Reproductive conflicts in honeybee colonies

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Abstract

In advanced eusocial hymenopteran societies workers have ovaries and can lay eggs, but are unable to mate. Workers are more related to their own offspring than to every other member of the colony. So worker reproduction contains both worker-worker and worker-queen conflict. The queen- worker conflict is discussed elsewhere, but if the queen mates with more than two males, worker policing should be selected to lower potential conflicts. However in the Cape honeybee it was predicted that worker policing is absent or less expressed than in other honeybee subspecies, because workers produce female offspring thelytokously. So laying workers and their offspring are nearly genetically identical, which results in the fact that other workers are as related to workers derived from eggs laid by the queen as laid by a worker.

However, worker reproduction may be costly and therefore worker policing could be an evolutionary adaptation in the Cape honeybee to lower the costs derived from laying worker activity. Indeed, Cape honeybee colonies show efficient egg removal behaviour, suggesting that other factors like colony efficiency could favour egg removal behaviour. Since egg removal behaviour is a colony phenomenon, factors that affect colony performance could also affect egg removal behaviour. Egg removal behaviour was considerably affected by environmental changes, indicating that other tasks have a higher priority than egg removal behaviour. Thousands of queenright colonies of the neighbouring subspecies (*A. m. scutellata*) were taken over by laying *A. m. capensis* workers, showing that *A. m. capensis* workers are facultative social parasites. These observations strongly indicate that laying workers of *A. m. capensis* are able to evade worker policing and the inhibitory effects of the queen pheromones, but what potential strategies could these laying workers use to increase the survival of their eggs and evade the queen?

On the one hand, egg removal behaviour is variable. One behavioural strategy of laying workers to achieve successful reproduction could be that they lay during periods with low egg removal behaviour. On the other hand, the inhibitory effect of the queen's pheromones diminishes with distance. Maybe the level of egg removal also depends, like the inhibitory effect of the queen pheromones, on the distance from the queen. Indeed, further away from the queen the effect of the queen pheromone and the level of egg removal is reduced, making successful worker reproduction possible. In both subspecies, *A. m. capensis* and *A. m. scutellata,* egg removal behaviour is reduced further away from the queen. In the case of *A. m.*

scutellata egg removal is lacking further away from the queen. This explains why colonies of *scutellata* are so prone to takeovers by laying Cape honeybee workers.

One question in the context of parasitic Cape honeybees is how they manage to get into the host colony. One way could be that they get into the colonies during a natural colony merger which is common in African bees. Two unrelated colonies merged and it took them only 24 hours to show effective integration. Because both colonies are unrelated, the potential reproductive conflict among workers should be more strongly expressed than in a normal colony, which is not the result of a merger. Therefore, the effect of nestmate recognition for eggs on the egg removal behaviour was investigated. The results suggest that workers recognise the origin of an egg and that the standard policing experiments overestimate the level of egg removal and only represent relative values. Moreover, the results show that colony specific components on the eggs are more important than a postulated queen egg marking pheromone. Finally, for the first time empirical evidence from a population of the parasitic laying Cape honeybee workers, invading thousands of colonies of *A. m. scutellata* in northern South Africa, for a short-sighted selection theory is presented.

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Chapter 1

General introduction

Eusocial insects societies, like honeybees (*Apis mellifera* L.), are characterized by an overlap of generations within a colony, co-operative brood care and reproductive division of labour (Wilson 1971; Michener 1974). Among the female individuals in a honeybee colony only one, the queen, normally reproduces, whereas all other females, the workers, are usually non-reproductive. These workers are unable to mate (Wilson 1971), but have ovaries and can lay unfertilised eggs (Bourke 1988; Ratnieks 1988; Bourke & Franks 1995; Crozier & Pamilo 1996), which normally develop into males, a process called arrhenotoky (Crozier 1975; Crozier & Pamilo 1996).

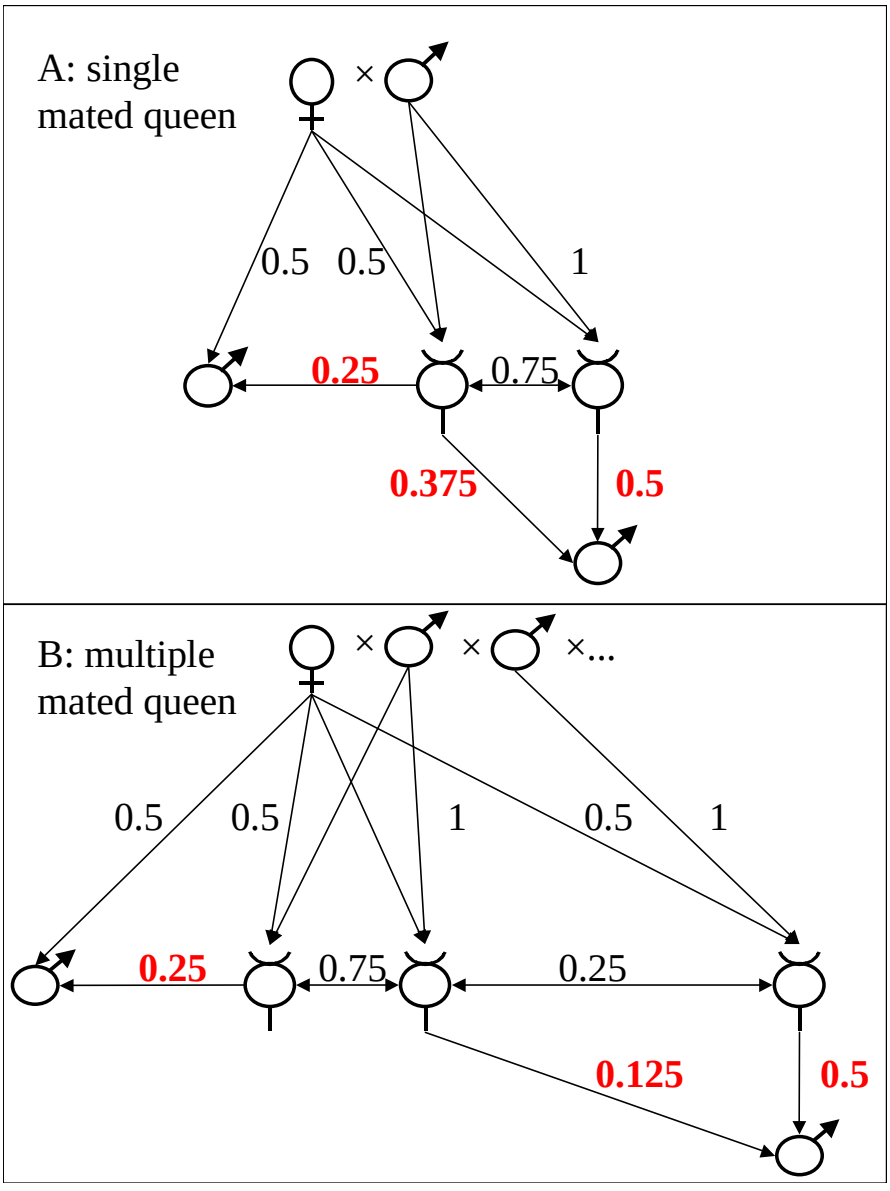
The existence of non-reproductives poses an evolutionary paradox between sterility and natural selection (Bourke & Franks 1995) in Darwin's theory of evolution. To solve this problem Darwin (1859) argued that natural selection in social insects acts on the colony rather than at an individual level (Moritz & Southwick 1992). Hamilton (1964) provided an explanation for the evolution of sociality with his kin-selection theory. He compared the costs of an individual (worker), the abdication or reduction of its own offspring, and the benefits of this individual with the increased survival of offspring of another individual (queen) in terms of the relatedness between them. So individuals can propagate their genes by aiding collateral kin, as well as by rearing their own offspring (Hamilton 1964).

The haplo-diploid sex determination mechanism in Hymenoptera leads to asymmetries in genetic relatedness among nest mates. In an insect society, where the queen only mates one time, workers are more related to their sisters (r=0.75) than to their own female offspring (r=0.5). In case of the production of males, workers are more related to their own offspring (r=0.5) than to their brothers (r=0.25) or nephews (r=0.375). So workers should prefer themselves ideally as the mother of males, then their sisters and then their mother queen progeny (Ratnieks & Keller 1998). This results in a potential conflict between queens and workers over male reproduction (Ratnieks & Reeve 1992; Fig. 1.1a). However, the number of matings of the queen should affect the preferred options of the workers in terms of worker reproduction (Moritz 1985; Crozier & Pamilo 1996) which then results in potential reproductive conflicts among workers (Fig. 1.1b).

There are several hypotheses why polyandry evolved in social insects (reviewed by Crozier & Fjerdingstad 2001). Honeybee queens mate with several males, typically with to 5-30 males (Adams et al. 1977; Estoup et al. 1994; Fuchs & Moritz 1999; Neumann et al.

1999a; Neumann & Moritz 2000; Palmer & Oldroyd 2000) which results in several groups within a colony which have different fathers (subfamilies, Laidlaw & Page 1986; Robinson & Page 1988) or patrilines (Getz 1991). As a consequence, colony members are related to each other by various degrees (Fig. 1.1b) and that results in a decrease in the average relatedness among colony members (Ratnieks 1988). So workers are still more related to their own male offspring, but if the queen mates with several drones, a worker is more related to its queen male offspring than to the male offspring of a randomly chosen worker (r=0.15, by a paternity of 10). It has therefore been argued that there is a potential reproductive conflict among workers (Ratnieks & Reeve 1992, Fig. 1.1b).

Fig. 1.1: Pedigree coefficients of relatedness among members of a honeybee colony when the queen mated with 1 drone (A) and with 10 drones (B, only two are shown). Red numbers indicate the genetic basis for potential reproductive conflict between the queen and workers (a) or among workers (b) over male reproduction. Arrows indicate the relatedness of one individual X to another individual Y, e.g. the probability that an individual Y is holding genes, which are identical to genes in X.



Behavioural mechanisms of manipulation that limit the reproductive success of individuals could reinforce the social harmony of a colony (Visscher & Dukas 1995) and if workers favour queen-produced over worker-produced eggs this could reduce the conflict potential among workers (Starr 1984; Seeley 1985; Woyciechowski & Lomnicki 1987; Ratnieks 1988).

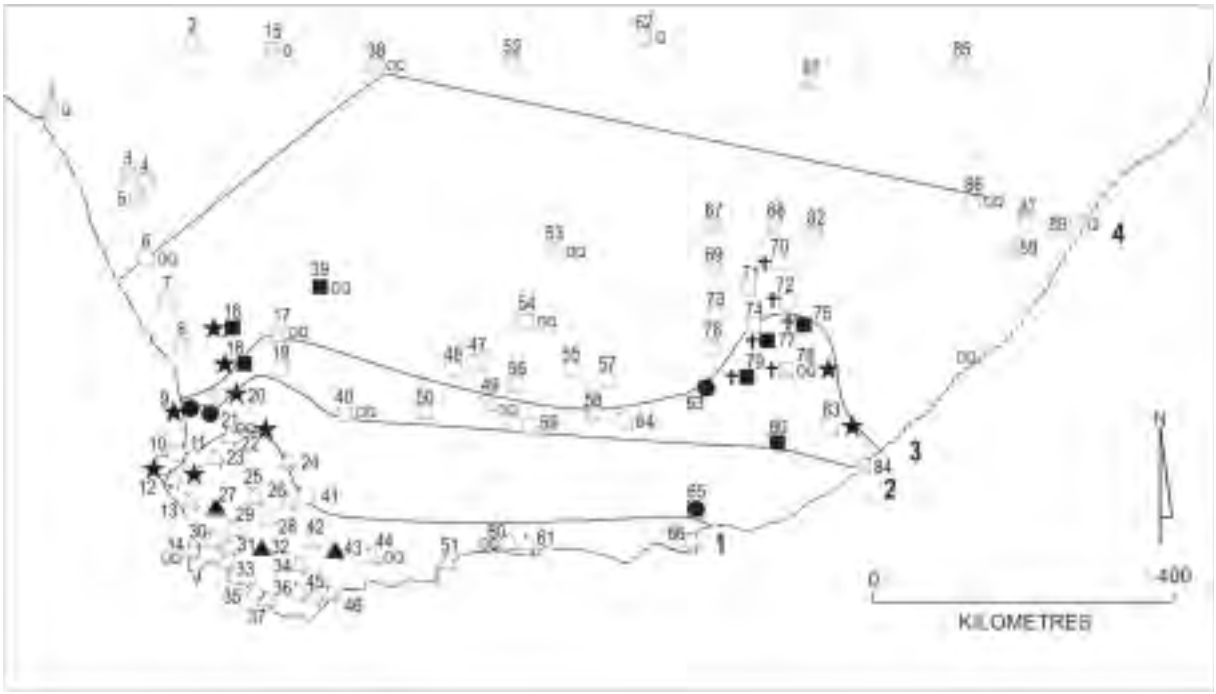
One way to achieve this situation would be to prevent reproduction by other workers such as by eating worker-laid eggs, a behaviour called worker policing (Ratnieks 1988; Ratnieks & Visscher 1989). Since there is evidence for a queen-marking pheromone for eggs (Ratnieks 1992; Ratnieks 1993; but see Katzav-Gozansky et al. 2001) workers are thought to be able to distinguish between queen and worker-laid eggs. Indeed, only a small proportion of the males produced in a honeybee colony are worker-derived (Visscher 1989; Visscher 1996), indicating that worker reproduction occurs only in low numbers. So worker reproduction is nearly absent in queenright honeybee colonies because workers favour queen-derived males over brothers and therefore prevent reproduction by other workers. However, the situation is different in *Apis mellifera capensis* (Fig. 1.3) where workers are able to lay unfertilised diploid eggs which develop into females called thelytoky (Crozier & Pamilo 1996; see Mackensen 1943 and Tucker 1958 for rare cases of thelytoky in other subspecies of *A. mellifera*).

A. m. capensis is native to the macchia-like fynbos region in the Western and Eastern Cape provinces of South Africa (Hepburn & Jacot Guillarmod 1991) and its colony life cycle, brood rearing (Hepburn 1992a) swarming and absconding (Hepburn 1993) are attuned to floral events in the fynbos biome. Over the years the Cape honeybee was described by several authors (DeGeer 1778; Eschscholtz 1821; Lepeletier de Saint-Fargeau 1836) and the name *Apis mellifera capensis* was given by Eschscholtz (1821), which was later supported by Ruttner (Ruttner 1975; Ruttner 1977). Alpatov tried first to separate *A. m. capensis* from its northern neighbour, *Apis mellifera scutellata* (Alpatov 1933; Alpatov 1940).

