	138[i]	244[i]	143 151	222	230[i]	131[i]	141	110	134 142	143	138[i]	250[i]	193	1	7	0.9922	0.125	0.193
I	186[i]	221	104[i]	114 119	126	244[i]	143	227[i]	230[i]	137	157[i]	110	134	143	140[i]	226[i]	189[i]	2	9	0.9980	0.215	0.234
J	174	221	96	114	126	259[i]	151	242[i]	230[i]	137	139[i]	110	140[i]	143	144[i]	245[i]	193	4	7	0.9922	0.138	0.199
K	174	221	96	114	126	308[i]	143	218[i]	237[i]	131[i]	139[i]	110	140[i]	143	140[i]	236[i]	188[i]	12	9	0.9980	0.132	0.196
L	176[i]	215	96	114	139[i]	238[i]	143	222	255[i]	131[i]	141	119[i]	148[i]	143	136[i]	234[i]	188[i]	10	10	0.9990	0.172	0.214
M	176[i]	215 221	96	114 119	126	244[i]	143 151	222	219[i]	137	161[i]	117[i]	134	143	134 142	236[i]	193	1	6	0.9844	0.125	0.193
N	186[i]	221	104[i]	125[i]	126	234[i]	143	227[i]	221[i]	137	139[i]	110	134	157[i]	146[i]	247[i]	188[i]	2	11	0.9995	0.128	0.194
O	174	221	96	114	126	308[i]	143	218[i]	230[i]	131[i]	143[i]	110	140[i]	143	140[i]	236[i]	188[i]	1	9	0.9980	0.125	0.193
P	174	221	96	114	126	259[i]	151	242[i]	230[i]	137	139[i]	110	140[i]	143	144[i]	242	193	5	6	0.9844	0.125	0.193

[i] denotes that the marker is informative for the given patriline.

When two alleles are given in a father's genotype cell, it means that they had an allele identical to the queen and it was not possible to distinguish between the two possibilities.

Probability of detection gives the probability that a son of a worker of that patriline could be distinguished from a queen's son.

4.2.2. How many worker patrilines produced the males?

Each worker's son inherits from 0 to 17 paternal alleles from his mother. (The actual number follows a binomial distribution with p = 0.5 and n = 17, assuming unlinked loci and fair meiosis). These paternal alleles allow us to determine which patriline a mother belongs to. However, because different fathers that mated to the same queen can have the same allele at a locus, the number of informative loci for assigning the mother worker's patriline is fewer than 17. We could determine the exact patriline origin of 75 of the 96 males reared above the excluder. 7 of the 16 patrilines produced males, but neither in equal proportions (χ^2; $p<0.01$) nor in proportions similar to their representation in the workers (χ^2; $p<0.01$, Figure 5.1). The effective number of patrilines contributing to male production was 4.21, considering only the 75 patriline-assigned males. In the 21 males who could not be assigned to a precise patriline, 6 other patrilines (C, D, F, H, P, O) could have produced males and one of them (patriline D or H) definitely produced at least one male (Table 5.3). Thus, at least 8 patrilines were producing males, only 7 of which could be named. In addition, it is possible that several of the 6 other patrilines also produced males.

Table 5.3. Assignment of the 96 males sampled from above the queen excluder to their mother's patriline.

Mother worker's patrilines	Number of males
B	17
G	10
I	27
J	4
K	2
L	14
N	1
B or C	1
D or H	***1***
G or K	7
G or L	2
J or P	*6*
C,D or H	***1***
B, C, F or I	*1*
B, C, D, G, H, K, L or O	*1*
not assigned	1
Total	96

Some of the males could not be assigned to a single patriline and could have been the offspring of workers of several patrilines, as indicated.
Bold Italics: Possible patrilines are different from the patrilines definitely involved in male production which means at least one additional patriline produced males.
Italics: both definitely male producing patrilines and others are possible.

4.2.3. How many individual workers produced the males?

Because males are haploid all the workers in one patriline inherit the same paternal alleles. These alleles, therefore, provide no information about whether two males from the same patriline of workers had the same or different mother workers. But maternal alleles (i.e. from the queen via a worker mother) can provide this information when the queen is heterozygous. A worker's son inherits the queen allele with a probability of 0.5 per locus. Workers can inherit one of two alleles per locus from their mother queen if she is heterozygous. If two workers of the same patriline have two different maternal alleles at a given locus and their father's allele is different from the queen's, their respective sons will inherit different detectable queen alleles from them at this locus. In this situation, it is possible to say that male offspring of the same patriline (via the paternal alleles) have different mothers. By examining all the informative loci in the males from one patriline, the minimum number of workers that could have produced these males can be estimated by finding the minimum number of unique combinations of maternal alleles. This method shows that at least 19 workers produced the 75 males, with at least 5 in patriline I and 5 in patriline B.

4.3. Males from below the queen excluder

Using the same methods, 11 of the 24 males from below the queen excluder were positively identified as workers' sons. The probabilities that one or more than one of the remaining 13 males are workers' sons are 9.4% and 0.45% respectively. The queen was, therefore, most likely the mother of 54% (probability 90%) of the males sampled from below the excluder. The 95% binomial confidence interval for this proportion is $\pm 1.96\sigma/\sqrt{n}$ which is ±4.1% (Sokal and Rohlf 1995). If an additional male was a worker's son (probability 9.4%), the proportion would be 50% ±4.1 (95% CI). In the colony, the presence of the queen excluder meant that workers had access to more drone cells than the queen and, therefore, workers produced around 75% of the male brood throughout the colony. In the absence of the excluder, it is likely that competition for cell space between workers and the queen would have brought the proportion down to the 54% observed below the excluder.

4.4. Relatedness of workers to males

Assuming that all fathers are unrelated to each other and to the queen, the relatedness between workers of non-reproducing patrilines and workers' sons is 0.125.

The relatedness between workers from anarchistic patrilines and worker produced males was slightly higher, 0.128 for patriline N up to 0.215 for patriline I (Table 5.1), or 0.159 on average. The mean relatedness between all workers and worker-derived males was 0.143. If we consider that the queen is the mother of 54% of the males in the colony, as suggested from the sample from below the excluder, we can also estimate the mean relatedness of the workers to all males produced in the colony. We used this estimate because it seems closer to what the proportion would have been if the queen excluder were not present in the colony. For non-anarchistic workers, this is 0.193. For anarchistic patrilines, it ranges from 0.194 for patriline N to 0.234 for patriline I (Table 5.1), with a mean of 0.208. If all workers are considered, the relatedness is 0.201. If the estimate of 75% of worker-derived males in the colony had been used, the mean relatedness of anarchistic patrilines to all workers would have been 0.227, which is still below 0.25. Patriline I produced the most males, 36% of the worker's sons. From the molecular data we determined that at least 5 workers of patriline I produced these males. If these workers were the only ones to reproduce in patriline I and did so equally, the mean relatedness of these individual workers to all males would be 0.238. Clearly then, there are no relatedness gains to the anarchistic workers as a collective, since 0.238 is still below 0.25, the relatedness to brothers.

5. Discussion

The genetic analyses confirm the field diagnosis of anarchy by showing that the workers were producing many of the colony's males. The analysis of the worker pupae demonstrated that 16 patrilines were present, that the effective paternity was 9.85 and that the mean relatedness was 0.30. This is a typical figure for *Apis mellifera* in which multiple paternity is the rule (Estoup et al. 1994, Oldroyd et al. 1997).

The 17 loci gave us the necessary power to show that all but one of the 96 males from above the queen excluder were definitely workers' sons. Because 1% of the workers' sons could not be distinguished from the queen's sons, the remaining male was probably also a worker's son. This shows that the presence of drone brood above the queen excluder is indeed indicative of worker laying, and that workers do not merely transfer queen's sons, eggs or larvae, from below the excluder. Previous studies (Ratnieks 1993, Ratnieks et al. in press) had implicitly made this assumption, and our study shows this to be reasonable.

Our results show that workers' sons were also being reared below the queen excluder making it highly unlikely that worker reproduction was caused by the isolation of workers above the excluder and away from the normal inhibition of ovary activation caused by the queen and her brood. Approximately half the males being reared below the excluder were workers' sons. This confirms that anarchy is a distinct reproductive syndrome in honeybee colonies rather than simply a manifestation of worker reproduction caused by the use of a queen excluder (Montague and Oldroyd 1998).

The minimum estimate of the number of workers that produced the 75 males assigned to patrilines was 19. This shows that multiple workers were responsible for male production in each of the 8 male-producing patrilines. In both patrilines I and B, which produced 27 and 17 males respectively, at least 5 workers contributed to the production of the males. Therefore, male production was not monopolised by just a few individual workers. Nineteen is probably a great underestimate of the actual number of mother workers for two reasons. First, we only analysed a sample of the males being reared. Second, the genetic methods did not always allow us to distinguish among mothers within the same patriline.

In contrast with the two other previously described naturally-occurring anarchistic colonies studied (Oldroyd et al. 1994, Montague and Oldroyd 1998) the males in our colony were sons of many worker patrilines. Eight of the 16 patrilines detected in the 94 workers analysed were also detected in the 96 workers' sons analysed. Even though some of these patrilines produced few males (Figure 5.1), the effective number of mother patrilines, 4.21, was well above one and about half the effective number of patrilines, 9.85. Different patrilines varied significantly in their production of males, and also differed from their numerical representation in the worker sample. For example, patriline I produced 36% (27 of 75) of the males but represented only 2.1% (2 of 94) of the workers whereas patriline K, which represented 12.8% (12 of 94) of the workers, produced only 2.7% (2 of 75) of the males (Figure 5.1). In other patrilines (G, L, B), male production is more in line with the number of workers in the patriline. From 6 to 8 of the patrilines produced no males. This variation among patrilines in male production provides further evidence for a genetic component to anarchistic behaviour (Oldroyd and Osborne 1999).

The use of many highly polymorphic DNA microsatellite loci allowed us to make a clear but necessarily incomplete picture of male production in the study colony. Importantly, our data show that the transfer of eggs across the queen excluder either

does not occur or is of negligible importance, thereby confirming that studies examining eggs laid above the queen excluder indeed demonstrate worker-laying. This is the first naturally-occurring anarchistic colony to be studied with many worker patrilines producing males. This has previously been observed only after active selection for anarchistic reproduction (Oldroyd and Osborne 1999). In our colony, the presence of many anarchistic patrilines suggests that the trait is, in part, maternally inherited, although the differences in male production among patrilines suggest that the fathers also influenced the phenotype of their daughters. In other words, the anarchistic phenotype may be influenced both by maternally and paternally derived genes, as is expected in diploid genetics. For example, patrilines may not share the same threshold values for signals that normally inhibit ovary activation. Similar differences between patrilines have already been demonstrated for oviposition and oophagy in queenless colonies (Robinson *et al*. 1990). Finding a naturally-occurring colony displaying such a trait confirms that anarchy has a complex genetic determinism (Oldroyd and Osborne 1999) and that both the maternal and paternal genotypes have an influence on the anarchistic phenotype, given that the two Australian anarchists with only one patriline producing males (Oldroyd et al. 1994, Montague and Oldroyd 1998) might have suggested a predominant effect of paternally-transmitted genes.

As a result of the high number of patrilines producing males, the mean relatedness of workers in anarchistic patrilines to the males being reared in the colony was below 0.25. Thus, not even the anarchistic workers benefited from worker reproduction. Anarchistic behaviour ceases to be beneficial even to anarchist patrilines when there are more than two effective anarchistic patrilines in the colony. In the study colony, anarchistic workers do not increase their fitness by reproducing and only their father's genes, which would not otherwise be present in the males produced, benefit from the worker reproduction caused. Anarchy is, therefore, costly for the workers of the colony and, as worker policing theory predicts, should be selected against by policing genes. However, it should be noted that anarchistic workers have a fitness advantage over non-anarchists within an anarchistic colony. When an anarchistic colony occurs the egg-layers will always have higher relatedness to the colony's males than the non-egg layers. The anarchistic trait is akin to a selfish gene (Hurst et al. 1996) that spreads at a cost to its host, which in this case is the whole colony. Why anarchy does not readily spread to high frequencies in the population remains a puzzle (Barron et al. 2001). But part of the answer is suggested by this study: if the anarchistic gene

does not cause any relatedness gains to the workers carrying it, modifiers will soon control worker reproduction, returning the population to the normal state of worker sterility in the presence of queen and brood.

Chapter 6

Worker policing persists in a hopelessly queenless honey bee colony (*Apis mellifera*)

1. Abstract

In queenright colonies of *Apis mellifera*, worker policing normally eliminates worker-laid eggs thereby preventing worker reproduction. However, in queenless colonies that have failed to rear a replacement queen, worker reproduction is normal. Worker policing is switched off, many workers have active ovaries and lay eggs, and the colony rears a last batch of male brood before dying out. Here we report a colony which, when hopelessly queenless, did not stop policing although a high proportion of workers had active ovaries (12.6%) and many eggs were laid. However, all these eggs and also worker-laid eggs transferred from another colony were policed. This unusual pattern was repeated eight weeks later by a second queenless colony made using worker bees from the same mother colony, which strongly suggests genetic determination.

2. Introduction

In queenright honey bee colonies, *Apis mellifera*, workers normally prevent each other from reproducing by worker policing (workers eat worker-laid eggs; Ratnieks and Visscher 1989, Barron et al. 2001). Worker reproduction is minimal in queenright colonies. Only 0.1% of the adult males are workers' sons (Visscher 1989) and only 0.01% of the workers have full-sized eggs in their ovaries (Ratnieks 1993). The situation is very different in queenless colonies. In a "hopelessly queenless" colony, that is one which has lost its mother queen and has failed to rear a replacement, many workers (5-24%) have fully-active ovaries with full-sized eggs and lay eggs (Page and Erickson 1988, Miller and Ratnieks 2001). Worker policing is switched off (Miller and Ratnieks 2001) so that the colony rears a final cohort of workers' sons (Page and Metcalf 1984, Seeley 1985, Winston 1987, Miller and Ratnieks 2001) before dying due to its dwindling workforce. Here we report an unusual colony which when hopelessly queenless did not rear any males. We investigated two different hypotheses for the

absence of male rearing: 1) absence of egg-laying by workers; 2) egg-laying by workers but persistence of worker policing. Our results support the second hypothesis and, although based on only one colony, are important because they show an interesting variant of the normal worker reproductive behaviour in *A. mellifera.* Such variants provide novel opportunities for studying reproductive conflict and the mechanisms underlying worker reproduction and policing in the honeybee.

3. Methods

3.1. Study colonies

On the 16th of May 2001 a populous queenright colony of honey bees on two brood chambers (mother colony) was divided (Chapter 3). Two weeks before the split, a queen excluder was inserted between the two chambers and additional frames of brood were transferred to the part of the colony without the queen. One colony contained the queen, brood (in both unsealed and sealed cells) and adult workers. The other (colony 1) became hopelessly queenless because it contained only sealed brood and adult workers so that the workers could not rear a replacement queen by emergency queen rearing from a young female larva (Seeley 1985). During the entire experiment, the colonies had a good supply of pollen and honey so that brood rearing was not affected by a lack of sufficient food supply. However, after 50 days and many colony inspections (2-3 times/week), very few eggs and no larvae had been observed in this colony while three other queenless colonies set up at the same time contained large numbers of worker-laid eggs. Because the absence of eggs was unusual, we investigated three reproductive characteristics: worker ovary activation, appearance of worker-laid eggs in drone cells, and worker policing (see below), to determine if workers were laying eggs and if worker policing had been switched off.

We also repeated our observations on a second queenless colony, set up on July 5th 2001, divided from the same mother colony. The queenless part (colony 2) was allowed to rear a new virgin queen. On the 30th of July (25 days after division), approximately 12 days after the new virgin queens emerged, the colony was inspected and the new queen, which had not started laying, was removed to make the colony hopelessly queenless. No unsealed brood were present in the colony at this time. This procedure, similar to Miller and Ratnieks (2001), mirrors the natural situation where the replacement queen dies during her mating flight, which takes place about one week or

more after pupal eclosion. As worker lifespan is c. 30 days in summer (15-38 days; Winston 1987), the second division involved an entirely new set of workers from the mother colony, i.e. the cohorts of workers in colony 1 and 2 were different but offspring of the same queen.

3.2. Quantifying worker ovary activation

Two samples of workers from each queenless colony were dissected to determine ovary activation. Colony 1 was sampled 50 and 67 days after division. At the second date the colony was on the verge of dwindling out and contained only c. 200 workers. Colony 2 was sampled 43 and 70 days after division (18 and 45 days after being made hopelessly queenless). Again, the final sample was made just before the colony dwindled out. Ovary activation was scored in three categories as low: largest oocyte < 0.8mm; medium: largest between 0.8 and 1.2mm; and high: largest >1.2mm (Miller and Ratnieks 2001, Chapter 3). Eggs in cells are approximately 1.3-1.8mm long (Ratnieks 1993, Winston 1987).

3.3. Quantifying egg-laying

Each hive contained one frame of drone cells (c. 3800 cells). Workers in queenless (Page and Erickson 1988) and queenright (Ratnieks 1993) colonies preferentially lay in drone cells. We studied the number of eggs appearing in these drone cells and their removal. For colony 1, we recorded the eggs on days 50-56 after division and on days 64-66, just prior to the colony death. For colony 2, we recorded eggs appearing in cells during 14 days between days 27-50 after division and on day 56 and 60, again just before the colony death.

At the beginning of each day's observation, we removed the drone frame and counted the number of eggs in cells. The position of these cells was recorded and on the next inspection (1 hour later) we determined if the eggs had been removed from them and if additional ones had appeared in other cells. This process was repeated after 2 hours and after 20 hours (next day) when a new record began.

3.4. Quantifying worker policing

Worker policing was assessed using an egg-removal bioassay in which 20 one-day old queen-laid eggs (taken from worker cells in an unrelated queenright colony) and 20 worker-laid eggs (from an unrelated queenless colony) were transferred into adjacent

cells in the drone comb. We used diploid queen-laid eggs which are easier to obtain for the trials as previous experiments have shown that male and female-destined queen-laid eggs are not treated differently by workers (Oldroyd and Ratnieks, 2000). The cells with eggs were checked after 1, 2 and 20 hours (methods of Ratnieks and Visscher 1989, Martin et al. 2002a, Miller and Ratnieks 2001, Chapter 3). Colony 1 was studied for 7 consecutive days (July 5-11, 50-56 days after division) and colony 2 on 13 days (between August 1 and 24, 27-50 days after division).

Egg-removal data after 20 hours were analysed using ANOVA. Rate of removal was analysed using repeated measures ANOVA. Data were normalised by arcsine transformation.

4. Results

4.1. Egg-laying and ovary activation

Larvae were not reared in either colony, although eggs appeared in cells (mean number of eggs observed per inspection of the drone frame: colony 1, 0.5; colony 2, 0.90). Both colonies had many workers with high (mean 12.6%) and medium (mean 9.7%) levels of ovary activation (Table 6.1).

Table 6.1. Worker ovary activation, length of largest oocyte, in the study colonies

		Ovary Activation Level (% workers)			
	Sample date (day after division)	1 (low) <0.8mm	2 (medium) 0.8-1.2mm	3 (high) >1.2mm	sample size
		Colony 1			
sample 1	05/07/02 (50)	82.1	12.5	5.4	56
sample 2	22/07/02 (67)	74.5	11.0	14.5	55
		Colony 2			
sample 1	17/08/02 (43)	77.0	9.0	14.0	100
sample 2	13/09/02 (70)	77.6	7.5	14.9	67

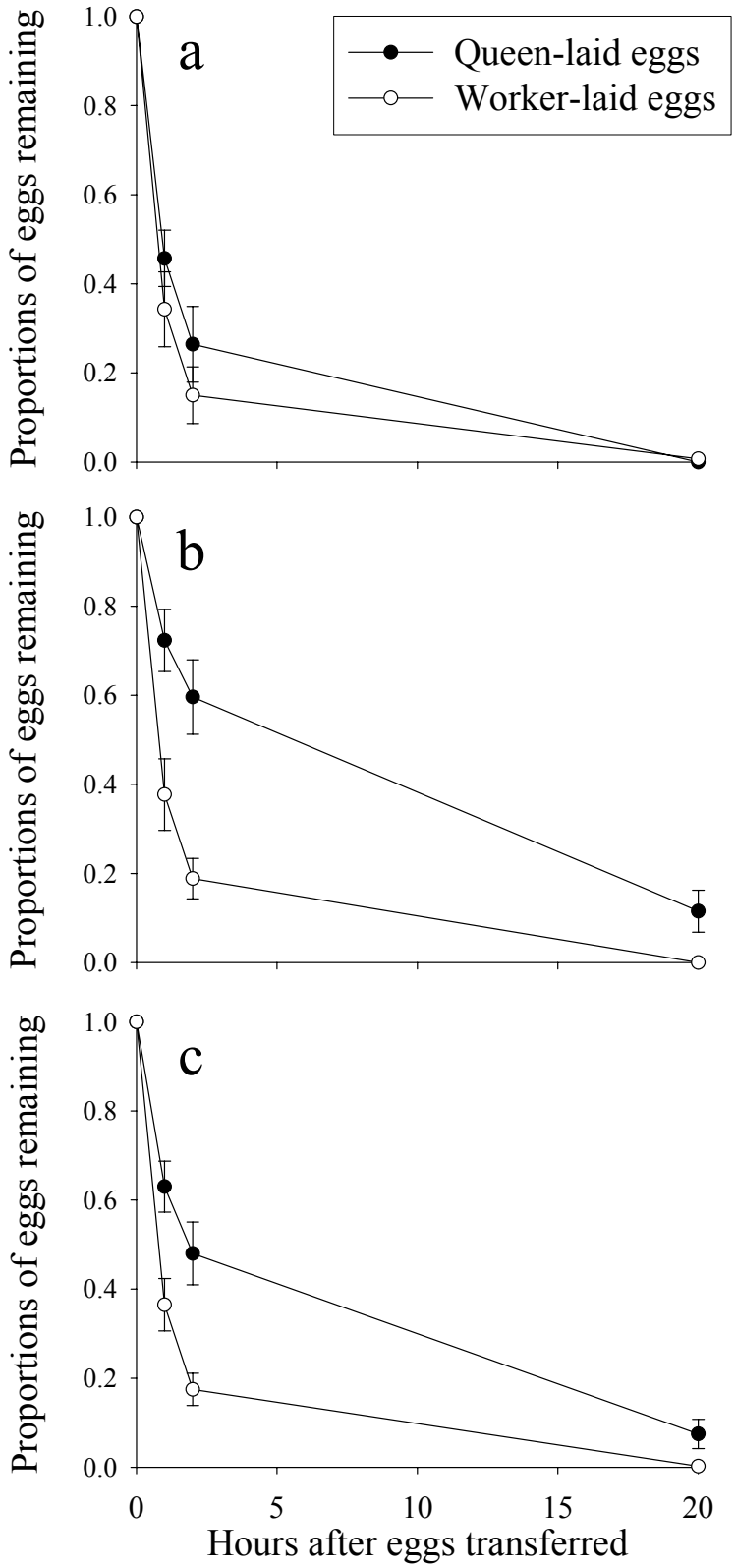


Figure 6.1. Mean proportions of eggs remaining after transfer to drone cells in the policing trials (± standard error of the mean) in a) colony 1, b) colony 2 and c) mean of both colonies.

4.2. Worker policing

Policing of worker-laid eggs was consistently effective. Most (37/58 = 64%) naturally-laid eggs appearing in combs were removed before the comb was checked again (1 hour) and 100% were removed within 20 hours (colony 1 n=15 eggs observed, 10 removed in <1h; colony 2 n=43, 27<1h).

In the experimental study of worker policing using worker-laid eggs from an unrelated colony, 100% were removed by 20h in all trials for both colonies (Figure 6.1). Almost all (mean 99.3%) of queen-laid eggs were also removed in colony 1. In colony 2, the removal of queen-laid eggs was initially low (27 days after division, 2 days after queenlessness, 40% eggs removed after 20h). However, in the following trials (day 28 and 29), this increased to 75% and 85% and to 100% on day 35. Subsequently, queen-laid egg removal remained high in colony 2 (mean 88.46% for all trials). Overall, in both colonies, there was no significant difference in the proportion of worker-laid eggs and queen-laid eggs remaining after 20 hours (ANOVA $F=3.819$, $n=40$, $p=0.58$), even though the queen-laid eggs were removed more slowly (Repeated measures ANOVA $F=10.482$, $n=40$, $p=0.03$, Figure 6.1).

5. Discussion

Our results clearly show that workers were laying eggs in the queenless study colonies but that all of these eggs were policed. At no time were any larvae seen and no males were reared. This is very different from previously studied hopelessly queenless colonies which all reared males (Miller and Ratnieks 2001, n=7; Page and Erickson 1988, n=5) and where hundreds of worker-laid eggs were present in a frame of drone cells c. 4 weeks after division (Miller and Ratnieks 2001). In contrast to normal hopelessly queenless colonies (Miller and Ratnieks, 2001), worker policing was not switched off. Hopelessly queenless colonies are effectively doomed whether they produce a last batch of males or not but workers, even non-reproducing workers, can enhance their inclusive fitness by rearing workers' sons. The study colony, therefore, was behaving maladaptively. It showed one trait -worker policing- adaptive in a queenright colony and another -ovary activation and egg laying- adaptive in a hopelessly queenless colony, but this combination is not adaptive in either queenless or queenright colonies for the colony as a whole. The proportion of workers with large oocytes (12.6%) was similar to normal hopelessly queenless colonies (5-24%; Miller

and Ratnieks 2001) and much higher than in queenright colonies (c. 0.01%; Ratnieks 1993).

It is interesting to note that queen-laid eggs were also removed, but at a lower rate. The reason for this is unclear. Workers in a hopelessly queenless colony should not encounter queen-laid eggs and maybe this causes the indiscriminate egg-eating. Miller and Ratnieks (2001) noted increased egg-removal in queenless colonies before worker policing broke down during the second and third weeks after division, which may be a sign of a conflict between workers over the onset of reproduction (Page and Robinson 1994). This increased egg-removal might have continued as workers did not stop eating eggs.

A wide range of reproductive variation has been documented in honey bee colonies. The best known variant is the anarchistic syndrome, in which workers activate their ovaries and lay eggs that evade policing in queenright colonies (Oldroyd et al. 1994, Oldroyd and Ratnieks 2000, Barron et al. 2001). In the parasitic Cape bee, *A. m. capensis*, one clone of workers that is invading African honey bee, *A. m. scutellata,* colonies also activates its ovaries and evades worker policing (Martin et al. 2002a). There is also much variation among *Apis mellifera* subspecies in the time it takes workers in queenless colonies to activate their ovaries (Ruttner and Hesse 1979) and between patriline variations in reproductive success have been documented in normal queenless colonies (Martin et al. 2004a).

Our study shows that worker policing did not break down at the colony level, but it does not tell us whether or not it persisted in all or only some of the workers. We expect that worker policing shows behavioural dominance (Craig 1980). That is, a colony with some workers that did not switch off their policing would still be a policing colony. This is because the act of killing eggs trumps not killing eggs. Behavioural dominance has been shown for hygienic behaviour in honey bees. A colony composed of 50% hygienic bees (Brown line) and 50% non-hygienic bees (Van Scoy line) is as hygienic as one of 100% Brown line bees (Trump et al. 1967).

Honey bee workers are known to differ genetically for many traits (anarchy, Oldroyd et al. 1994, chapter 5; egg-laying and ovary activation, Robinson et al. 1990; guarding and undertaking, Robinson and Page 1988). Because our results were repeatable using a second set of worker bees from the same mother colony, this suggests that the persistence of worker policing behaviour has a genetic component. This genetic variant concerns only the policing behaviour because the workers activated their ovaries

and laid eggs in the normal way. This suggests that the reproductive changes in worker bees in queenless colonies involve a number of genes, which can vary both in their expression (i.e., between queenright and queenless colonies) and in their occurrence (i.e., some workers in the study colony had a rare genotype which affected their policing behaviour). Reproductive mutants such as the one studied here are of great potential use in investigating both mechanistic and ultimate questions in worker reproduction and worker policing in the honey bee (Barron et al. 2001), which is itself a model system for studying conflict and cooperation in social groups.

Chapter 7

The influence of cell size and intra and inter colony variability on worker policing in the honey bee, *Apis mellifera.*

1. Abstract

In the honey bee, workers prevent each other from reproducing by eating worker-laid eggs, a phenomenon known as worker policing. Few studies so far have documented the variation among colonies and other factors that influence policing. Here I conducted a large scale experiment to study the influence of egg type (queen-laid and worker-laid), cell size (drone or worker cells), colony and trial on the removal rates of eggs in a classic worker policing bioassay. Although considerable variation was found between trials, differences were found among discriminator colonies with some being consistently slow or fast at killing worker-laid eggs. Colonies with higher rates of egg removal also ate more queen-laid eggs in error, suggesting a cost to worker policing. However, this appears to be offset by the confirmed higher policing rate in drone cells than in worker cells, where workers preferentially lay.

2. Introduction

In the honeybee, *Apis mellifera*, workers cannot mate but retain ovaries and can still lay unfertilised eggs which typically develop into males if reared. However, worker reproduction is rare in queenright colonies because few workers lay eggs (Ratnieks 1993) and most of the eggs that they lay are policed (eaten) by other workers (Ratnieks 1988, Ratnieks and Visscher 1989). Workers discriminate between queen-laid and worker-laid eggs (Ratnieks and Visscher 1989) probably due to a queen-produced egg-marking pheromone (Ratnieks 1995).

Workers in queenright colonies lay eggs preferentially in drone cells (Ratnieks 1993), as do workers in queenless colonies (Page and Erickson 1988). Laying in worker cells is presumably selected against because drones reared in worker cells are smaller, and may have lesser mating success given the intense scramble competition for queens by many pursuing males. Because males greatly outnumber queens this intensifies the competition. Drones perform no tasks in the colony and their morphology bears the hallmarks of strong sexual selection for competitively pursuing queens, such as large thorax and eyes (Snodgrass 1956).