Various authors described the distribution of *A. m. capensis*, some of them described smaller areas (Kerr & de Portugal-Araújo 1958; Anderson 1963; Guy 1976; Ruttner 1976a; Ruttner 1976b; Ruttner 1976c), some described wider areas for the occurrence of the Cape honeybee (Tribe 1983; Hepburn & Crewe 1990; Hepburn & Crewe 1991). Since the honeybees in South Africa represent a continuous population, it is difficult to separate this population on the subspecies level (Hepburn & Radloff 2002). However, in the following, the term "*A. m. capensis*" describes honeybees which mainly exhibit thelytokous worker reproduction and which are derived from the *A. m. capensis* area defined by Hepburn et al.

(1998). From their findings *A. m. capensis* is distributed from the Western Cape to Port Elisabeth in the Eastern Cape Province (Fig. 1.2).

Fig. 1.2: Map of southern Africa illustrating distributions of morphometric and non- morphometric features of *A. m. capensis*, *A. m. scutellata* and zone of introgression. Line 1 = northern limit of *A. m. capensis* morphocluster; line 2 = southern limit of *A. m. scutellata* morphocluster; line 3 = northern limit for thelytokous parthenogenesis; line 4 = northern limit for 100% frequency of the *A. m. capensis* haplotype P_0QQa. Open triangle = *A. m. capensis* morphocluster; closed triangles = high morphometric variance; open squares = *A. m. scutellata* morphocluster; closed squares = high morphometric variance; open circle = morphometric hybrids, closed circles = high morphometric variance in hybrids; stars = high sting pheromone variance; Q = *A. m. scutellata* P_0Qa haplotype; QQ = *A. m. capensis* P_0QQa haplotype; crosses = area of high mitochondrial and nuclear DNA variance. Oblique line \ = thelytokous parthenogenesis present, / = absent; (unmodified from Hepburn et al. 1998)

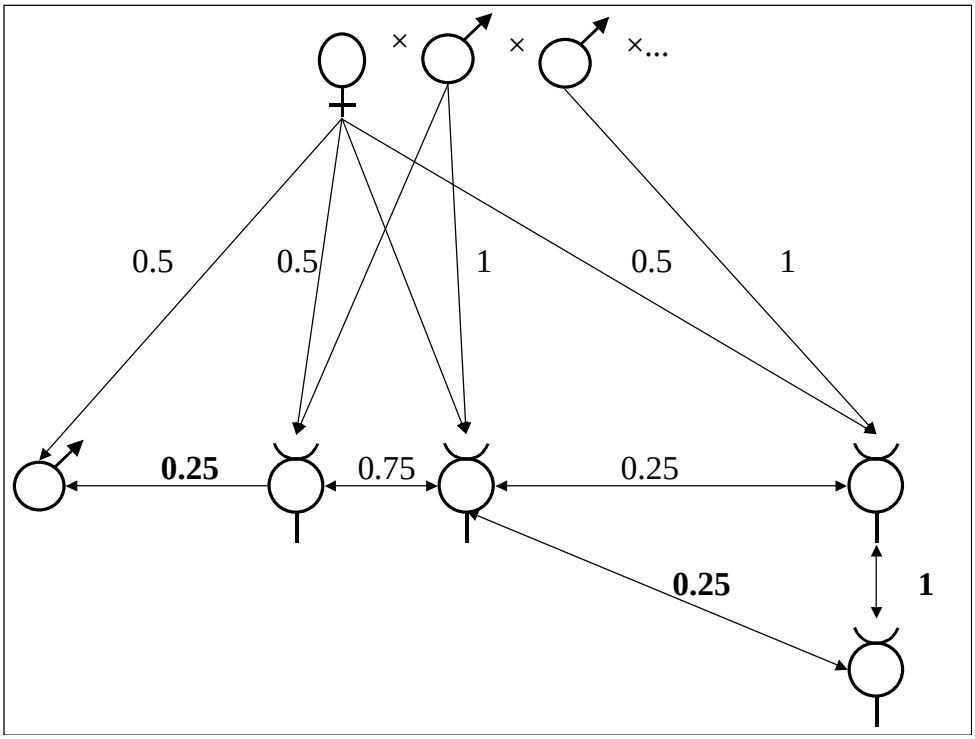


Thelytokous worker reproduction in Cape honeybees has long been known (Onions 1912; reviewed by Hepburn & Crewe 1991 and Hepburn & Radloff 1998). It has been shown that during meiosis the secondary oocyte fuses with a polar body (Verma & Ruttner 1983). This automictic parthenogenesis following meiosis was suggested by Tucker (1958), only through crossing over (Greeff & Villet 1993) could exchange be possible. However, recombination through crossing over is rare (Slobodchikoff & Daly 1971; Moritz & Haberl 1994; Solignac et al. 2001), so that workers and their offspring are almost genetically identical (Kryger 2002).

This thelytokous worker reproduction in *A. m. capensis* has consequences for the average relatedness among colony members. In particular, workers lay female eggs and workers are as related to other workers' daughters as to the queen's daughters. But an individual laying worker is still more related to her own offspring (clonal daughter r = 1) than to the average queen's offspring (r = 0.3, paternity of 10). Therefore, on relatedness grounds

alone one might expect that *A. m. capensis* workers would either not police each other or would do so less effectively than in other *A. mellifera* subspecies (Greeff 1996), because worker's sisters and nieces are related to them by the same degree. So the basis for potential reproductive conflict resulting in arrhenotokous honeybees from the fact that workers are more related to the offspring of their mother than that of other workers is lacking in a subspecies with thelytokous worker reproduction (Fig. 1.3). Indeed, brood is frequently observed in hive boxes located above the queen excluder, a part of the hive in which the queen cannot enter to lay eggs, in queenright Cape honeybee colonies (Pettey 1922; Hepburn & Radloff 1998; Tribe & Allsopp 2001; personal observations) and this brood is actually the female offspring of workers (Moritz et al. 1999). The question arises whether worker policing is expressed in the Cape honeybee or is it lacking. This problem is dealt with in **Chapter 2**. Worker policing could be favoured if it increases the efficiency of the colony. Hence, the occurrence of worker policing could depend on a trade off between the cost of policing and the cost of worker reproduction (Greeff 1996). For example, the colony efficiency could be lowered, if the laying of additional eggs in a cell reduces the overall efficiency of brood rearing. The existence of worker policing in *A. m. capensis* would support the hypothesis that worker reproduction is costly.

Fig. 1.3: Pedigree coefficients of relatedness among members of a Cape honeybee colony when the queen mated with 10 drones (only two are shown) and thelytokous worker reproduction occurs. Arrows indicate the relatedness of one individual X to another individual Y, e.g. the probability that an individual Y is holding genes which are identical to genes in X.



So it may be that worker policing is displayed in the Cape honeybee on colony efficiency grounds alone. The high degree of variation among queenright *A. m. capensis* colonies in terms of successful worker reproduction (Pettey 1922; Hepburn et al. 1991) indicates that the cost of worker policing sometimes extend the benefits of worker policing, but sometimes the benefits outweigh the costs. There is evidence (Pettey 1922; Hepburn et al. 1991) that the intensity of worker policing is much more variable in *A. m. capensis* than in other honeybee subspecies with arrhenotokous worker reproduction, where worker policing is favoured by both relatedness and colony efficiency grounds. So a more fragile ratio of costs and benefits for worker policing could be more easily affected by environmental changes.

Rainfall, wind, humidity and temperature are highly important environmental factors affecting honeybee colonies (Luterbacher 1974) which trigger e.g. foraging (Luterbacher 1974), brood warming (Himmer 1926), nursing behaviour (Blaschon & Crailsheim 2001) and other activities of house bees (Riessberger et al. 1998). Ecological studies of honeybee behaviour must be conducted with bees living under natural conditions (Seeley 1985). This methodological principle takes into account the moulding of honeybee behaviour by natural selection and the often remarkable precise "fit" between bee behaviour and the external environment (Seeley 1985). Thus, it might well be that environmental factors affecting colony performance also have an effect on worker policing. The benefits of worker policing are lower in the Cape honeybee (Greeff 1996), so it could be expected that potential environmental parameters which affect overall colony performance are more readily expressed in the Cape honeybee than in *A. m. scutellata*, a subspecies with arrhenotokous worker reproduction. **Chapter 3** addresses the question whether worker policing in the Cape honeybee is affected by changing environmental conditions and **Appendix 3.1** investigates the same potential environmental effect in *A. m. scutellata*.

The reproductive conflict among honeybees (Ratnieks & Reeve 1992) can be lifted to a higher level if workers are unrelated. After absconding (non-reproductive swarming) natural mergers of unrelated swarms are commonplace in African honeybees (Hepburn & Radloff 1998). Colonies may subsequently merge with each other on tree congregations (up to 55 queenright swarms, Herman 1922; 11 queenright swarms, Hepburn & Whiffler 1988) or with established colonies (Walter 1939; Hepburn 1993; Hepburn & Radloff 1998). Potential reproductive conflict after a merger is higher compared to a colony where all workers are the offspring of one queen, because in the new unit workers which are not related to the queen have an inclusive fitness of zero. These workers could gain direct fitness through reproducing. This potential conflict could result in a behaviour, that they still act as single colonies in terms

of division of labour and spatial distribution, within the new colony. **Chapter 4** addresses the questions of how long after a merger between two unrelated swarms does it take to become a social unit and what are the consequences of the merger on the division of labour. Furthermore, the possible benefits of mergers of unrelated swarms will be discussed from an evolutionary perspective.

The recent transfer of many *A. m. capensis* colonies into the area of *A. m. scutellata* (Allsopp 1992; Johannsmeier 1992; Lear 1992; Allsopp & Crewe 1993) resulted in massive invasions and takeovers of *A. m. scutellata* colonies by *A. m. capensis* laying workers (Allsopp & Crewe 1993). This indicates that *A. m. capensis* workers are facultative social parasites (Velthuis et al. 1990; Hepburn & Allsopp 1994) and that they are able to reproduce in the presence of a queen. Furthermore, the observations of Pettey (1922) and the findings of Moritz et al. (1999) show that *A. m. capensis* workers not only reproduce in colonies of other subspecies (Onions 1912; Guy 1975; Tribe 1981; Moodie 1983; Lundie 1954; Johannsmeier 1983; Koeniger & Wurkner 1992; Woyke 1995) but also in queenright colonies of their own subspecies. So the question arises how workers, especially in the case of the Cape honeybee, achieve successful reproduction despite the presence of the queen?

It has been shown that the pheromones of a queen's mandibular glands have an inhibitory effect on the ovarial development of workers (de Groot & Voogt 1954; Pain 1955; Butler 1959a). Moreover the substances produce by the mandibular glands not only inhibit ovary development, they also trigger many other aspects of colony organisation (Kaminski et al. 1990). These inhibitory effects could either be the result of selection of queen dominance or kin selected interest of workers (see Seeley 1985; Keller & Nonacs 1993; for a discussion on this issue), however worker fecundity clearly depends on the behavioural interactions of workers with the queen (Moritz et al. 2001). Nevertheless, as indicated by frequently observed brood above queen excluders in queenright Cape honeybee colonies (Pettey 1992; Tribe & Allsopp 2001; personal observations) workers are apparently able to evade both queen pheromonal control and possibly worker policing (Chapter 2).

One strategy laying Cape honeybee workers could employ to avoid worker policing or to reduce the effect of worker policing on their eggs is by laying eggs which show more queen-like removal rates, comparable to findings in anarchistic honeybees (Oldroyd & Ratnieks 2000). The term anarchistic honeybees describes the phenomenon of higher laying worker activity and the raising of their offspring to maturity in queenright colonies of the European subspecies of *A. mellifera* (Oldroyd et al. 1994) compared to "normal" European colonies where about 1 male in a 1000 seems to be worker-derived (Visscher 1989). Another

way for laying workers to increase the survival of their eggs arises from the fact that egg removal behaviour is variable (Chapter 3).

Egg removal behaviour is subject to environmental variation leading to periods of low and high levels of egg removal behaviour (Chapter 3). Since worker policing only takes place in the egg stage (Ratnieks & Visscher 1989) worker-laid eggs only need to survive until they become larvae. These three days after oviposition might well fit in a time window of low egg removal during a period of bad weather. However, if that is a strategy of workers to avoid worker policing, workers must be able to discriminate between periods of low and high levels of egg removal and lay their eggs during periods of low egg removal. **Chapter 5** investigates whether workers are able to discriminate between periods of low and high egg removal behaviour, and if workers prefer to lay their eggs during a time window of low level of egg removal behaviour.

Besides the fact that workers have to avoid worker policing behaviour, they also have to evade the inhibitory effects of the queen pheromones. Indeed, some of the workers, so called pseudoqueens, develop their ovaries and emit a queen-like pheromonal signal themselves (Crewe & Velthuis 1980; Hepburn & Crewe 1991) indicating that they escaped the inhibitory influence of the queen. One way to achieve this is by avoiding close proximity to the source of the pheromone, i.e. the queen. Moritz et al. (2001) showed that workers of *A. m. capensis* tend to stay further away from the queen than do workers of *A. m. scutellata*. Workers with a queen-like pheromone bouquet avoid the queen, a behaviour which might reduce the inhibitory effect on ovary development (Moritz et al. 2002). Moreover there is evidence that *A. m. capensis* pseudoqueens are spatially separated (Lattorff et al. 2001) indicating that workers not only avoid the queen, but also other highly developed workers.

In large commercial colonies emergency queen rearing (Swart et al. 2001) and laying worker activity (Hepburn & Radloff 1998) have been reported, which could be the result of an inefficient transfer (Moritz et al. 2001) of the queen pheromone by messenger bees (Velthuis 1972; Seeley 1979). So one way for a worker to achieve successful reproduction is to stay away from the queen; because the suppression of ovarial development caused by the release of pheromones by the queen is reduced the further away the worker is from the queen (Moritz et al. 2002). Another way would be to avoid worker policing. Such a possible strategy is investigated in Chapter 5. Since the spatial distance from the queen has an effect on the ovary development of workers and the fact that the queen substance stabilises the colony, stimulates brood rearing, comb building, hoarding, and foraging (Butler 1954; Butler 1959b; Butler et al. 1961; Butler et al. 1964) it might also influence worker policing behaviour. So if worker

policing behaviour decreases with increasing distance from the queen, workers which stay away from the queen not only put themselves in the position to possibly develop their ovaries (Neumann & Hepburn 2002) and pheromones (Moritz et al. 2002), they also increase the chances of the survival of their eggs because these eggs, laid further away from the queen, would be less exposed to well expressed egg removal behaviour by other workers. So if worker policing is less well expressed further away from the queen one alternative strategy (to the strategy investigated in Chapter 6) would be for workers to avoid the queen and lay their eggs further away from her. In **Chapter 6** I will investigate the effects of distance from the queen on egg removal behaviour and if workers prefer to lay their eggs further away from the queen.

The unique traits of the Cape honeybee, such as higher numbers of pseudoqueens than in colonies of European subspecies of *A. mellifera* (Hepburn & Allsopp 1994), thelytokous worker reproduction (Onions 1912), the rapid development of ovaries and a queen-like pheromonal bouquet (Hemmling et al. 1979) especially after queen loss (Wossler 2002), the parasitic behaviour of some pseudoqueens (Onions 1912; Neumann et al. 2001; Neumann & Hepburn 2002) and the recent transfer of a high number of *A. m. capensis* colonies into the native distribution of *A. m. scutellata* (Allsopp 1995) inevitably set-up conditions for reproductive conflict between colonies of two subspecies of *A. mellifera*. Moreover, besides the usurpation of numerous colonies of *A. m. scutellata* (Hepburn & Allsopp 1994) it also lead to reproductive conflict among these parasitic Cape honeybee workers. It has been shown that the *A. m. capensis* population in the area of *A. m. scutellata* is genetically identical (Solignac et al. 2001; Kryger 2002), possibly originating from a single laying *A. m. capensis* worker (Kryger 2002).

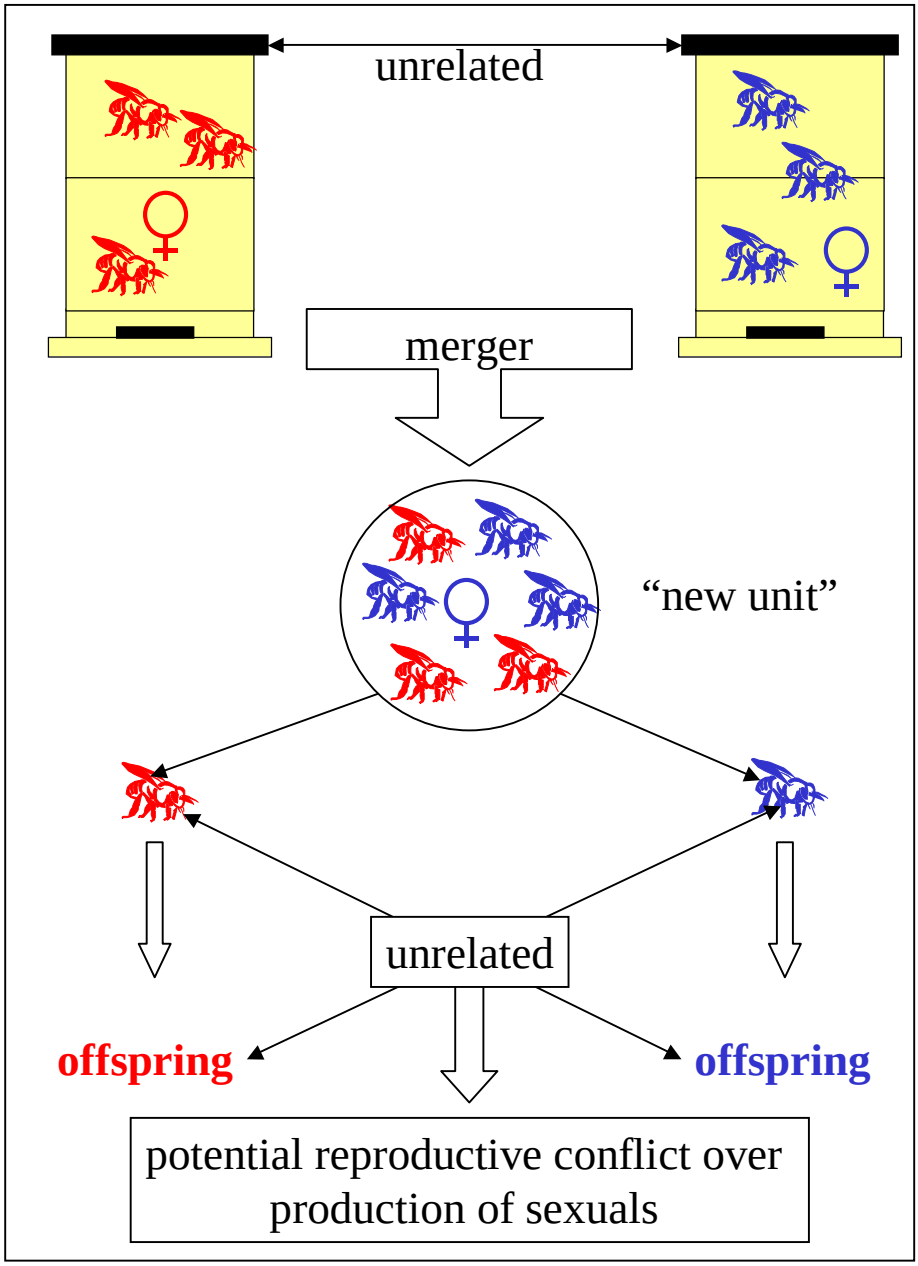
Since Cape honeybee workers reproduce thelytokously (Onions 1912) and crossing over is rare (Moritz & Haberl 1994), this *A.m. capensis* trait in the *A. m. scutellata* area is not only the result of reproductive competition among different colonies, nor among subfamilies or patrilines, it is the result of reproductive competition among different genotypes. The multiple infestations due to migratory beekeepers (Lundie 1954; Johannsmeier 1983; Allsopp 1995) combined with a series of traits of *A. m. capensis* that reflect important behavioural, physiological and genetic pre-adaptations for intraspecific social parasitism (Neumann & Hepburn 2002) sets the conditions for reproductive competition among different genotypes. The short-sighted selection hypothesis for parasite virulence predicts that winners of within-host competition (resulting from multiple infestations) are poorer at transmission to new hosts (Schmid-Hempel 1998). **Chapter 7** will investigate whether there are evidences within the *A.*

m. capensis population in northern South Africa for the short-sighted selection hypothesis (Schmid-Hempel 1998) which has been suggested to occur in HIV, polio and bacterial meningitis in man (Schmid-Hempel 1998).

In general the ability to recognise nestmates and non-nestmates is essential to maintain colony integrity (Crozier & Pamilo 1996) and not only in a population where natural mergers are common (Hepburn & Radloff 1998) or in populations where traits of parasitic honeybee workers occurs, as in *A. m. capensis* populations (Allsopp & Crewe 1993; Hepburn & Allsopp 1994; Neumann et al. 2001; Neumann & Hepburn 2002). The ability to recognise eggs of non-nestmates, queen or worker-laid, would play a major role in the reproductive competition among unrelated workers. The recognition ability would not only be necessary in terms of a host defence mechanism against parasitic honeybees (Neumann & Hepburn 2002), but could also be used after a merger (Chapter 4; Hepburn & Radloff 1998) to recognise unrelated eggs and remove them. After a merger (Chapter 4) it does not matter whether the workers are related to the queen or not, if worker reproduction takes place at least one part of the colony members are unrelated to this offspring (Fig. 1.4).

So if workers are able to recognise unrelated eggs and remove them, that would on average increase the proportions of their own genes within the offspring. In the case of arrhenotokous worker reproduction, workers could compete over the production of drones (Crozier & Pamilo 1996); whereas if they reproduce thelytokously, they compete over the reproduction of females (queens and workers). Indeed, in the context of queen rearing it has been shown that honeybee workers can discriminate between unrelated (alien) and related (native) queen-laid eggs (Visscher 1986). Moreover, there is evidence for a queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995), which should enable workers to discriminate between queen and worker-laid eggs and so reduce recognition errors during the egg removal which could result in the removal of queen eggs and the non-removal of worker-laid eggs (Reeve 1989).

Fig. 1.4: Schematic drawing of the basis for potential reproductive conflicts among workers after a natural merger.



The cues of this putative queen pheromone are most likely to be a product of the Dufour´s gland (Ratnieks 1995; but see Katzav-Gozansky et al. 2001). Dufour´s gland is diverse in its biosynthetic capabilities and the secretion comprises a multi-component blend (Katzav-Gozansky et al. 1997). One of the postulated functions in non-apis bees is to chemically mark the precise location of the nest. Because the secretion of the Dufour´s gland include hydrocarbons (Katzav-Gozansky et al. 2000), it may also secret colony specific components or nestmate recognition cues as well (Breed 1998). The Dufour´s gland opens into the dorsal vaginal wall (Billen 1987) so that the eggs come into contact with the secretion

probably during the oviposition process. So on the surface of eggs cues occur which encode for the maternity of the eggs (queen or worker-laid, Ratnieks 1995) and for the origin of the eggs (colony specific cues, Breed 1998) laid either by a nestmate (native) or a non-nestmate (alien). Several policing studies (Ratnieks & Visscher 1989; Ratnieks 1995; Oldroyd & Ratnieks 2000; Halling et al. 2001; among others) used partly or exclusively eggs laid by alien queens and workers to evaluate egg removal behaviour. To interpret egg removal behaviour in a more detailed way it is important to know what proportion of the level of egg removal is due to the method (the transfer of the eggs), nestmate recognition cues and cues of a postulated queen pheromone. Otherwise it may be that the difference in the removal rates of queen-laid and worker-laid eggs either over or underestimate the real levels of egg removal. **Chapter 8** investigates the effects of nestmate recognition of eggs on egg removal behaviour in the Cape honeybee and *A. m. scutellata*. Moreover, it will distinguish how many eggs are removed because of the method, colony specific cues and caste specific cues.

Chapter 2

Cape honeybees police worker-laid eggs despite the absence of relatedness benefits

Abstract In the Cape honeybee, *Apis mellifera capensis*, workers lay diploid (female) eggs via thelytoky. In other *Apis* species and other *A. mellifera* subspecies workers lay haploid (male) eggs via arrhenotoky. When thelytokous worker reproduction occurs, worker policing has no relatedness benefit because workers are equally related to their sister workers' clonal offspring and their mother queen's female offspring. We studied worker policing in *A. m. capensis* and in the arrhenotokous African honeybee *A. m. scutellata* by quantifying the removal rates of worker-laid and queen-laid eggs of both subspecies. Discriminator colonies of both subspecies policed worker-laid eggs of both their own and the other subspecies. Because there is no relatedness benefit to worker policing in *A. m. capensis*, the occurrence of worker policing in this subspecies strongly suggests that worker reproduction is costly and that policing is maintained because it enhances colony efficiency. In addition, because both subspecies policed each others eggs it is probable that the mechanism used in thelytokous *A. m. capensis* to discriminate between queen-laid and worker-laid eggs is the same as in arrhenotokous *A. m. scutellata*.

2.1. Introduction

Inclusive fitness theory (Hamilton 1964) has been successful in explaining and predicting social behaviour (Jarvis 1981; Bourke & Franks 1995; Crozier & Pamilo 1996; Ratnieks et al. 2001). Hamilton's rule, Br > C, shows the condition under which a social action is favoured in terms of the benefit to the recipient, the cost to the actor, and their genetic relatedness. Genetic relatedness is a key parameter and can now be measured with comparative ease and accuracy (Queller et al. 1993; Crozier et al. 1997; Pamilo et al. 1997; Ross 2001). Many empirical studies confirm the importance of genetic relatedness in social evolution (e.g. Sundström 1994; Foster & Ratnieks 2000; among others). However, it is unlikely that any comparable technological breakthrough will facilitate the measurement of costs and benefits (Ratnieks et al. 2001). One solution to investigating the importance of costs and benefits in social evolution is to chose study systems in which relatedness is held constant but costs and benefits vary. Worker reproduction and worker policing in the Cape honeybee, *Apis mellifera capensis*, is one such system.

In most eusocial Hymenoptera workers cannot mate but retain ovaries and can lay eggs (Ratnieks 1988; Bourke & Franks 1995; Crozier & Pamilo 1996). Unfertilised worker-laid eggs are normally haploid (arrhenotoky, Crozier 1975; Crozier & Pamilo 1996) and develop into males if reared. However, in a few species (currently 6 ants and *A. m. capensis* are known; Wenseleers & Billen 2000) workers lay unfertilised diploid eggs which develop into females (thelytoky, Crozier & Pamilo 1996; see Mackensen 1943 and Tucker 1958 for rare cases of thelytoky in other subspecies of *A. mellifera*). *A. m. capensis* is native to the fynbos region in the Western and Eastern Cape provinces of South Africa (Hepburn & Jacot Guillarmod 1991). Thelytokous reproduction by Cape honeybee workers has been long been established (Onions 1912, reviewed by Hepburn & Crewe 1991 and Hepburn & Radloff 1998) and many of the genetic details are also understood (Verma & Ruttner 1983; Moritz & Haberl 1994; Greeff 1996; Solignac et al. 2001). Although the thelytoky is via automictic parthenogenesis following meiosis (Verma & Ruttner 1983), recombination through crossing over is rare (Moritz & Haberl 1994). Thus, workers' offspring are almost genetically identical.

Workers' sons are seldom reared in queenright European honeybee colonies (Visscher 1989; Visscher 1996). This is because few workers have active ovaries (Ratnieks 1993) and the eggs they lay are eliminated by worker policing (Ratnieks 1988). Honeybee workers in queenright colonies eat eggs laid by other workers (Ratnieks & Visscher 1989; Ratnieks 1993;

Visscher 1996). Queen-laid and worker-laid eggs are probably discriminated by means of a queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995).

Worker policing is selected for in a population of arrhenotokous social Hymenoptera on relatedness grounds alone when each colony has a single queen mated to more than two males (Ratnieks 1988; Foster & Ratnieks 2001a). *Apis mellifera* queens are typically mated to 5-30 males (Estoup et al. 1994; Fuchs & Moritz 1999; Neumann et al. 1999a; Neumann & Moritz 2000; Palmer & Oldroyd 2000). As a result, worker policing is beneficial on relatedness grounds because workers are on average less related to other workers' sons (nephews r = 0.15, for an effective paternity of 10) than to the queen's sons (brothers r = 0.25, Fig. 1.1). However, in *A. m. capensis* the situation changes significantly (Fig. 1.3). In particular, workers lay female eggs and are as related to other workers' daughters as to the queen's daughters. But an individual laying worker is still more related to her own offspring (clonal daughter r = 1) than to the average queen's offspring (r = 0.3, assuming an effective paternity frequency of 10). Therefore, on relatedness grounds alone one might expect that *A. m. capensis* workers would either not police one another or would do so less effectively than in other *A. mellifera* subspecies (Greeff 1996). Indeed, brood is frequently observed in hive boxes located above the queen excluder, a part of the hive in which the queen cannot enter to lay eggs, in queenright Cape honeybee colonies (Pettey 1922; Hepburn & Radloff 1998; personal observations). Moritz et al. (1999) have shown that this brood is the female offspring of workers.