One cost of worker policing is the mistaken removal of queen-laid eggs. One way of reducing this cost would be to police eggs in worker-sized cells less harshly, given that workers lay eggs only in drone cells (Wattanachaiyingchareon et al. 2002). Recent evidence shows that this is in fact the case (Halling and Oldroyd 2003). However, Halling and Oldroyd did not include queen-laid eggs as a control and did not fully investigate intercolony variability and other factors, such as variation among trial days and the variation in erroneous removal of queen-laid eggs, which might influence the rate of policing and its possible costs.

In order to gain a deeper understanding of variation in the rate of worker policing, we performed two experiments using 6 discriminator colonies and 45 trials in total. This allowed us to investigate the influence of four factors, namely egg-type (queen-laid vs. worker-laid eggs), cell type (drone cells vs. worker cells), test colony and trial. We found considerable variability in policing rate for all four factors and significant differences between discriminator colonies and cell type. These data give an indication that more effective policing may cause a colony-level cost by removing some queen-laid eggs but that one mechanism by which this cost may be reduced is via increased targeting of eggs in drone cells.

3. Material and methods

The experiments were conducted using 6 colonies in two different countries in October 2000 and July-August 2001. Similar but not identical methods were used in both sets of experiments to assess the factors influencing policing. For this reason, the methods for each experiment are presented separately, but the results were pooled for the statistical analyses.

3.1. Experiment 1

Experiment 1 was conducted at the Laboratory of Apiculture and Social Insects, Sheffield UK, during the summer of 2001 using *Apis mellifera* colonies of European races, predominantly *A. m. mellifera* (the native "black bee" subspecies). One-day-old (0-24h) queen-laid eggs were obtained by confining the queen of a populous colony on an empty worker frame with queen excluder mesh. The frame was changed every day. Queens lay fertilised eggs in worker-sized cells. Fertilised eggs were used for convenience. Previous research has shown that policing workers do not treat fertilised and unfertilised queen-laid eggs differently (Oldroyd and Ratnieks 2000) in policing bioassays. (Fertilised eggs develop into females, or into diploid males. Unfertilised eggs develop into haploid males). To obtain worker-laid eggs, we removed the queens from two colonies and prevented the rearing of replacement queens. Approximately one month later workers started to lay eggs. One-day-old (0-24h) worker-laid eggs were obtained by introducing an empty frame of drone-sized cells into each colony each day. Each trial used the eggs from a single queenright colony (queen-laid eggs) and a single queenless colony (worker-laid eggs).

Three queenright discriminator colonies (D2, D3, and D4) were set-up following standard methods for policing bioassays (Ratnieks and Visscher 1989, Ratnieks 1995, chapter 3) in which a test frame is sandwiched between frames of young brood above a queen excluder in a populous queenright hive. The test frame had equal areas of worker and drone cells built on drone and worker cell foundation purchased from beekeeping supply companies. The test frame was put in place at least 24h before a trial.

40 queen-laid and 40 worker-laid eggs were transferred into each test frame, 20 eggs of each per cell size, using modified forceps (Taber 1961). The number of eggs remaining was observed after 1, 2 and 20 hours. The two sides of the frame were used for alternate trials. Ten trials were made per discriminator colony giving 30 trials in total. Trials were carried out from July 2nd to August 8th. 7 paired trials (i.e., on the same day) were made for colonies D3 and D4 and 3 for colonies D2 and D3.

3.2. Experiment 2

Experiment 2 was conducted at the University of Pretoria, South Africa, during October 2000 using colonies of African bees, *A.m. scutellata*. The set-up was the same as in Experiment 1 except that two test frames were used, one with worker cells and one

with drone cells. Three discriminator colonies were used (H1, H2, H3) and each trial was repeated five times for each colony. The numbers of eggs remaining after 2, 6 and 20 hours were recorded. All three colonies were trialled on the same 5 consecutive days.

3.3. Data Analysis

In order to compare all colonies and all factors in the same analyses, we fitted an exponential decay curve ($y = e^{-bt}$), where t is elapsed time and *b* a factor that affects decay rate per unit time, to the egg removal rate for each treatment for each trial. The number of eggs remaining follows this decay pattern well (Visscher 1996). This also reduces the problem of the normality of the data set caused by the use of proportions. Factorial analyses of variance were then performed on the decay parameter, *b*, using STATISTICA 6.0. Higher *b* means a greater removal rate. Figure 7.1e shows the decay curve for different values of *b* as a comparison to the actual data (Figure 7.2).

4. Results

The mean results for each discriminator colony in each of the two experiments are presented in Figure 7.2. In total we studied the removal of 900 eggs for each of the four treatments. That is, worker-laid eggs and queen-laid eggs in both worker and drone cells.

4.1. Worker-laid vs. queen-laid eggs

The rate of removal of queen-laid eggs (fitted value of *b*: 0.055±0.106, mean and s.d., range [0.0005-0.75]) was always smaller and less variable than the removal rate of worker-laid-eggs (1.546±1.447, range [0.05-4.61]) (Figure 7.1a). After 20h, the proportions of queen-laid eggs remaining were always higher than for worker-laid eggs remaining (56.2±26.4% vs. 3.9±8.4%, mean and s.d.) and had a much lower coefficient of variation (47% vs. 215%, 100*(standard deviation/mean)).

4.2. Variation among discriminator colonies and trials within discriminator colonies

There was significant variation in the rates of policing between discriminator colonies (range of means for worker-laid eggs [0.74-3.46]; $F(5,78)=18.74$, $p<0.001$), with colony D3 (mean $b=3.46$) having a significantly higher rate than all the others, as can be seen in Figure 7.1b and 7.1d (range [0.74-1.26]; Tukey HSD test) and also to a

lesser extent in the rate of queen-laid egg removal (range of means for queen-laid eggs [0.018-0.128]; F(5,78)=2.88, p=0.02), with colony D3 (*b*=0.128) differing from D2 and D4 only (*b*= 0.027and 0.018 respectively; Figure 7.1b Tukey HSD test).

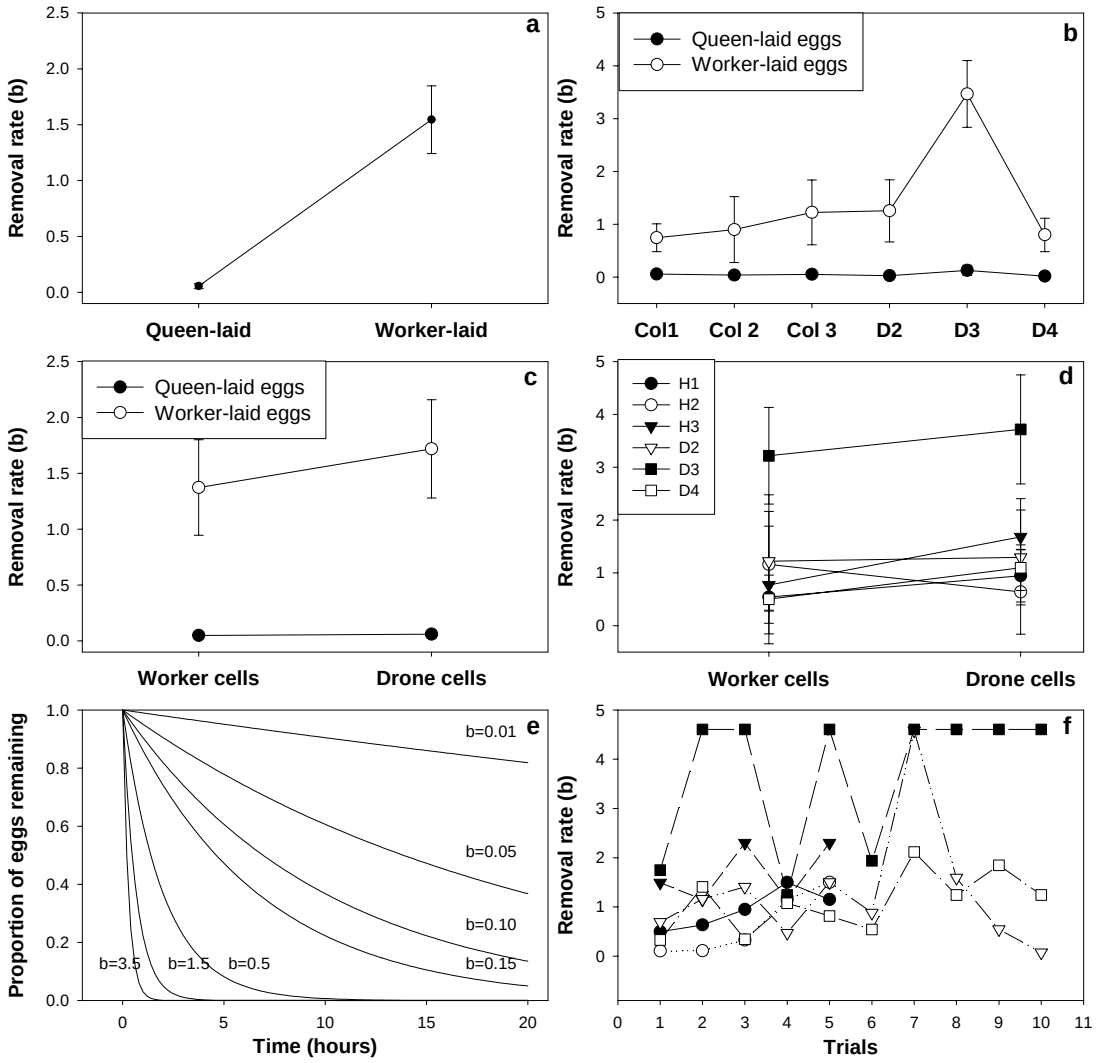


Figure 7.1. Egg removal rates. Error bars represent the 95% confidence level. a) Overall rate of removal for worker-laid eggs and queen-laid eggs pooled over all trials and discriminator colony and (b) for each discriminator colony. c) Overall removal rates of queen-laid and worker-laid eggs in worker and drone cells. d) Worker-laid egg removal rates in worker and drone cells for each discriminator colony. e) Exponential decay curve for a range of *b* values similar to the ones observed in the experiments. f) Removal rates of worker-laid eggs in drone cells over all the trials (10 for colonies D2, D3 and D4 and 5 for colonies H1, H2 and H3).

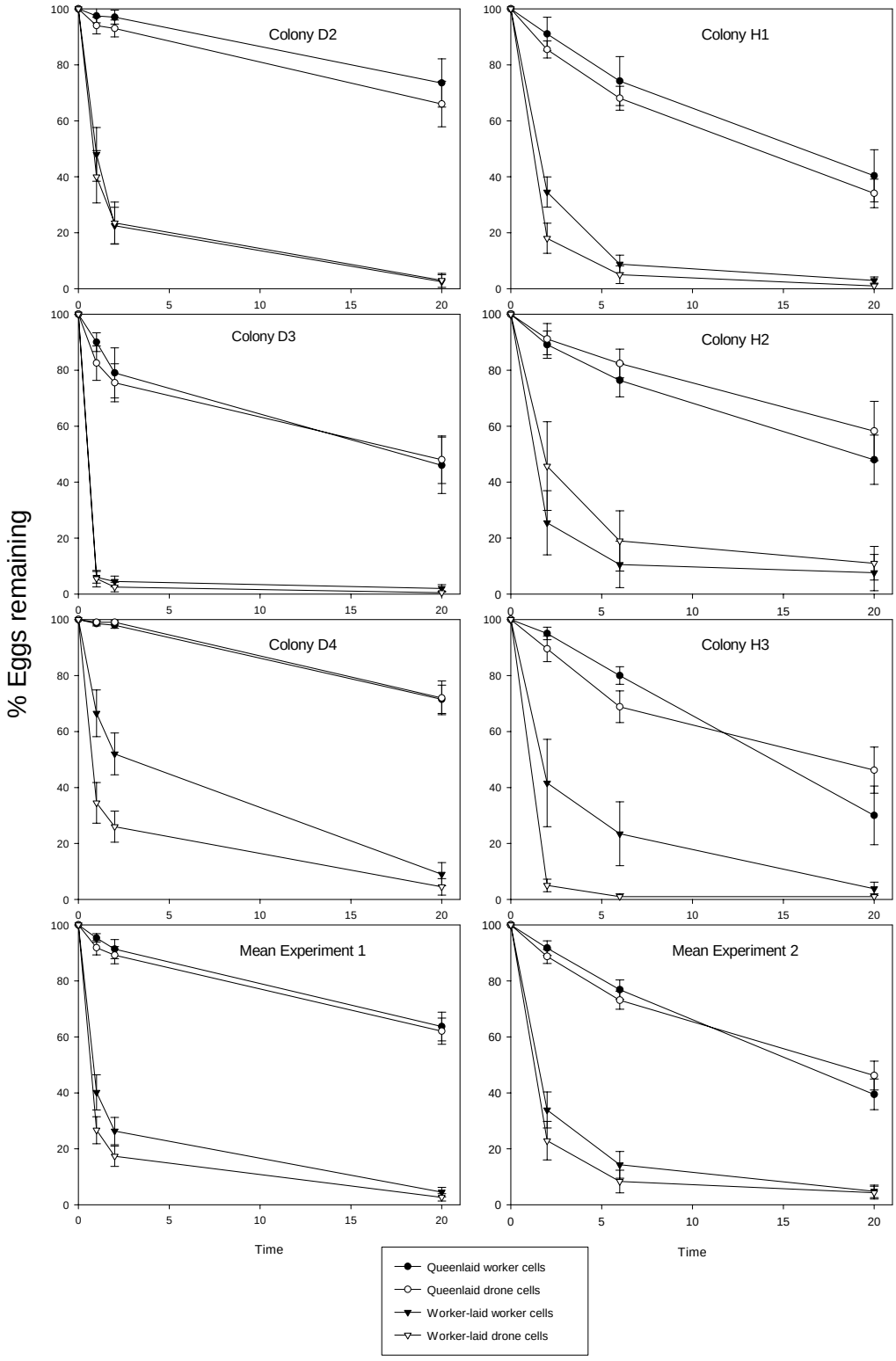


Figure 7.2. Mean (±s.e.) percentages of eggs remaining in each treatment for each discriminator colony (D2-D4, H1-H3) and grand mean for each experiment.

Variation was also apparent in the individual trials performed on each colony, as shown in the removal rates of worker-laid eggs (Figure 7.1f). There was no clear effect that could explain this variation, as colonies trialled on the same days did not vary in a

consistent manner. In experiment 2, there was a trend for the policing rate to increase during the consecutive days of the trials. When fitted with a linear regression, the trend was significant for colony H2 (R2=0.88; p=0.018) and marginally significant for colony H3 (R2=0.73; p=0.065; Figure 7.1f). No such trend appeared for experiment 1.

4.3. Cell size

In all but one colony (H2) across both experiments worker-laid eggs were removed more rapidly from drone cells (Figure 7.1d, Figure 7.2). The mean removal rate of worker-laid eggs for all colonies was 1.37±1.42 (mean and s.d.) in worker cells and 1.72±1.46 in drone cells. The effect of cell size was, however, not significant when all discriminator colonies were considered ($F(1,156)=2.09$, $p=0.15$). Colony H2 removed eggs in worker cells faster. When this colony was excluded from the analysis, there was a significant effect of cell size on the removal rates ($F(1,140)=4.21$, $p=0.04$) and a marginally significant effect of the interaction between cell type and egg type ($F(1,140)=3.84$, $p=0.05$). This is confirmed by the fact that when only worker-laid eggs were analysed, the effect of cell size was significant ($F(1,70)=4.06$, $p= 0.047$) but not when queen-laid eggs were considered alone ($F(1,70)=0.20$, $p=0.66$).

5. Discussion

Our results show that there is a large and consistent difference in egg-removal rate between worker-laid eggs and queen-laid eggs, but that there is also considerable variability in egg removal rates. This variability can be observed between trials within discriminator colonies, between discriminator colonies and also between drone and worker cells.

The difference between discriminator colonies observed in this study has also been found in other studies, as in the 4 colonies studied across 10 trial days by Ratnieks (1995). This variation could be caused by factors linked with the regulation of brood rearing in the colonies, environmental factors like the weather and food availability, colony size, or genetic differences among the colonies. It is unlikely that the population of the different colonies can explain these differences as all discriminator colonies were populous and of similar strength. Although the between trial variability (Figure 7.1f) suggests some environmental influence, it is more likely that the genetic make-up of the

different colonies influences policing and leads to some colonies policing more effectively. In two colonies, there was an increase in the policing efficiency while they were repeatedly tested (from 0.50 to 1.15 for H1 and 0.11 to 1.50 for H2). This might have been explained by an ability of some colonies to increase their worker allocation to policing as worker-laid eggs repeatedly appear in the colony. However, the absence of this trend in the other colonies makes it unlikely.

Worker policing has been shown to vary widely, and with a genetic basis, in rare reproductively variant colonies, including anarchistic colonies (Oldroyd and Ratnieks 2000) and in a hopelessly queenless colony which did not switch off policing (chapter 6). It is, therefore, likely that less dramatic genetically determined variation occurs in normal colonies. In colony D3, a higher rate of policing was accompanied by a higher erroneous removal of queen-laid eggs (Figure 7.1b). This also suggests a cost to more effective policing, as was suggested by Wattanachaiyingchareon et al. (2002). This is further supported by the positive correlation (Pearson correlation p=0.015, n=21) between the number of eggs remaining after 2 hours in this study and other policing studies (Oldroyd and Ratnieks 2000, Ratnieks 1995, Ratnieks and Visscher 1989, Figure 7.3).

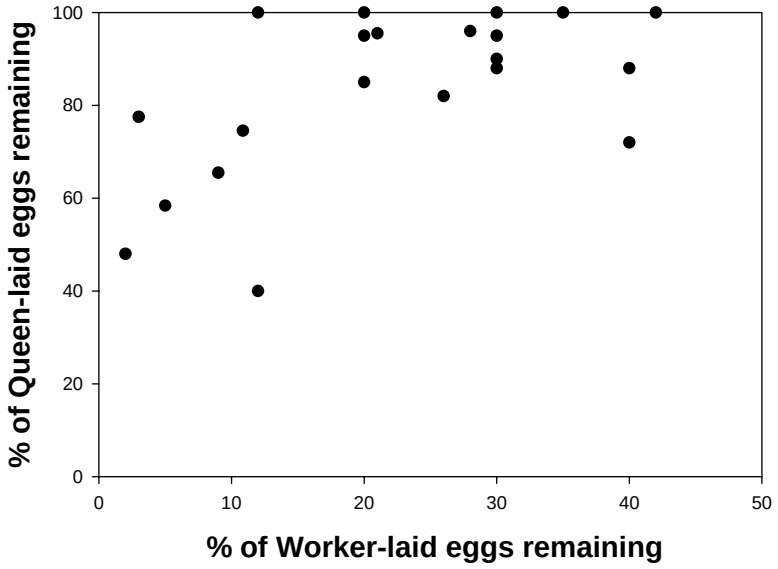


Figure 7.3. Mean proportion of worker-laid and queen-laid eggs remaining after 2 hours in colonies of this study and in Ratnieks 1995, Oldroyd and Ratnieks 2000 and Ratnieks and Visscher 1989 (n=21).

The results also evidence some differences in the policing rate between cell sizes. Surprisingly, one of the colonies followed the reverse pattern to that predicted, removing worker-laid eggs faster in worker cells than in drone cells (Figure 7.2). The reason for this is unknown. The other colonies followed the predicted pattern, which had already been observed by Halling and Oldroyd (2003), with worker-laid eggs being removed faster from drone cells (Figure 7.1c and 7.1d). The higher removal rate in drone cells could be due to police workers preferentially targeting drone cells because workers in queenright colonies only lay eggs in drone cells (Ratnieks 1993) and that the cost of wrongly removing female destined queen-laid eggs in worker cells is thus reduced (Wattanachaiyingchareon et al. 2002). The regulation of male brood rearing could also play a part, as well as the larger size of drone cells which means that there will be fewer cells to check per unit area of comb. In contrast to the different rates of worker-laid eggs removal, the removal rates of queen-laid eggs did not differ between the two cell types (Figure 7.1c). The fact that the rate of mistakes remains constant between cell sizes questions the effectiveness of targeting worker-laid eggs in drone cells more to reduce the costs of policing.

In conclusion, this study has shown that considerable variation occurs in the rate of policing in different discriminator colonies. Higher rates of worker-laid eggs removal (more effective policing) were associated with higher rates of queen-laid eggs removal, leading to a probable cost to the colonies. Higher rates of worker policing in drone cells would seem to reduce this cost. However queen-laid eggs were not removed in error less in worker cells, which weakens the support for this hypothesis.

Chapter 8

Lack of nepotism in honey bee (*Apis mellifera*) interactions between workers and young queens imprisoned in queen cells during swarming.

1. Abstract

Nepotism is an important potential conflict in animal societies. However, clear evidence of nepotism in the rearing of queens in social insects is limited and controversial. In the honey bee, *Apis mellifera*, multiple mating by queens leads to the presence of many patrilines within each colony. When the colonies reproduce through swarming, workers rear a number of new queens, only a few of which will ultimately head a colony. Workers can potentially increase their inclusive fitness by nepotistically favouring full-sister over half-sister queens during the queen rearing and elimination process. Most studies have focused on interactions between workers and immature queens (eggs, larvae) or adult queens who have exited their queen cells. However, adult queens often remain in their queen cells for up to one week after emerging from their pupa. In this situation, workers prevent the queens from emerging, feed them, and protect them from other emerged queens. This stage in queen rearing is, therefore, one in which nepotism could occur. The current study is the first to investigate the kinship between workers and adult queens who have not emerged from their queen cells. We observed the full suite of behaviours expected during this phase of colony reproduction. Although there was no evidence for nepotism in the worker-queens interactions, there was a non-random distribution across patrilines of the workers interacting with the queen cells. In addition, in one colony we found differential treatment of fostered (non-kin) queen cells.

2. Introduction

Insect societies are typically non-clonal and this leads to a wide range of potential reproductive conflicts, including conflicts over sex-ratio (Trivers and Hare 1976), male production (Ratnieks 1988), caste fate (Wenseleers and Ratnieks 2004) and queen rearing (Visscher 1993). Nepotism should play an important role in the resolution of these conflicts. However, clear evidence of nepotism in social insects is limited and controversial (see Hannonen and Sundström 2003, for an example in ants). Multiple mating by honey bee (*Apis mellifera*) queens leads to the presence of many patrilines (paternal subfamilies within the single matriline) within each colony (Estoup et al., 1994, Palmer and Oldroyd 2000). This causes potential conflict over queen-rearing because it creates relatedness asymmetries between the workers who rear the queens and the young queens themselves, with workers being either full sisters (r = 0.75) or half sisters (r=0.25) to these queens (Visscher 1986). When colonies reproduce through swarming, workers rear approximately 10-20 new queens, but only a few of these (1-4) will ultimately head a colony. Workers can potentially increase their inclusive fitness if they nepotistically favour full-sister queens or disfavour half-sister queens during the queen rearing and elimination process (Visscher 1998). However, if workers' recognition ability of full-sister queens against half-sister queens is error prone, or if nepotism is costly to the colony as a whole because it reduces queen quality or number, then nepotism would not be selected or selected for only weakly (Ratnieks and Reeve 1991, Tarpy et al. 2004).

When the primary swarm leaves with the old (mother) queen, the new (sister) queens are immature. When these queens mature into adults, they compete to head a new colony, either by leaving with a secondary swarm or by becoming the queen in the established nest site. In both cases the interests of individual queens may be different to those of the workers (Visscher 1993). The process of queen elimination has been described extensively (Bruinsma et al. 1981, Butler 1623, Fletcher 1978, Grooters 1987) and theoretical work shows that it could be an important stage for queen-queen, worker-queen and worker-worker conflict (Visscher, 1993). However, the precise role and importance of worker behaviour in the outcome of the process have only recently been studied in detail (reviewed in Tarpy et al. 2004). The elimination process is characterized by numerous queen-queen interactions, including queen fights in the form of "duels" between adult queens, "assassinations" in which a pupal queen in her cell is

killed by an adult queen free in the colony, vibratory signals made by adult queens (piping) and queen-worker interactions (vibration signals, aggressive behaviour, feeding). These interactions suggest that workers could play an important role in the queen selection process, motivated either by nepotism or "quality control" unconnected with nepotism (Tarpy et al. 2004). Tarpy and Fletcher (1998) found that queens that were sisters of the workers had an advantage in winning duels over unrelated queens. However, Gilley (2003) found that in colonies with naturally-mated queens, aggressive behaviour by workers was not more directed towards half sister queens. Queen quality had little influence on worker-queen interactions and survival (Gilley et al. 2003, Schneider and DeGrandi-Hoffman 2003, Tarpy et al. 2000). Despite these many studies, our understanding of the queen elimination process remains incomplete.

All studies of the influence of workers on the selection of the new queens have focused on interactions between workers and immature queens (Châline et al. 2003, Noonan 1986, Page et al. 1989, Schneider and DeGrandi-Hoffman 2002, Visscher 1998) or between workers and adult queens who have exited their special queen cells (Gilley 2001, Gilley 2003, Tarpy and Fletcher 1998). However, adult queens often remain in their queen cells for up to one week (Bruinsma et al. 1981, Fletcher 1978, Grooters 1987) before exiting into the colony. During this time workers cluster on each cell containing an adult queen and feed the queen through slits in the tip of the cell (Figure 8.1c) which are then resealed. They vibrate the queen cells and prevent the queens from exiting by repairing openings in the cells. Workers sometimes even press their head against the tip of the queen cell to prevent the queen from exiting while other workers close the cell (Fletcher 1978). They also protect the queens by aggressively preventing access by queens who have already left their cells (Gilley 2001). In natural queen rearing during swarming, all queens eventually exit their cells or are killed by another queen. Adult queens communicate during this process through vibratory signals (quacking from imprisoned queens and tooting from queens free in the colony, collectively known as piping, Kirchner 1993, Simpson and Cherry 1969), which influence queen exit from cells (Bruinsma et al. 1981, Grooters 1987). In addition to relatedness, individual queens and workers may differ in their interests with regard to the time of exiting queen cells, fighting and whether the colony should divide further by producing secondary swarms (Visscher 1993). Nevertheless, nepotism could be involved in the confinement of the queens as by doing so workers prevent emerged queens from attacking the cells, thus protecting the imprisoned queens. This allows the

queens to mature in the cells before emerging. Consequently, the later the queen emerges, the more likely she is to win the remaining fights with queens possibly weakened by previous fights and to inherit the original colony.

Here we present the first investigation of the effect of kinship on interactions between workers and adult queens who have not exited from their queen cells. To do this, we used an apparatus which allowed us to ensure that newly-emerged adult queens were imprisoned in their own cells for 4 days, as occurs naturally. This experimental set-up allowed us to recreate a secondary swarming situation where queens are confined in their cells by workers, and so allowed prolonged behavioural observations of the workers interacting with the confined queens to be made. During the experiments, queens often tried to emerge by cutting an opening through the tip of their cell (Figure 8.1d) but the workers always tried to close the hole. We observed the full suite of behaviours normally expected during this phase of colony reproduction, including piping from the queens. Although there was no evidence for nepotism in the worker-queen interactions, the workers interacting with the queen cells were not randomly distributed across patrilines. In addition, in one colony we found differential treatment of fostered (non-kin) queens.

3. Material and methods

3.1. Study species

We studied three populous colonies of *A. mellifera mellifera*. Colony 1 was studied in early July 2003 (experiment 1) and colonies 2A and 2B were studied simultaneously in August 2003 (experiment 2). Colonies 2A and 2B were paired to allow the cross-fostering of queen cells. The exceptionally good summer weather in 2003 resulted in a prolonged swarming season which continued well into August.

3.2. Colony setup

All study colonies were prepared identically for the behavioural observations. Colonies were fed sucrose syrup continuously for two weeks to increase brood rearing and create swarming conditions. Queen rearing was then initiated in each colony by dry-grafting 1-2 day-old worker larvae from each colony into plastic queen cups, which were returned to their own colony between frames of young brood for rearing.

After ten days, the then sealed queen cells were removed from their colonies and given an apparatus that prevented queens from exiting their cells (Figure 8.1d). The apparatus consisted of three thin copper wires interwoven to form a six-legged star. In the centre we attached a small piece of acetate sheet using new (white) wax taken from the colony in which the cell was reared (arrow in Figure 8.1d). The apparatus prevented the queen from exiting through the tip of the cell, the normal exit location, while the side of the cell was still accessible to workers who could interact with the queen through slits made around the perimeter of the tip, as occurs naturally. Tests had previously confirmed that this apparatus allowed normal interactions between workers and confined queen.

The modified cells were then returned to their colonies for 24 hours which gave the workers time to embed the wires into the wax of the cell. At the same time, queen cells from colony 2A and 2B were randomly chosen and cross-fostered between the two colonies. The cells had therefore been built in their original mother colonies. After this period, we simulated swarming by removing the queen and approximately half the workers but no brood. The remaining part of the hive containing the brood was fitted with an observation box with a removable panel on one side (Figure 8.1a). This formed an integral part of the nest cavity and allowed the observation of all the queen cells, which were attached to two wooden bars in the observation box (Figure 8.1b). The box also contained two frames of unsealed brood (larvae, eggs). In experiment 2, a second set of queen cells were initiated 5 days after the first set to increase the sample size. After the observations on the first set had been completed, the second set was transferred to the observation box.

3.3. Observations

The observations were carried by double blind protocol as kinship was only determined afterwards using microsatellite markers. During each day of observations we sampled workers performing one of the three following behaviours on queen cells showing openings.

1) Closing: the worker repaired slits made in the queen cell by the queen trying to exit (Figure 8.1d).

2) Feeding: the worker was performing trophallaxis with the queen who had her tongue extended through a slit or small hole in the queen cell (Figure 8.1c).

3) Vibrating: The worker grabbed the queen cell with her legs and then performed a short dorso-ventral abdominal vibration (1-3s) on the queen cell. This behaviour is believed to regulate queen exit (Bruinsma et al. 1981).