Hamilton's rule for worker policing in *A. m. capensis* can be written as follows:

$$Br_q > Cr_w$$

$$B/C > r_w / r_q$$

$$B/C > 1$$

where r_q and r_w are the relatedness of police workers to the queen's and other workers' female offspring, and B and C are the relative efficiencies (i.e. total reproduction) of colonies with and without worker policing. Clearly, worker policing is favoured if it increases the efficiency of the colony. Colony efficiency could be lowered, for example, if the laying of additional eggs in a cell reduces the overall efficiency of brood rearing. The existence of worker policing in *A. m. capensis* would support the hypothesis that worker reproduction is costly.

In this study we investigated whether worker policing occurs in *A. m. capensis* by quantifying the removal rates of worker-laid and queen-laid eggs of both *A. m. capensis* and of the neighbouring arrhenotokous subspecies *A. m. scutellata* in queenright discriminator

colonies of both subspecies. The results show that both *A. m. capensis* and *A. m. scutellata* police their own as well as the other subspecies' worker-laid eggs.

2.2. Methods

2.2.1. Sampling colonies and experimental design

Queenright study colonies of *A. m. capensis* were obtained near Port Elizabeth, within their native range in the Eastern Cape province in southern South Africa. Queenright *A. m. scutellata* colonies were from the Pretoria area, within their native range. These localities were chosen because morphometrically and physiologically pure *A. m. capensis* and *A. m. scutellata* occur there. (Hepburn & Radloff 1998; Hepburn et al. 1998; and Hepburn & Radloff 2002 reviewed the distribution and biology of these two subspecies.)

The colonies were placed in two study apiaries in Grahamstown, South Africa. The *A. m. scutellata* apiary was set apart, >1km, from any other bee hives to minimize intersubspecific drifting and/or dispersing (Neumann et al. 2000a; Neumann et al. 2001), which may result in social parasitism by thelytokous laying workers (Hepburn & Allsopp 1994; Beekman et al. 2000; Neumann et al. 2001). Both *A. m. capensis* and *A. m. scutellata* colonies were studied in order to compare between African subspecies with arrhenotokous and thelytokous worker reproduction (Hastings 1989; Hepburn & Crewe 1991; Neumann et al. 2000b). The experimental set-up followed standard methods for investigating worker policing via egg removal (Ratnieks & Visscher 1989; Ratnieks 1995; Oldroyd & Ratnieks 2000). We used colonies of both subspecies as discriminator and egg-source colonies. The discriminator and the source colonies for queen-laid eggs were headed by the same queens throughout the experiment. The source colonies for worker-laid eggs were made queenless two weeks before egg-removal trials started. All colonies were housed in two-box hives with a queen excluder between the boxes.

2.2.2. Quantifying egg-removal rates

In February 2000 we compared the removal rates of queen-laid and worker-laid eggs of both subspecies on the same five sequential days**.** We used haploid, male, eggs laid by *A. m. scutellata* queens and workers and diploid, female, eggs laid by *A. m. capensis* queens and workers. The difference in the sex of the eggs studied does not interfere with the results, because our primary aim was to compare the removal rates of queen-laid and worker-laid eggs within each subspecies. For both the *A. m. capensis* and *A. m. scutellata* discriminator

colonies we used test frames with both drone and worker cells, because *A. m. capensis* workers naturally lay diploid eggs mainly in worker cells (Neumann et al. 2000b) and workers of arrhenotokous subspecies lay eggs mainly in drone cells (Page & Erickson 1988; Ratnieks 1993). The drone cells were used for male eggs laid by *A. m. scutellata* workers and *A. m. scutellata* queens. The worker cells were used for female eggs laid by *A. m. capensis* workers and *A. m. capensis* queens. Following standard procedures (Ratnieks & Visscher 1989; Ratnieks 1995; Oldroyd & Ratnieks 2000) the test frames were placed above the queen excluder in each queenright discriminator colony and sandwiched between two frames containing brood of all ages (eggs, larvae and pupae). The other frames in this upper box contained a mixture of empty cells, honey and pollen. For each discriminator colony (N=2 for *A. m. scutellata* and N=3 for *A. m. capensis*) we used a single test comb which was initially placed into the hive two days before egg removal trials began.

Eggs were removed from the source colonies using special forceps (Taber 1961) and introduced into the discriminator colonies following standard methods (Ratnieks & Visscher 1989; Ratnieks 1995; Oldroyd & Ratnieks 2000). Queen-laid eggs were obtained from below the excluder in the queenright source colonies (N=1 for *A. m. scutellata* and N=3 for *A. m. capensis*). Because *A. mellifera* queens only lay fertilized eggs in worker cells and unfertilised eggs in drone cells (Ratnieks & Keller 1998), we were able to obtain male eggs from drone cells in the queenright *A. m. scutellata* colonies and female eggs from worker cells in the queenright *A. m. capensis* colonies. Worker-laid eggs were obtained from the queenless colonies (N=1 for *A. m. scutellata* and N=2 for *A. m. capensis*). After the eggs had been transferred, each test comb was placed into its discriminator colony and then briefly removed after 2, 4 and 24 hours to determine which eggs were still present. We compared the removal rates of worker-laid and queen-laid eggs both within and between subspecies using Kruskal-Wallis ANOVAs and Mann Whitney U-tests (with Bonferroni adjusted levels of significance) using Statistica. The relevant raw is shown in Appendix 2.1.

2.3. Results

The time elapse between the transfer of eggs and their removal was determined for 2480 eggs. The percentage of remaining eggs per egg source in the two subspecies of discriminator colonies are shown in Fig. 2.1 and Fig. 2.2. The results of the Mann-Whitney U-tests are shown in Table 2.1. *A. m. capensis* and *A. m. scutellata* discriminator colonies removed worker-laid eggs of their own subspecies significantly faster than queen-laid eggs of their own

subspecies. Likewise, in both subspecies of discriminator colonies, worker-laid eggs of the other subspecies were removed significantly faster than queen-laid eggs of the other subspecies. Worker-laid eggs of *A. m. scutellata* were removed significantly faster in *A. m. capensis* discriminator colonies than in *A. m. scutellata* colonies. However, *A. m. capensis* worker-laid eggs were not removed significantly faster in the *A. m. scutellata* than in *A. m. capensis* discriminator colonies. Finally, there were no significant differences among the individual discriminator colonies of both subspecies in the removal rates of worker-laid eggs of their own subspecies after 24 hours (*A. m. scutellata*: Mann Whitney U-test, U=5, ns.; *A. m. capensis*: Kruskal-Wallis ANOVA, H=3.42, n.s.). Mann Whitney U-test was used in case of the *A. m. scutellata* discriminator colonies and not a Kruskal-Wallis ANOVA because of the number of *A. m. scutellata* discriminator colonies (N = 2).

2.4. Discussion

The data clearly show that queenright *A. m. capensis* and *A. m. scutellata* colonies police both their own worker-laid eggs as well as those of the other subspecies. While *A. m. capensis* discriminator colonies removed worker-laid eggs of *A. m. scutellata* faster than *A. m. scutellata* colonies, *A. m. capensis* worker-laid eggs were not removed faster in *A. m. scutellata* discriminator colonies. Within each subspecies, there were no significant differences among discriminator colonies.

Our data suggests that the two African subspecies are very similar in their general behavioural pattern to European bees, i.e. worker-laid eggs are less acceptable than queen-laid eggs, but there are some differences. In the *A. m. scutellata* discriminator colonies substantially more worker-laid eggs of both *A. m. scutellata* and *A. m. capensis* remained after 24 hours (20%) compared to colonies of European subspecies (1%, Ratnieks & Visscher 1989) and *A. m. capensis* (1% of *A. m. scutellata* worker-laid eggs and 10% of *A. m. capensis* worker-laid eggs). Thus, it is possible that either policing is lower, or worker-laid eggs are more acceptable in African than in European subspecies. Moreover, less queen-laid eggs remained in the *A. m. capensis* colonies after 24 hours (20%) compared to earlier studies on European honeybees (45%, Ratnieks & Visscher 1989) and to *A. m. scutellata* (40%). This may be due to nestmate recognition of queen-laid eggs, which is well expressed in African honeybee subspecies (Chapter 9).

Our observations of effective worker policing in the Cape honeybee is contrary to theory which shows no benefit of worker policing in this subspecies (Greeff 1996). But this

prediction is based on relatedness grounds alone (Greeff 1996). Our observation of policing is also partly in contrast to the other existing empirical study (Moritz et al. 1999). However, in this study, even if most worker-laid eggs were removed some may have remained and it would have been these which were detected using DNA microsatellites (Moritz et al. 1999). Thus, Moritz et al. (1999) showed that some workers' eggs are reared but they did not show that worker policing is absent. The level of egg-removal observed in this study are in accordance with our casual observations of the study colonies which showed that no larvae were being reared above the queen excluders in the test colonies immediately prior to or during the experimental period. Since the presence of brood above the queen excluder occurs frequently in Cape honeybees (Pettey 1922; Tribe & Allsopp 2001; personal observations), it was quite possible that worker policing would prove to be less effective than we actually observed. The combination of Moritz et al. (1999) with our data indicates that effective worker policing does occur in *A. m. capensis* but is sometimes absent or not fully effective in preventing worker reproduction. Indeed, there is considerable phenotypic variation for this trait in *A. m. capensis* populations. While some colonies of *A. m. capensis* may show worker-derived brood above the excluder, others in the same apiary do not (Pettey 1922; personal observations). Given that variation among *A. m. capensis* workers for other reproductive traits has already been shown (Hillesheim et al. 1989; Hepburn et al. 1991; Hepburn 1994), this is perhaps not surprising.

Why does worker policing still occur in *A. m. capensis*? Earlier theoretical studies (Greeff 1996) may have missed a critical piece in the cost benefit analysis for policing in the Cape honeybee. The intensity of policing should be dependent on a trade off between the cost of policing and the cost of worker reproduction to overall colony efficiency and reproduction. Given that policing probably costs very little, because eggs are held in open cells which workers are regularly checking anyway, the costs derived from unhindered worker reproduction might easily be higher than the costs of policing. Thus, a large efficiency gain is not needed to favour worker policing. In the *A. m. capensis* situation the gain need only be marginal. Even if a queen is single mated, so that policing of worker-laid eggs has a relatedness cost, policing is still favoured and colony efficiency increases by 20% (Ratnieks 1988). A recent theoretical study (Foster & Ratnieks 2001a) shows that worker policing can be even more easily selected for at a mating frequency of one as part of a sex allocation biasing strategy of workers. That is, workers want to eliminate males thus creating a female biased sex ratio, and the only eggs they know to be male are workers' sons.

What are the possible costs of worker reproduction in honeybees? There are two different potential cost issues involved in worker reproduction in queenright colonies:

1. Reduced brood rearing efficiency: Honeybee nests have a limited brood rearing area which constrains the number of eggs laid by the queen, given that a queen typically will not lay an egg in a cell already containing an egg (Ratnieks 1990). Worker egg laying, when abundant, is characterized by multiple eggs per cell. A worker will lay an egg in a cell that already contains an egg (Gary 2000; Tribe & Allsopp 2001, Fig. 9.1), with the earlier eggs frequently being crushed or knocked down by the abdomen of the laying worker in the process. Only one larva can be reared to adulthood in a single cell and additional larvae are eaten by workers leading to costs associated with cannibalism (Elgar & Crespi 1992). Thus, when many workers lay eggs it may simply take longer for any cell to yield a worker. If it took just 1 additional day to rear a worker per cell this would lead to a 5% reduction in the maximal rate of colony build up, given an egg-adult stage of 19 days in workers of the Cape honeybee (Hepburn & Radloff 1998).

2. Work rate of laying workers: Laying Cape honeybee workers in queenless and queenright groups do not participate as much in hive duties, such as brood rearing, compared to subordinate workers (Moritz & Hillesheim 1985; Hillesheim et al. 1989). Thus, a high frequency of laying workers may also reduce colony productivity (Hillesheim et al. 1989). This aspect may be less relevant because worker policing by oophagy does not directly stop or penalize egg-laying workers. It may cause a reduction in worker egg-laying over evolutionary time, but for it to be selected for there has to be an immediate benefit in the colony with policing. Reproductive dominance seems to be strongly genetically determined (Moritz & Hillesheim 1985; Moritz et al. 1996). Therefore, almost clonal (Moritz & Haberl 1994; Solignac et al. 2001) laying worker offspring are predisposed to develop into laying workers. Thus, worker policing via oophagy may limit the establishment of such laying worker matrilines in queenright colonies, constituting an immediate benefit for policing colonies. Alternatively, but not mutually exclusive, worker-worker aggression in queenright colonies, which is directed towards nestmates with developed ovaries (Visscher & Dukas 1995), might also restrict the establishment of such laying worker matrilines.

There is considerable variation in egg-laying behaviour by Cape honeybee workers (Allsopp 1995). While in some laying worker colonies the brood nest is virtually indistinguishable from that of a queen (because a single egg is laid per cell; Tribe 1981, personal observations), others show the typical pattern of multiple eggs per cell as the colonies in our study (personal observations). This suggests that it is possible in Cape

honeybees to have a low level of worker reproduction, which does not interfere with brood rearing. Thus, the costs need not be high for a colony if the amount of worker reproduction is low.

Our data are a clear indication that worker policing can be selected for to reduce the efficiency costs of worker reproduction. The study shows this because in Cape bees there are no relatedness costs or benefits. Also in the common wasp, *Vespula vulgaris*, relatedness alone cannot explain selection for worker policing (Foster & Ratnieks 2001b) because paternity is close to 2, at which workers are equally related to queen's sons and other workers' sons. The occurrence of worker policing in *V. vulgaris* also suggests, that worker policing is selected for due to other factors, such as the colony-level benefits of reducing reproductive conflict (Ratnieks 1988; Frank 1995; Keller 1999). In both wasps and honeybees there is evidence of policing when there is no relatedness benefit (Cape honeybees, present study; *V. vulgaris*, Foster & Ratnieks 2001b). In wasps, policing also occurs when there is a relatedness cost (*Vespa crabro*, Foster & Ratnieks 2002) and the occurrence of policing also strongly suggests costs of worker reproduction (Foster & Ratnieks 2001), but further study is needed to identify the nature of these costs, and the colony-efficiency benefits of worker policing.

The Cape honeybee is a particularly convincing example. Thelytoky causes a change in kin structure, rendering relatedness neutral with respect to worker reproduction (see above, Fig. 1.3). Thus, if worker policing were not beneficial in *A. m. capensis* it should be evolutionarily lost. That is, workers should accept eggs laid by other workers. Loss of policing would be a simple adaptation. In fact, loss of policing occurs commonly in queenless *A. mellifera* colonies (Miller & Ratnieks 2001), which have failed to rear an emergency replacement queen. Worker-laid eggs are accepted and reared into a final cohort of males before the colony dwindles in population and dies (Page & Erickson 1988). The results also show that both races are able to police worker-laid eggs of the other subspecies, indicating that the same underlying mechanism is used for worker policing. Arrhenotoky (Ruttner 1992), multiple paternity (Neumann & Moritz 2000; Palmer & Oldroyd 2000) and worker policing (*A. mellifera*, Ratnieks & Visscher 1989; *A. florea*, Halling et al. 2001; *A. cerana*, Oldroyd et al. 2001) appear to be ancestral in *Apis*, indicating that thelytoky is a derived condition in *A. m. capensis* and arose in a clade in which worker policing occurred.

In conclusion, we hypothesize that worker policing still exists in the Cape honeybee due to colony efficiency grounds. This illustrates that relatedness grounds alone cannot always predict the reproductive characteristics of insect societies. The application of the inclusive fitness theory requires knowledge of costs, benefits and relatedness.

Table 2.1: Removal rates of eggs from different source colonies after 24 hours in the two subspecies of discriminator colonies. In all cases source and discriminator colonies were not identical. The results of the Mann Whitney U-Tests are shown. The Bonferroni adjusted level of significance is p = 0.025 (C = *A. m. capensis*, S = *A. m. scutellata*, + = queenright, - = queenless, n.s. = not significant).

Type of comparison	Egg source	Discriminator colonies	Trend	Egg source	Discriminator colonies	U-value	p-value
worker-laid vs. queen-laid	C-	C	Faster	C+	C	46	0.007
	S-	S	Faster	S+	S	19.5	0.021
	S+	C	Slower	S-	C	28.5	<0.001
	C+	S	Slower	C-	S	17	0.012
worker-laid vs. worker-laid	S-	S	Slower	S-	C	12	<0.001
	C-	C	Faster	C-	S	63	n.s.

Fig. 2.1: Number of remaining queen-laid and worker-laid eggs of *A. m. capensis* and *A. m. scutellata* after 0, 2, 4 and 24 hours in queenright *A. m. capensis* discriminator colonies. The mean number of remaining eggs [%] and standard deviations are shown.

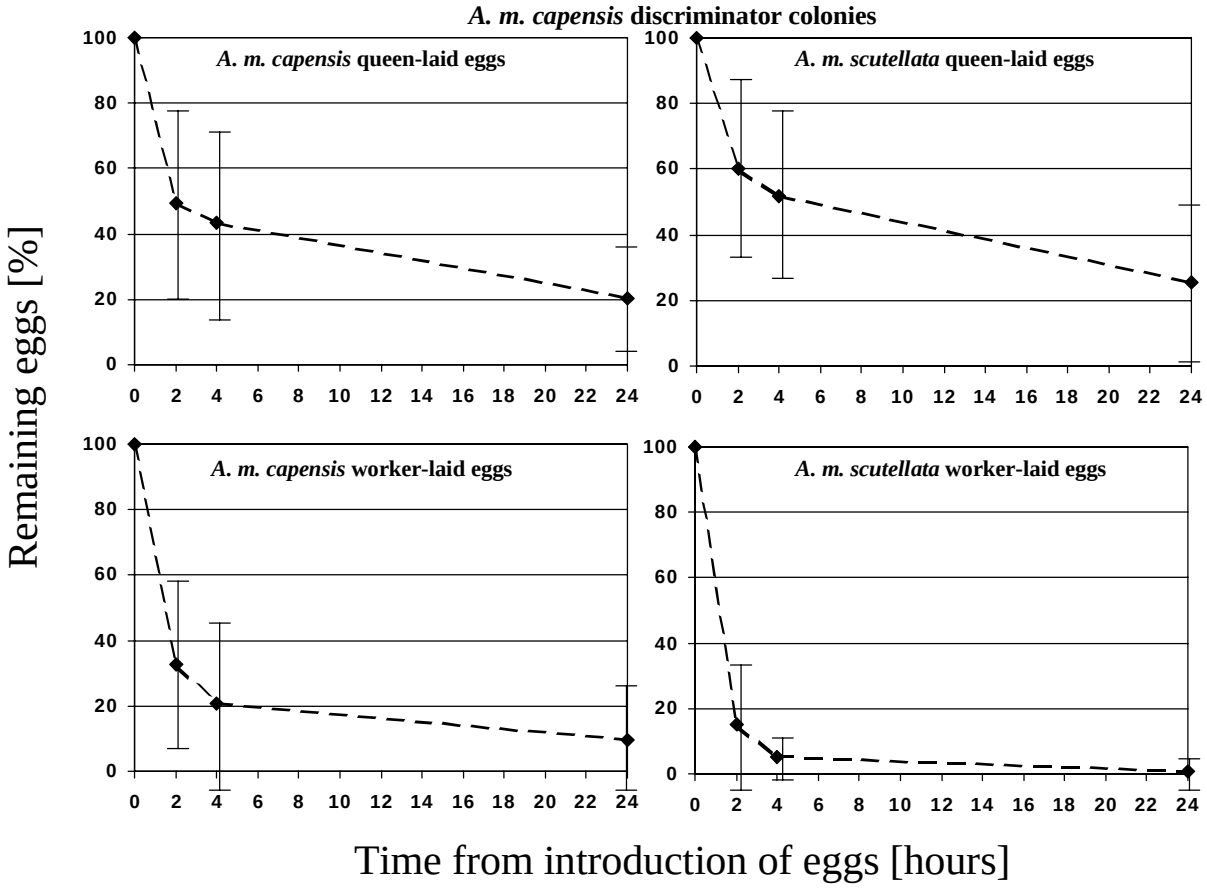
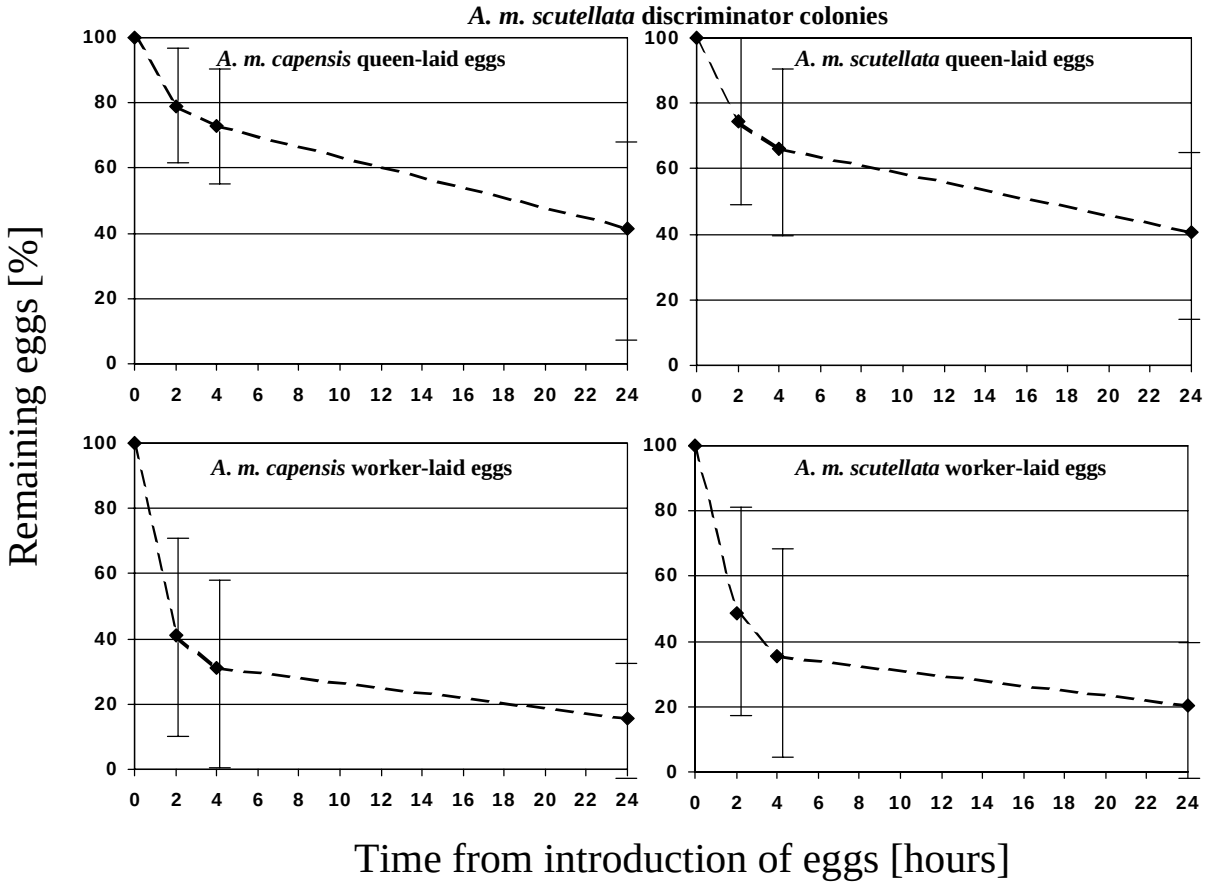


Fig. 2.2: Number of remaining queen-laid and worker-laid eggs of *A. m. capensis* and *A. m. scutellata* after 0, 2, 4 and 24 hours in queenright *A. m. scutellata* discriminator colonies. The mean number of remaining eggs [%] and standard deviations are shown.



Appendix 2

Appendix 2.1: Egg removal data after 2, 4, and the next day for the *A. m. capensis* and *A. m. scutellata* discriminator colonies.

Experimental day	Discriminator Colony	Type of egg	0 hours	2 hours	4 hours	Next day
1	*capensis* 5	Queen *capensis*	20	14	10	4
1	*capensis* 5	Queen *scutellata*	20	14	14	3
1	*capensis* 5	Worker *capensis*	20	8	6	4
1	*capensis* 5	Worker *scutellata*	20	9	1	0
1	*capensis* 6	Queen *capensis*	20	3	2	2
1	*capensis* 6	Queen *scutellata*	20	0	0	0
1	*capensis* 6	Worker *capensis*	20	6	5	0
1	*capensis* 6	Worker *scutellata*	20	3	1	0
1	*capensis* 7	Queen *capensis*	20	5	5	4
1	*capensis* 7	Queen *scutellata*	20	10	6	4
1	*capensis* 7	Worker *capensis*	20	5	4	2
1	*capensis* 7	Worker *scutellata*	20	1	0	0
1	*scutellata* 1	Queen *capensis*	20	17	16	7
1	*scutellata* 1	Queen *scutellata*	20	17	13	7
1	*scutellata* 1	Worker *capensis*	20	13	10	0
1	*scutellata* 1	Worker *scutellata*	20	14	15	5
1	*scutellata* 2	Queen *capensis*	20	13	13	9
1	*scutellata* 2	Queen *scutellata*	20	17	14	7
1	*scutellata* 2	Worker *capensis*	20	6	1	0
1	*scutellata* 2	Worker *scutellata*	20	10	2	2
2	*capensis* 5	Queen *capensis*	20	16	15	4
2	*capensis* 5	Queen *scutellata*	20	17	17	9
2	*capensis* 5	Worker *capensis*	20	10	2	0
2	*capensis* 5	Worker *scutellata*	20	1	0	0
2	*capensis* 6	Queen *capensis*	20	8	7	2
2	*capensis* 6	Queen *scutellata*	20	17	7	5
2	*capensis* 6	Worker *capensis*	20	9	4	0
2	*capensis* 6	Worker *scutellata*	20	0	0	0
2	*capensis* 7	Queen *capensis*	20	13	11	10
2	*capensis* 7	Queen *scutellata*	20	9	5	0
2	*capensis* 7	Worker *capensis*	20	13	6	0
2	*capensis* 7	Worker *scutellata*	20	0	0	0
2	*scutellata* 1	Queen *capensis*	20	8	8	7
2	*scutellata* 1	Queen *scutellata*	20	8	8	8
2	*scutellata* 1	Worker *capensis*	20	10	9	6
2	*scutellata* 1	Worker *scutellata*	20	6	4	4
2	*scutellata* 2	Queen *capensis*	20	20	20	14
2	*scutellata* 2	Queen *scutellata*	20	8	7	7
2	*scutellata* 2	Worker *capensis*	20	2	0	0
2	*scutellata* 2	Worker *scutellata*	20	4	3	1
3	*capensis* 5	Queen *capensis*	20	14	13	9
3	*capensis* 5	Queen *scutellata*	20	17	16	12
3	*capensis* 5	Worker *capensis*	20	3	2	2
3	*capensis* 5	Worker *scutellata*	20	2	0	0
3	*capensis* 6	Queen *capensis*	20	7	7	7
3	*capensis* 6	Queen *scutellata*	20	10	6	4
3	*capensis* 6	Worker *capensis*	20	0	0	0
3	*capensis* 6	Worker *scutellata*	20	2	1	1
3	*capensis* 7	Queen *capensis*	20	7	5	1
3	*capensis* 7	Queen *scutellata*	20	7	3	0
3	*capensis* 7	Worker *capensis*	20	13	12	1

Appendix 2.1 continues

Experimental day	Discriminator Colony	Type of egg	0 hours	2 hours	4 hours	Next day
3	*capensis* 7	Worker *scutellata*	20	6	2	0
3	*scutellata* 1	Queen *capensis*	20	20	19	18
3	*scutellata* 1	Queen *scutellata*	20	20	18	13
3	*scutellata* 1	Worker *capensis*	20	19	17	6
3	*scutellata* 1	Worker *scutellata*	20	19	17	6
3	*scutellata* 2	Queen *capensis*	20	17	15	6
3	*scutellata* 2	Queen *scutellata*	20	9	9	7
3	*scutellata* 2	Worker *capensis*	20	0	0	0
3	*scutellata* 2	Worker *scutellata*	20	0	0	9
4	*capensis* 5	Queen *capensis*	20	11	9	1
4	*capensis* 5	Queen *scutellata*	20	16	16	4
4	*capensis* 5	Worker *capensis*	20	4	3	0
4	*capensis* 5	Worker *scutellata*	20	9	2	0
4	*capensis* 6	Queen *capensis*	20	13	12	5
4	*capensis* 6	Queen *scutellata*	20	16	16	14
4	*capensis* 6	Worker *capensis*	20	8	4	0
4	*capensis* 6	Worker *scutellata*	20	4	2	1
4	*capensis* 7	Queen *capensis*	20	10	9	9
4	*capensis* 7	Queen *scutellata*	20	14	14	1
4	*capensis* 7	Worker *capensis*	20	0	0	0
4	*capensis* 7	Worker *scutellata*	20	5	3	0
4	*scutellata* 1	Queen *capensis*	20	18	18	8
4	*scutellata* 1	Queen *scutellata*	20	19	16	10
4	*scutellata* 1	Worker *capensis*	20	14	11	8
4	*scutellata* 1	Worker *scutellata*	20	17	15	11
4	*scutellata* 2	Queen *capensis*	20	13	11	1
4	*scutellata* 2	Queen *scutellata*	20	17	17	2
4	*scutellata* 2	Worker *capensis*	20	10	8	5
4	*scutellata* 2	Worker *scutellata*	20	10	7	1
5	*capensis* 5	Queen *capensis*	20	18	18	6
5	*capensis* 5	Queen *scutellata*	20	14	14	3
5	*capensis* 5	Worker *capensis*	20	5	4	4
5	*capensis* 5	Worker *scutellata*	20	3	2	0
5	*capensis* 6	Queen *capensis*	20	9	7	3
5	*capensis* 6	Queen *scutellata*	20	15	11	7
5	*capensis* 6	Worker *capensis*	20	9	8	8
5	*capensis* 6	Worker *scutellata*	20	1	1	1
5	*capensis* 7	Queen *capensis*	20	5	2	1
5	*capensis* 7	Queen *scutellata*	20	16	16	10
5	*capensis* 7	Worker *capensis*	20	5	5	5
5	*capensis* 7	Worker *scutellata*	20	0	0	0
5	*scutellata* 1	Queen *capensis*	20	15	13	13
5	*scutellata* 1	Queen *scutellata*	20	15	17	9
5	*scutellata* 1	Worker *capensis*	20	6	4	3
5	*scutellata* 1	Worker *scutellata*	20	15	8	5
5	*scutellata* 2	Queen *capensis*	20	17	13	0
5	*scutellata* 2	Queen *scutellata*	20	19	13	11
5	*scutellata* 2	Worker *capensis*	20	2	2	0
5	*scutellata* 2	Worker *scutellata*	20	2	0	0

Chapter 3

Environmental effects on worker policing in the Cape honeybee (*Apis mellifera capensis* Esch.)