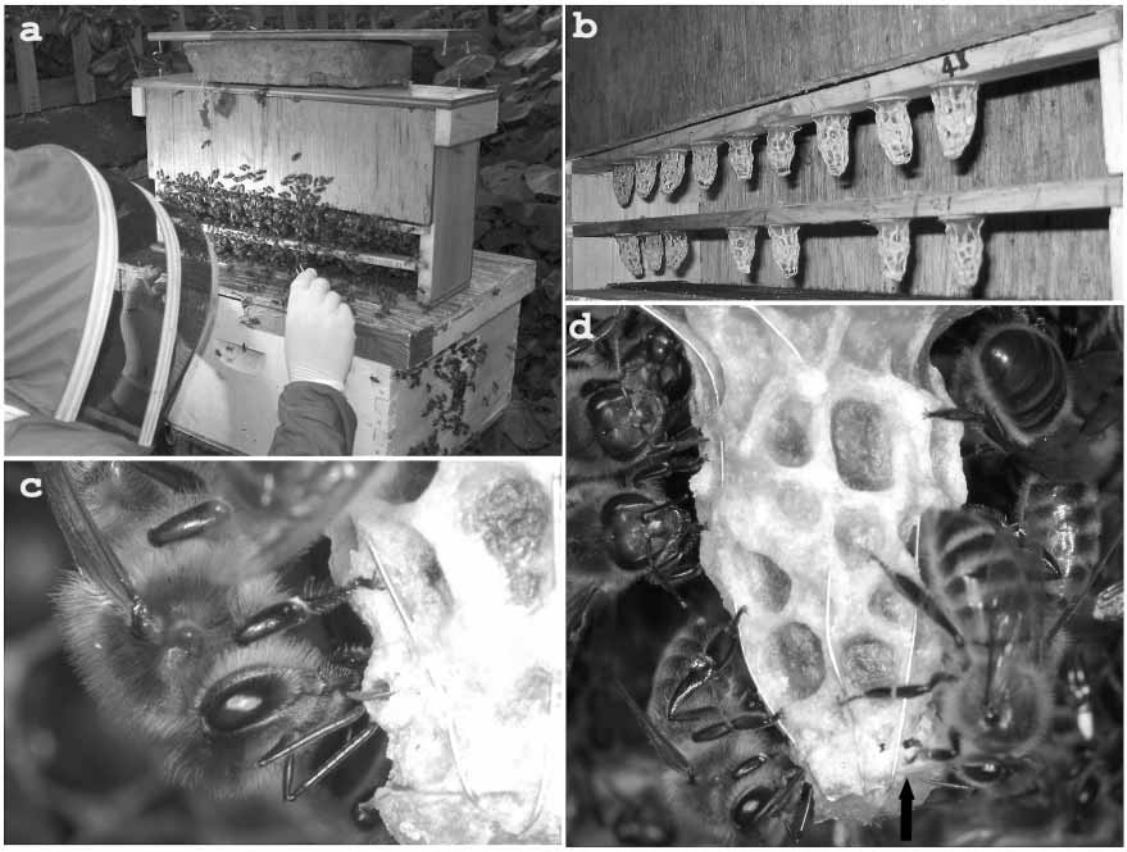


Figure 8.1. Photographs of the experimental set-up and worker behaviours on adult queens confined in their queen cells. a) Experimental set-up with the modified cover on the main brood box of the colony to which is attached the modified three-frame observation box with the queen cells accessible through an opening. b) Queen cells attached to the wooden bars in the observation box ready to be fitted on the test colony. c) Worker feeding a queen through an opening in the queen cell. The queen's tongue is visible. d) Queen cell fitted with the star apparatus to prevent queen exit. The piece of acetate is visible at the end of the arrow. Openings made by the confined queen trying to emerge can be seen and the worker on the right is in the process of closing these.

Before the collection of each worker, we made sure that the worker had been directly in contact with the queen with its antennae through the slits in the cell. This was possible because workers would often be seen staying on the same queen cell for a long time. Workers interacting with the queens and queen cells were very active and so were not confused with other workers. Typically, one worker would first be seen closing the cell, then contacting the queen inside the cell, then vibrating the cell and going back to close the cell. Feeding workers would show the same pattern. For this reason, the three

behaviours recorded appeared to belong to the same repertoire of interactions with the queens and queen cells.

Workers were stored frozen at –20C individually in eppendorf vials until genetic analyses. We aimed to collect 30 or more workers observed interacting with each queen cell. Collection of samples ended after 4 days for each set of queens. After the observations, the queen cells were opened and the queens inspected for any physical deformation and frozen at -20C for genetic analyses.

3.4. DNA microsatellite analysis

To determine the kin structure of the colonies, we used polymorphic DNA microsatellite markers. In addition to the sampled workers and queens, we also analyzed 94 newly-emerged workers and 92-94 adult workers per colony taken randomly at the start of observations from the comb next to the queen cells. These samples allowed us to assess the number of mating and effective paternity in the study colonies and to test for patriline differences in the probability of performing the observed behaviours.

DNA was extracted from the antennae using chelex®100 (chapter 4, Walsh et al. 1991). PCR reactions were performed as described in chapter 4. The products were multiplexed and visualized using an Applied Biosystems ABI 3730 capillary sequencer and analyzed with the dedicated software GeneMapper v 3.0.

To reduce the number of markers used, we first screened the young worker sample and the queens at 6 microsatellite loci: A107, A14, A29, A76, Ap33 and B124 (Baudry et al. 1998, Estoup et al. 1994). Having determined the patriline structure of each colony, we then chose 3 marker loci sufficient to distinguish all patrilines in each colony, plus one extra locus for added confidence in patriline assignment. The markers used were A107, A14, A29 and A76 for colony 1, and A107, A29, A76 and Ap33 for colonies 2A and 2B.

The rest of the workers were genotyped at these four markers and each worker was then assigned to a particular patriline. A few adult workers that could not be assigned as the colony queen's progeny were considered to have drifted from other colonies. In some cases, they could be assigned to another experimental colony as the colonies were all located in the same apiary.

3.5. Statistical analyses

The sampling technique, which did not permit constant observation of the cells, resulted in relatively few vibrating and feeding events (see results). The higher proportion of closing behaviour could also be due to the presence of the apparatus which prevented even the most motivated queens to emerge. Because of the small sample sizes for feeding and closing, we pooled all the behavioural data per queen for the statistical analysis. This allowed more powerful tests. This pooling of data is biologically reasonable because we often saw individual workers performing a combination of behaviours (i.e. vibrating and closing or feeding and closing). For safety, we still confirmed the overall results for each behaviour by comparing the proportion of full-sister interactions with the expected proportion from a random sample of bees (calculated with the effective paternity) with a chi-square test (Table 8.2).

At any one time, many workers were interacting with each queen cells. The sampled workers thus constitute only a small subset of the interacting workers. This means that any differences in numbers of workers of a particular patriline would also reflect the frequency at which workers of this patriline interacted with the queen cells.To test for differences in the distribution of patrilines in the different groups of workers (young, random, interacting with the queen cells and interacting with cross-fostered queen cells), we used a Fisher's exact test with an exact method using the program 'Monte Carlo RxC 2.2' developed by W. Engels, University of Wisconsin. When differences between groups were significant, we analyzed differences in individual patriline proportions using a chi-square test with Yates' correction. Because differences in the patriline proportions were observed (see results), we could not use the two control samples to test for nepotism in the interacting workers. Instead we used the overall distribution of patrilines across all queens in the interacting worker samples.

To test for nepotism between workers and each queen, we used a 2x2 chi-square test with Yates' corrections to compare the proportion of interacting workers of the same patriline as the focal queen, versus the proportion of interacting workers of that patriline interacting with all the queens of other patrilines. We then tested for each colony and for all queens for an overall trend in workers interacting with full-sister queens by using Gilley's (2003) nepotism index. In this case the index was the difference between the proportion of full-sisters of the focal queen interacting with this queen and the proportion of workers from the same patriline interacting with queens of other patrilines. We used a two-tailed Z-test to detect significant differences. We also

calculated the effect sizes for each of the tests performed using Cohen's d (Cohen 1988), calculated with Becker's formulae (1988). We did not perform a retrospective power analysis as their use is now considered inconclusive and flawed (Nakagawa and Foster, in press).

4. Results

4.1. Behavioural observations

During the experiments, 24 out of the 82 queen cells transferred to the colonies contained dead queens and were not attended by workers. These deaths probably happened during the fitting of the star apparatus, or before, as the queens inside these cells were unemerged pupae of various ages.

In experiment 1, we observed 15 cells with live queens in colony 1. In experiment 2 we observed 22 queens in total in colony 2A, 4 of which were cross-fostered, and 21 in colony 2B, 3 of which were cross-fostered.

We genotyped 2026 workers performing feeding (208, 10.3%), closing (1715, 84.6%) and vibrating behaviours (103, 5.1%) on 58 queen cells. The mean number of workers sampled per queen cell was 34.8 ± 9.8 (mean ± s.d.). Throughout the observations the queens could be heard piping, mostly quacking but sometimes tooting, which suggests that they were behaving in a normal way while being experimentally confined to their cells.

4.2. Colony kin structure

The queens of the three colonies had mated with 12, 11 and 20 males (Table 8.1) with short-term effective paternity frequencies of 9.46, 6.00 and 13.43 respectively, calculated from the young worker sample (Table 8.1).

The random sample of workers was significantly different from the sample of young workers in both colony 2A and 2B (Table 8.1). The sample of interacting workers was also significantly different from the random sample of workers for all three study colonies. When examining the cause of these differences, between 3 and 6 patrilines differed significantly between the two samples (Figure 8.2B), either by being over or under-represented in the sample of workers interacting with queen cells. The most striking difference is for patriline F in colony 2A which was 29.8% of the random sample but 48.3% of the cell-attending workers. Patriline E of colony 2B which was overrepresented in the interacting worker sample also contributed to 9 of the 13 drifted

workers interacting with queen cells in colony 1. Fewer or zero drifted workers were found in the other colonies (Table 8.1).

Table 8.1. A. Kin structure, overall differences in the different worker samples and drifted workers in the colony. Sample sizes are in brackets. B. Proportion of workers performing the three observed behaviours towards full-sisters in the three colonies. In bold are significant deviations from the expected random distribution.

A.	Colony 1	Colony 2A	Colony 2B
Number of matings	12 (721)	11 (981)	20 (935)
Short-term effective paternity	9.46 (94)	6.00 (94)	13.43 (94)
Young workers/ Random workers	NS (94/94)	P<0.001 (94/94)	P<0.03 (94/92)
Interacting workers/ Random workers	P=0.02 (529/94)	P<0.001 (768/94)	P<0.001 (727/92)
Workers interacting own queen/cross-fostered queens	No cross-fostered queens	P=0.024 (660/108)	NS (630/97)
Number of drifted workers	13/529	4/768	0/727
B. % of workers interacting with full-sister queens			
Feeding	14.3 (77)	18.5 (54)	14.7 (61)
Closing	14.3 (399)	15.5 (582)	7.23 (553)
Vibrating	9.4 (53)	18.2 (22)	**31.6 (19)**

Table 8.2. Nepotism index in each colony and overall. Cross-fostered queens are not included. The effect size is calculated using Cohen's d (1988)

Nepotism index (sample size)	Colony 1 (n=15)	Colony 2A (n=18)	Colony 2B (n=18)	Overall (n=52)
mean±s.d.	-0.007±0.055	0.015±0.059	-0.005±0.097	0.001±0.072
probability	P=0.59	P=0.27	P=0.83	P=0.89
Effect size (Cohen's d) (±95%CI)	-0.13±1.00	0.26±0.95	-0.05±0.94	0.02±0.55

4.3. Nepotism

The frequencies of interactions between full-sisters for each behaviour (Table 8.2) were not different from an expected random distribution, except in one case. This is for vibrating in colony 2B. However, the small sample of bees (n=19) for this case suggests this is an artefact. This absence of difference bolsters the fact that the three behaviours can be collapsed in a single "interacting" category.

There was no significant effect of relatedness on the probability of interacting with individual queens confined in their cells (Figure 8.2A). There was also no significant overall trend towards nepotism in individual colonies or overall, as measured by the nepotism index (Gilley 2003, Table 8.2). The large sample size of the interacting

workers makes it highly unlikely that this result is a false negative for anything other than a weak effect since the effect sizes (d) were in all cases small (Table 8.2; Cohen 1988).

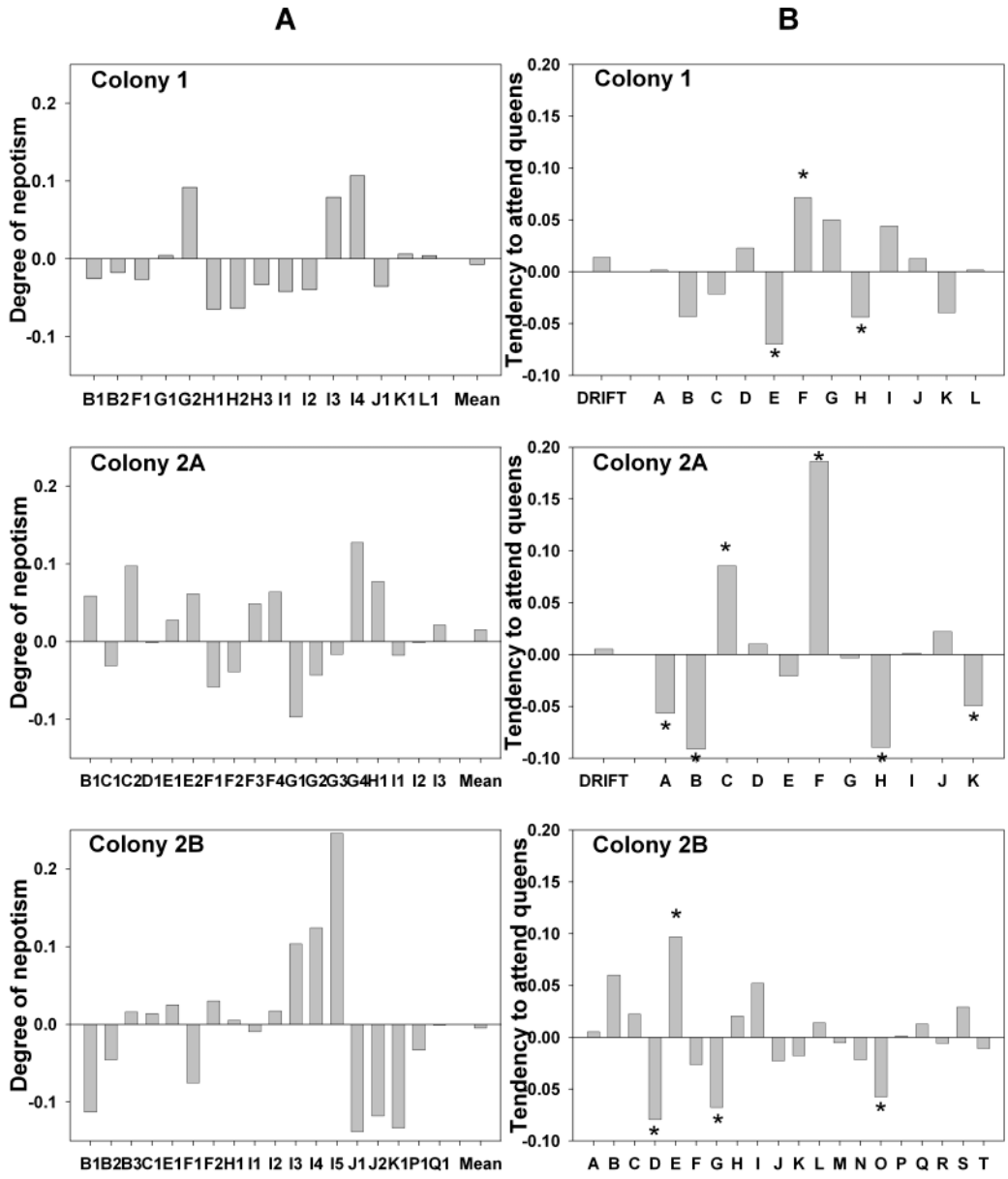


Figure 8.2. Degree of nepotism towards individual confined queens and tendency of workers of different patrilines to attend confined queens in general in the three experimental colonies. A. Degree of nepotism of interacting workers, represented by the difference in the proportion of interacting workers who are full-sisters of the confined queen versus their proportion in the workers interacting with half-sister queens (Gilley (2003) nepotism index). Cross-fostered queen cells, none of which had full-sister in the colony, are not shown. B. Differences in the proportions of workers of each patriline interacting with cells versus their representation in random adult worker sample. Overall difference is significant for all three colonies. Individual significant differences are marked with a *.

4.3.1. Cross-fostered queens

When we compared the distribution of workers interacting with their sister queens against unrelated cross-fostered queens in the paired colony experiment, there was a significant difference only in colony 2A ($p=0.024$). This was mainly because workers of patriline F were less likely to interact with cross-fostered queens ($p=0.0004$).

5. Discussion

Our results clearly show that although workers of different patrilines differ significantly in their likelihood of interacting with adult queens imprisoned in their cells, nepotism almost certainly does not occur. The absence of nepotism is further confirmed by the absence of any nepotistic trends in subsets of the overall data. There was no significant nepotistic effect in *any* of the study colonies, or towards *any* single confined queen. The large sample size, both in terms of the 58 confined queens and the mean of 35 interacting workers per queen, and the small effect sizes make our conclusion robust. Furthermore Gilley (2003), in a study of the influence of relatedness on worker aggression towards queens, using a power analysis found that a lower sample size of 20.37±9.96 (mean ± s.d.) allowed him to detect all but weak and biologically insignificant nepotism.

The experimental set-up closely mirrored natural conditions and resulted in behaviours, of both confined queens and interacting workers, which are naturally observed. The situation only differed by the absence of a "roaming" queens in the colony, and the fact that we "helped" workers keeping the queens in their cells. However, workers cannot directly assess the presence of an emerged queen in the colony and rely on other cues like queen piping. Because queens were frequently piping during the experiment, it is highly likely that workers were behaving normally. This fact was also supported by previous observations of two observation hives which produced two and three secondary swarms spontaneously, with the queens remaining within their cells for up to 6 days. No behavioural differences were seen between the study colonies or the two observation hives. The protection of queen cells from emerged queens which could potentially be nepotistic as well could not be observed because no emerged queens were present. However, the same set of workers probably would have protected the queen cells as interacting workers were very active on the cells. This makes us confident that the absence of nepotism was not an experimental artefact but a true

reflection of what occurs naturally. In one instance (vibrating in colony 2B) there was a significant difference in the proportion of full-sisters performing the behaviour. Although the small sample size and the single occurrence of this make us doubt the relevance of this result, further experiments could be needed to confirm this.

There were differences in the tendencies of different patrilines to interact with the queen cells, both in over and under representation. In colony 2A, the most abundant patriline (F) was overrepresented in interacting workers, and this caused the mean relatedness between the workers interacting with a confined queen to increase from 0.42 to 0.53. However, no similar trend occurred in the other two colonies, where representation of the patrilines in the workers interacting with the queens was not linked to relative abundance in the colony. These marked differences could be due to genetic differences among patrilines in their tendency to perform different tasks. This has been documented for other behaviours like guarding and undertaking (Robinson and Page 1988). This result also emphasizes the fact that when investigating nepotism, great care has to be taken in the selection of the controls, as differences in the probability to perform a behaviour can lead to erroneous conclusion as to whether nepotism occurs or not. An example of this in this study is patriline F in colony 2A, which represented more than 50% of the interacting workers and who would have given positive nepotism towards F queens had we considered the newly-emerged worker sample as a control.

Interestingly, the workers in colony 2A seemed able to discriminate between queens from their own colony and cross-fostered queens. However, because the queen cells were built in a different colony, discrimination may have been mediated by wax odour rather than by the queen inside. Wax odours are known to influence honey bee nestmate recognition (Breed et al. 1998). Other studies using cross-fostered queens (Tarpy and Fletcher 1998) found a significant effect of kinship on aggression by workers towards queens, which disappeared when workers were confronted only with full-sister and half-sister queens (Gilley 2003).

Extreme multiple paternity, although increasing potential reproductive conflicts between patrilines (Visscher 1986), may also hamper recognition and increase the cost of nepotism by decreasing the probability of encountering a full-sister queen (Ratnieks and Reeve 1991). This may explain why nepotism studies using unnaturally low number of patrilines (2-3 or unrelated bees, Noonan 1986, Page et al. 1989, Schneider and DeGrandi-Hoffman 2002, 2003) tend to find an effect while studies done with naturally mated queens (10-20 patrilines, Gilley 2003) tend not to.

Another factor that could influence queen care is queen quality. Specialist workers could preferentially take care of higher quality queens regardless of kinship. We were unable to correlate any pattern of interaction to the quality of queens as measured by wing length or fresh weight. Future research might study the influence of queen piping on cell attendance and the interactions of workers with confined queens.

In conclusion, our data mirror most previous research on honey bees, but in the novel context of interactions with confined queens, in showing that the potential reproductive conflict in queen rearing caused by multiple paternity does not seem to translate into detectable nepotism (Tarpy et al. 2004).

Chapter 9
Learning and discrimination of individual cuticular hydrocarbons by honey bees (*Apis mellifera*)

1. Abstract

In social insect colonies, recognition of nestmates, close kin, caste and reproductive status is crucial both for individuals and for the colony. The recognition cues used are thought to be chemical with the hydrocarbons found on the cuticle of insects often being cited as particularly important. However, in honey bees (*Apis mellifera*) the role of cuticular hydrocarbons in nestmate recognition is controversial. Here we report the use of the proboscis extension response (PER) conditioning paradigm to determine how well honeybees learn, and discriminate between long-chain linear alkanes and (*Z*)-alkenes present on the cuticle of worker bees. We found large differences in both the learning of and discrimination between different cuticular hydrocarbons and that the compounds tested could be classified into those which the bees learnt well (mostly alkenes) and those which they did not (alkanes and some alkenes). These well learnt alkenes may constitute important compounds used as cues in the social recognition processes.

2. Introduction

For colonial living organisms, being able to distinguish between colony and non-colony individuals has a number of advantages. For example, in the social insects the ability to recognise nestmates helps them to prevent intra- and interspecific parasitism and the theft of colony resources (Breed 1998). It also enables context specific behavioural modifications by colony members such as reproductive dominance (Heinze et al. 2002, Endler et al. 2004). Individual recognition is achieved mainly by chemical communication in social insects (Breed 1998) and the chemical composition of these cues is starting to be elucidated. One of the groups of compounds thought to play an

important role in recognition are the long-chain hydrocarbons on the cuticle which protect insects against desiccation (Gibbs 2002).

Many correlation studies have shown a wide variation in the cuticular hydrocarbon profiles between individuals from different colonies (Breed 1997). Hydrocarbons have been shown to play a part in nestmate recognition in some ant species (Boulay et al. 2000, Lahav et al. 1999) and also in the recognition of reproductive status (Cuvillier-Hot et al. 2002, Endler et al. 2004). They have also been shown to be the cues for nestmate recognition in social wasps (Panek and Gamboa 2000), with branched alkanes and alkenes likely to be more important recognition cues than linear alkanes (Dani et al. 2001).

However, in honey bees, the evidence for the role of hydrocarbons in nestmate recognition is more controversial. There are colony differences in the composition of cuticular hydrocarbons which have been reported to separate nestmates and even full-sisters from half-sisters (Arnold et al. 2000). Supplementation experiments, that is the modification of an individual profile by the addition of specific compounds, have shown an effect on nestmate recognition for some alkanes like hexadecane and octadecane (Breed and Stiller 1992) but these are absent on the cuticle of worker bees. Alkenes such as Z-(9)-tricosene which are present on the cuticle have an effect on nestmate recognition by guard bees while other compounds like dodecane, tricosane and pentacosane do not (Breed 1998). Recently Dani et al. (submitted) showed that supplementation of alkenes rather than alkanes modified the recognition cues of worker honey bees and caused them to be rejected from their own colony. Furthermore, Breed et al. (2004) postulated that although hydrocarbons may play a part, fatty acids are more important recognition cues used by honey bees. Thus, the role of hydrocarbons on nestmate recognition is still a controversial issue.

In the laboratory, honeybees can learn to associate olfactory stimuli with a sucrose reward, according to the proboscis extension response (PER) conditioning paradigm (Kuwabara 1957, Bitterman et al. 1983). When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis. Other odours and stimuli presented to the antennae do not usually release such a reflex in naive animals. However, if an odour is presented immediately before sucrose solution (forward pairing), an association is formed and the odour will subsequently release the PER in following tests. This effect relies on classical (Pavlovian) conditioning (Bitterman et al. 1983), with the odour as the conditioned stimulus (CS) and the sucrose

solution as the reinforcing unconditioned stimulus (US). This paradigm has been used to study the olfactory discrimination abilities of bees and has shown that they can differentiate between many odours (Vareschi 1971). Therefore, PER conditioning is an ideal way to investigate candidate recognition cues, because it allows direct investigation of whether different individual compounds are perceived and discriminated by honeybees. For this application, differential conditioning, in which an odour is rewarded with sucrose solution (positive conditioned stimulus CS+) and another odour is presented without reward (negative conditioned stimulus CS-), is the ideal conditioning procedure, because it determines both whether bees can both perceive the odours and discriminate between them. PER differential conditioning allows the screening of individual compounds and differences in the ability of bees to learn and discriminate between them indicates candidate compounds possibly important for recognition among the dozens present on the cuticle. With this assay, Getz et al. (1986, 1988) showed that workers can discriminate adult, larvae, and eggs using volatile and contact chemicals and Getz and Smith (1987) demonstrated that bees can discriminate between different mixtures of tricosane and pentacosane. More recent work by Fröhlich et al. (2000, 2001), using different fractions of non-polar and polar compounds, showed that bees could not discriminate the hydrocarbon profiles of different comb waxes, and drone and worker cuticular waxes and concluded that compounds other than hydrocarbons were more likely to be involved in recognition.

In this paper, we use PER differential conditioning to determine the discriminatory and learning abilities of bees presented with individually synthesized long-chain alkanes and alkenes present on the cuticle of worker bees and representing around 80% of a typical worker profile. By clarifying the bees' ability to perceive cuticular hydrocarbons with different structures we aimed to determine if bees can use hydrocarbons as recognition cues in general and to predict which specific compounds are more likely to be used in nestmate recognition.

3. Material and methods

3.1. Bees

Workers were collected at random from the top box of a populous colony occupying two Langstroth hive boxes and containing c. 20000 bees. Collected workers were kept for 30 minutes at 33^{o}C in groups of ten to starve them. They were then chilled

until motionless and placed into plastic straws and held in by means of a pin inserted between the thorax and the abdomen which immobilized them without harming them. This allowed free movement of the head, antennae and forelegs of the workers. Workers were then starved for an additional three hours in the restraining device prior to the beginning of the experiments.

3.2. Preparation of odours

Aliquots of 320 µg of each compound were made up in crimp caps vials. Before each experiment, these aliquots were suspended in 160 µl hexane and then 10 µl were evaporated on a glass rod (heat-sealed Pasteur pipette) so that the topmost 1cm of each rod was coated by 20 µg of compound. Sixteen glass rods could be made with each aliquot which were used within two days, eight per day. Rods were kept in an oven at 60^{o}C for at least 15 minutes prior to testing to ensure that all compounds were liquid when tested. A random sample of glass rods (8) were analysed on the GC-MS after use in the conditioning experiments, and all proved to still have at least 98% pure initial compounds, showing that no contamination occurred during the experiments.

3.3. PER Conditioning

Experiments were performed in a temperature-controlled room kept at 25^{o}C. Each bee, restrained in a straw, was placed in a wooden rack with regularly-spaced slots 4 cm apart and kept there during all experiments. We used differential PER conditioning procedures, in which one hydrocarbon is rewarded (CS+), and another hydrocarbon is unrewarded (CS-). Bees received 6 CS+ presentations and 6 CS- presentations in the following pseudo-randomized order: -++-+--+-++-. The sequence always started with a CS- presentation and then a CS+ presentation, so that possible spontaneous responses to the two odours could be recorded prior to the first presentation of the sugar. After conditioning, bees were subjected to a control trial with a blank rod treated with hexane only, to make sure that the bees were responding to the compound and not the mechanical stimulation (Figure 9.1).

During CS+ trials, the hydrocarbon was presented for 6 s by touching the antennae of the bee with the hydrocarbon glass rod. Three seconds after onset of the CS, the antennae were contacted with a 30% sucrose solution (w/w). The subsequent proboscis extension was then rewarded by feeding the bee with a drop of the same solution.

During CS- trials, bees were presented with an odour in the same way but without the subsequent presentation of sucrose. The interval between trials was 15 min. Individuals showing spontaneous responses at the first presentation of the CS- were discarded from the experiment. Individuals showing responses to the CS+ at the second trial were recorded but then discarded in the analyses since later responses of such individuals could not be interpreted as purely associative. Furthermore, only bees that showed a normal proboscis extension when stimulated with the US of sucrose in at least half (i.e. in more than 3) of the CS+ trials were kept for the following steps of the experiments.

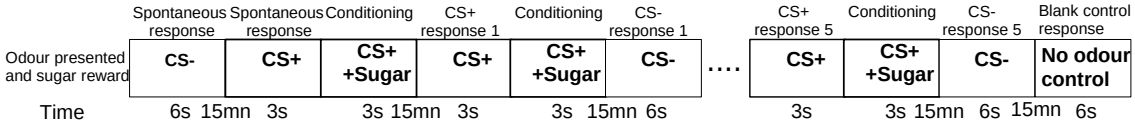


Figure 9.1. Experimental design for the conditioning procedure, including the PER responses recorded (Spontaneous responses, 2 of the 5 responses to CS+ and CS- and control trial). The length of odour and sugar presentation and interval between trials is also included. Bees responding spontaneously and to the control trial were discarded from the analyses, as were bees not responding to the sugar reward (conditioning) more than three times.