Abstract Egg removal rates for queen and worker-laid eggs were evaluated for 4 queenright *Apis mellifera capensis* colonies (where unmated laying workers produce female offspring) using standard methods for 6 sequential days. Worker-laid eggs were removed significantly faster than queen-laid eggs confirming earlier results that *A. m. capensis* is able to police worker-laid eggs. These data were analysed with respect to ambient weather conditions (rainfall, average temperature, humidity and wind speed) at the test locality. Egg removal rates for worker-laid eggs were significantly lowered with an increase in rainfall and a decrease in temperature. However, the egg removal of queen-laid eggs were not affected by changing weather conditions. Thus, our results indicate that the environment significantly affects worker policing in the Cape honeybee and suggest that environmental factors should be considered in future studies on worker policing.

3.1. Introduction

In the genus *Apis* unmated workers are able to reproduce by parthenogenesis. Workers of almost all subspecies of *Apis mellifera* produce haploid male sexuals arrhenotokously (Ruttner 1992; but see Mackensen 1943 and Tucker 1958 for very rare exceptions). However, the majority of laying workers of the Cape honeybee, *A. m. capensis*, produce diploid female offspring thelytokously (Onions 1912; Hepburn & Crewe 1991; Neumann et al. 2000b). In some colonies of *A. m. capensis* workers may exhibit both forms of parthenogenesis (Pettey 1922; Hepburn & Crewe 1991). The production of adult males by workers in queenright colonies of European honeybee subspecies is rare (Visscher 1989), because few workers have active ovaries (Velthuis 1970) and if eggs are laid they are eaten due to worker policing (Ratnieks 1988; Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996).

Queen-laid and worker-laid eggs may be recognised by means of a queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995; but see Katzav-Gozansky et al. 2001). Worker policing is selected for in a population of social Hymenoptera with arrhenotokous worker reproduction on relatedness grounds alone when each colony has a single queen mated to more than two males because workers in colonies with multiply mated queens are less related to other workers' sons than to the queen's male offspring (Ratnieks 1988). *A. mellifera* shows an extreme level of polyandry (Neumann et al. 1999a; Neumann et al. 1999b; Neumann & Moritz 2000; Palmer & Oldroyd 2000; Strassmann 2001). However, when workers' offspring are thelytokously-produced females, they are as related to other workers' daughters as to the queen's daughters.

Therefore, on relatedness grounds alone one might expect that *A. m. capensis* workers would either not police or would do so less effectively than other honeybee races (Greeff 1996). Indeed, brood can be worker-derived in queenright Cape honeybee colonies (Moritz et al. 1999). However, the results given in Chapter 2 showed that queenright colonies of the Cape honeybee are capable of worker policing, suggesting that unlimited worker reproduction is not free of costs. Thus worker policing might be favoured by selection in the Cape honeybee on colony efficiency grounds alone (Chapter 2) because there are no relatedness benefits (Greeff 1996). The high degree of

variation among queenright *A. m. capensis* colonies in terms of successful worker reproduction (Pettey 1922; Tribe & Allsopp 2001) indicates that the cost/benefit ratio for worker policing is much more fragile than in other honeybee subspecies with arrhenotokous worker reproduction, where worker policing is favoured by both relatedness and colony efficiency grounds.

Ecological studies of honeybee behaviour must be conducted with bees living under natural conditions (Seeley 1985). This methodological principle takes into account the moulding of honeybee behaviour by natural selection and the often remarkable precise fit between bee behaviour and the external environment (Seeley 1985). Thus, it might well be that environmental factors affecting colony performance also have an effect on worker policing. Rainfall, wind, humidity and temperature are highly important environmental factors for honeybee colonies (Luterbacher 1974) that trigger e.g. foraging (Luterbacher 1974), brood warming (Himmer 1926), nursing behaviour (Blaschon & Crailsheim 2001) and other activities of house bees (Riessberger et al. 1998).

Since there are lowered benefits of worker policing if laying workers reproduce thelytokously (Greeff 1996, Chapter 2), we expect potential environmental effects to be more readily expressed in the Cape honeybee compared to subspecies with arrhenotokous worker reproduction. Thus, *A. m. capensis* is an ideal model organism to test the following hypothesis: Given that there is any effect of environment, we expect a decrease in worker policing during unfavourable weather conditions in queenright Cape honeybee colonies. In this study we tested this hypothesis by evaluating egg removal rates for queen and worker-laid eggs in queenright colonies of *A. m. capensis* and evaluate this data in terms of ambient weather conditions prevailing during the experimental period.

3.2. Materials and methods

3.2.1. Worker policing behaviour

In November 2000 five queenright and two queenless *A. m. capensis* colonies were placed in a test apiary in Grahamstown, South Africa. All discriminator and egg source

colonies were unrelated and housed in standard Langstroth hives made of two brood boxes separated by a queen excluder. Worker policing was evaluated using the standard method for determining egg removal rates (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) with the following modifications. The test combs (standard Langstroth deep frames, consisting only of worker cells) were placed in the four queenright discriminator colonies to avoid any potential influence of the comb (Breed et al. 1995) on egg removal behaviour. Then, the colonies were given two days to settle before the experiments began to prevent absconding (Hepburn et al. 1999). One queenright and the two queenless colonies were used as egg sources for the queenright discriminator colonies.

On a daily basis 80 queen-laid fertilized eggs (diploid females) were obtained from worker cells of the queenright source colony and 80 worker-laid diploid eggs (thelytokously produced females) were obtained from worker cells of the two queenless source colonies. All eggs obtained from the source colonies were initially transferred on glass slides, which were placed in a closed Styrofoam box [30×20×20cm] and provided with moist tissue to avoid dehydration of the eggs (FLW Ratnieks, personal communication). Then, 20 worker-laid eggs and 20 queen-laid eggs were transferred onto each of the test combs. One test comb was returned to above the queen excluder and sandwiched between frames containing all stages of brood (Ratnieks 1993). All egg transfers were performed using special forceps (Taber 1961) in a nearby laboratory (less than 200m from the test apiary). The test combs were transported between the test apiary and the laboratory in a closed hive. The experiment was carried out for six sequential days (27.Nov.-02.Dec. 2000). After 24 hours the test combs were briefly removed and remaining eggs were counted.

3.2.2. Environmental conditions

Daily measurements of rainfall [mm], average temperature [°Celsius], humidity [%] and wind speed [m/s] were used to evaluate the environmental conditions at the test locality during the experimental period. The measurements were performed by the Climate Information Section, South African Weather Bureau using the meteorological station in Grahamstown in close proximity to the test apiary (less than 100m distance).

3.2.3. Data analysis

Mann-Whitney U tests were performed to test for differences in the removal rates of queen and worker-laid eggs. Correlation and stepwise regression analyses were used to quantify the effects of average temperature, rainfall, humidity and wind speed on the numbers of remaining worker-laid eggs in the test cells after 24 hours. Bonferroni adjustment to the level of significance was used in the correlation analysis to ensure that the level of significance did not exceed 0.05 (Miller 1981). The same test was performed with the number of queen-laid eggs to investigate a general environmental effect on egg removal. All analyses were performed using Statistica©. Egg removal data obtained by Chapter 2 for three other queenright *A. m. capensis* discriminator colonies on 5 sequential days in Grahamstown (07.Feb.-11.Feb.2000) were also incorporated and reanalysed in this study. The weather data (absent in Chapter 2) for these results were also obtained from the Climate Information Section, South African Weather Bureau. The relevant raw data is shown in Appendix 3.2.

3.3. Results

A total number of 960 eggs (480 queen-laid and 480 worker-laid) were tested for egg removal. Significantly more queen-laid eggs (mean ± SD: 6.41 ± 2.51; 32.05%) than worker-laid eggs (mean ± SD: 3.67 ± 3.60; 18.35%; $Z = 2.41$, $P < 0.002$) remained in the test cells after 24 hours.

The correlation analysis revealed a significant positive correlation between the number of remaining worker-laid eggs and rainfall ($r=0.60$, $P<0.0001$, Fig. 3.1) and a significant negative correlation between the number of remaining worker-laid eggs and the average temperature ($r=-0.56$, $P<0.0001$, Fig. 3.2). No significant correlations were found between the number of remaining worker-laid eggs with wind speed and with humidity ($P> 0.05$). The correlation analysis revealed no significant influence of weather parameters on the number of remaining queen-laid eggs ($P>0.05$).

The stepwise regression analysis on the worker-laid eggs confirmed these results (Table 3.1). The environmental variables that entered the model in order of magnitude

of the coefficients of determination (R^2) were rainfall, average temperature and humidity (R^2=0.53, P<0.0001). The stepwise regression analysis revealed no significant influence of environmental variables on the number of remaining queen-laid eggs (Table 3.2).

3.4. Discussion

Our data show a significant environmental effect on worker policing in the Cape honeybee because removal rates for worker-laid eggs were lowered by an increase in rainfall and a decrease in temperature. However, there were no significant environmental effect on removal rates for the queen-laid eggs. This strongly suggests that worker policing and worker reproduction are not independent of environmental conditions. This is an entirely novel aspect of worker policing which has not been investigated in previous studies. Moreover, we can confirm earlier results that *A. m. capensis* is able to police worker-laid eggs (Chapter2).

The data clearly support our hypothesis that worker policing is less expressed during unfavourable weather conditions. Analogously, brood cannibalism for diploid drone larvae seems to be strongly environmentally determined (Polaczek et al. 2000). Since worker policing is basically a colony phenomenon (Ratnieks et al. 2002), it is not surprising that factors affecting total colony performance also have an effect on worker policing. Unfavourable weather conditions cause the foraging force to stay within the hive (Ribbands 1953) and especially rain results in a decrease of nursing behaviour of nurse bees towards younger larvae and eggs (Blaschon & Crailsheim 2001). Despite the higher number of foraging workers present in the colony during inclement weather we found a decrease in worker policing. Thus, our results suggest that foragers did not participate in egg removal as much as did house bees that regularly check brood cells.

Lower temperatures cause an increase in brood warming (Seeley 1985) which is mainly performed by house bees (Lindauer 1952). Nurse bees mainly take care of older larvae during unfavourable weather conditions (Blaschon & Crailsheim 2001). Thus, the observed decrease in worker policing might be explained by a task shift of the house bees to primarily performing brood caring of older larvae (Blaschon & Crailsheim

2001) and a decrease of inspecting cells with young larvae and eggs (Blaschon & Crailsheim 2001) or being inactive (Riessberger et al. 1998) during unfavourable weather conditions instead of removing eggs. This potential mechanism, which might explain the observed decrease in worker policing, should be the same for all honeybee colonies.

Fewer queen-laid and more worker-laid eggs remained in this study compared to previous ones (Ratnieks & Visscher 1989; Oldroyd & Ratnieks 2000). Thus, it is unlikely that any systematic error has affected our results because we would expect the same trend for both types of eggs unless worker-laid eggs are more viable than queen-laid eggs. Only 32% of the tested queen-laid eggs remained in the test cells after 24 hours. That is smaller than the proportion of queen-laid eggs remaining in other studies on worker policing (e.g. 45% Ratnieks & Visscher 1989; 30% Oldroyd & Ratnieks 2000). The relatively poor acceptance for queen-laid eggs in this study might be related to nestmate recognition for eggs in African honeybees (Chapter 9). More efficient nestmate recognition for eggs in African subspecies compared to European ones seems plausible in light of the different life history strategy in Africa, involving frequent non-reproductive swarming (absconding, Hepburn et al. 1999) and subsequent colony mergers (Hepburn & Whiffler 1988, Chapter 4), resulting in reproductive conflicts between unrelated colonies.

However, 11% of the worker-laid eggs remained in the test cells after 24 hours. That is considerably higher than the proportion of worker-laid eggs remaining in other studies on worker policing (e.g. 1% Ratnieks & Visscher 1989; 2% Oldroyd & Ratnieks 2000) and indicates a weaker policing ratio between worker and queen derived eggs in the Cape honeybee. Because Cape honeybees police worker-laid eggs on colony efficiency grounds alone (Chapter 2), it seems not surprising that this behaviour is less well expressed than in honeybee subspecies with arrhenotokous worker reproduction (Greeff 1996). Indeed, our data agree well with earlier studies on worker policing (Greeff 1996; Moritz et al. 1999) and field observations that brood above the excluder is common in queenright Cape honeybees (Pettey 1922; Tribe & Allsopp 2001; personal observations).