It was not possible to test every pairing of all the compounds of interest, or to test each odour pair on each day. We therefore divided the experiments into 5 groups of 3 or 4 hydrocarbons that could be tested simultaneously in a randomised way during subsequent days. For each pair of hydrocarbons tested, each hydrocarbon was presented both as the rewarded hydrocarbon (CS+) and as the unrewarded hydrocarbon (CS-) simultaneously for different bees because of possible discrimination asymmetries. Experiments involving six hydrocarbon combinations were run on 7 consecutive days and experiments involving 12 pairs lasted 14 consecutive days. In this paper when a pair of hydrocarbons is noted as A+/B- the first compound is the CS+ and the second the CS-.

In total, 2012 worker bees were used in the experiments. 297 (14.7%) responded spontaneously to the CS- at the first trial and were discarded from the experiments. 197 (11.4%) additional bees responded spontaneously to the CS+ and 130 (7.6%) to the control test and were discarded from the analysis, with some workers responding to both (1.6%). 47 bees (2.5%) did not respond to the unconditioned stimulus more than 3

times and were also discarded. Overall, 643 bees were rejected (32%) and the results were obtained from 1369 worker bees, with a mean (± s.d.) of 32.6±2.4 workers per hydrocarbon pair.

3.4. Hydrocarbons Tested

In order to obtain meaningful comparisons between compounds with potential roles in recognition, we chose alkanes and alkenes among the compounds most abundant on the honey bee cuticle (Blomquist et al. 1980, Carlson et al. 1989, McDaniel et al. 1984, Francis et al. 1989, Wakonigg et al. 2000). These were the alkanes heptacosane (C27); nonacosane (C29); hentriacosane (C31); and the alkenes 9(*Z*)-pentacosene (9-C25:1); 9(*Z*)-heptacosene (9-C27:1); 8(*Z*)-nonacosene (8-C29:1); 9(*Z*)-nonacosene (9-C29:1); 9(*Z*)-hentriacosene (9-C31:1); 10(*Z*)-hentriacosene (10-C31:1); 10(*Z*)-tritriacosene (10-C33:1) representing respectively, 19.5, 14.2, 10.1, 1.8, 1.8, 2.7, 13.0 and 15.8% of foragers cuticular hydrocarbons as reported by McDaniel et al. 1984 (alkenes differing by double-bond positions pooled as they were not separated in the original paper), with a pooled total of 78.9% of the total hydrocarbons present on the cuticle.

Table 9.1. Questions addressed during the experiments, odours involved for each questions and experiments addressing each question.

Questions	**Odours**	**Expts**
1) Can bees discriminate between alkanes of different chain length?	C27, C29, C31	1
2) Can bees discriminate between alkenes of different chain lengths with the same double bond position?	9C25:1, 9C27:1, 9C29:1, 9C31:1	2,4,5
3) Can bees discriminate alkanes from alkenes with the same chain length	9C27:1, C27, 9C29:1, C29, 9C31:1, 10C31:1, C31	3,4
4) Can bees discriminate alkenes on the basis of double bond position?	8C29:1 *vs*. 9C29:1, 9C31:1 *vs*. 10C31:1	4,5
5) Can bees discriminate between alkenes with different chain length and double bond position?	9C25:1, 10C33:1, 8C29:1, 9C29:1, 10C31:1	4,5
6) Can bees discriminate alkanes from alkenes with different chains lengths?	C29 *vs*. 9C27:1, C31 *vs*. 9C29:1	3,4

Linear alkanes were purchased from Fluka (Sigma Aldrich Company Ltd). Alkenes were synthesised following standard Wittig procedures following methods already described (Dani et al. 2001). As previously reported (Dani et al. 2001), the *Z* geometrical purity of all alkenes was greater than 98% as assessed by GC-MS.

The rationale behind the choice of odours used for each experiment was to test for differences in discriminatory abilities of compounds differing in chain-length,

chemical nature (alkanes vs. alkenes) and double-bond position (Table 9.1 for questions addressed by each experiments). However, we chose not to use the structure of Table 9.1 to present the results as more meaningful divisions appeared (see results).

3.5. Statistical analyses

Since bees were subjected to 6 trials with the CS+ (rewarded compound) and 6 trials with CS- (unrewarded compound), and only bees not showing a spontaneous response to the CS+ or the CS- were kept, bees could give between 0 and 5 responses to each stimulus. To check whether bees significantly preferred the CS+ over the CS-, we used a Wilcoxon test for matched pairs.

Two indexes were used to get a more detailed analysis of the responses: The first was a *learning index* used to characterise the learning abilities of individual bees with the different odours. The index is defined by the number of conditioned responses (CS+) associated with an odour. For graphic purposes, we represented it as a mean proportion of the 5 possible responses (Figure 9.4A). The index range is thus between 0 and 1 with 1 representing a very good learning ability.

We also used a *discrimination index*, defined as $\frac{(CS+)-(CS-)}{(CS+)+(CS-)}$

where CS+ is the number of responses to the CS+ and CS- is the number of responses to CS-. This gives an index between -1 and 1, with 0 meaning that the bee responded equally to the CS+ and to the CS-, thus showing no discrimination. A value of 1 would mean that a bee responded only to the CS+, thus showing total discrimination. Numbers below one mean that the bee showed more responses to the CS- than to the CS+. We used a Kruskal-Wallis test to test for significant differences by comparing indexes for individual bees across odours or odour pairs for each experiment. When significant, it was followed by two-by-two comparisons using the Noether method (Scherrer 1984) with Dunn-Sidák threshold corrections.

4. Results

4.1. Spontaneous responses, learning index and compounds groups

Over a total of five experiments, 20 hydrocarbon pairs were tested both ways (i.e. with each hydrocarbon as the CS+ and the CS-; 40 tested pairs) with one pair (9C29:1 vs. 9C31:1) being repeated in two combinations of hydrocarbons (Figure 9.4). The proportions of spontaneous responses observed in the first CS- conditioning trial varied between 0.01 for C31 and 0.23 for 9C29:1 (Figure 9.2) and there was an overall significant difference in these responses (Chi-square=19.65, df=9, p=0.02). Five compounds, including all the alkanes and two alkenes, 9C31:1 and 10C33:1 gave low levels of spontaneous responses while the other alkenes elicited higher levels of spontaneous responses (Figure 9.2).

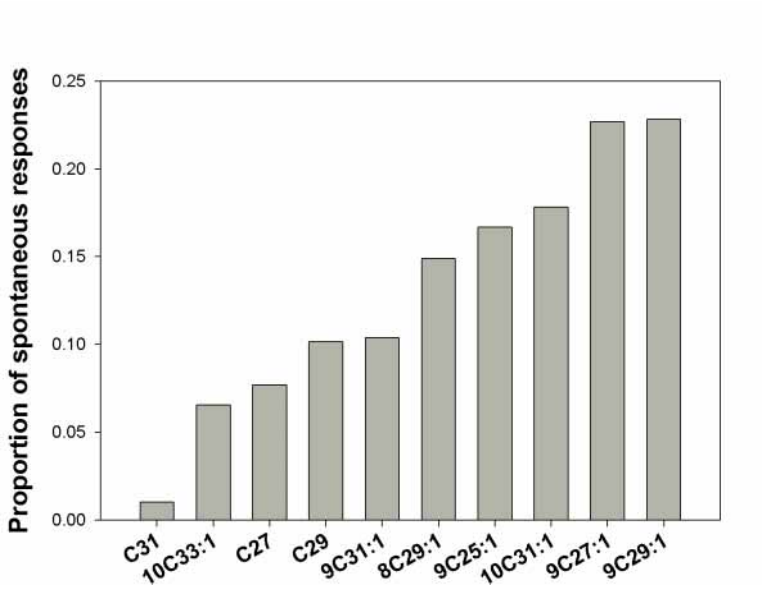


Figure 9.2. Proportion of spontaneous responses to the tested hydrocarbons at the first trial of the experiments. Results from all five experiments have been pooled.

There were also significant differences in the learning success of bees with the different compounds used as CS+ (Figure 9.3). Alkanes were generally poorly learnt, as shown by the curves for CS+ and CS- responses (Figure 9.3 first three columns), and by the low learning indexes (Figure 9.4A, 1,3,4) which were always below 0.61. Two alkenes, 9C31:1 and 10C33:1 showed similar low learning performance (Figure 9.3, respective columns), and low learning indexes, below 0.61 (Figure 9.4A, 2, 4, 5). Alkane indexes did not differ significantly from each other or from that of 9C31:1, and although 10C33:1 was not tested together with these compounds, its learning index differed significantly from that of other alkenes such as 9C29:1 and 9C25:1.

All the other alkenes showed high learning curves, reaching 80-100% conditioned responses (Figure 9.3 see respective columns), and had mean learning

indexes between 0.67 and 0.92 (Figure 9.4A, 2-5). The learning indexes of these alkenes did not differ between each other, but were significantly higher than those of alkanes or of 9C31:1 and 10C33:1 (Figure 9.4A, 2-5). Within experiments or overall, there were no significant differences in the learning indexes of individual CS+ odours according to the odour presented as CS- except for 9C31:1 in experiment 2 where the mean learning index when the CS- was 9C29:1 (0.52) was different from when 9C27:1 was the CS- (0.62; Kruskal-Wallis test, $p=0.047$; all the other tests gave p values above 0.3, see columns in Figure 9.3). This indicates that the learning indexes observed for odours when used as CS+ did not vary according to the CS-, therefore validating the use of the learning index.

We observed that the clear differences between odours concerning learning success mirrored the trend observed in the spontaneous responses, since the five least-well learnt hydrocarbons (the three alkanes and the two alkenes 9C31:1 and 10C33:1) were also those that showed the lowest spontaneous responses (Figure 9.2). The clear-cut difference observed between hydrocarbons in learning performance suggested that it was meaningful for further analysis to divide the hydrocarbons into two groups according to the learning success: the alkanes and 9C31:1 and 10C33:1 in a low learning index group (LL) and the other alkenes in a high learning index group (HL).

4.2 Discrimination success and asymmetries

Out of the 40 tested pairs, 13 pairs of hydrocarbons gave non-significant discrimination results (Wilcoxon matched pairs test), with bees responding with similar probability to the CS+ and to the CS- (Figure 9.3). Nine of these involved pairs in which a LL compound was the CS+ (Figure 9.3; Figure 9.4B patterned white and light grey bars). In the four cases where both odours were HL compounds, the pairs could not be discriminated whichever way they were tested, and they involved the compounds with the highest learning indexes, 9C29:1 vs. 9C27:1 and two very close compounds with regards to formula, 9C29:1 and 8C29:1 (Figure 9.3, columns 9C27:1 to 9C29:1; Figure 9.4B, patterned dark grey bars in experiment 2 and 5).

Although in most cases (27 out of 40), bees could discriminate between the CS+ and the CS-, there were important differences in the magnitude of this discrimination. We therefore used the discrimination index which quantifies such differences to get a better picture of bees' discrimination ability regarding different cuticular hydrocarbons (Figure 9.4B). Two different situations were observed with regards to the symmetry of

odour discrimination. In 10 out of 20 pairs, a hydrocarbon pair gave symmetrical results when the rewarded and unrewarded hydrocarbons were exchanged.

In the ten other pairs, bees' discrimination success depended on which hydrocarbon was rewarded in the pair, one combination being well discriminated and the other not, causing an asymmetry. Some of the most striking asymmetries were between alkanes and alkenes, e.g. 9C29:1 *vs.* C29 (Experiment 3) or 10C31:1 *vs.* C31 (Experiment 4) and between short chain and long chain alkenes e.g. 9C25:1 and 10C33:1 (Experiment 5). Here again, the asymmetries greatly depended on the type of odours used. We therefore evaluated discrimination success depending on how well odours were learnt (LL and HL odours, see above).

4.2.1. LL vs. LL odours

For pairs of LL hydrocarbons, discrimination was always very low (see for instance how close together the CS+ and CS- curves are for different alkanes in Figure 9.3). Discrimination indexes were therefore also low, ranging from 0.11 to 0.55, without any significant differences between them. The biggest difference in a pair was for 9C31:1 *vs.* C31, with bees discriminating better when the alkene was rewarded. Altogether, bees discriminated poorly between alkanes (Figure 9.3 upper left, Figure 9.4B, 1), and no clear trend in the discrimination index appeared between them (range 0.11-0.36, Figure 9.4B, 1).

4.2.2 LL vs. HL odours

When a HL odour was rewarded (CS+) against a LL hydrocarbon (CS-), the discrimination index was always high, between 0.70 and 0.84 when the LL hydrocarbon was an alkane or 10C33:1 and between 0.38 and 0.53 for 9C31:1. When the LL hydrocarbon was rewarded the discrimination was low, between -0.14 and 0.45. These differences caused a systematic significant asymmetry between the discrimination indexes for the two hydrocarbons of a pair. In all but one of the 12 pairs tested, the discrimination index for HL+/LL- situations was significantly higher than that for LL+/HL- situations (see respectively the black and white bars in Figure 9.4B, 2-5). The remaining pair was 9C27:1 vs. C29 where C29+/9C27:1- did not differ from the alkene+/alkane- indexes.

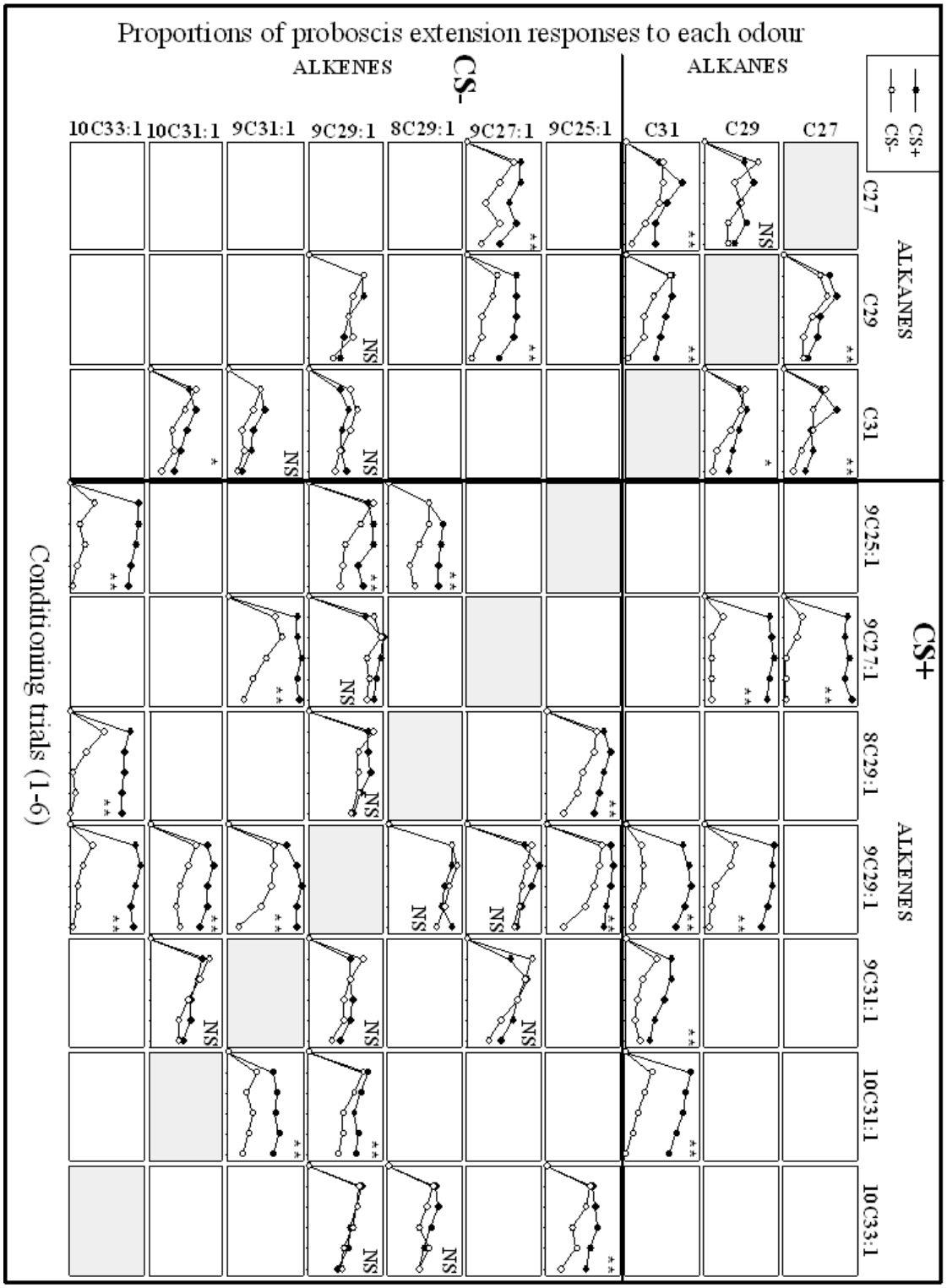


Figure 9.3. Proportions of proboscis extension responses to the CS+ and the CS- during differential conditioning experiments between each odour pair. Significant differences between the response curves are indicated by * $p<0.05$; ** $p<0.01$ (Wilcoxon test for matched pairs).

4.2.3 HL vs. HL odours

When both hydrocarbons had a high learning index (HL), discrimination was generally low. The discrimination index was low between these HL alkenes, ranging between 0.03 and 0.35, and never significantly differed between odour pairs.

5. Discussion

In this work, we used 10 hydrocarbons, representing almost 80% of the honey bee cuticular hydrocarbon profile, and a PER conditioning technique to evaluate how well bees learn and discriminate these compounds. Our results show that bees can discriminate between most of the cuticular hydrocarbons tested but that there are clear differences in learning and discrimination abilities according to the nature of the compounds. There appears to be a clear divide between alkanes and alkenes, with alkenes being generally much better learnt than alkanes.

Honey bees are known to be able to learn a very wide range of odours in an appetitive (PER experiment) context (Vareschi 1971, Menzel 1985, Laska et al. 1999). In particular, they can learn odours with a strong pheromonal value (queen pheromonal compounds, alarm pheromones, social aggregation pheromone, Vareschi 1971, Smith and Menzel 1989, Smith 1991, Sandoz et al. 2001) or even initially aversive odours (von Frisch 1965, Kriston 1971). Thus, the efficiency with which bees learn odours in the PER conditioning context gives us important information about how well such odours are perceived independently of any biological value they might have. We thus think that the odours which were not learned efficiently by bees in our experiments are odours that are not well detected by the bee nervous system, i.e. not very salient odours. This happened mainly with alkanes. This may not be surprising as alkanes have only one distinguishing feature, the length of their carbon chain, whereas alkenes have at least three distinguishing features, the bend of the double bond, the length of the short chain between one terminus and the double bond and the length of the long chain between the other terminus and the double bond (Table 9.2). What is striking is that the two LL alkenes 9C31:1 and 10C33:1 have long chains of 21 and 22 carbon atoms respectively. This, coupled with the fact that the HL 10C31:1 is distinguishable from the LL 9C31:1 strongly suggests that there is a link between how well the molecules are perceived and the long carbon chain length (Table 9.2). We can thus speculate that a critical binding

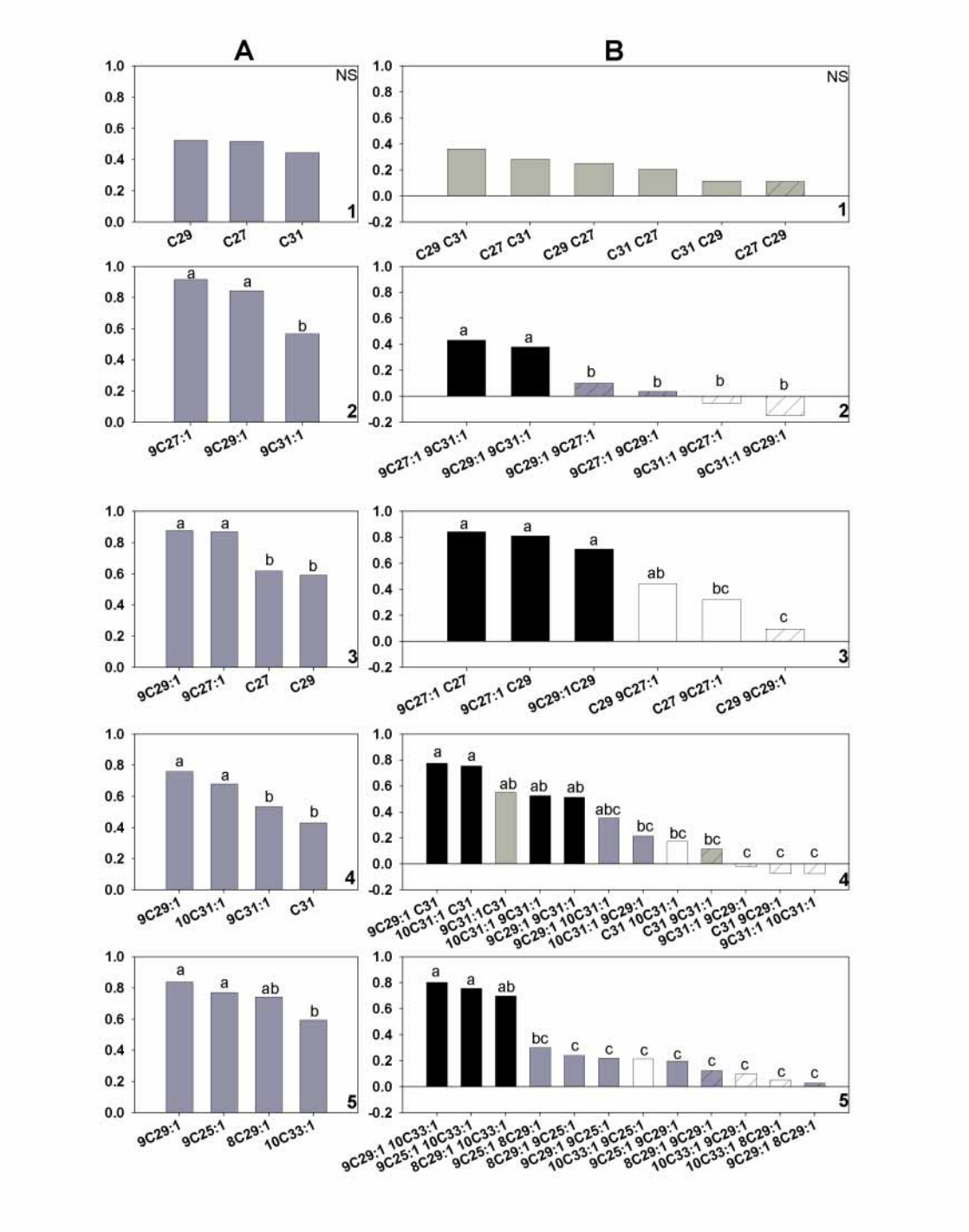
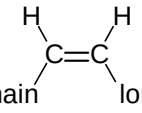


Figure 9.4. Discrimination and learning ability for the different odours and odour pairs in the five experiments, numbered 1-5. NS indicates a non-significant overall difference. Different letters indicates significantly different values for experiments where the overall Kruskall-Wallis test was significant. **A. Learning index** for individual compounds. **B. Discrimination index** for all the odour pairs tested. The first odour of each pair is the rewarded odour. The colour of the bars indicates the type of comparison between well-learned (HL) and less well-learned odours (LL): black HL+/LL-, Dark grey: HL+/HL-, Light grey: LL+/LL-, White: LL+/HL-. The significance of the discrimination test is indicated by an unpatterned bar. For patterned bars, the test was non-significant, except for 9C31:1+/9C29:1 in experiment 5 where the bees responded significantly more to the unrewarded compound (see text).

protein in the olfaction system may only bind compounds with a maximum long chain length of 20 carbon atoms.

Table 9.2. Structural features of tested alkenes. The two alkenes with low learning index (LL) are characterised by a long chain of more than 20 carbons.

Alkene			Average learning index
9C25:1	$H_{17}C_8$	$C_{15}H_{31}$	0.82
9C27:1	$H_{17}C_8$	$C_{17}H_{35}$	0.91
8C29:1	$H_{15}C_7$	$C_{20}H_{41}$	0.80
9C29:1	$H_{17}C_8$	$C_{19}H_{39}$	0.85
9C31:1	**$H_{17}C_8$**	**$C_{21}H_{43}$**	**0.60 (LL)**
10C31:1	$H_{19}C_9$	$C_{20}H_{41}$	0.77
10C33:1	**$H_{19}C_9$**	**$C_{22}H_{45}$**	**0.65 (LL)**

Calcium imaging experiments, as carried out on the honey bee brain, allow odour-evoked activity in olfactory brain areas to be recorded (Joerges et al. 1997, Faber and Menzel 2001), giving some insight into how odours are perceived by the brain. In the antennal lobe, the first relay of the olfactory pathway, odours have been shown to elicit glomerular response patterns (Joerges et al., 1997) based on a code which is conserved between individuals (Galizia et al. 1999, Sachse et al. 1999). Since these responses emphasize the activity of sensory neurons (Galizia and Menzel 2001), and because sensory neurons carrying one type of receptor seem to all project to the same glomerulus (Voshall 2000), calcium imaging of the antennal lobe gives us an idea of the sensitive range of possible olfactory receptors on the bees' antennae. In one study, Sachse et al. (1999) presented bees with C5-C13 hydrocarbons. Results showed that odour-evoked responses were only obtained for the shortest-chained alkanes (C5 to C9), in which very few glomeruli responded, which also responded to several other oxygenated compounds with the same chain lengths (alcohols, aldehydes, ketones). No signals appeared for the longer-chained alkanes (C10 to C13), and very long-chained alkanes (like our C27, C29 or C31) were not tested. The results obtained by Sachse et al. (1999) suggest that, at least on the surface of the antennal lobe which is accessible to optical imaging studies (about 40 glomeruli out of the 165 present in the lobe), no glomerulus is specifically sensitive to alkanes, and none responds to long-chained alkanes. Because the olfactory code is thought to be highly redundant (Galizia et al. 1999, Galizia and Menzel 2001), it could be that long-chain alkanes bind only non-specifically onto odour receptors, and do not therefore give rise to very salient or clear

neural representations. In this case, they would represent poor substances to act as nestmate recognition cues (see below). However, this remains a hypothesis, since not all regions of the lobe have yet been explored and long-chain alkanes and alkenes specifically have not been tested.

The implications of our results in the context of nestmate recognition are profound. First, we believe that the compounds which were not well learned are highly unlikely to have any role in chemical communication. Interestingly, these compounds include the most abundant compounds on the bee cuticle, namely alkanes and the longer-chain alkenes like 10C33:1 (74 to 91% of the compounds we tested on the cuticle; McDaniel et al. 1984). Recent supplementation experiments of cuticular hydrocarbons (Dani et al. submitted) and experiments in other species (*Polistes*, Dani et al. 2001) have also confirmed that these compounds are not likely to be used for nestmate recognition.

On the other hand, the ability of workers to learn shorter-chained alkenes below 29 carbons makes these compounds good candidates for recognition cues. In our experiments, however, these structurally similar compounds were not always discriminated well and when they were the discrimination indices were generally low. This generalisation phenomenon is however not uncommon for biologically active compounds like pheromones, even when their composition is very different like the honey bee alarm pheromones 2-heptanone and isoamylacetate (Sandoz et al. 2001). It is possible that in a context other than the appetitive context of conditioning, like for instance while guarding at the entrance to the hive, the bees could be more motivated to discriminate between these compounds or that the bees would class compounds together in groups. Moreover, we do not have data on how bees would respond to mixtures of the different odours and discrimination could be increased in this case, as was found by Getz and Smith (1987) in an experiment using C23 and C25. Of particular interest would be to test alkenes with the same carbon number but different double-bond position (like 9C31:1 and 10C31:1), the proportions of which have been shown to change according to race but could also vary between colonies (Carlson et al. 1989).

In contrast to our results, Fröhlich et al. (2000, 2001) have found, using PER conditioning, that the hydrocarbon fraction of different comb waxes and cuticular waxes are not discriminated by honey bees and hence these authors conclude that hydrocarbons cannot be used as cues for nestmate recognition. We believe that the experiments done by these authors cannot allow such a conclusion. In their work, they

have tested the cuticular extracts from two different castes, namely males and workers, coming from the same colony. The colony signature being the same could explain the absence of discrimination in the learning experiments. Moreover, chemical properties and recognition ability can also change between the compound alone and the compound in a mixture of different compounds, and this can be influenced by the solid or liquid phase of the compounds (Gibbs 2002). An indication of this comes from the fact that bees examined by guards increase their thorax temperature, possibly to improve chemical communication (Stabentheiner et al. 2002). This could also explain why some experiments (Fröhlich 2000, 2001) failed to show any discrimination of hydrocarbons in some experimental conditions at room temperature.

In conclusion, our experiments have shown differences in the learning and discrimination ability of cuticular hydrocarbons by honey bees. The most common compounds on the cuticle (alkanes and long-chained alkenes) are learnt least well, which could mean that such compounds are not used for recognition and probably only have a role against desiccation. Less common compounds, like shorter-chained alkenes, were well learnt and easily differentiated. This suggests that bees could have the ability to use such compounds in a recognition context. Recent work on two ant species would confirm the role of these compounds in recognition contexts. Three alkenes (C29:1, C31:1 and C33:1) have been found to be characteristic of queen cuticular profile in *Linepithema humile* (de Biseau et al. 2004) and internally branched alkanes and alkenes, with shorter straight carbon chains, are predominant in the post-pharyngeal gland of *Pachycondyla villosa*. This gland is believed to be used in the transfer of recognition cues between nestmates and its composition in hydrocarbons was significantly different from the cuticle where alkanes and externally branched alkanes also occurred (Lucas et al. 2004).

The PER conditioning approach is a useful method of filtering through the many compounds present on the cuticle to select compounds likely to be acting as recognition cues. Further tests should include PER conditioning with mixtures of the well-learned compounds. Such tests should be linked with correlational and supplementation studies in order to improve our understanding of nestmate recognition in bees.

Chapter 10
General Conclusion

1. Worker policing and worker reproduction

1.1 Variation and deviation in worker reproduction

In chapters 5-7, large variations in the colony phenotype in relation to worker reproduction and worker policing were investigated. In chapters 5 and 6, two atypical colonies were described, one (chapter 5) having worker reproduction in a queenright colony ("anarchy"), the other (chapter 6) absence of worker reproduction in a hopelessly queenless colony. In the British anarchist colony (chapter 5), a detailed genetic analysis showed that workers in many patrilines were the mothers of the males being reared despite the presence of the queen. This contrasted strongly with the previously described naturally occurring Australian anarchist colonies where only one patriline of worker monopolised worker reproduction which increased the inclusive fitness of both individual worker layers and their patriline (Oldroyd et al. 1994, Montague and Oldroyd 1998). The high number of patrilines reproducing in the studied colony has two implications. First, in the colony studied in chapter 5, only individual anarchistic workers gained some inclusive fitness benefits, at the expense of the whole colony and even the non-reproducing workers from their patrilines, making the anarchistic phenotype costly and an example of a tragedy of the commons (Hardin 1968, Wenseleers and Ratnieks 2004). Second, it shows that anarchistic traits can be maternally as well as paternally inherited and that the genetic determinism of this phenotype is complex as not all patrilines reproduced and those that did did so in different proportions. These two points help explain why the anarchistic phenotype is so rare, because of its cost and because it probably stems from a combination of unlinked genetically determined traits (Osborne and Oldroyd 1999) which have to be brought together in the same colony for the workers to lay acceptable eggs and for the anarchistic colony phenotype to be observed.

In the hopelessly queenless colony that did not produce males (chapter 6), the workers behave maladaptively by showing a trait, worker policing, which prevented them from rearing a last batch of males before the colony dwindled to death. Workers

still activated their ovaries but as worker policing probably shows behavioural dominance (Craig 1980), then if only a few workers (or a few patrilines) do not stop policing, then this is probably enough to prevent worker reproduction. This colony type does not seem to be as rare as anarchy, and appears to occur in about 1 in 20-50 colonies (personal observation) versus 1 in 1000 or more for anarchy (Barron et al. 2001). While policing in a queenless situation is not normal, policing is of course adaptive in queenright colonies. A trait which is selected for in a queenright situation might lead to a maladaptive phenotype in a queenless situation, especially if the selective pressure on queenless colonies is lower, as is the case because queenless colonies are rarer than colonies with a queen leading to fewer genes being transmitted through queenless colonies than queenright colonies to the next generation.

In chapter 7, variation within the typical range of colony phenotypes was observed, including a discriminator colony which removed worker-laid eggs at a greater rate than the other colonies. This suggests that considerable phenotypic variation in worker policing probably also occurs within the normal range of colony phenotypes. This study also provided evidence for a cost to more effective policing in terms of greater errors, namely the removal of queen-laid eggs. We also confirmed that this cost may be offset by a more stringent policing in drone cells, the type of cell in which workers in queenright colonies lay eggs, as was also shown by Halling and Oldroyd (2003). However, because error rates did not vary between worker cells and drone cells, the significance of this may not be great.

1.2 The Chemistry of the regulation of worker reproduction

Chemical communication is important in the regulation of much of the functioning of honey bee colonies (Breed 1998). The queen plays an important role in the regulation of worker reproduction. The queen produces pheromones, which include the mandibular pheromone and the queen esters from the Dufour's gland (Katzav-Gozansky et al. 1997, 2001), which act as an honest signal of her presence (Seeley 1985) and fertility, thereby inhibiting worker ovary activation. Anarchistic workers are less sensitive to this pheromone and are more likely to activate their ovaries despite the presence of a queen (Oldroyd et al. 2001b). The ability to produce queen-specific pheromone is, however, not limited to the queen. Bio-synthetic pathways are conserved between the queen and workers and may differ only in a few steps. The end products of these pathways can be used in different contexts. For example, 9-ODA, the main

component of queen mandibular pheromone, is produced by a very similar biochemical pathway as that used by workers to produce 10-HDA, a fatty acid used as larval food (Plettner et al. 1996). Workers in queenless colonies are known to be able to switch to the production of 9-ODA.

Similarly, another conserved bio-synthetic pathway produces both the queen esters and a worker alarm pheromone, 11-Eicosenol (Martin and Jones 2004) in the Dufour's gland. Here again, workers are able to switch from one pathway to another and some workers in queenless colonies produce the queen ester but not 11-eicosenol. The control of this pathway seems to be linked with reproduction, and anarchistic workers, which evade worker policing, are able to produce the queen esters while living in a queenright colony (Martin et al. 2004c, Appendix 1.2), thus making their eggs less likely to be policed. In the hopelessly queenless colony which continued to police worker-laid eggs (chapter 7), workers do not seem to be able to produce any esters, and although 11-Eicosenol production is reduced, no worker with active ovaries produced esters (Martin, unpublished results). This could be linked to the observed phenotype of not switching off policing, although it probably is not its cause. Policing is also dependent on a queen egg-marking chemical (Martin et al. 2004b). If this chemical varies in quantity between worker-laid and queen-laid eggs rather than by presence or absence, there is a possibility that differences in the detection thresholds among workers could make the egg-removal rate vary between colonies (chapter 7) and could cause some costly policing errors. With the bee genome now sequenced and a lot of the bio-synthetic pathways linked with reproduction described, a fruitful approach in the study of mechanisms involved in worker reproduction would be a genomic one, in order to document the differences in the expression of the genes involved in these pathways. When the egg-marking pheromone is finally discovered, its occurrence in *Apis* and other social Hymenoptera promises a large advance in the understanding of intracolony conflict. The ability of workers to synthesize it or not will be elucidated, and this should make it possible to determine whether the signal can be easily be made by workers, and if there are any costs to doing this such as not making some chemical needed for colony function (e.g., an alarm pheromone) or if workers who do cheat are killed or aggressed.

2. Nepotism and recognition

2.1 Nepotism in queen rearing

The data in chapter 8 and other recent evidence (Gilley 2003, reviewed in Tarpy et al. 2004), confirm earlier studies indicating that nepotism is either absent or weak in the honey bee. The increased likelihood of larvae of certain patrilines to be reared as queens under emergency queen rearing conditions is the only sign of any preferential rearing being expressed (Osborne and Oldroyd 1999, Châline et al. 2003), although the exact mechanism behind this is still unknown. One probable reason for the absence or weakness of nepotism is the cost to the colony, for example if its expression reduces colony efficiency or even jeopardizes the rearing of a new queen (Tarpy et al. 2004). Other reasons could be that nepotistic interactions in a patriline should be counter balanced by nepotism in the other patrilines, making its expression unapparent (Page et al. 1989), and the absence of sufficient cues to reliably recognise closer kin within colonies which would lead to too many errors (Ratnieks and Reeve 1991). A recent study on *Polistes* and *Vespa* wasps (Dani et al. 2004) showed that this could indeed be the case. A further indication of unreliability in relatedness cues, especially in multiply mated species, is that many studies with unnaturally low numbers of patrilines or unrelated queens have found evidence of differential treatment (Tarpy and Fletcher 1998, Noonan 1986), while a repeat of them in a natural situation did not (Gilley 2003, Appendix 2.2). In chapter 8, we found evidence for genetic specialisation in caring for the queen cells with adult queens in them, and in the colony with the lowest number of patrilines, a differential treatment of cross-fostered queen cells. This again suggests that, were the cues available, honey bee workers would be likely to express nepotism.

2.2 Cuticular hydrocarbons discrimination

Although it seems unlikely that worker bees can reliably discriminate full sisters from half sisters, they are better at the less challenging recognition decision made by guard bees at the colony entrance in discriminating between nestmates and non-nestmates. The cues that guards use are still unclear but hydrocarbons seem to be likely candidates. In chapter 9, using the proboscis extension bioassay (PER), I have shown that there is a large difference between hydrocarbons in terms of the ability of workers to learn them, with lighter alkenes being learnt better, and to discriminate between them. The PER bioassay seems, therefore, a good technique for screening the cuticular

compounds found on honey bees and to select candidates for further behavioural bioassays, in mixture or not. Chemical analyses would confirm if these show the necessary variability between colonies. Further analyses could include calcium imaging, which would document the perception of the cues at the neurological level, and supplementation experiments to evidence the effects of these chemicals at the behavioural level. Ultimately, the best evidence would be to recreate a colony odour on non-nestmate workers so that they are accepted with the same probability as nestmates.

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Appendix 1

In this appendix, I have included four papers which resulted from collaborative work with Dr. Stephen Martin and which all have policing and egg chemistry as their main subject. They are

Appendix 1.1: Martin SJ, Jones GR, Châline N, Middleton H, Ratnieks FLW (2002) Reassessing the role of the honeybee (*Apis mellifera*) Dufour's gland in egg marking. *Naturwissenschaften*, **89**, 528-532.

Appendix 1.2: Martin SJ, Châline N, Oldroyd BP, Jones GR, Ratnieks FLW (2004c) Egg marking pheromones of anarchistic worker honeybees (*Apis mellifera*). *Behavioural Ecology*, **15**, 839-844.

Appendix 1.3: Martin SJ, Jones GR, Châline N, Ratnieks FLW (2004d) Role of hydrocarbons in egg recognition in the honeybee. *Physiological Entomology*, **29**, 395-399.

Appendix 1.4: Martin SJ, Châline N, Jones GR, Ratnieks FLW (2004b) Searching for the egg-marking signal in honeybees. *Journal of Negative Results, Ecology and Evolutionary Biology*, **1**, in press.

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SHORT COMMUNICATION

Stephen J. Martin · Graeme R. Jones · Nicolas Châline · Helen Middleton · Francis L. W. Ratnieks

Reassessing the role of the honeybee (*Apis mellifera*) Dufour's gland in egg marking

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Abstract Dufour's gland secretion may allow worker honeybees to discriminate between queen-laid and worker-laid eggs. To investigate this, we combined the chemical analysis of individually treated eggs with an egg removal bioassay. We partitioned queen Dufour's gland into hydrocarbon and ester fractions. The bioassay showed that worker-laid eggs treated with either whole gland extract, ester fraction or synthetic gland esters were removed more slowly than untreated worker-laid eggs. However, the effect only lasted up to 20 h. Worker-laid eggs treated with the hydrocarbon fraction were removed at the same rate as untreated eggs. The amount of ester which reduced the egg removal rate was far higher than that naturally found on queen-laid or worker-laid eggs, and at natural ester levels no effect was found. Our results indicate that esters or hydrocarbons probably do not function as the signal by which eggs can be discriminated.

Introduction

The ability of worker honeybees to distinguish between queen-laid and worker-laid eggs is well established (Ratnieks and Visscher 1989; Oldroyd and Ratnieks 2000; Katzav-Gozansky et al. 2001). This egg recognition is fundamental to the mechanism of worker policing, by which workers kill eggs laid by fellow workers but leave queen-laid eggs. Only 0.1% of adult males in a queenright colony are workers' sons (Visscher 1989), despite 7% of male eggs being worker-laid (Visscher 1996). However, the nature of the differences between queen-laid and worker-laid eggs remains unknown.

There is little physical difference between the shape or length of worker-laid (1.53±0.11 mm, Miller and Ratnieks 2001; 1.49±0.05 mm, Ratnieks 1993) or queen-laid (1.53–1.63 mm, Snodgrass 1925) eggs. In fact, there is considerable variation among eggs from the same source. A queen can lay eggs that vary almost two-fold in length (DuPraw 1961) yet all develop normally. In addition, worker-laid eggs can sometimes evade policing (Oldroyd and Ratnieks 2000; Martin et al. 2002). The lack of physical differences between egg types and the fact that honeybees possess an intricate chemical communication system (Free 1987), means that egg discrimination is probably chemically based. Furthermore, any signal is universal since workers can discriminate between worker-laid and queen-laid eggs even if the eggs are from another colony (Ratnieks and Visscher 1989).

Previous studies (Ratnieks 1995; Katzav-Gozansky et al. 2001) have focused on the Dufour's gland, which has long been considered one source of secretions which coat eggs (Bordas 1895; Trojan 1930). The Dufour's gland secretion is composed of esters and hydrocarbons (Katzav-Gozansky et al. 1997, 2001), both of which may have a pheromonal function in social insects (Francke et al. 1984; Breed and Stiller 1992). Together with Ratnieks (1995), who found that worker-laid eggs treated with Dufour's gland extract were policed more slowly, these factors collectively suggest that the Dufour's gland is a source of egg-marking chemical pheromone. However, the chemical plasticity of the Dufour's gland means that egg-laying workers are also able to produce queen-specific esters (Katzav-Gozansky et al. 1997, 2001). Nevertheless, this does not normally prevent worker-laid eggs from being removed, which suggests that esters from the Dufour's gland may not play a key role in egg recognition. Katzav-Gozansky et al. (2001) also found that when either Dufour's gland extract or a synthetic blend of the gland's esters were applied to worker-laid eggs, it did not make them more acceptable,

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thus challenging the interpretation of previous observations (Ratnieks 1995).

In this study we investigate the effect of hydrocarbons and esters found in the Dufour's gland on the removal of worker-laid eggs by combining an egg removal bioassay with the surface chemistry of individual eggs.

Method

Honeybee colonies

The study was conducted during the summer of 2001 in Sheffield, UK using colonies of *Apis mellifera*. Eggs aged 0–24 h were obtained from worker cells in a queenright colony's (queen-laid) or drone cells in a colony which had been maintained in a queenless state for over 1 month (worker-laid). Three unrelated, queenright colonies were used as discriminator colonies for egg removal bioassays.

Chemical analysis

All GC-MS (gas chromatography–mass spectrometry) analyses were performed in a splitless mode on a 5890 Hewlett Packard gas chromatograph coupled with a 5970 quadrupole mass spectrometer (70 eV electron impact ionisation). A 15 m × 0.25 mm ID column with a 0.25 μm thickness bonded BP5 stationary phase (SGE) was used. The GC oven was programmed from an initial temperature of 150°C (3 min) to a final temperature of 325°C (2 min) with a ramp rate of 10°C/min. The injection port was held at 250°C and the transfer line at 300°C. Helium carrier gas flowed at a rate of 1 ml/min.

Preparation of Dufour's gland fractions

Virgin honeybee queens were reared and held in cages in a colony for at least 8 days following emergence before their Dufour's gland was removed. Each gland was individually extracted into 100 μl of hexane by sonication for 30 min. The extracts of five glands were combined and with vial washings made up a total volume of 600 μl. Then 3 μl of this solution was injected onto the GC-MS for analysis (Fig. 1a) and the remainder loaded onto a silica chromatography column (0.5 g SiO_2, silica gel for flash chromatography, conditioned with hexane). Next 1.5 ml of hexane was pressurised through the column and four 0.5 ml fractions were collected. The fourth fraction was analysed by GC-MS and was found to contain no hydrocarbons. The elution solvent was then changed to dichloromethane and a further three 0.5 ml fractions containing the esters were collected. All fractions were then analysed by GC-MS. All fractions containing hydrocarbons or esters were combined separately. The hydrocarbon and ester fractions were then divided equally into five vials, so that each contained one gland equivalent of either fraction, before being evaporated. Prior to applying to eggs for the bioassay each fraction was redissolved in 0.5 ml of hexane, the same dilution used for the Dufour gland extracts.

Ester synthesis

Three of the main esters identified on both the surface of queen-laid eggs and in queen Dufour's glands (S.J. Martin and G.R. Jones, unpublished) were synthesised. These were: hexadecyl hexadecanoate, hexadecyl hexadecenoate and tetradecyl tetradecanoate. Three different concentrations of each ester (1 μg/μl, 0.1 μg/μl, 0.01 μg/μl) were prepared for bioassay.

Application of chemicals to eggs

Queen-laid and worker-laid eggs were transferred to a glass slide using a blunt needle. This allowed approximately 0.5 μl of hexane containing the test substance to be applied to the surface of each egg via a 10 μl Hamilton syringe. This was approximately 1/40 queen-gland equivalent.

Chemical analysis of treated eggs

To ensure that our method had altered the surface chemistry of treated worker-laid eggs, during each bioassay we collected individual eggs in sealed glass capillary tubes. These were analysed by the standard technique of crushing the egg within the capillary tube in the GC-MS using a Keele injector (Morgan 1990).

Egg removal bioassay

Groups of 20, untreated queen-laid eggs, treated and untreated worker-laid eggs were transferred from glass slides into adjacent worker cells in test frames from each discriminator colony. Each test frame was then replaced into the discriminator colony above the queen-excluder between two frames of open brood. The numbers of eggs remaining after 1, 2 and 20 h were recorded. Each trial quantified the removal of 20 eggs from each class, in one of the three discriminator colonies. As one colony (D3) was found to police eggs very rapidly, eggs were also counted after 0.5 h for this colony, although these data were not used.

Statistical analysis

All data were arcsine transformed to satisfy the assumption of normality before one-way factorial ANOVA was used to confirm that the main variation was among the egg sources at each observation time.

Results

Effect of hexane solvent

There was no significant difference at any of observation times (P always >0.2) between the removal rates of untreated queen-laid (n=181) or worker-laid (n=101) eggs and queen-laid (n=225) or worker-laid (n=100) eggs treated with hexane in any of the discriminator colonies. Thus the solvent had no effect on egg removal rates.

Egg surface chemistry

We successfully separated whole Dufour's gland extract (Fig. 1a) into hydrocarbon (Fig. 1b) and ester (Fig. 1c) fractions. When these fractions were applied to worker-laid eggs (Fig. 1d) the amount of hydrocarbons and esters on the egg increased (Fig. 1e) as evidenced by the GC-MS data. The amounts of esters on the worker-laid eggs treated with both whole Dufour's gland extract and the ester fraction were both greater than naturally found on either worker-laid or queen-laid eggs. The addition of either the hydrocarbon fraction or whole Dufour's gland extract to worker-laid eggs increased hydrocarbon levels

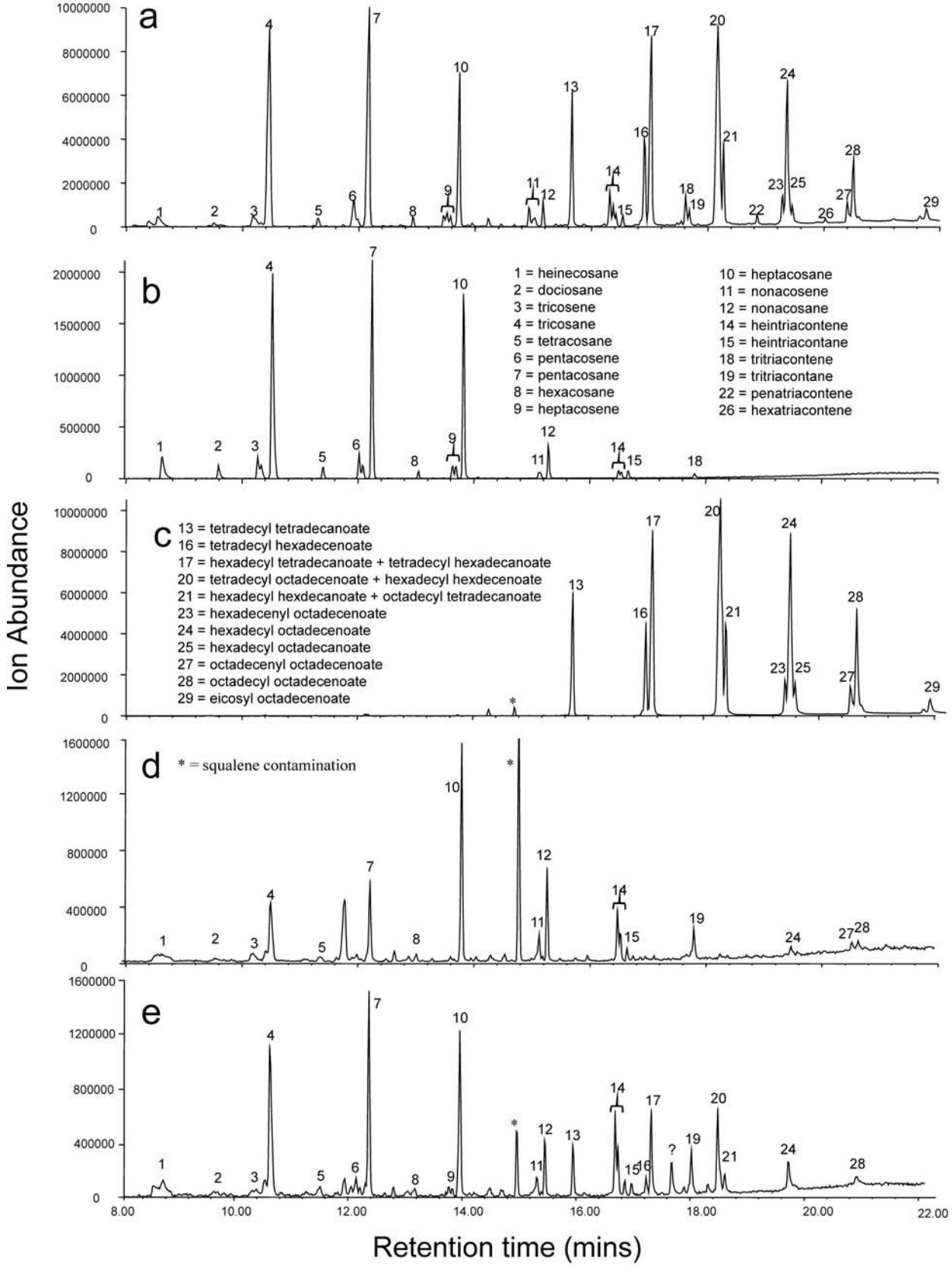

Fig. 1 Gas chromatograms showing the main chemicals found in the Dufour's gland of a virgin queen (**a** whole gland) and its fractions (**b** hydrocarbon, **c** esters), on individual worker-laid eggs (**d** untreated) and (**e** treated with whole Dufour's gland extract)

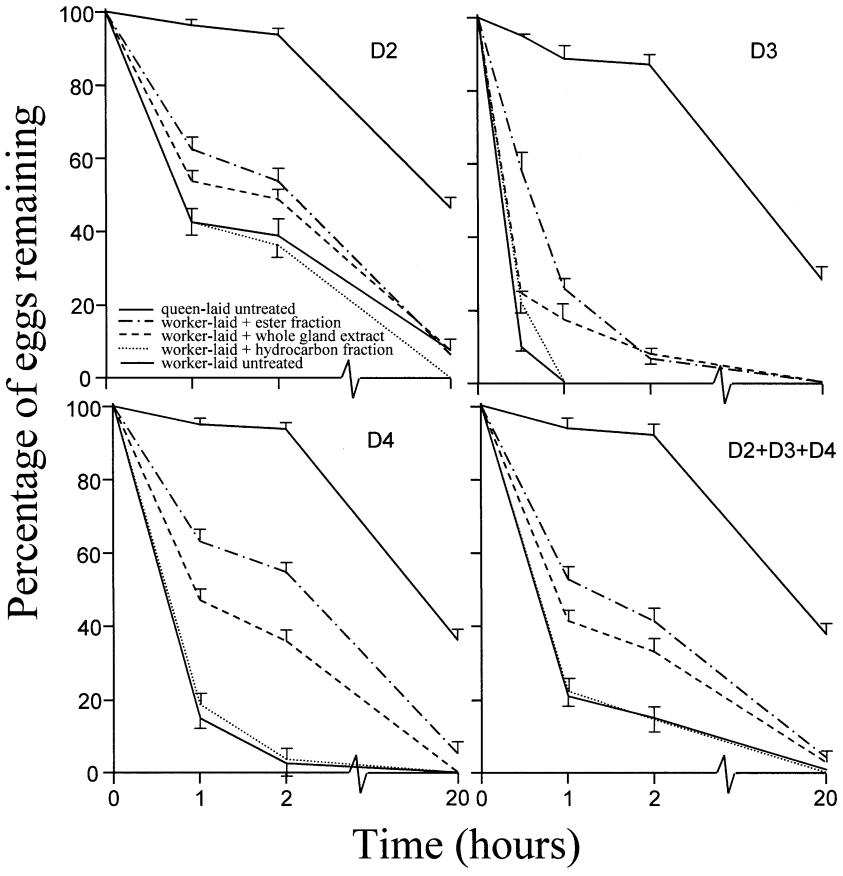

Fig. 2 Rates of removal of treated and untreated worker-laid eggs from worker cells in three discriminator colonies. The standard error bars are given

(Fig. 1e), with the amount of tricosane and pentacosane reaching that found naturally on queen-laid eggs.

Bioassay of Dufour's gland and gland fractions

During 11 separate trials, a total of 1,066 eggs were transferred (212 queen-laid, 215 worker-laid eggs, and three groups of 213 worker-laid eggs treated with whole gland extract, hydrocarbon or ester fractions). In each discriminator colony the rate of egg removal consistently followed the order: worker-laid untreated = worker-laid + hydrocarbon fraction >worker-laid + whole gland extract >worker-laid + ester fraction >queen-laid eggs (Fig. 2) despite the rate of removal varying among colonies and increasing in all colonies during the study. There was no significant difference at any of observation times (P always >0.9) between the removal rates of untreated worker-laid eggs and those treated with hydrocarbon fraction. Worker-laid eggs treated with the ester fraction were removed significantly slower than untreated worker-laid eggs after 1 h ($F_{1,20}$=8.9; P=0.007) and 2 h ($F_{1,20}$=5.5; P=0.030). However, this difference became non-significant after 20 h ($F_{1,20}$=1.9; P=0.182). Queen-laid egg survival after 20 h was 38% similar to other experiments (Ratnieks and Visscher 1989; Martin et al. 2002) using this methodology.

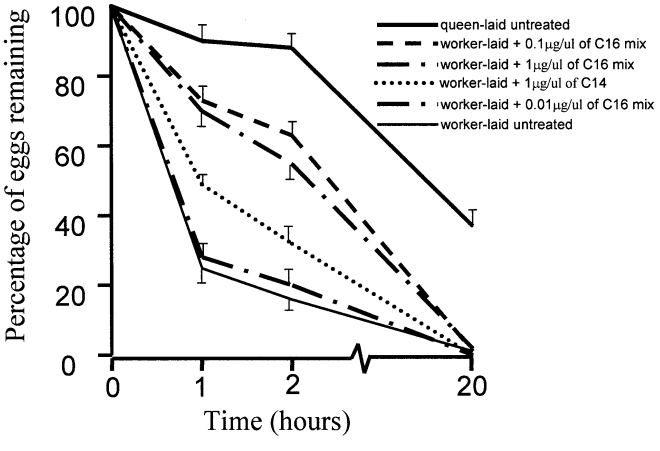

Fig. 3 Rates of removal from worker cells in three discriminator colonies of worker-laid eggs treated with either hexadecyl hexadecanoate plus hexadecyl hexadecenoate (C16 mix) or tetradecyl tetradecanoate (C14) in comparison to untreated worker-laid and queen-laid eggs. The standard error bars are given

Bioassay of synthesised esters

Figure 3 shows that the synthesised esters hexadecyl hexadecanoate plus hexadecyl hexadecenoate (C16 mix) had a similar effect to the whole ester fraction and a stronger effect than tetradecyl tetradecanoate. Although

there was little difference between the C16 mix at the 1 μg/μl and 0.1 μg/μl concentrations, there was no effect at the 0.01 μg/μl dilution which was similar to untreated worker-laid eggs. Again, all effects on the worker-laid eggs disappeared after 20 h (Fig. 3).

Discussion

Our results show that worker-laid eggs treated with either the whole Dufour's gland extract or ester fraction were initially removed at a lower rate, while the hydrocarbon extract had no effect despite increasing the amounts of tricosane and pentacosane to that found normally on queen-laid eggs. Ratnieks (1995) also found that queen Dufour's gland temporarily reduced removal of worker-laid eggs but again after 20 h little or no difference remained. Therefore, the findings of this study are consistent with those of both Ratnieks (1995) and Katzav-Gozansky et al. (2001), who found that worker-laid eggs treated with Dufour's gland extract were removed at the same rate as untreated worker-laid eggs after 24 h. In addition, we could reproduce the temporary delay in egg removal by using synthetic esters found in queen Dufour's gland. Again the effect was gone after 20 h as also found by Katzav-Gozansky et al. (2001), who used isopropyl tetradecanoate. This was despite the esters tested still being present on the egg as detected by the GC-MS, which rules out possible evaporation of the esters which was not expected due to their non-volatile nature.

Unnaturally high amounts of esters, applied using both queen Dufour's gland extract and synthetic esters, have the ability to delay the removal of worker-laid eggs, perhaps by making them appear more queen-like or by interfering with worker policing in some other way. Low amounts of esters <0.01 μl, similar to those found on queen-laid eggs, had no effect. It is unclear why having high amounts of esters results in fewer eggs being removed but this effect was consistently observed in each discriminator colony although over different time periods, which may reflect differences in the number of policing workers, threshold levels or accuracy of discrimination. The esters and hydrocarbons stored in the queen's Dufour's gland do not appear to be the signal by which worker-laid and queen-laid eggs are distinguished by policing workers under natural conditions. The role of these chemicals and the nature of the chemicals which do mediate egg recognition await further study.