Our results also indicate that in *A. m. capensis* the benefits derived from worker policing on colony efficiency grounds alone (Chapter 2) do not outweigh the costs

involved with less efficient brood caring during unfavourable weather conditions. Assuming that the costs of worker policing and the benefits derived on colony efficiency grounds are the same in *A. m. capensis* and subspecies with arrhenotokous worker reproduction, one might expect that environmental effects are less readily expressed in the latter. This seems plausible, because benefits derived from relatedness grounds in arrhenotokous subspecies might be higher than the costs of worker policing, regardless of changing environmental conditions. However, this remains to be tested.

We conclude that worker policing is another honeybee colony phenomenon, which is influenced by environmental conditions. In the Cape honeybee, costs involved with worker policing under unfavourable weather conditions seem to be able to override benefits derived from colony efficiency grounds. We also suggest that environmental factors should be included in further studies on worker policing.

Table 3.1: Results of the stepwise regression analysis with the number of remaining *A. m. capensis* worker-laid eggs after 24 hours as the dependent variable. Significant P-values are in italics.

Regression model R^2=0.61, *P<0.0001*	**Independent variables**	**Coefficient**	**t-value**	**R^2**	**P-level**
	Intercept	5.92	3.33		*0.002*
Step 1	Rainfall	1.15	4.86	0.53	*0.0023*
Step 2	Average temperature	-0.22	-2.51	0.61	*0.016*

Table 3.2: Results of the stepwise regression with the number of remaining *A. m. capensis* queen-laid eggs after 24 hours as the dependent variable. Significant P-values are in italics (n.s.= not significant).

Regression model R^2=0.05, P=0.17	**Independent variables**	**Coefficient**	**t-value**	**R^2**	**P-level**
	Intercept	9.19	3.61		*0.000892*
Step 1	Average temperature	-0.19	-1.41	0.05	n.s.

Fig. 3.1: Correlation between daily rainfall and number of worker-laid eggs remaining in the test cells in seven queenright *A. m. capensis* discriminator colonies (data of this study and Chapter 2, shadows indicate multiple data points).

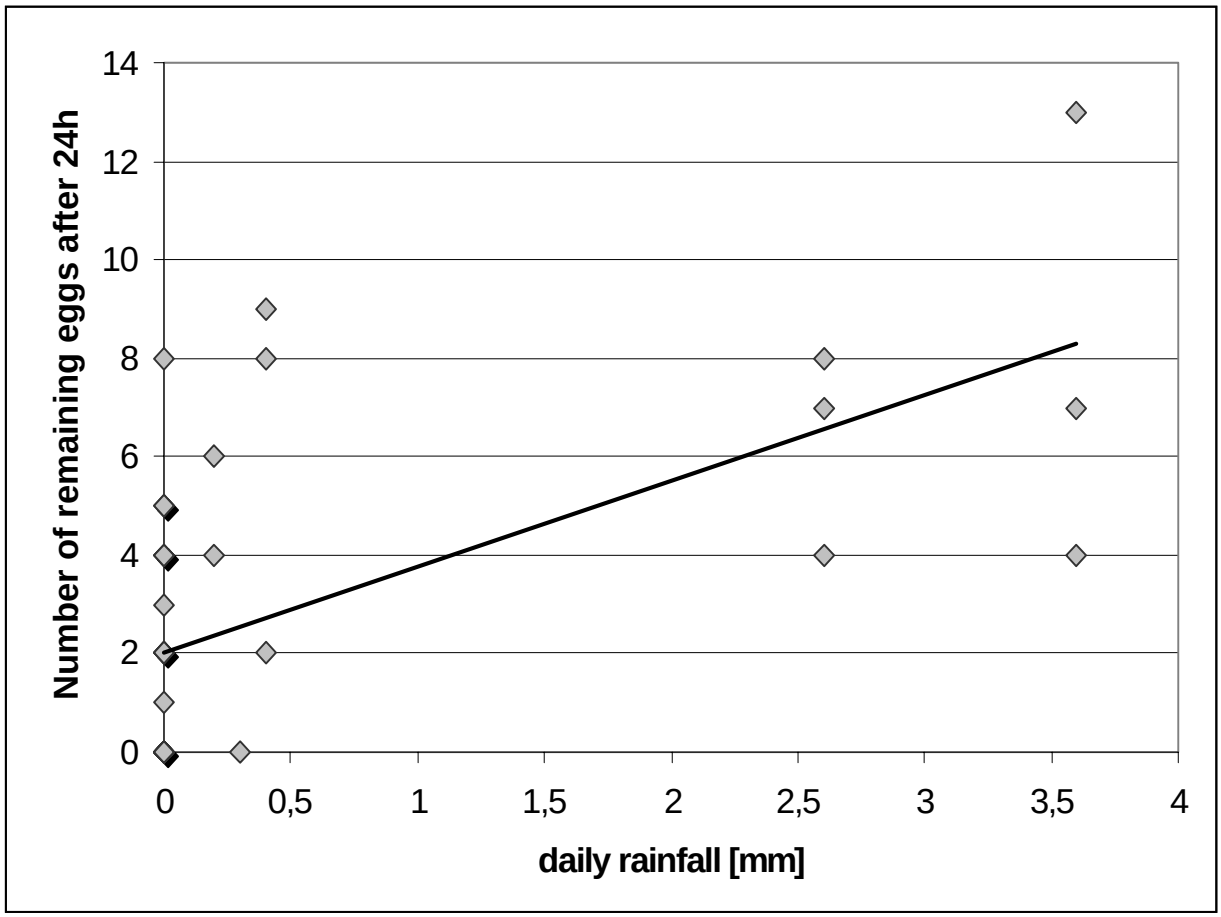
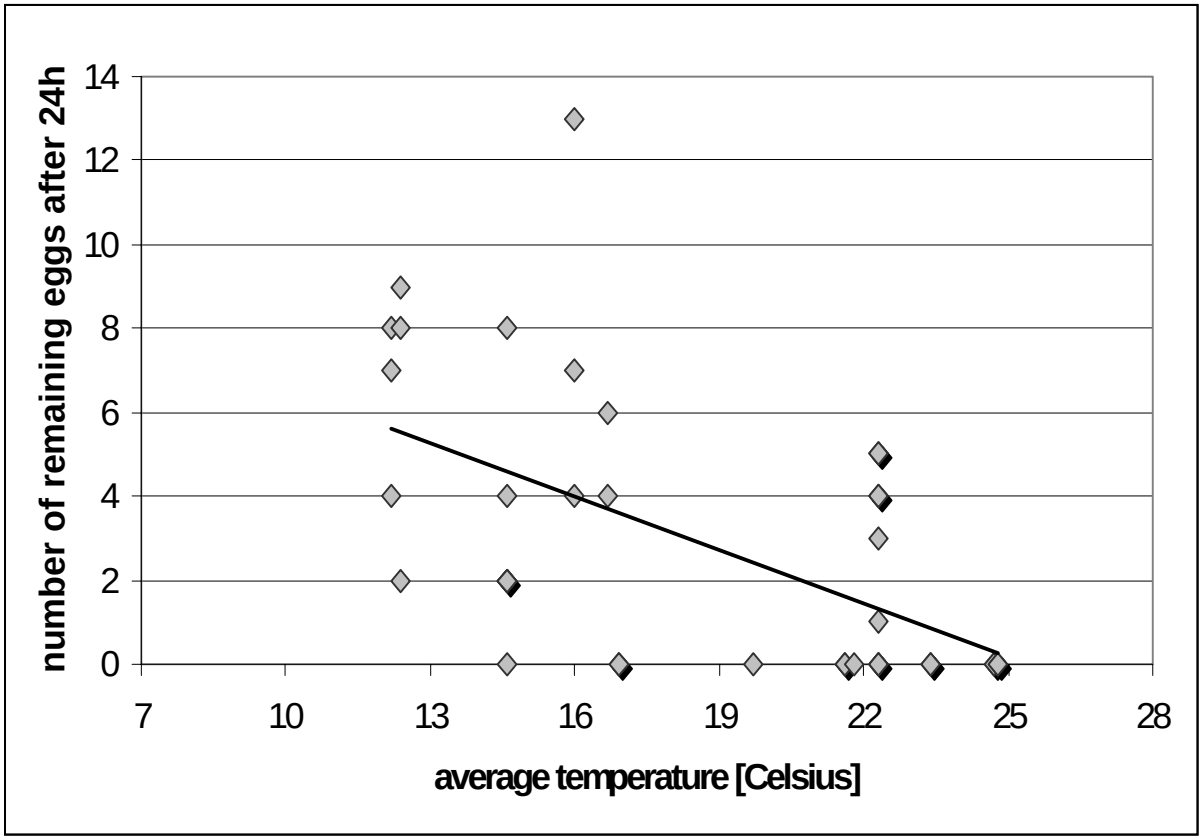


Fig. 3.2: Correlation between daily average temperature and number of worker-laid eggs remaining in the test cells in seven queenright *A. m. capensis* discriminator colonies (this study and Chapter 2, shadows indicate multiple data points).



Appendix 3

Appendix 3.1:
A note on environmental effects on worker policing in the African honeybee, *Apis mellifera scutellata* (Lepeletier)

Environmental factors such as rainfall, wind and temperature are important for honeybee colonies (Luterbacher 1974) and may trigger activities of foragers (Luterbacher 1974) and house bees (Himmer 1926; Riessberger et al. 1998). The environment also affects worker policing because the level of egg removal rates for worker-laid eggs of the Cape honeybee decreased during unfavourable weather conditions (Chapter 3).

We tested whether the environment also affects worker policing in the African honeybee *A. m. scutellata* by evaluating egg removal rates (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) on 3 subsequent days for queen and worker-laid eggs in 3 queenright colonies of *A. m. scutellata* as discriminator colonies and 1 queenright and 1 queenless colony as egg sources. After 24 hours the test combs were briefly removed and the remaining eggs were counted. This data was combined with ambient weather conditions (daily measurements of rainfall [mm], average temperature [°C] and wind speed [m/s], the humidity data was not available) prevailing at the testing locality during the experimental period (07.12-09.12.2000). Only the egg removal data obtained from the earlier experiment for two *A. m. scutellata* (Chapter 2) were analysed with the respect to the weather data.

Mann-Whitney U tests showed that queen-laid eggs (mean ± sd of remaining eggs: 5.89 ± 2.37) were removed significantly more slowly than worker-laid eggs (mean ± sd of remaining eggs: 2.56 ± 1.67; $Z = 2.78$, $P < 0.01$). A linear model, which was used for partial correlation and multiple regression analysis in order to quantify the effects of average temperature, rainfall and wind speed on the numbers of remaining eggs in the test cells, revealed no significant effects (Table 3.3). However, in *A. m. capensis* the regression analysis and in particular the wind speed was significant (Table 3.3).

This study confirms earlier results (Chapter 2) that *A. m. scutellata* is able to police worker-laid eggs. However, the data show no significant environmental effect on

worker policing in *A. m. scutellata*, a honeybee subspecies with arrhenotokous worker reproduction as opposed to earlier data on the thelytokous Cape honeybee (Chapter 3). Since it is likely that the costs of worker policing and the benefits derived on colony efficiency grounds are the same in both subspecies (Chapter 2), the results suggest that the benefits derived from relatedness grounds in *A. m. scutellata* are much higher than the costs of worker policing, regardless of the costs resulting from changing environmental conditions. However, it might well be that I measured egg removal rates in a too narrow range of environmental factors to reveal the full potential effects of environmental factors on policing in *A. m. scutellata*. Long-term studies on worker policing in arrhenotokous honeybee subspecies including a wider range of environmental factors would be necessarily to establish this point.

Table 3.3: Results of the stepwise regression analysis with the number of remaining *A. m. scutellata* queen and worker-laid eggs after 24 hours as the dependent variable. Significant P-values are in italics (n.s.= not significant).

Worker-laid eggs					
Regression model R^2=0.18, P=0.07	**Independent variables**	**Coefficient**	**t-value**	**R^2**	**P-level**
	Intercept	4.47	5.61		*0.000032*
Step 1	Rainfall	-1.32	-1.92	0.18	n.s.
Queen-laid eggs					
Regression model R^2=0.23, P=n.s.	**Independent variables**	**Coefficient**	**t-value**	**R^2**	**P-level**
	Intercept	-3.56	-0.46		n.s.
Step 1	Wind speed	0.74	1.77	0.17	n.s.
Step 2	Average temperature	0.39	1.11	0.23	n.s.

Appendix 3.2: Egg removal data after 24 hours for the *A. m. capensis* discriminator colonies and the environmental conditions. Average temperature [°C], wind speed [m/s], rainfall [mm], relative humidity [%]

Discriminator colony	Type of egg	0 hours	24 hours	Temperature	Wind speed	Rain	Humidity
C1	queen	20	1	12.2	3	2.6	84.5
C1	queen	20	3	12.4	4	0.4	92.0
C1	queen	20	11	14.6	5	0	86.3
C1	queen	20	8	16.0	3	3.6	84.8
C1	queen	20	6	16.9	2	0	95.0
C1	queen	20	7	22.3	2	0	55.6
C1	worker	20	2	14.6	5	0	86.3
C1	worker	20	2	16.9	2	0	95.0
C1	worker	20	0	22.3	2	0	55.6
C1	worker	20	4	12.4	4	0.4	92.0
C1	worker	20	8	12.2	3	2.6	84.5
C1	worker	20	7	16.0	3	3.6	84.8
C2	queen	20	9	12.2	3	2.6	84.5
C2	queen	20	9	12.4	4	0.4	92.0
C2	queen	20	0	14.6	5	0	86.3
C2	queen	20	0	16.0	3	3.6	84.8
C2	queen	20	8	16.9	2	0	95.0
C2	queen	20	0	22.3	2	0	55.6
C2	worker	20	0	14.6	5	0	86.3
C2	worker	20	2	16.9	2	0	95.0
C2	worker	20	0	22.3	2	0	55.6
C2	worker	20	4	12.4	4	0.4	92.0
C2	worker	20	7	12.2	3	2.6	84.5
C2	worker	20	13	16.0	3	3.6	84.8
C3	queen	20	9	12.2	3	2.6	84.5
C3	queen	20	10	12.4	4	0.4	92.0
C3	queen	20	11	14.6	5	0	86.3
C3	queen	20	7	16.0	3	3.6	84.8
C3	queen	20	0	16.9	2	0	95.0
C3	queen	20	9	22.3	2	0	55.6
C3	worker	20	0	14.6	5	0	86.3
C3	worker	20	3	16.7	2	0	95.0
C3	worker	20	0	22.3	2	0	55.6
C3	worker	20	5	12.4	4	0.4	92.0
C3	worker	20	9	12.2	3	2.6	84.5
C3	worker	20	4	16.0	3	3.6	84.8
C4	queen	20	11	12.2	3	2.6	84.5
C4	queen	20	6	12.4	4	0.4	92.0
C4	queen	20	12	14.6	5	0	86.3
C4	queen	20	7	16.0	3	3.6	84.8
C4	queen	20	1	16.9	2	0	95.0
C4	queen	20	9	22.3	2	0	55.6
C4	worker	20	0	14.6	5	0	86.3
C4	worker	20	0	16.9	2	0	95.0
C4	worker	20	0	22.3	2	0	55.6
C4	worker	20	6	12.4	4	0.4	92.0
C4	worker	20	8	12.2	3	2.6	84.5