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Egg marking pheromones of anarchistic worker honeybees (*Apis mellifera*)

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In honeybees, worker policing via egg eating enforces functional worker sterility in colonies with a queen and brood. It is thought that queens mark their eggs with a chemical signal, indicating that their eggs are queen-laid. Worker-laid eggs lack this signal and are, therefore, eaten by policing workers. Anarchistic worker honeybees have been hypothesized to circumvent worker policing by mimicking the queen egg-marking signal. We investigated this phenomenon by relating chemical profiles of workers and their eggs to egg acceptability. We found that the ability of some workers (anarchistic workers in queenright colonies and deviant workers from a queenless colony) to lay more acceptable eggs is due to them producing significant amounts of queen-like esters from their Dufour's gland. These esters appear to be transferred to eggs during laying and increase egg survival. However, these esters cannot be the normal queen egg-marking signal, as they are generally absent from queen-laid eggs and only increase the short-term persistence of worker-laid eggs, because only 7–30% of anarchistic worker-laid eggs persisted to hatching versus 91–92% of queen-laid eggs. All workers can produce some esters, but only workers that greatly increase their ester production lay more acceptable eggs. The production of esters appears to be a flexible response, as anarchistic workers reared in queenless colonies did not increase their ester production, while some deviant workers in queenless colonies did increase their ester production. *Key words:* anarchy, *Apis mellifera*, esters, laying workers, social insects, worker policing. *[Behav Ecol 15:839–844 (2004)]*

Reproductive division of labor is a central feature of all social insects; that is, the queen monopolizes egg-laying, and workers refrain from, are coerced not to, or have lost the ability to reproduce. In honeybees (*Apis mellifera*), workers have functional ovaries, but in the presence of the queen, i.e., in a 'queenright' colony, the ovaries are inactive in ~99.9–99.99% of workers (Ratnieks, 1995), and the queen dominates the egg production. However, if the queen is removed or lost from a queenright colony and the workers fail to rear a new queen, the colony becomes 'queenless.' In queenless colonies 5–24% of the workers activate their ovaries and lay unfertilized eggs that develop into males (drones) (Miller and Ratnieks, 2001).

A consequence of the haplodiploid sex determinism is that workers are more related to their own sons (0.5) than to the queen's sons (0.25). This can cause conflict within the colony between the queen and workers over male production, as there is a strong incentive for workers to lay their own eggs. Male production in a queenright honeybee colony accounts for 3–10% (Seeley, 1985) of total brood production by the queen, while the small (0.1%) worker population with developed ovaries (Ratnieks, 1993) accounts for about 7% of the male eggs produced (Visscher, 1996). However, only one adult male in 1000 is a worker's son (Visscher, 1989). This low survival of workers' sons is the result of worker policing (Ratnieks, 1988), where workers detect and kill worker-laid eggs (Barron et al., 2001; Ratnieks and Visscher, 1989). The lack of physical differences between surface structure of queen-laid and worker-laid eggs (Katzav-Gozansky et al., 2003b) and the intricate chemical communication system used by honeybees (Free, 1987) both suggest that egg recognition is chemically based. Worker policing in the honeybee is one of the best examples of social control overcoming individual selfishness (Ridley, 1997).

Worker policing can be quantified by placing worker-laid eggs from a queenless colony along with some control queen-laid eggs taken from an unrelated colony into a queenright discriminator colony. Normal worker-laid eggs are invariably removed within 2 h, whereas 90–95% of control queen-laid eggs remain after 2 h (Martin et al., 2002a,b).

Occasionally queenright honeybee colonies occur where most males are worker- rather than queen-derived (Châline et al., 2002; Montague and Oldroyd, 1998; Oldroyd et al., 1994). These colonies were dubbed 'anarchistic' (Oldroyd et al., 1994) because they represented colonies that were not well policed. Workers in anarchistic colonies are able to lay eggs that evade the normal policing mechanisms, which result in large numbers of workers' sons being reared. Oldroyd and Ratnieks (2000) hypothesized that evasion is mediated by the ability of anarchistic workers to mimic the queen egg-marking signal.

As anarchistic colonies are very rare in nature, probably less than one per thousand (Barron et al., 2001), most research on the anarchistic traits has been conducted on a line of anarchistic honeybees maintained through instrumental insemination at the University of Sydney (Oldroyd and Osborne, 1999). In queenright colonies of this line, up to 18% of workers at any one time will have activated ovaries (Barron et al., 2001), but here we refer to any workers of this strain as 'anarchistic,' i.e., it describes the genotype not the phenotype.

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When anarchistic worker-laid eggs are transferred into a non-anarchistic colony, along with queen-laid eggs and worker-laid eggs taken from a queenless colony, the survival rate of the anarchistic worker-laid eggs is midway between that of queen-laid and worker-laid eggs (Beekman and Oldroyd, 2003; Oldroyd and Ratnieks, 2000). However, anarchistic workers reared in a queenless colony lay eggs that have a survival rate only slightly above that of wild-type workers reared in queenless colonies (Beekman and Oldroyd, 2003). This suggests that only anarchistic worker-laid eggs from queenright colonies have an increased acceptability.

The aim of this study was to investigate how queenright anarchist workers produce eggs that are more acceptable than those laid by queenless wild-type workers. To achieve this we used four lines of investigation. First, we studied the long-term persistence of anarchistic worker-laid eggs versus queen-laid eggs, as previous comparative bioassays have only been carried out over a 24-h or shorter period (Beekman and Oldroyd, 2003; Oldroyd and Ratnieks, 2000). This will give an indication of how queen-like the anarchistic worker-laid eggs are, because the viability of worker-laid and queen-laid eggs are similar (Ratnieks and Visscher, 1989), so any differences in persistence will be the result of egg removal. Second, we studied the differences in Dufour's gland secretions, which may be the source of a queen egg-marking pheromone (Ratnieks, 1995), associated with ovary activation in anarchistic and wild-type workers reared in queenright and queenless colonies. Third, we investigated the differences in the surface chemicals found on eggs laid by queens, anarchistic, and wild-type workers. Finally, we studied wild-type workers from a queenless colony, which were also capable of laying more acceptable eggs than usual. We used workers from this colony to directly link chemical changes in the Dufour's gland secretion to changes in egg acceptability.

MATERIALS AND METHODS

Colony composition and locations

All anarchistic colonies were from the anarchistic line that is maintained at the University of Sydney by inseminating daughters of queens from anarchistic colonies with the semen from sons of anarchistic workers (Oldroyd and Osborne, 1999). In addition to anarchistic colonies studied/sampled in Sydney, three anarchistic colonies were established in the United Kingdom by importing anarchistic queens from Sydney, introducing them in wild-type colonies, and waiting 6–8 weeks until all the wild-type workers were replaced by those from the anarchistic queen. Wild-type colonies were standard European strains of *A. mellifera*. Standard egg removal bioassays (Martin et al., 2002b; Ratnieks and Visscher, 1989) established that the survival of anarchistic worker-laid eggs from the queenright colonies established in the United Kingdom were similar (unpublished data) to that previously found (Beekman and Oldroyd 2003; Oldroyd and Ratnieks, 2000), that is, between that of queen-laid and wild-type worker-laid eggs.

Experiment 1: long-term persistence of queen-laid versus anarchistic worker-laid eggs

In August 2002, five similar-sized colonies established in Sheffield were used to obtain combs containing 0–12 hour-old eggs laid by one of two wild-type queens or anarchistic workers from the three anarchistic colonies. These were obtained by enclosing a wild-type queen on a comb of worker cells using a 'comb cage' or by placing a comb of drone cells above a 'queen excluder' in a queenright anarchistic colony. The structure of the comb cage and queen excluder allows the free movement of workers but not the larger queen, restricting the queens' movements to a single comb (cage) or lower part of the colony (excluder). After 12 h the combs were removed and the position of each egg marked on an acetate sheet laid over the comb. The combs were then returned to the same colonies above a queen excluder, between frames of eggs and young brood to ensure that test combs were part of the natural brood area and covered with large numbers of workers.

The test combs were removed after 1, 2, 3, and 4 days and examined with reference to the acetate sheets to determine the number of original eggs still present. Any eggs not hatching within five days were classified as non-viable.

Experiment 2: ovary activation and analysis of Dufour's gland secretions

During 2001, ~1000 wild-type workers and ~1000 anarchistic workers were reared in their natal queenright colonies in Sydney. Prior to pupal emergence, brood combs were placed in an incubator and newly emerged workers were paint-marked on the thorax. Then, ~500 marked wild-type and ~500 marked anarchistic workers were placed either back into their queenright colonies or into a wild-type queenless colony. Fourteen days later the marked workers were collected, frozen in dry ice, and transported to Sheffield. From each group, 100–300 individuals were dissected to obtain individuals with fully activated ovaries (containing a series of clearly defined oocytes) or non-activated ovaries (thread-like ovarioles). Workers showing intermediate levels of ovary activation were recorded but were not analyzed chemically. From a subset of each group the Dufour's gland and later setose membrane were dissected by removing the sting apparatus with blunt forceps. The Dufour's gland was removed by severing it at its base using fine forceps. The setose membrane, an area of cuticle covered in dense hairs lying over the sting bulb, was removed using fine forceps. These were placed separately into small soft glass tubes, flame-sealed and frozen. These samples were analyzed by crushing them inside the injector port of a GC-MS (gas chromatography–mass spectrometry) machine using a 'Keele-injector' (Morgan, 1990). Initially, only Dufour's glands were analyzed, but as the study progressed the setose membranes were analyzed, as these provided a more reliable measure of the chemical state of the workers, because the chemicals secreted by the Dufour's gland accumulate on the setose membrane (Martin SJ and Jones GR, personal observations). The Dufour's gland and setose membranes of both virgin and egg-laying queens were also dissected and analyzed as for the workers to act as a comparison.

Experiment 3: differences in egg surface chemistry

Eggs were analyzed from three queenright wild-type, three queenright anarchistic, and three queenless wild-type colonies established in Sheffield. Pooled egg samples were obtained by placing 100 queen-laid, worker-laid (queenless colony), or anarchistic worker-laid (queenright colony) eggs into 100 μl of hexane for 30 min. The solvent was then evaporated under nitrogen to ~2 μl before injection into a GC-MS machine for analysis. Individual eggs were also collected from the same colonies by placing them separately into small soft glass tubes, and they were analyzed in the same way as the Dufour's glands. To reduce the pseudo-replication caused by taking more than one sample of eggs from a colony, several weeks elapsed between sampling.

Experiment 4: egg survival and chemical analysis of F4 workers

In June 2002, a queenless wild-type colony (F4) at Sheffield was found producing eggs that had unusually high survival when transferred to queenright discriminator colonies. As the level of egg-laying by workers in this colony was very high, we were able to remove a drone comb from the colony, observe a worker laying an egg, and then retrieve both the egg for use in a policing bioassay and the worker that laid the egg for chemical assay. In addition, we determined the chemical profile of a small number of randomly sampled individual eggs with any eggs that survived 20 h in the bioassay. This allowed us to compare the chemistry of F4 workers and the acceptability of their eggs at the level of individuals, rather than at the group level (e.g., worker-laid eggs vs. queen-laid eggs).

Chemical and statistical analyses

We investigated three chemical groups known to be associated with the tip of the abdomen of both egg-laying workers and queens (Katzav-Gozansky et al., 1997; Martin et al., 2002b). These are the ubiquitous hydrocarbons, both alkanes and alkenes (which act as an anti-desiccation barriers); the esters produced by the Dufour's gland act as a queen signal and cause retinue behavior in workers (Katzav-Gozansky et al., 2003a) (for details of 10+ esters involved see Katzav-Gozansky et al., 2003a; Martin et al., 2002b) and the long-chain alcohol, eicosenol which is part of the worker alarm pheromone (Pickett et al., 1982).

GC-MS analyses were performed in a splitless mode on a 5890 Hewlett Packard GC coupled with a 5970 quadruple MS (70eV electron impact ionization). A 15 m × 0.25 mm ID column with a 0.25 μm thickness bonded BP5 stationary phase (SGE) was used. The GC oven was programmed with an initial temperature of 170°C to 260°C with a ramp rate of 10°C/min, then with a ramp rate of 20°C/min to a final temperature of 325°C, which was held for 2 min. The injection port and transfer line were held at 250°C and 300°C, respectively. The carrier gas was helium at 1 ml/min. All compounds were identified by comparison of retention times and mass spectra with synthetic standards held at Keele University. The peak area of each compound was calculated for each sample and then normalized by calculating the percentage abundance for that sample. The total percentages for each of the three chemical groups were then averaged and the standard deviation calculated for each set of samples. The normalized proportions were Arcsine transformed prior to any statistical analysis in order to satisfy the assumptions of homogeneity of variance and normality of residues. All ANOVAs were accompanied by post-hoc pairwise comparisons using the Tukey HSD test. Ideally, in experiment 2 an ANOVA using differences between types of workers (anarchistic vs. wild type), colony state (queenright vs. queenless), and ovary activation would have been performed. However, the extreme rarity of wild-type workers in queenright colonies caused an unbalanced design. Therefore, we conducted two ANOVAs, one comparing workers without ovary activation and the other comparing workers with ovary activation.

RESULTS

Experiment 1: long-term persistence of queen-laid versus anarchistic worker–laid eggs

The survival of 1206 queen-laid eggs in two colonies and 2222 anarchistic worker-laid eggs in three colonies were measured (Table 1). We found that 91–92% of the queen-laid eggs persisted long enough to hatch versus only 7–30% of the anarchistic worker-laid eggs.

Table 1
Cumulative proportional survival (%) of queen-laid and anarchistic worker-laid eggs in their own queen-right colonies

		Wild-type queen-laid		Anarchistic worker-laid		
Day	Stage	Colony 1 $n = 675$	Colony 2 $n = 531$	Colony A $n = 329$	Colony B $n = 1196$	Colony C $n = 697$
1	egg	99	99	78	83	76
2	egg	98	98	57	72	49
3	egg	95	96	51	41	30
4	larvae	91	92	30	16	7

The number of eggs studied (n) in each colony is also given.

Experiment 2: ovary activation and analysis of Dufour's gland secretions in workers

We dissected 419 anarchistic (316 from queenright and 103 from queenless colonies), 325 wild-type workers (171 from queenright and 154 from queenless colonies), and eight wild-type queens (Table 2). As expected (Barron and Oldroyd, 2001), the level of ovary activation was greater in anarchistic workers than in wild-type workers and greater in queenless colonies than in queenright colonies (Table 2). Chemical analysis of 57 anarchistic (24 from queenright and 33 from queenless colonies) and 38 wild-type workers (12 from queenright and 26 from queenless colonies) revealed that all individuals, irrespective of ovary activation or queen status of the colony in which they had been reared, had predominantly C21-C33 hydrocarbons in their Dufour's glands and on their setose membranes (Table 2). In non-laying anarchistic ($n = 21$) and wild-type ($n = 28$) workers, from both queenless and queenright colonies, the only other compound present in high amounts was eicosenol. The esters normally present in the queen's Dufour's gland were present only in trace amounts (<0.7%) in all four groups of non-laying worker bees (Table 2). Although there was a marginally significant difference in the ester levels among the groups of non-laying workers (ANOVA, $F = 3.1$, df = 3,37, $p = .04$), no significant differences ($p > .05$, Tukey HSD test) between any of the groups could be found. However, there was a highly significant difference (ANOVA, $F = 46.9$, df = 2,34, $p < .0001$) in the proportion of esters present in the groups of workers with activated ovaries. The anarchistic workers from the queenright colony had a significantly higher proportion of esters ($p < .01$, Tukey HSD test) than either anarchistic or wild-type workers from queenless colonies (Table 2). This corresponds well with the finding that only laying anarchistic workers in queenright colonies can lay acceptable eggs. This appears to be associated with ester production from the Dufour's gland, which is normally only a characteristic of queens, whether they are egg-laying or not (Table 2).

Experiment 3: differences in egg surface chemistry

The proportion of esters were significantly different (ANOVA, $F = 5.67$, df = 2,12, $p = .018$) between the three pooled egg samples, with the amount on the anarchistic worker-laid eggs (queenright colonies) significantly higher ($p < .05$, Tukey HSD test) than present on both wild-type queen-laid or wild-type worker-laid (queenless colony) eggs (Table 3). The ester proportions on wild-type queen-laid and worker-laid (queenless colony) eggs were not significantly different ($p > .05$, Tukey HSD test) (Table 3).

Table 2

(a) Ovary activation: number and proportion of dissected workers that had inactive, partly active, or fully active ovaries

	Anarchistic workers		Wild type workers		Wild type queens
Colony condition (number dissected)	Queenright (316)	Queenless (103)	Queenright (171)	Queenless (154)	Queenright (8)
Inactive (%)	91	59	100	93	—
Partly active (%)	3	6	0	0	—
Fully active (%)	6	35	0	7	—

(b) Chemical analyses of setose membrane and/or Dufour's gland from workers and queens whose ovaries were either inactive or fully active

	Anarchistic workers				Wild type workers			Wild type queens	
Colony condition (number dissected)	Queenright (316)		Queenless (103)		Queenright (171)	Queenless (154)		Queenright (8)	
Full ovary activation	No	Yes	No	Yes	No	No	Yes	No (virgin)	Yes
Material and number analyzed	Setose 10	Setose 14	Setose 3 (Dufour's 8)	Setose 13 (Dufour's 9)	Setose 12	Setose 11 (Dufour's 5)	(Dufour's 10)	(Dufour's 4)	Setose 3 (Dufour's 1)
% Hydrocarbons	62.4 ± 4.1	63.5 ± 14.6	55.2 ± 5.0 (73.3 ± 20.9)	70.6 ± 13.0 (90.6 ± 6.0)	41.0 ± 6.0	46.0 ± 6.4 (63.9 ± 20.5)	(62.3 ± 12.4)	(74.6 ± 6.8)	44.8 ± 2.2 (72.1)
% Esters	0.4 ± 0.4	36.5 ± 14.6	0 (0.7 ± 1.0)	3.7 ± 4.3 (2.7 ± 3.6)	0.1 ± 0.2	0.1 ± 0.2 (0.6 ± 0.5)	(0.8 ± 1.5)	(25.4 ± 6.8)	55.2 ± 2.2 (27.9)
% Eicosenol	37.2 ± 4.3	0	44.8 ± 5.0 (26.0 ± 21.0)	25.7 ± 14.9 (6.7 ± 8.0)	58.9 ± 6.0	53.9 ± 6.4 (35.5 ± 20.8)	(36.9 ± 13.1)	(0)	0 (0)
Increased egg acceptability	—	Yes*	—	No*	—	—	No*	—	Yes

* With respect to wild-type worker-laid eggs, see Beekman and Oldroyd (2003).

The mean percentage ± SD are given for each chemical group found either on the setose membrane or in the Dufour's gland.

When individual eggs were analyzed, there was again a significant difference in ester proportions between the four groups (ANOVA, $F = 13.94$, df = 3,32, $p < 00001$). No significant difference ($p > .05$, Tukey HSD test) was found between the ester proportions on wild-type queen-laid and wild-type worker-laid (queenless colony) eggs, or between anarchistic worker-laid (queenright colonies) and F4 worker-laid (queenless colony) eggs. However, the anarchistic worker-laid eggs (queenright colonies) and F4 worker-laid eggs had significantly higher proportions of esters ($p < .01$, Tukey HSD Test) than the wild-type queen-laid or wild-type worker-laid (queenless colony) eggs. In all groups the range of ester proportions on eggs showed considerable variation (Figure 1).

Experiment 4: egg survival and chemical analysis of F4 workers

From colony F4 we measured the survival of 16 newly laid eggs and the ester levels on the setose membranes of the corresponding 16 F4 workers that laid them. This revealed a positive relationship between the proportion of esters on the worker's setose membrane and the survival of her egg (Figure 2). F4 workers whose eggs were removed rapidly, i.e., before the 2 h check, had significantly lower (t test, $t = 4.2$, df = 13, $p < .001$) ester levels than workers whose eggs were removed after the 2 h and before the 20 h check. Only one egg survived 20 h, and the proportion of esters on its surface was the highest level seen in this experiment (Figure 1). This egg was laid by an F4 worker with the corresponding ester level of 55% on the setose membrane, which is high relative to the other laying workers (Figure 2). Three F4 workers produced no esters, and their eggs were removed rapidly, whereas the eggs laid by F4 workers producing higher proportions of esters had an increased, but not guaranteed, chance of survival.

DISCUSSION

Our results show that anarchistic workers in queenright colonies and rare queenless wild-type workers (F4 colony) can increase survival of their eggs by greatly increasing ester

Table 3

Proportions (% ± SD) of hydrocarbons, eicosenol, esters, and arcsine transformed values for the esters, extracted from the pooled samples of 100 egg surfaces

	n	Hydrocarbons	Eicosenol	Esters	Esters transformed values
Wild-type queen	5	98.2 ± 1.8	0.1 ± 0.1	1.7 ± 1.8	1.0 ± 1.2
Wild-type worker (queenless colony)	5	97.8 ± 2.5	0.1 ± 0.1	2.1 ± 2.5	1.2 ± 1.6
Anarchistic worker (queenright colony)	5	92.2 ± 3.6	0.3 ± 0.1	7.6 ± 3.6	4.3 ± 2.3

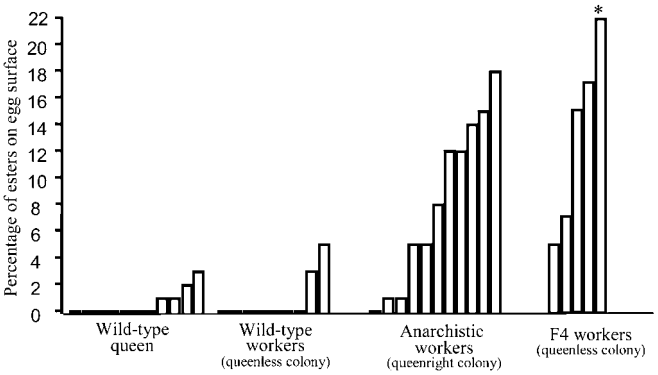

Figure 1
Natural variation in the ester levels relative to all hydrocarbons, esters, and eicosenol detected on the surface of individual eggs laid by wild-type queens, wild-type workers, anarchistic workers, and F4 workers. An asterisk (*) indicates the egg that survived 20 h in the bioassay.

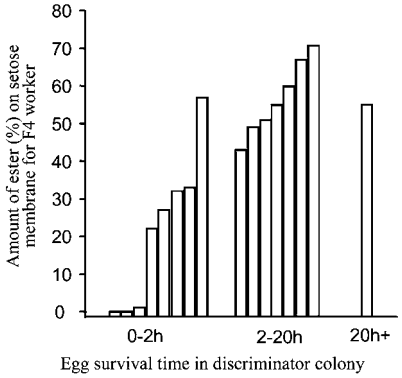

Figure 2
The relationship between amount of ester on the setose membrane of egg-laying F4 workers and the time their eggs survive in an unrelated queenright discriminator colony, as determined by checking for the presence or absence of the eggs at 2 h and 20 h.

production from their Dufour's glands, some of which gets transferred to their eggs. The levels of esters on the egg surface of anarchistic workers from queenright colonies and F4 workers were far higher than that found on queen-laid or worker-laid eggs in a random sample of eggs from queenless wild-type colonies other than F4. This corresponds with the greatly increased levels of esters found in the Dufour's gland or on the setose membrane in the F4 workers and anarchistic workers from the queenright colony. Increased egg acceptance can also result from the application of esters to wild-type worker-laid eggs either via extracts of a queen's Dufour's gland (Martin et al., 2002b; Ratnieks, 1995) or by using individual or mixtures of synthesized esters (Martin et al., 2002b). However, since all egg-laying workers can produce some esters (this study and Katzav-Gozansky et al., 1997), it is not just the simple ability to produce queen-like esters per se that increases egg survival (Martin et al., 2002b), but also the production of greatly increased amounts of esters. The reduced ester levels in the anarchistic workers reared in a queenless colony explains why their eggs are only slightly more acceptable than wild-type worker-laid eggs (Beekman and Oldroyd, 2003). There is no clear relationship between the colony state (queenless vs. queenright) and laying more acceptable eggs, because workers from both queenless (F4 workers) and queenright (anarchistic workers) colonies had high ester levels while others from queenless (wild-type and anarchistic workers) colonies have a much lower ester production (but see Beekman and Oldroyd, 2003).

Although we have demonstrated a close relationship between ester production by workers and initial egg acceptance, we emphasize that esters are not the signal by which worker honeybees normally discriminate between queen-laid and worker-laid eggs. Despite queens having high proportions of esters in their Dufour's glands (Table 1; Katzav-Gozansky et al., 1997; Martin et al., 2002b), these esters are absent or present only in low amounts on queen-laid eggs (Table 2 and Figure 1). The reason queen-laid eggs and anarchistic/F4 worker-laid eggs have such different ester levels may be the result of the rate at which the two castes lay eggs, which in queens can be 100 to 1000 times greater than that of a worker. In addition, the bursa copulatrix is poorly developed in workers, resulting in slower egg-laying (10 s in a queen vs. 60+ s in a worker; Page and Erickson, 1988; Perepelova, 1928) with eggs often sticking to the worker sting area during oviposition. This does not occur with queens (S.J.M., personal observations). Thus, the chance of an egg being coated with products of the Dufour's gland during oviposition is much greater in a worker than in a queen.

It appears that the increased acceptability of anarchistic- and F4 worker-laid eggs results from the application of esters during laying and not from mimicking a queen egg-marking signal. These esters, to which workers are attracted (Katzav-Gozansky et al., 2003a), might somehow disguise the true identity of worker-laid eggs, making them more acceptable to workers. Because of the wide variation in ester levels found on anarchistic eggs (Figure 1), despite all anarchistic workers from queenright colonies producing high proportions of esters (Table 1), the variable amounts of esters on anarchistic worker-laid eggs may explain why a high proportion them (70–93%) are removed during development. Despite this, the acceptability of eggs laid by anarchistic workers is still far greater than that of wild-type workers, and this, coupled with high levels of worker egg production and reduced policing (Oldroyd and Ratnieks, 2000), is sufficient to produce the very high proportion of worker-derived male brood found in anarchistic colonies (Châline et al., 2002; Montague and Oldroyd, 1998).

These results support Katzav-Gozansky et al.'s (2002) idea that a worker's Dufour's gland cannot produce an egg-marking pheromone but may under certain conditions produce queen-like esters. The effect of esters on policing workers is unclear, but what is certain is that if mimicry is taking place it is not a duplication of the queen's signal.

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Role of hydrocarbons in egg recognition in the honeybee

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Abstract. Despite worker-laid and queen-laid honeybee eggs having significantly different hydrocarbon profiles, bioassays and chemical supplementation studies show that changing the hydrocarbon profile does not affect egg identity. Furthermore, full-sized eggs that are tested just before being laid or just after being laid have similar hydrocarbon patterns but are treated differently in egg-discrimination bioassays with only the laid eggs surviving. This suggests that hydrocarbons play no role in the egg-recognition system in honeybees.

Key words. Alkanes, alkenes, *Apis mellifera*, honeybee, hydrocarbons, recognition.

Introduction

Communication is the common bond that holds all societies together, whether it is visual, oral or chemical. All social insects employ a complex chemical communication system, which mediates orientation, foraging, defence, brood-care, reproductive hierarchy and kin recognition. Many pheromones have been identified in honeybees (Free, 1987) and ants (Hölldobler & Wilson, 1990), but the nature of those responsible for individual recognition remains elusive (Vander Meer & Morel, 1998). Cuticular hydrocarbons consist of mainly long-chained linear alkanes and smaller amounts of alkenes and methyl branched alkanes. They are biologically stable, found in all life stages of arthropods and have the primary function of providing protection from desiccation (Lockey, 1988). However, in social insects, cuticular hydrocarbons are widely assumed to be used in both nest mate and kin recognition (Singer, 1998). In honeybees, cuticular hydrocarbons profiles are partly genetically based (Page *et al.*, 1991; Arnold *et al.*, 1996) but can be affected by reproductive status (Katzav-Gozansky *et al.*, 1997). Because cuticular hydrocarbons are highly variable between the sexes and castes in honeybees (Page *et al.*, 1991; Arnold *et al.*, 1996), this makes them ideal candidates for use in chemical communication (Smith & Breed, 1995).

The ability of honeybees to discriminate between worker-laid and queen-laid eggs, known as worker policing (Ratnieks & Visscher, 1989), was used to investigate the role of cuticular hydrocarbons in the egg-recognition system. Correlational studies were combined with data from discrimination bioassays and chemical supplementation data (Martin *et al.*, 2002). In light of the findings obtained, the potential role that cuticular hydrocarbons play as recognition compounds in honeybees is discussed.

Materials and methods

Individual honeybee (*Apis mellifera*) queen-laid or worker-laid eggs were obtained from queen-right or queen-less colonies, respectively, maintained at the University of Sheffield. Eggs were collected just before being laid (i.e. full-sized eggs dissected from the ovaries), just after being laid (i.e. eggs collected from queens held in a cupped hand or removed from a brood cell immediately after a worker or queen was seen to lay them), and up to 24 h after being laid normally. Each egg was sealed in a small glass tube and stored in a freezer until analysed. Some egg samples became contaminated with squalene from being handled but, as levels were always small, any squalene was ignored for subsequent analysis. Treated eggs in the chemical

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supplementation study were also handled in the same way. All eggs were analysed by crushing them inside a gas chromatography–mass spectrometer (GC–MS) using a Keele injector (Morgan, 1990). GC–MS analyses were performed in a splitless mode on a 5890 Hewlett Packard GC (Wilmington, Delaware) coupled with a 5970 quadruple MS (70 eV electron impact ionization). A 15 m × 0.25 mm inner diameter column with a 0.25 μm thickness bonded BP5 stationary phase was used. The GC oven was programmed with an initial temperature of 170 °C to 260 °C with a ramp rate of 10 °C min^{-1}, with a subsequent ramp rate of 20 °C min^{-1} to a final temperature of 325 °C, which was held for 2 min. The injection port and transfer line were held at 250 °C and 300 °C, respectively. The carrier gas was helium at 1 mL min^{-1}. All compounds were identified by comparison of retention times and mass spectra with synthetic standards. The peak area of each compound was calculated for each sample and then normalized by calculating the percentage abundance for that sample, and the mean values were determined. Differences in hydrocarbons patterns between the various groups were investigated further using discriminant analysis (Monnin *et al.*, 1998). The details and data of the egg-discrimination bioassays have been published separately (see Martin *et al.*, 2002 together with unpublished data) but, in brief, eggs from various sources were transferred into a test frame from an unrelated honeybee colony. The speed of egg removal was recorded by inspecting the test frame after 1, 2 and 20 h.

Results

Odd chain-length linear alkanes (C23–C31) were the dominant hydrocarbons on the surfaces of both queen and worker-derived eggs (Table 1, Fig. 1). Significant differences were found in the profile of the linear alkanes between full-sized worker and queen eggs just before being laid (Figs 1 and 2) (i.e. those dissected from the ovary). However, egg-discrimination bioassays showed that only queen-laid eggs (just laid, $n = 4$; 0–24-h old, $n > 100$) were not removed, whereas all worker-laid eggs (just laid, $n = 22$; 0–24-h old, $n > 100$) and all full-sized eggs taken from the ovaries (queen, $n = 19$; worker, $n = 7$) were removed within 2 h. These results have been supported in similar studies conducted in Australia (M. Beekman, unpublished data). Thus, despite full-sized eggs in the queen's ovary having a similar hydrocarbon profile to those just laid, they were treated very differently. The initial caste differences in hydrocarbon profiles disappear as their profiles merge during the first 24 h in the colony (Table 1, Figs 1 and 2), although this fails to protect the worker-laid eggs because they are always removed. In addition, even when the hydrocarbon profile of worker-laid eggs was artificially changed to be queen-like (Fig. 2) by the addition of a hexane extract of a queen Dufour's gland, these treated eggs were still removed (Martin *et al.*, 2002).

Discussion

It is well established that honeybees can differentiate between worker-laid and queen-laid eggs. The recognition is certainly chemically based because no physical differences can be found between the two egg types (Martin *et al.*, 2002; Katzav-Gozansky *et al.*, 2003). The correlational studies suggest that fluctuations in the proportion of C25 and C27 (Figs 1 and 2b) may be critical in allowing honeybees to discriminate between egg types. However, this is not supported by the egg-discrimination bioassays or the chemical supplementation studies (Martin *et al.*, 2002). This strongly suggests that the cuticular hydrocarbons, and

Table 1. List of hydrocarbons detected on the surface of various classes of eggs produced by queens and workers. The table shows the mean percentage ± SD for each class of egg. Only compounds with > 0.5% relative abundance are considered.

		Queen-laid eggs			Worker-laid eggs		
Peak no.	Compound	In ovary ($n = 19$)	Just laid ($n = 13$)	*c.* 24-h old ($n = 12$)	In ovary ($n = 7$)	Just laid ($n = 4$)	*c.* 24-h old ($n = 20$)
1	C21 heinecosane	–	–	2 ± 2	–	–	1 ± 2
2	C22 docosane	–	–	5 ± 3	–	–	3 ± 3
3	C23 : 1 tricosene	1 ± 2	2 ± 4	2 ± 1	–	2 ± 1	2 ± 1
4	C23 tricosane	45 ± 8	29 ± 10	22 ± 9	42 ± 15	23 ± 4	18 ± 3
5	C25 : 1 pentacosene	2 ± 1	3 ± 3	3 ± 2	–	4 ± 1	4 ± 8
6	C25 pentacosane	22 ± 3	21 ± 6	24 ± 3	23 ± 9	14 ± 2	13 ± 3
7	C26 hexacosane	–	–	1 ± 2	–	–	1 ± 1
8	C27 : 1 heptacosene	1 ± 1	2 ± 1	1 ± 1	–	3 ± 1	1 ± 1
9	C27 heptacosane	13 ± 3	18 ± 3	18 ± 3	33 ± 11	23 ± 7	25 ± 7
10	C28 octacosane	–	–	1 ± 1	–	–	1 ± 1
11	C29 : 1 nonacosene	2 ± 1	4 ± 3	2 ± 1	–	4 ± 1	6 ± 3
12	C29 nonacosane	2 ± 1	4 ± 2	4 ± 1	2 ± 2	7 ± 1	8 ± 4
13	C31 : 1 heintriacontene	7 ± 4	9 ± 5	7 ± 1	–	9 ± 3	3 ± 5
14	C31 heintriacontane	1 ± 1	2 ± 1	2 ± 1	–	2 ± 1	7 ± 3
15	MeC31 methyl-heintriacontane	–	–	–	–	–	2 ± 2
16	C33 : 1 tritriacontene	4 ± 3	6 ± 3	6 ± 1	–	9 ± 3	5 ± 3

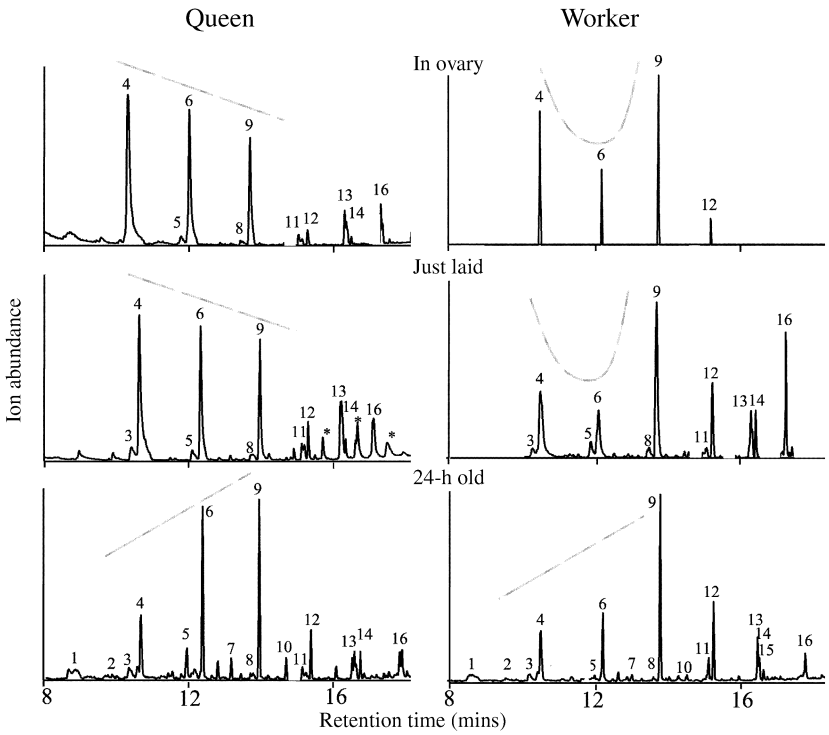

Fig. 1. Comparisons of typical chromatograms from the three egg types, full-sized eggs from the ovary just before being laid, eggs just after being laid and eggs laid approximately 24-h earlier. The asterisks represent esters that can contaminate the eggs when the queen is forced to lay an egg outside the colony.

specifically the linear alkanes, do not play a role in egg discrimination. This idea is supported by the findings of other studies, which have often been overlooked.

Pickett *et al.* (1982) found that fractions containing mainly the linear alkanes (C23, C25 and C27) or synthetic linear alkanes C23 and C25 gave no significant electroantennogram response, which measures receptor potentials of olfactory neurones in the honeybee antenna, whereas eicosenol, an alcohol-based worker alarm pheromone (Pickett *et al.*, 1982), gave a significant response. This would explain why honeybees discriminate between different waxes using esters and alcohols but not using cuticular hydrocarbons (Fröhlich *et al.*, 2001), based on experiments using the classic conditioning of the proboscis extension reflex response. Furthermore, no glomerular (neural) responses in the antennal lobe of honeybees were obtained for linear alkanes of less than C10 whereas a wide range of short-chained (C5–C10) alcohols, aldehydes and ketones elicited both strong neural responses (Sachse *et al.*, 1999) and can be discriminated by free-flying honeybees (Laska *et al.*, 1999). The reason why nonvolatile linear alkanes are not used as recognition cues may be because they have no electronegative atoms (such as oxygen or nitrogen) (Hauptmann, 1987), that their hydrophobic properties reduce interactions with the receptors, or that the alkanes could be coded in glomeruli not included in the study of Sachse *et al.* (1999).

By contrast to these findings are two often cited studies. Breed & Stiller (1992) demonstrated that honeybees only showed a response to the linear alkanes C16 and C18 but admitted that neither of these chemicals actually occur in wax or on honeybees, whereas linear alkanes that do occur (e.g. C29) had no significant effect. In addition, the concentrations of substances used in this study (10 μg/bee) are very high compared with natural abundance levels. Getz & Smith (1987) also demonstrated that honeybees appeared to be able to discriminate between two specific blends of the linear alkanes C23 and C25 (90% C23 : 10% C25 from 100% C25 or 10% C23 : 90% C25). However, they also found that honeybees were unable to discriminate between the two pure compounds (100% C23 from 100% C25) or several other various mixes of the two compounds. Getz & Smith (1987) stated that textural differences between the various mixes could have caused sensory modalities of touch to confound those of olfaction and that care must be taken in interpreting their results. The role of texture in determining the action of compounds has been suggested by the ant repellents used by wasps, where it appears that it is not the actual chemical but its physical state (liquid or solid) that is crucial to its function (Dani *et al.*, 2003).

The apparent inability of honeybees to use linear alkanes in recognition has also been found in the social paper wasp *Polistes dominulus* were linear alkanes were found to have

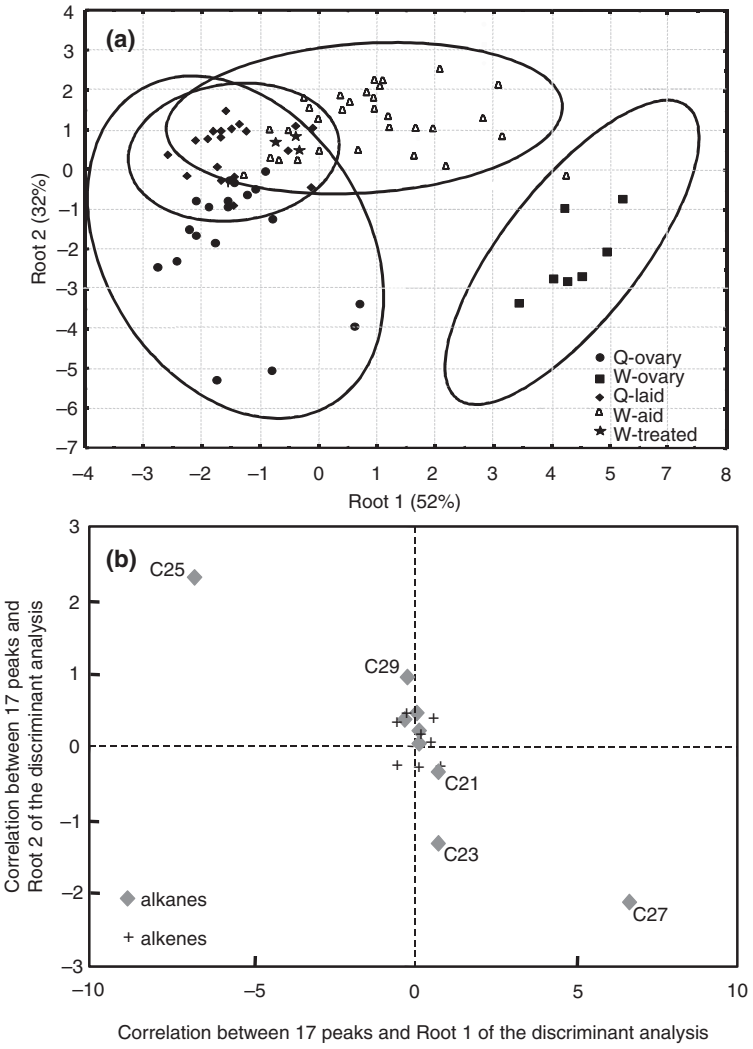

Fig. 2. (a) Discriminant analysis of seven full-sized worker (square) and 19 full-sized queen (circle) eggs just before being laid with 24 worker-laid (triangle) and 25 queen-laid eggs (diamonds) that were laid 0–24 h earlier. The three asterisks represent three worker-laid eggs treated with a queen's hydrocarbon fraction to make these eggs more queen-like. The analysis compared the 16 major peaks (after standardization) given in Table 1. Envelopes represent the 95% confidence ellipses. (b) Factor structure coefficients for the 16 peaks used in the comparison are given, with grey diamonds representing alkanes and crosses representing alkenes. Only the names of the key compounds responsible for group separation in the discriminant analysis are given. The eigen values are Root 1 = 6.1 and Root 2 = 2.2.

no effect upon their recognition response, whereas wasps treated with methyl-branched alkanes or alkenes were attacked (Dani *et al*., 2001). Furthermore, differences between three termite (*Macrotermes falciger*) phenotypes were mainly based on alkenes, not alkanes, and these differences were associated with the level of aggression between the three groups (Kaib *et al*., 2002). The branched alkanes and alkenes are far better candidates for recognition compounds than the linear alkanes because they posses a more defined conformation due to the double bond or branch position.

Despite the extensive research conducted on hydrocarbons during the past 20 years, there is still no unequivocal behavioural evidence in honeybees that cuticular hydrocarbons, especially the abundant linear alkanes, can be detected as recognition signals. Furthermore, no actual biochemical mechanism of recognition has yet been determined for any social insect (Breed, 1998). The discovery of specific kin-recognition chemicals would represent a major breakthrough in the field, but better bioassays, the testing of a much wider range of compounds at biologically realistic levels and the use of a wider range techniques, such as electroantennograms and the proboscis extension reflex, are required to select potential compounds from the vast number of possible compounds, rather than relying solely on correlational studies. Recently, a proteinaceous molecule was found in fire ant queens *Solenopsis invicta* that informs workers when to execute sexual larvae (Klobuchar & Deslippe, 2002) and may represent a major step forward in widening the search for recognition compounds.

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Appendix 1.4 Searching for the egg-marking signal in honeybees

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Key words: *Apis,* esters, hydrocarbons, recognition, worker policing

Running title: Egg-marking signal in honeybees

Abstract

Behavioural bioassays have shown that worker honeybees can distinguish between worker-laid and queen-laid eggs. By eating worker-laid eggs nest-mate workers prevent each other from reproducing, a behaviour known as worker policing. However, the recognition cue used by worker honeybees to discriminate between worker-laid and queen-laid eggs remains elusive. This study describes various experiments that were conducted in an attempt to elucidate the nature of the recognition cue. No physical differences were found between worker-laid and queen-laid eggs in SEM studies when magnified up to 2500 times, indicating that the cue is probably chemical in nature. We confirmed that the signal probably resides on the queen-laid and not the worker-laid eggs, since a worker-laid egg is protected when placed in contact with a queen-laid egg. We conducted a series of standard egg-removal bioassays in queenright colonies using queen-laid and worker-laid eggs treated with a wide range of potential recognition chemicals, solvents, buffers or gland extracts. The aim was either to disrupt or remove the egg-marking signal from queen-laid eggs, or to add the signal to worker-laid eggs. Nevertheless, we were unable to alter the egg-marking signal on queen-laid eggs or remove it from queen-laid eggs and place it on worker-laid eggs in the vast majority of the trials. Two candidate signals, esters from the queen and eicosenol from the worker, were tested, however they were shown not to be the cues used by workers. This indicates that the egg-marking signal in honeybees is remarkably robust and consists of

a chemical or group of chemicals not previously associated with chemical signalling in social insects.

1. Introduction

The majority of social bees, wasps and ants have a haplo-diploploid sex determination system in which fertilized eggs become female and unfertilized eggs become male. Although the female worker caste has lost the ability to mate in most social insects, workers often retain functional ovaries from which unfertilized (male) eggs can be produced. In honeybees, due to the haplo-diploploid sex determination system and multiple mating, workers are more related, on average, to the queen's sons than to the workers sons and therefore, benefit from rearing only the queen's sons. This has resulted in the evolution of mechanisms to prevent workers from reproducing, which include the removal of worker-laid eggs by other workers. This phenomenon, called worker policing, was first predicted on relatedness grounds (Ratnieks 1988) and then discovered in the honeybee *Apis mellifera* (Ratnieks & Visscher 1989). Worker policing relies on the ability of workers to discriminate accurately between worker-laid and queen-laid eggs. However, despite the existence of an egg-marking pheromone being postulated over 15 years ago (Ratnieks 1988) neither the pheromone nor the gland from which it is derived has yet been found. The Dufour's gland was at first thought to be the source of the pheromone (Ratnieks 1995), but this was later shown to be unfounded (Martin *et al*. 2002a). The aim of this study was to determine the information mechanism by which workers are able to discriminate between queen-laid and worker-laid eggs. Using standard egg-removal bioassays (Ratnieks & Visscher 1989; Martin *et al*. 2002a, b), we conducted a series of experiments to first disrupt or remove the putative chemical information cue from queen-laid eggs, and then to supplement the surface of worker-laid eggs with candidate compounds from various gland extracts. Additionally, we examined the bursa copulatrix of the queen sting region to determine the possible source and properties of the eggs marking signal.

2. Material and methods

All studies were conducted during the summers of 2001, 2002 and 2003 in apiaries at the Laboratory of Apiculture and Social Insects, University of Sheffield, UK, using

honeybee (*A. mellifera*) colonies. Because queen-laid eggs are treated identically regardless of their gender in egg removal bioassays (Oldroyd & Ratnieks 2000), we conducted our experiments mainly using fertilized queen-laid eggs as these were easier to obtain throughout the summer. Therefore, eggs were obtained primarily from worker cells in queen-right colonies, although sometimes unfertilized eggs from drone cells were used to confirm equal treatment of haploid eggs. Worker-laid eggs were obtained from queenless colonies with many laying workers. Each bioassay (see 2.1. for details) compared the number of untreated eggs against the number of treated eggs remaining after 2 and 20 h.

2.1. Standard worker policing bioassay

Typically 20-30 or occasionally 10-15, individual eggs were removed from cells with either a blunt needle or adapted (Taber) forceps (Collins 2002) and placed upright onto a glass slide (Fig. 1). In this position, eggs could easily be treated using a variety of methods (see 2.4, 2.5, 2.6). Eggs were then transferred from the glass slide into adjacent drone cells from an unrelated queenright discriminator colony using a test frame. Occasionally test frames containing the smaller worker cells were used. A frame in a honeybee colony contains around 3000 hexagonal wax cells that are used repeatedly to either rear brood or store pollen or honey. The test frame contains only empty cells and is typically placed into the discriminator colony several days prior to experimentation to acquire the colony odour. After the eggs have been transferred into the test frame, it is reinserted into the discriminator colony between two honeybee frames containing eggs and larvae and above the queen-excluder, a device that confines the queen to lower part of the colony. Therefore, only the workers have access to the upper part of the colony where the test frame was placed. The numbers of eggs remaining after typically 2, 4, 6, 20 h were recorded in over 150 separate trials. During the course of the study, a total of nine different discriminator colonies were used, typically three to four each year. Each year worker-laid eggs were sourced from two to three queenless colonies, while queen-laid eggs were sourced from two to three queenright colonies. The colonies with the highest egg-laying rates where chosen to provide the eggs for the bioassays.

In all bioassays we compared the removal of treated eggs with that of untreated eggs of the same type, e.g. queen-laid eggs treated with hexane vs. untreated control queen-laid

eggs. When only one bioassay was conducted (only two cases) we used a paired t-test to make the comparison using the number of eggs at 0 h and after 2 h in each group. In all other cases the percentage of eggs remaining after 2 h in each group was calculated in relation to the number at the start of the bioassay. Then the mean percentage and standard deviation was calculated for each group of eggs in that trail. We then used an unpaired t-test to compare the mean and SD of the untreated with the treated group. In the experiment to determine the caste origin of the signal (see 2.3.) we used an ANOVA and post-hoc Tukey tests as we were comparing the difference between the three types of egg-pairs at the same time.

2.2. Egg surface morphology

To detect any surface physical differences between queen-laid and worker-laid eggs, five eggs of each type were coated with 25-30 nm of gold using an Edwards S150B sputter coater and viewed with a Philips PSEM501B scanning electron microscope set at an accelerating voltage of 30KV.

2.3 Caste origin of the signal

To determine which egg type (queen-laid vs. worker-laid) may be marked with the egg recognition signal, we measured in three separate trials the removal rates of untreated egg pairs that we had placed in each cell, in contact with each other. Two different discriminator colonies were used, one once and one twice on different days. The egg pairings were queen-queen, worker-worker and worker-queen. Our hypothesis was if worker-laid eggs were marked, then a worker-queen egg pair would be policed (killed and removed from cell), whereas if queen-laid eggs were marked, a worker-queen egg pair would not be policed because the worker-laid egg would be protected by the presence of the queen egg. We measured the removal rates of single eggs as a control to determine whether the presence of two eggs in a cell influences the results.

2.4. Effect of solvents on eggs

Before treating the eggs with egg or gland extracts, we conducted a series of bioassays to confirm that our main solvent, hexane did not affect short-term egg viability. We also

checked if other solvents (acetone, dichloromethane, di-ethyl ether) have any affect. Because all these solvents are non-polar compounds, we were also able to determine whether the egg discrimination signal is likely to be a non-polar chemical. We used two application methods. The drop method involves applying a 0.5 µl droplet of solvent to the egg surface using a 10 µl Hamilton syringe. The capillary method (Ratnieks 1995) entails holding a glass capillary tube containing the solvent over the egg for 10-20 s. After solvent application, eggs were transferred from the glass slide into the test frame.

2.5. Effect of egg, tissue and gland extracts on removal rates

To help discover the origin of the egg-marking signal we investigated the effect of various extracts on egg removal rates. The 'queen-laid egg' extract was an attempt to transfer the egg marking signal via the solvent from queen-laid to worker-laid eggs. As the concentration of the signal may be low in the egg extract we made an extract of the queen's Dufour's gland as extracts of this gland had been shown to effect egg removal rates (Ratnieks, 1995). To further investigate the role of the Dufour's gland we tested extracts of the setosa membrane, a region of hairy cuticle found at the base of the sting where the secretions of the Dufour's gland appear to become concentrated (Martin *et al.* 2004a), and fractionated the queen's Dufour's gland extract into a hydrocarbon and ester fractions. In addition, we speculatively tested extracts of the queen spermathecal and manbidular glands, as both produce chemicals unique to the queen, and a worker setosa membrane extract as it contains a chemical unique to workers. All test extracts were made by placing queen eggs (n=50), a setosa membrane, a Dufour's gland or a spermathecal gland in 100 µl of hexane for around 30 min. For the queen manbidular gland extract we used queen manbidular pheromone purchased from Pherotech.

All extracts where applied to the eggs on the glass slide using the drop method. In addition to being an easier method, Gas-Chromatography analysis revealed that the drop method deposits more extract on the egg surface than the capillary method (unpublished data).

2.6. Signal disrupters

The queen Dufour's gland and worker setosa membrane extract produced two potential candidate signals, esters from the queen and Z-11-eicosenol from the workers. Therefore, at Keele University we synthesize the main esters found in the queen's Dufour's gland (Martin *et al*. 2002a) and Z-11-eicosenol which is the main chemical found on the workers setosa membrane (Pickett *et al*. 1982). As both these compounds are caste specific they both are excellent candidates for identifying egg origin. In addition, to the synthesized compounds we applied a range of chemicals using the drop method to queen-laid and worker-laid eggs in an attempt to remove or disrupt any chemical cues or signals used in egg recognition (see Table 1 for list of substances, application method and number of trials). For substances with slower acting reactions, such as Trypsin, which degrades proteins, we used the wash method. This involved immersing the eggs in the test substance in a glass well for approximately 20mins prior to transferring them into the test frame.

Because the largest differences in egg removal rates were observed primarily during the first two hours after treated eggs had been introduced into the test colonies, we have presented, for the most part, the results obtained at the 2 h mark. We believe the results at 2 h represent best the effect of egg treatment on worker policing, while minimising the effect of the treatments on egg viability, which we found in some cases damaged the eggs (see 3.6). Each trial contained a corresponding control of untreated queen-laid and worker-laid eggs in the same frame as the treated eggs to standardise for the variation in speed and accuracy of worker policing between trials and colonies. Also these controls ensured that egg removal was not prevented by chemical toxicity or repulsion, since a slower removal rate of treated worker-laid eggs would occur if repulsion was occurring.

2.7. Physical effects of solvents

Eggs laid by a queen pass over a region covered in dense hairs known as the bursa-copulatrix or copulatory organ. There is evidence that eggs must pass over this region in order to be recognized as queen-laid eggs; workers eat full-sized eggs dissected from the ovaries but spare freshly laid eggs (Martin *et al*. 2004b). To determine the possible source and properties of the egg-marking chemical, we removed the entire sting region of eight queens and compared SEMs (see 2.2.) of bursa-copulatrices that were untreated or that had been placed in hexane, dichloromethane or aceto-nitrile for 30 min. As the

oily layer coating the hairs of the bursa-copulatric may contain the egg marking single we were attempting to see which of the three solvents were best able to dissolve it.

3. Results

3.1. Standard worker policing bioassay of untreated eggs

Comparing only the untreated eggs used to standardise each of the 167 bioassays conducted during three years, significantly more untreated queen-laid eggs (x=91%, sd+/-12 %, n= 3340 eggs) remained after two hours (unpaired t-test, $p<0.0001$) than untreated worker-laid eggs (x=15%, sd+/-20 %, n=3340 eggs). However, the speed with which policing occurred varied between individual discriminator colonies throughout the season (see Fig. 2 for a typical example).

3.2. Egg surface morphology

The surfaces of the five queen-laid and five worker-laid eggs were indistinguishable by-eye at high (x1250-x2500) magnifications (Fig. 3), with both egg types showing the typical polygonal areas and discrete rugosities on the egg surface. The density of polygons varies across the surface of all eggs, but their diminutive size makes it unlikely that the honeybees can detect the polygon walls or surface rugosities.

3.3. Caste origin of the signal

Egg-pair trials were conducted to determine whether the recognition signal really is on queen-laid eggs, as has been commonly assumed, or whether the signal may actually be found on worker-laid eggs. Individual analyses of each bioassay conducted (n=3) showed that the number of eggs of the same type in a cell (one vs. two) had no effect ($p>0.7$) on the rate at which they were removed, given that the persistence of worker-laid (single vs. paired) or queen-laid (single vs. paired) eggs was not significantly different ($p>0.7$). However, there was a significant difference between the three types of egg-pairs (ANOVA, $F=17.4$, $df=8$, $p=0.003$) (Fig. 4.). Post-hoc Tukey tests showed that there was no significant difference between the queen-queen and queen-worker egg-pair while there was a significant difference ($p<0.05$, $df=2$) between both the

queen-worker vs. worker-worker egg-pair, and also between queen-queen vs. worker-worker egg-pairs as expected.

3.4. Effect of solvents on eggs

Treating eggs with hexane, acetone, dichloromethane or di-ethyl ether using either drop or capillary methods did not decrease the persistence of the queen-laid eggs or increase the persistence of worker-laid eggs when compared with untreated eggs (Table 1). However, queen-laid eggs washed in hexane for 24 h were all removed within 2 h, presumably because the structure of the eggs had been irrevocably damaged.

3.5. Effect of egg, tissue and gland extracts on removal rates

Treatment with extracts from queen-laid eggs and queen spermathecal gland had no significant effect on the persistence of worker-laid eggs. However, extract of queen Dufour's gland increased the persistence of worker-laid eggs (Table 1). The Dufour's gland fraction that contained the esters had a similar positive effect on egg persistence whereas the fraction containing the hydrocarbons did not (see Martin *et al*. 2002a). The ability of esters to decrease policing of worker-laid eggs was confirmed by using a synthetic ester (Table 1). The only extract to produce a significant reduction in the persistence of queen-laid eggs was that of the worker setosa (Table 1). Although eicosenol is an excellent candidate for identifying egg origin as it only occurs on the worker setosa membrane (Martin *et al*. 2004a), the application of Z-11-eicosenol with concentrations ranging from 10^{-3} to 10^{-7} to queen-laid eggs had no effect on egg persistence (Table 1).

3.6. Signal disrupters

None of the chemicals we tested affected the persistence of queen-laid eggs after two hours, although some of the treatments, e.g., 10% Sodium Hydroxide (NaOH), probably killed eggs. We noticed that queen-laid eggs in trials involving chemicals such as acid or NaOH were frequently non-viable, due to either the toxic nature of treatment. Nevertheless, these eggs often remained in the cells for several hours or days before workers removed them. We found that the removal rate of eggs were either not affected

(by 10% acetic acid; NaOH 1% and 0.1%) or increased (by NaOH 10%) and confirms that our treatments did not discourage workers from removing the eggs. The persistence of queen-laid eggs washed in the enzyme trypsin suggests that proteins are not involved in egg recognition system. Therefore, the only chemicals able to disrupt the egg recognition signal were the esters, while all the other chemicals tested (Table 1) had no effect.

3.7. Physical effects of solvents

Scanning electron images show that the oily secretion covering the hairs on (Fig. 5b) the bursa-copulatrix of the queen (Fig. 5a) was not dissolved after exposure to non-polar solvents such as hexane or dichloromethane, or by aceto-nitrile a polar solvents, but becomes a solid film (Fig. 5c).

4. Discussion

Egg transfer bioassays (this study; Ratnieks & Visscher 1989, Oldroyd & Ratnieks 2000), comb-section transfers (Katzav-Gozansky *et al*. 2001; Calis *et al*. 2003), and natural observations (Ratnieks 1993; Visscher 1996; Martin *et al*. 2002b) have shown that honeybee workers are able to discriminate worker-laid eggs and eat them. In this study, on average, 85% of worker-laid eggs versus 9% of queen-laid eggs were eaten by workers in the discriminator colonies within the first 2 h after eggs were introduced. The small loss of queen-laid eggs is most likely due to damage sustained (Collins 2002) during the egg transfer process. However, despite conducting bioassays using the same egg sources on the same day, the speed of policing sometimes varied between discriminator colonies (Fig. 2), as has been seen in previous studies (see Ratnieks 1995; Oldroyd & Ratnieks 2000). These differences usually disappeared when a discriminator colony was used repeatedly. This may be a function of workers adjusting to the abnormally high density of worker-laid eggs suddenly appearing in their colony. No physical differences were observed between the surface of queen-laid and worker-laid eggs, which confirms previous findings (Katzav-Gozansky *et al*. 2002), and strongly suggests that egg discrimination is chemically mediated. We also confirmed that the signal is most likely found on queen-laid and not worker-laid eggs as has been assumed previously (Ratnieks 1988), since worker-laid eggs are protected when placed in contact

with a queen-laid egg. If the signal was on the worker-laid egg were would have predicted an increase in removal of the queen-laid eggs placed next to worker-laid eggs but this did not occur. This also helps to explain the slightly increased persistence of worker-laid eggs that had been rubbed against queen-laid eggs (Ratnieks 1992). It remains unclear, however, why the worker setosa extract reduced the persistence of queen-laid eggs. The setosa membrane is a platform for release of many alarm pheromones (Lensky *et al*. 1995). Residuals of these alarm pheromones may have influenced worker perception of queen-laid eggs by masking the signal. However, eicosenol, a major chemical found on the worker setosa membrane and acts as a long term alarm pheromone (Pickett *et al*. 1982), had no effect on the rate of egg removal (Table 1). As workers only produce eicosenol it potentially could have been the cue by which worker-laid eggs are identified. Despite the large number of bioassays we conducted using a wide range of solvents and gland extracts, only the ester fraction from the queen's Dufour's gland and synthetic esters produced an increase the persistence of worker-laid eggs as reported previously (Martin *et al*. 2002a). Because esters are either absent or present in only small amounts on queen-laid eggs (Martin *et al*. 2002a), they are unlikely to be the egg-marking pheromone. Esters are, however, utilised by egg-laying worker anarchistic bees to prevent their eggs from being removed (Martin *et al*. 2004c). The role of the esters appears to be part of the complex queen honeybee pheromonal bouquet (Katzav-Gozansky *et al*. 2003) and may be used by the egg-laying anarchistic workers to mask the true identity of their eggs.

The inability of our studies to transfer the signal from queen-laid eggs to worker-laid eggs using non-polar solvents such as hexane suggests that the signal is unlikely to be a non-polar compound. This would also explain why the removal rate of worker-laid and queen-laid eggs treated with different non-polar solvents remained unaffected, that is treated queen-laid eggs remain while treated worker-laid eggs are removed. This suggests that the signal is likely to be a non-polar compound. Furthermore, our inability to disrupt or remove the signal by treating queen-laid eggs with a wide variety of compounds indicates how robust the signal is. We believe the main barrier preventing the identification of the egg-marking signal is finding a solvent with which to dissolve the signal so that it may be transferred from queen-laid eggs to worker-laid eggs. Thus far, the oily secretion found on the queen bursa-copulatrix where we believe the eggs are marked, does not dissolve in hexane or dichloromethane (non-polar solvents), or in

aceto-nitrile a polar solvent. Non-polar compounds, such as hydrocarbons, have long been thought to be the most likely candidates for a wide range of chemical signals in social insects (Singer 1998). However, their role in honeybee egg recognition has recently been questioned (Martin *et al*. 2004b) and other classes of compounds such as fatty acids (Breed 1998) and proteins (Klobuchar & Deslippe 2002) are now being investigated. All current evidence indicates that the honeybee egg-marking signal may also belong to one of these classes of compounds.

Acknowledgements

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Figure 1. Rows of queen-laid (top) and worker-laid (bottom) eggs on a glass microscope slide ready for being treated with solvents and then placed into cells.

Figure 2. Differences in the speed of removal of untreated queen-laid eggs (dotted bars) and untreated worker-laid eggs (solid bars) in two different discriminator colonies (D2 and D3) over a 15 day period.

Figure 3. Scanning electron microscope images of the surface of queen-laid (left) and worker-laid (right) eggs (x1250), scale bar = 10 μm.

Fig. 4. Mean percentage (+ SD) of single eggs and egg pairings (worker-laid + worker-laid, queen-laid + queen-laid, queen-laid + worker-laid) that remained in cells after 2 and 20 h. Three separate trails were conducted. Each trial compared groups of 20 single and 20 pairs of each egg combination.

Fig. 5. Scanning electron microscope images of (a) the queen bursa-copulatrix region (*bc*) along which the eggs pass during laying, (b) the oily secretions on the hairs lining the untreated bursa-copulatrix, and (c) the changes when the region is immersed in hexane, dichloromethane or aceto-nitrile. Scales bar represent 100μm (a) or 10μm (b and c).

Table1. Summary of the egg-discrimination bioassays. The approximate number of eggs used in each trial can be estimated by dividing the total number of eggs by the number of trails. The effect after 2 h of the treatment is compared with untreated eggs. The direction of the effect (+ or -) is also given. If the egg recognition chemical were present in the treatment, then the expected direction of the treatment is positive on worker-laid eggs (+, an increase in persistence via an supplement affect). However, if the signal is been removed or blocked by the treatment then a negative effect on queen-laid eggs (-, a decrease in persistence via an removal or masking affect) is excepted. Therefore, if the treatment decreases the persistence (-) of worker-laid eggs, this is probably due the treatment killing the eggs and not connected with the egg-recognition system

Compound	Method	Queen-laid eggs			Worker-laid eggs treated		
		# eggs	# trials	effect after 2h compared with untreated eggs	# eggs	# trials	effect after 2h compared with untreated eggs
Solvents							
Hexane	drop & capillary	340	18	ns	200	10	Ns
Acetone	drop	38	2	ns	20	1	Ns
Dichloromethane	drop	88	4	ns			
Di-ethyl ether	drop	89	4	**p=0.02 (+)**			
Egg, tissue and gland extracts							
Queen eggs	drop	30	2	ns	186	10	Ns
Queen setosa membrane	drop	115	6	ns			
Queen spermathecal gland	drop				30	2	Ns
Queen Dufour's gland (QDG)	drop				433	22	**p=0.01 (+)***
QDG hydrocarbon fraction	drop				251	13	Ns
QDG ester fraction	drop				247	13	**p=0.02 (+)***
Queen manbidular gland	drop				28	2	Ns
Worker setosa membrane	drop	252	13	**p=0.009 (-)**			
Signal disrupters							
Synthesised ester C16/C16:1	drop				120	6	**p=0.02 (+)***
Eicosenol (10^{-4} concentration)	drop	35	3	ns			
Aldhyde (all conc combined)	drop	80	4	ns			
Acetic acid 10%	drop	30	3	ns	20	2	Ns
NaOH 10%	drop	40	4	ns	36	4	**p=0.02 (-)**
NaOH 1% & 0.1%	drop	56	4	ns	36	2	Ns
Trypsin (35°C)	wash	16	1	ns			
Buffers, PBS, pH 9.2 & pH 4.2	wash	38	3	ns			

* see Martin *et al*. 2002a

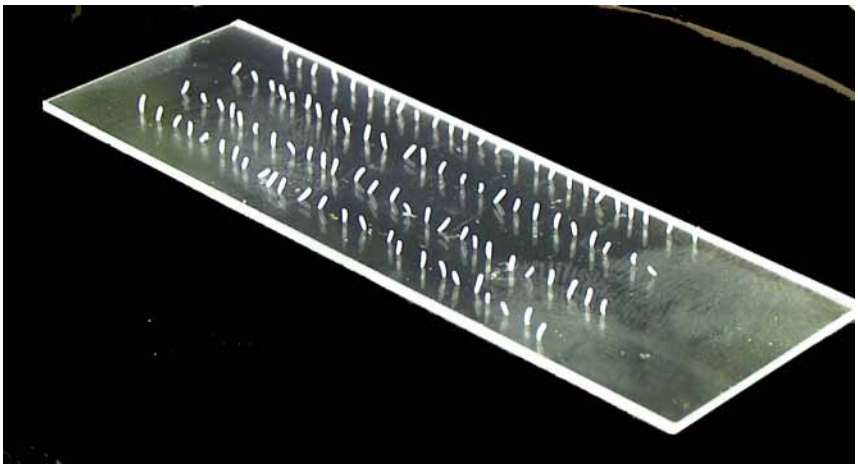
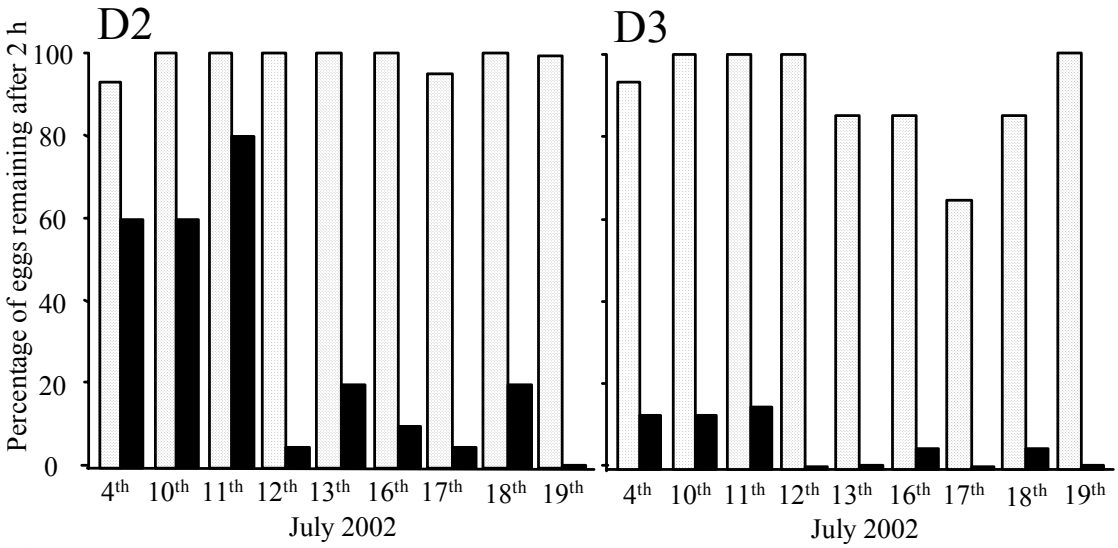


Fig.1



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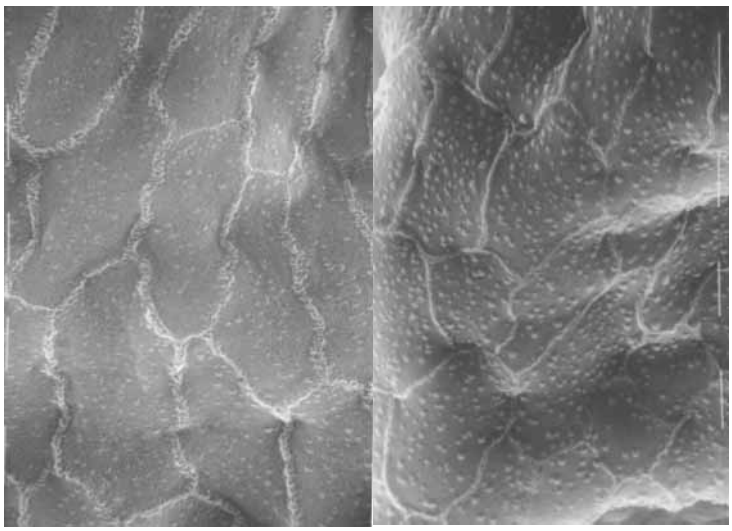

Fig. 3

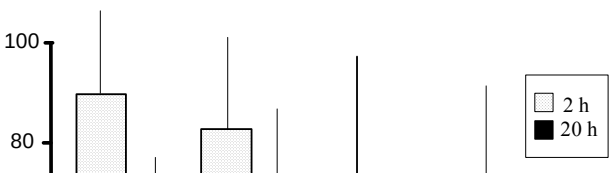

Fig. 4

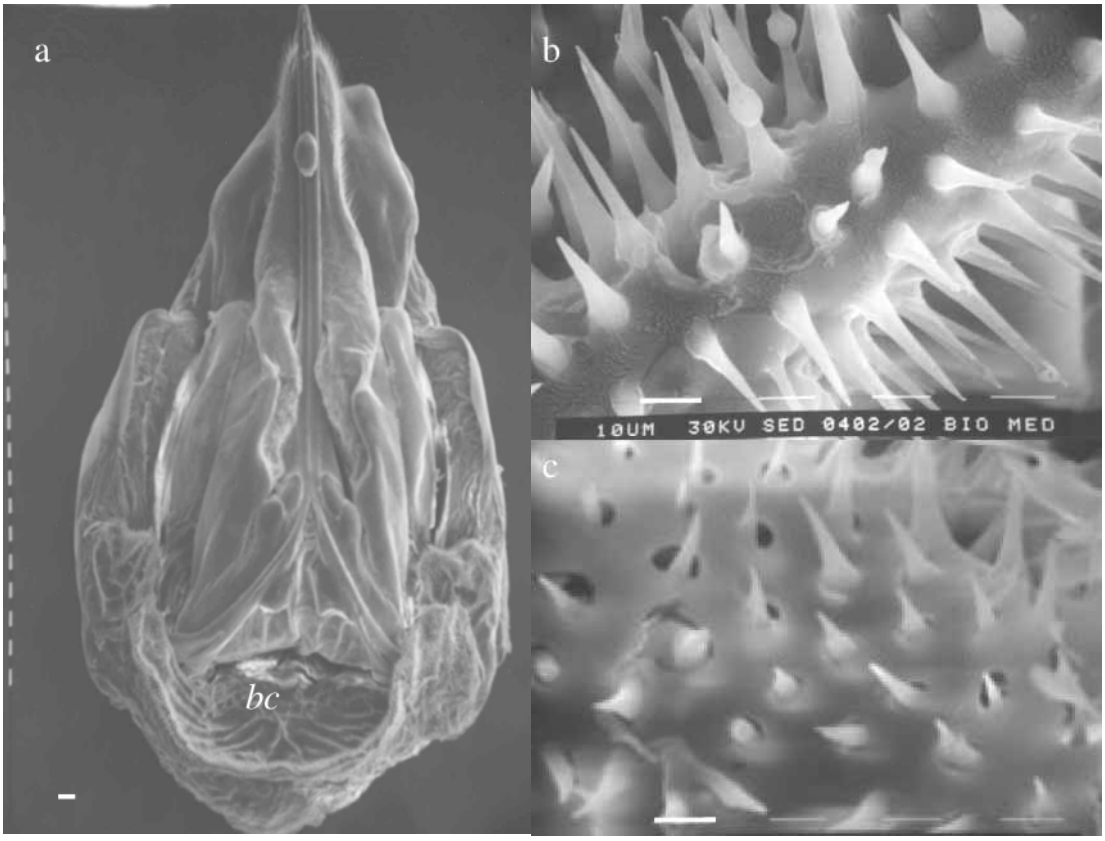

Fig. 5

Appendix 2

In this Appendix, I have included two papers on nepotism in emergency queen rearing which, although the research was done years ago, I wrote during my PhD.

Appendix 2.1: Châline N, Arnold G, Papin C, Ratnieks FLW (2003) Patriline differences in emergency queen rearing in the honey bee, *Apis mellifera*. *Insectes Sociaux,* **50**, 234-236.

Appendix 2.2: Châline N, Arnold G (Submitted) Lack of nepotism during queen larval feeding in emergency queen rearing in the honey bee (*Apis mellifera*)

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DOI 10.1007/s00040-003-0664-6

Insectes Sociaux

Research article

Patriline differences in emergency queen rearing in the honey bee, *Apis mellifera*

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Summary. In the polyandrous honey bee, *Apis mellifera*, 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 (emergency queen rearing). Using DNA microsatellite markers we determined the patriline of emergency queens reared in two colonies headed by naturally-mated queens before being made queenless. We found that some patrilines were reared more than others in one colony, but not in the other. These differences between colonies suggest that selective rearing is not always present and this might explain the mixed results of previous nepotism studies in the honey bee.

Key words: Apis mellifera, nepotism, kin recognition, queens, microsatellites.

Introduction

Queen honey bees, *Apis mellifera*, mate with many males (Estoup et al., 1994; Arnold et al., 1996; Oldroyd et al., 1997). Within a colony, workers are related to new queens by 0.75 (full-sister) or 0.25 (half-sister). New queens are reared in three contexts: swarming, supersedure of a failing queen, and emergency (if the mother queen dies suddenly). In emergency queen rearing, only a few larvae among the numerous ones available in worker cells are selected by workers to rear as queens. These larvae are then fed royal jelly and the worker cell is transformed into a queen cell. Because of relatedness differences, workers can potentially gain a threefold increase in inclusive fitness if they can cause a full-sister to head a new colony instead of a half-sister (Visscher, 1998). This can lead to reproductive conflict between nestmates if workers can discriminate between half-sister and full-sister larvae. Arnold et al. (1996, 2000) have shown that the cuticular hydrocarbons of adult workers vary more among patrilines than within patrilines which suggests that there is sufficient variability for workers to discriminate between full and half-sisters, at least for some patrilines. Despite numerous studies, evidence of nepotism in honey bee queen rearing remains controversial and only weak nepotism, or absence of nepotism, has been observed (reviewed in Breed, 1994; Visscher, 1998). Early studies of kin recognition were hindered by a lack of sufficient genetic markers to assess kinship in colonies headed by naturally-mated queens, which have many patrilines. The use of DNA microsatellites now allows the precise assessment of kinship in natural colonies (e.g. Estoup et al., 1994; Palmer and Oldroyd, 2001; Châline et al., 2002) and has greatly facilitated the study of kinship and reproductive conflict in honey bees (e.g. Franck et al., 2002; Tilley and Oldroyd, 1997; Wattanachaiyingcharoen et al., 2002).

Three studies using microsatellites have so far studied nepotism in emergency queen rearing. Tilley and Oldroyd (1997) have shown that in long-established queenless colonies with repeated introductions of young brood, some patrilines were preferentially selected. Osborne and Oldroyd (1999) suggest that this unequal representation of patrilines could be caused by nepotism. On the other hand, Franck et al. (2002) failed to find any patriline differences between worker and queen proportions.

We conducted a similar experiment to that of Tilley and Oldroyd (1997). The patrilines of emergency queens reared after queen removal were determined in two colonies. There was a significant difference between patriline representation in workers and emergency queens in only one colony. These results show that selective queen rearing sometimes occurs and sometimes does not and helps explain why mixed results have been obtained previously.

Material and methods

Sample collection

Two *Apis mellifera* colonies (A and B) with brood and a one-year-old queen kept at the Laboratoire de Neurobiologie Comparée des Invertébrés (LNCI, INRA-CNRS, 91440 Bures-sur-Yvette, France) were studied in 1997. Emergency queen rearing was initiated by removing the queens. The colonies were kept queenless until they contained sealed queen cells. The queen pupae were then collected and placed at –20 °C for DNA analyses. At the same time as the queen pupae were removed, we also removed a comb of sealed brood from each colony and placed it in an incubator at 35 °C to collect emerging workers. The workers and the queens came from eggs laid at the same time. The mother queens were then reintroduced to their respective colonies. Five weeks later, when the queen had resumed normal egg-laying, we repeated the process above. We repeated the procedure a third time on colony A but the second reintroduction of colony B's queen failed. We did not collect additional samples of workers as Estoup et al. (1994) and Tilley and Oldroyd (1997) have shown that colony kin composition remains stable over short periods.

DNA extraction and microsatellite analyses

DNA was extracted according to Garnery et al. (1990) using proteinase K and phenol. To determine the patrilines of queens and workers, we used three highly polymorphic microsatellite markers (A76, A29 and B124 identified by Estoup et al., 1994). Polymerase chain reactions followed Estoup et al. (1994). The patriline of each sample was determined using standard procedures (Estoup et al., 1994).

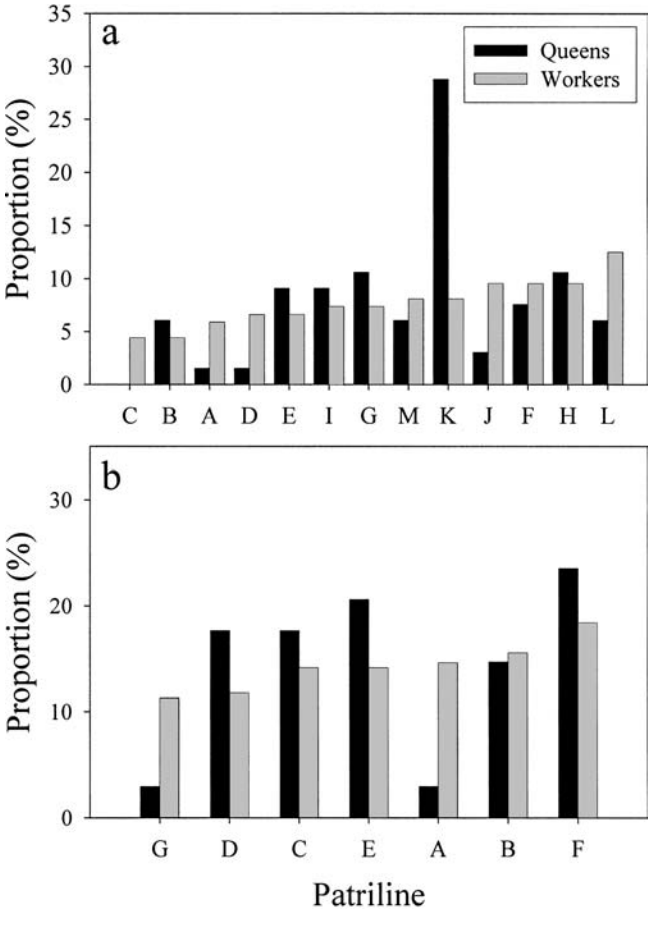

Figure 1. Percentage of each patriline in the worker and queen samples from colony A (a) and B (b).

Statistical analyses

We pooled the samples of queen larvae from the different trials for each colony because the sample sizes of some trials were very low (8 and 14). Because the expected values in the contingency tables were low, we analysed the data using Fisher's exact test with an exact method using the program 'Monte Carlo RxC 2.2' developed by W. Engels, University of Wisconsin.

Results

We analysed 136 workers and 66 queens (respectively 14, 28 and 24 for each trial) in colony A and 212 workers and 34 queens (respectively 26 and 8) in colony B. Colony A had 13 patrilines and colony B had 7. There was a significant difference in the patriline composition of queens and workers in colony A ($p = 0.008$, Fisher's exact test), but not in colony B ($p = 0.194$, Fisher's exact test) (Fig. 1). In colony A, one patriline, K, accounted for 19 of the 66 queens (28.8%). If this patriline is removed from the analysis, no significant differences can be found in the distribution of queens and workers of the other patrilines in colony A ($p = 0.281$ Fisher's exact test).

Discussion

Our results show that in emergency queen rearing the proportion of emergency queens and workers can differ in their patriline composition, but that the pattern is not consistent among colonies. It also seems that only a few patrilines are preferred over others, such as patriline K in colony A. Our results confirm, although with slightly different methods, the findings of Tilley and Oldroyd (1997). Contrary to Tilley and Oldroyd (1997), the queen was reintroduced in the test colonies and allowed to re-establish normal egg-laying between samples. Thus, our experiment allowed the different trials to be done in a more natural situation with the queens present in the colonies before each sample.

Our study does not investigate why some patrilines are reared more than others and the actual reason is unclear. It could result from nepotism by rearing workers, or because larvae vary heritably in their attractiveness as potential queens ("royalty alleles"; Osborne and Oldroyd, 1999). However, Osborne and Oldroyd (1999) failed to find any evidence for the "royalty alleles" when rearing queens in related and unrelated colonies. In our study, both possibilities could explain why patriline K, one of 13 patrilines and representing only 8% of the workers, was highly preferred, representing 28% of the queens in colony A. The variability in the expression of preferences between colonies also occurred in Osborne and Oldroyd's (1999) study. This suggests that patriline preference, be it caused by nepotism or "royalty alleles", is a weakly expressed and relatively rare and polymorphic

trait in the honey bee. The number of queens reared from each patriline was not related to its relative size (Fig. 1), which refutes the hypothesis that the patriline with the most workers dominates queen rearing and preferentially rear full-sister larvae (Tilley and Oldroyd, 1997).

Arnold et al. (2000) have shown that, in a colony environment, cuticular hydrocarbon profiles of adult workers from different patrilines often become less distant while some patrilines remain very distinct and this could explain disparities between the different patrilines if a similar pattern occurs in larvae. Such polymorphism between nepotistic and non-nepotistic patrilines can be maintained if kin discrimination is associated with some costs to colony efficiency (Ratnieks and Reeve, 1991) and it would be the same for royalty alleles. Our study adds some evidence for the presence of reproductive competition in queen rearing in the honey bee, at least during emergency queen rearing, but it also shows that much variation occurs among colonies and so may help explain the mixed results in the previous studies.

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Appendix 2.2 Scientific note on the lack of nepotism in queen larval feeding during emergency queen rearing in a naturally mated honey bee colony

Submitted to Apidologie

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Honey bee colonies consist of many patrilines (Estoup et al., 1994) and workers can potentially increase their inclusive fitness during queen replacement if they can favour a full-sister queen (Visscher, 1998). There is evidence of preferential rearing of some patrilines in emergency queen rearing (Tilley and Oldroyd, 1997; Osborne and Oldroyd, 1999; Châline et al., 2003), although the reason for this is unclear. Some patrilines could act nepotistically or a "royalty allele" could make some larvae more attractive to workers (Osborne and Oldroyd, 1999). Early studies on behavioural interactions during queen rearing used colonies with limited numbers of patrilines and showed weak or no evidence of nepotism (Breed et al. 1984, Noonan, 1986; Page et al., 1989). To avoid the problem of unnaturally low number of patrilines and the use of artificial insemination, we used microsatellites to analyse the kinship of feeding workers and queen larvae during emergency queen rearing, for the fist time in a colony with a naturally mated queen.

The experiment was carried out in double blind in a queenless observation hive containing individually marked bees. This was done by marking bees emerging from two brood combs of the test colony over 6 consecutive days. Before the experimental colony set-up, the marked bees were returned to their mother colony to ensure a normal ontogeny. Twenty-four hours before the set-up, 50 queen cells were initiated in a queen rearing colony by grafting larvae from the test colony. Thirteen of these were then attached to a brood comb from the test colony and transferred in an observation hive with 2000 of the marked workers. During the experiment, three additional queen cells

were built naturally.

Each queen cell was observed individually for 1/2h every day until capping. The total observation time was 21h30. The bees feeding each queen larvae were recorded, as this seemed the best behaviour for the expression of nepotism. The queen pupae (16) and marked adult workers (1300 left due to drifting and predation) were then collected together with a control sample of worker larvae. The patriline of each sample was determined with three microsatellite markers (A76, A29 and B124, Estoup et al., 1994).

There were 21 patrilines in the colony (effective paternity=11.65). The queens belonged to 9 patrilines. During the observations, 172 workers performed 183 feedings. Of these 172 workers, 97 could be retrieved (107 feedings). The repartition of feeding nurses (n=97) in the different patrilines was not significantly different from the control sample (n=136; p=0.21, Fisher's exact test using the program 'Monte Carlo RxC 2.2' by W. Engels, University of Wisconsin). The overall proportion of workers feeding queens of their own patriline was 0.065 which is not different from the random chance of encountering a full sister with a paternity of 11.65 (0.086; χ^2 P>0.1, df=1, n=107). A correspondence analysis showed no association between the queens and nurses patrilines.

Our results did not show any tendency of workers to bias their behaviour towards closer kin. This suggests that no nepotism occurred in this colony during emergency queen rearing, and confirms the results of other studies (Breed et al., 1984, 1994). Because the sample size was small relative to the number of patrilines, it is possible that weakly expressed nepotism could not be seen. Page et al. (1989), with colonies containing three patrilines, found that a likely mechanism for the observed bias was that some patrilines are overrepresented in the nurses and alter the outcome of queen rearing, but no such specialist patrilines were found here. The use of grafted larvae could also be the reason for a lack of nepotism as the initial larval selection process may be where the differences occur (Visscher 1998). After this step, the risk of failing to rear a queen may be too costly for any bias to be expressed (Tarpy et al., 2004). Our study is the first behavioural study of queen rearing using a naturally mated queen. Although the sample size was necessarily small, it confirms the findings of earlier studies that nepotism is probably absent or weakly expressed during this process (Tarpy et al., 2004).

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Appendix 3

Quantification of honey bee mating range and isolation in semi-isolated valleys in the Peak District National Park, England, as revealed by paternity analysis using DNA microsatellites

Submitted to Conservation Genetics

Running title: Honey bee mating in semi-isolated valleys

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Abstract

Controlled mating of virgin queens is important in selective breeding of honey bees, *Apis mellifera* and can be achieved both by instrumental insemination and by natural mating between selected drones and queens in isolated sites, such as islands or mountain valleys, with little gene flow via drones flying from outside colonies . This study used DNA microsatellite markers to determine the mating range of native black bees (*Apis mellifera mellifera*) in two adjacent semi-isolated valleys (Edale and Hope Valley) located in the Peak District National Park, England, in order to determine their suitability as isolated mating locations and for the conservation of native honey bees. Three apiaries were set up in Edale, a valley free of honey bees prior to the experiment and three in Hope Valley, an adjacent valley where the beekeepers predominantly keep *A. m. m.* In each of these apiaries, twelve colonies headed with virgin queens and two drone producing colonies were set up. The virgin queens were allowed to mate naturally

with drones from the colonies we had set up and from colonies owned by local beekeepers in Hope Valley. After mating, samples of worker larvae were taken from the 41 queens which mated successfully and genotyped (16 per queen) at 11 DNA microsatellite loci. Paternity analyses were then carried out (using the genotypes of the drone mother queens and the worker larvae), to determine mating distances and isolation. An average of 10.2 fathers were detected in the 16 progeny, and the mean effective mating frequency of the mated test queens was estimated to be 17.7, which is a normal figure for honey bees. 90% of matings occurred within 7.5km (distance between hives holding drone and queen) and 50% within 2.5km. The maximal mating distance recorded was 15 km. Queens and drones from the two valleys, Edale and Hope Valley, do occasionally mate, showing that Losehill mountain ridge between the two valleys does not provide isolation. Nevertheless, in the most isolated part of Edale 60% of all matings were to the 6 drone producing hives set up in Edale. The mating distance is similar to the size of Hope Valley making Hope valley a reasonable location for conserving and breeding black bees so long as the whole valley is involved in selection. Edale is reasonably isolated from Hope Valley and by increasing the number of drone-producing hives in Edale from 6 to approximately 20, it should be possible to ensure that 80-90% of all matings are "local", that is to drones from colonies in Edale.

Key Words: geneflow, honey bee conservation, mating distances, paternity analysis, polyandry, social insects, Peak District, National Park, *Apis mellifera mellifera*