Appendix 3.2 continues

Discriminator colony	Type of egg	0 hours	24 hours	Temperature	Wind speed	Rain	Humidity
C4	worker	20	4	16.0	3	3.6	84.8
C5	queen	20	6	19.7	5	0.3	77.8
C5	queen	20	4	21.6	3	0	88.6
C5	queen	20	4	22.3	5	0	82.9
C5	queen	20	9	24.7	3	0	94.2
C5	queen	20	1	24.8	3	0	83.9
C5	worker	20	4	21.6	3	0	88.6
C5	worker	20	0	22.3	5	0	82.9
C5	worker	20	2	24.7	5	0	94.2
C5	worker	20	0	24.8	3	0	83.9
C5	worker	20	4	19.7	5	0.3	77.8
C6	queen	20	3	19.7	5	0.3	77.8
C6	queen	20	2	21.8	3	0	88.6
C6	queen	20	2	22.3	5	0	82.9
C6	queen	20	7	24.7	3	0	94.2
C6	queen	20	5	24.8	3	0	83.9
C6	worker	20	0	21.8	3	0	88.6
C6	worker	20	0	22.3	5	0	82.9
C6	worker	20	0	24.7	5	0	94.2
C6	worker	20	0	24.8	3	0	83.9
C6	worker	20	8	19.7	5	0.3	77.8
C7	queen	20	1	19.7	5	0.3	77.8
C7	queen	20	4	21.8	3	0	88.6
C7	queen	20	10	22.3	5	0	82.9
C7	queen	20	1	24.7	3	0	94.2
C7	queen	20	9	24.8	3	0	83.9
C7	worker	20	2	21.8	3	0	88.6
C7	worker	20	0	22.3	5	0	82.9
C7	worker	20	1	24.7	5	0	94.2
C7	worker	20	0	24.8	3	0	83.9
C7	worker	20	5	19.7	5	0.3	77.8

Appendix 3.3: Egg removal data after 24 hours for the *A. m. scutellata* discriminator colonies and the environmental conditions. Average temperature [°C], wind speed [m/s] and rainfall [mm].

Discriminator colony	Type of egg	0 hours	24 hours	Temperature	Rain	Wind
S 1	queen	20	7	19.7	0.3	5
S 1	queen	20	8	21.8	0	3
S 1	queen	20	13	22.3	0	5
S 1	queen	20	10	24.7	0	3
S 1	queen	20	9	24.8	0	3
S 1	worker	20	5	19.7	0.3	5
S 1	worker	20	4	21.8	0	3
S 1	worker	20	6	22.3	0	5
S 1	worker	20	11	24.7	0	3
S 1	worker	20	5	24.8	0	3
S 2	queen	20	7	19.7	0.3	5
S 2	queen	20	7	21.8	0	3
S 2	queen	20	7	22.3	0	5
S 2	queen	20	2	24.7	0	3
S 2	queen	20	11	24.8	0	3
S 2	worker	20	2	19.7	0.3	5
S 2	worker	20	1	21.8	0	3
S 2	worker	20	9	22.3	0	5
S 2	worker	20	1	24.7	0	3
S 2	worker	20	0	24.8	0	3
S 3	queen	20	6	23.2	2.6	2
S 3	queen	20	4	20.3	0.6	1
S 3	queen	20	8	20.9	1.1	1
S 3	worker	20	2	23.2	2.6	2
S 3	worker	20	3	20.3	0.6	1
S 3	worker	20	2	20.9	1.1	1
S 4	queen	20	6	23.2	2.6	2
S 4	queen	20	3	20.3	0.6	1
S 4	queen	20	5	20.9	1.1	1
S 4	worker	20	2	23.2	2.6	2
S 4	worker	20	5	20.3	0.6	1
S 4	worker	20	3	20.9	1.1	1
S 5	queen	20	9	23.2	2.6	2
S 5	queen	20	9	20.3	0.6	1
S 5	queen	20	3	20.9	1.1	1
S 5	worker	20	0	23.2	2.6	2
S 5	worker	20	5	20.3	0.6	1
S 5	worker	20	1	20.9	1.1	1

Chapter 4
The natural merger of two Cape honeybee colonies (*Apis mellifera capensis*)

Natural mergers of honeybee colonies are commonplace in tropical Africa (Hepburn & Radloff 1998), but their consequences on organizational structure are unknown. Here we determine the spatial distribution and division of labour of workers *(Apis mellifera capensis* Esch.) following a merger of two colonies. Two unrelated colonies (each ~3000 bees) were placed in three-frame observation hives. It was a serendipity that labelled workers were present in both colonies as they merged, so that this study represent a natural case study rather than an experimental artefact, regardless of a sample size of one. When workers emerged from the sealed brood of each colony, they were individually labelled and reintroduced into their respective mother hives. They are referred to as cohorts A and B, each comprising 300 workers of the same age. The behaviours and positions of all labelled workers and queens were recorded twice daily for 24 days (Kolmes 1985; Pirk et al. 2000). On day 14 colony B was dequeened, left its nest and merged with colony A on day 15.

4357 individual behavioural acts (48 different tasks) and 2263 queen-worker distances (1422 before and 841 after merger) were recorded for 360 labelled bees (Appendix 4). Severe fighting initially occurred at the nest entrance when the merger began but no aggression occurred once the workers of colony B had entered the nest of colony A. No significant differences in total activity (all tasks/idleness) and mean queen-worker distances of individuals bees were observed between the cohorts A and B before and after merger (Table 4.1a). However, total activity decreased and queen-worker distances increased after merger for the individual bees of both cohorts (Table 4.1a). There were significant differences among and between tasks of cohorts A and B before and after merger (Table 4.1b). While some tasks increased and others decreased, the patterns of changes between cohorts differed (Table 4.1b). Daily counts of queen-worker distances were significantly different on four occasions before the merger but only once 24 hours after the merger (Appendix 4.3, 4.5), demonstrating effective cohort integration. Also workers of both cohorts were similarly distributed throughout the nest after the merger.

On queen removal cohort B workers did not attempt to re-queen but immediately merged with colony A. This may seem puzzling from an evolutionary perspective because the inclusive fitness of queenless workers is zero in the new unit. However, mergers are frequent

in tropical honeybees (Hepburn & Radloff 1998) and could be adaptive because workers may gain direct fitness through laying own eggs. Alternatively, but not mutually exclusive, the merger of two colonies could be adaptive if the colonies are related. There are evidences for colony aggregation in *Apis mellifera* (Taber 1979, Oldroyd et al 1995) and that colonies in these clusters can be headed by potentially related queen (i.e. parent-offspring or supersisters; Oldroyd et al 1995). If that is the case a natural merger of related colonies would benefit both colonies in terms of inclusive fitness and therefore could be evolutionary adaptive.

The lower levels of activity and the immediate increase in colony size after the merger probably reduce *pro rata* survival costs (Hepburn & Radloff 1998). The origin of merging bees may matter, because task shifts differed in the two cohorts. This might be partially ascribed to age-related division of labour; however, this does not explain the substantial shifts observed both within and between the cohorts before and after the merger. Possibly, workers changed tasks as a result of different behavioural thresholds and task specialization (Moritz & Page 1999). Thus, the possible acquisition of more efficient genetic specialists (Fuchs & Moritz 1999) may also contribute to reducing pro rata costs in the new unit. The task shifts and worker distribution suggest that many bees responded to a different colony environment in the new unit, presumably necessary for social integration.

Table 4.1: Proportional comparisons for (a) individual workers and (b) whole cohorts A and B before and after merger. Differences in total activity and mean queen-worker distances for individual bees were analysed with Mann Whitney U-tests. Z-tests of proportions were used to test for significant differences in the task performances of the whole cohorts **A** and **B**: (i) for each cohort and the new colony and (ii) to assess frequency changes of performances before and after merger between cohorts A and B. Only those behaviours are shown, where significant results have been obtained. Significant results are indicated with * for P<0.01 and ** for P<0.001 using Bonferroni adjustments (N= sample size, P= significance level, F= frequency, new colony = **A + B** combined).

(a) Individual bees

	Before merger					After merger					Before vs. After	
											A	**B**
	Cohort **A**	N	Cohort **B**	N	P	Cohort **A**	N	Cohort **B**	N	P	P	P
Total activity	0.51±0.33	170	0.51±0.28	180		0.29±0.30	143	0.33±0.36	118		**	**
Mean queen-worker distance	30.1±14.2	167	31.1±11.9	175		43.3±13.9	132	44.7±16.4	106		**	**

(b) Whole cohorts

	Cohort **A**			Cohort **B**			Changing patterns			New colony Vs. **A** **B**		
	Before	After		Before	After		**A**	**B**				
Task	F	F	P	F	F	P			P	F	P	P
Walk	213	209	*	246	123	*	-4	-123	*	332	*	*
Idleness	478	903	*	612	681	*	+425	+69	*	1584	*	*
Groom self	49	15	*	78	14	*	-34	-64	*	29	*	*
Inspecting empty/egg cell	58	24	*	104	22	*	-34	-82	*	46	*	*
Inspecting honey cell	20	23		38	9	*	+3	-29	*	32		*
Build comb	1	8		3	7		+7	+4		15	*	
Groom other worker	24	8	*	27	4	*	-16	-23		12	*	*
Get groomed	4	0		10	0	*	-4	-10		0	*	*
Lateral shake	1	0		5	1		-1	-4		1		*
Dorsoventral abdominal vibration	5	0	*	2	0		-5	-2		0	*	
Begging for food	2	0		2	22	*	-2	+20	*	22	*	*
Attend queen	6	0	*	1	0		-6	-1		0	*	
Antennate with worker	52	26	*	61	12	*	-26	-49	*	38	*	*
Run (move faster ~3 cm/s)	4	0		12	1	*	-4	-11		1	*	*
Forage	1	1		0	4	*	0	+4		5		
Wax chain	0	15	*	0	10	*	+15	+10		25	*	*

Appendix 4

Appendix 4.1: List of individual performed tasks (modified after Kolmes 1985) by workers and the corresponding numbers.

1. **walk**
2. **idleness, stand in hive**
3. **inspect or feed larvae**
4. **mouth wax brood**
5. **groom self**
6. **into empty or egg cell**
7. **into honey cell**
8. **build comb**
9. **cap brood**
10. **groom other worker**
11. **get groomed**
12. **feed worker**
13. **get fed**
14. **lateral shake**
15. **DVAV, dorso-ventral-abdominal vibration**
16. **dance**
17. **attend dance**
18. **beg for food**
19. **attend queen**
20. **antennated with worker**
21. **chew on hive**
22. **extend mouthparts**
23. **fan wings**
24. **guarding**
25. **attend worker**
26. **run**
27. **manipulate propolis**
28. **stand on landing board**
29. **feed on provided sugar**
30. **foraging**
31. **feed on provided water**
32. **wax chin**
33. **orientation flight**

Tasks which were not observed

34. **into pollen cell**
35. **mouth wax on honey**
36. **feed queen**
37. **unload pollen**
38. **laying eggs**
39. **uncap brood**
40. **undertaker**
41. **cap honey**
42. **aggression towards the queen**
43. **aggression towards other worker**
44. **dead**
45. **chew at pollen on other worker**
46. **groom dance**
47. **mouth wax on pollen**
48. **attacking parasites**

Appendix 4.2: The individually performed tasks of every labelled worker from both colonies before the merger (only observed behaviours before the merger are shown).

Colony	Worker ID	Task																												
		1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
5	B 1	2	6			2	1												2										
5	B 2	2	9			1						1							1										
5	B 4	1	4																										
5	B 5	1	5		1		2												1										
5	B 7	2	1		1	1							1																
5	B 9	2	5						1	1									1										
5	B 10	3	1						1																				
5	B 12		3				1		2																				
5	B 13	1	3																								1		
5	B 14	2	7		1	1	1																						
5	B 15	2																											
5	B 16					1																							
5	B 17		2																1										
5	B 18	4	5							1			1						1										
5	B 19	1	7		1	3	1																						
5	B 20	1																											
5	B 21	2	2						1																				
5	B 22	2	13			1	1												1										
5	B 23	5	8					1	1											1									
5	B 24	1	1																										
5	B 25	3	6		1	2	1																						
5	B 26		2		1														1										
5	B 28		1		1		1												1					1					
5	B 29				1	1													1										
5	B 30	1																											
5	B 31		2		2	1													1										
5	B 32	4	10			1													1					1					
5	B 33		1																										
5	B 34	1	9		1				1																				
5	B 35		4			2				1									1										
5	B 36	6	5			1			1																				
5	B 37	3	2		1	1	1												1										
5	B 38	1	4			2	1													1									
5	B 39	1																	1										
5	B 40		2																										
5	B 41	2	7		1	1																							
5	B 42	1	1			2																							
5	B 43	1	1				1													1									
5	B 44	2	6		3	3	1		1	1									1										
5	B 45	1	2																										
5	B 46		3																1										
5	B 47		3																	1									
5	B 48		6		1																								
5	B 49	2	8		2		1												1	1									
5	B 50	4	6			1				1									1					1					
5	B 51	2	9		1																								
5	B 53	1	6			2																		1					
5	B 54	1	5		3	3	1																						
5	B 55		1			1																							
5	B 56	1	2		2	1			2																				
5	B 57	1	4			1	1																	1					
5	B 58		1																										
5	B 59	2	5			2		1																					

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
5	B 60																		1				1					1	
5	B 61	2	3																										
5	B 62		4			1								1															
5	B 63		4			1																							
5	B 64	2	4	1	1	3	1																	1					
5	B 65	1	1			1																							
5	B 66	2	10		2	1														1									
5	B 67								1																				
5	B 68					1																							
5	B 69		2		1														1	1		1							
5	B 70		2		2																								
5	B 71	3	5		1	2													1										
5	B 72	2				3													1					1					
5	B 73	1																						1					
5	B 74	4	10				1												1										
5	B 75	2	7		2	1			1																				
5	B 76		3		1	1													1										
5	B 77	6	5			1																							
5	B 78	4	5			1	1											1											
5	B 79	4	3		1	1	2												1										
5	B 80	1	2																1										
5	B 81		1							1																			
5	B 82	4	6	1		1													1										
5	B 84	1																											
5	B 85	3	14			2			1	1									1					1					
5	B 86		1																										
5	B 87	2	2		1														1										
5	B 88	1																											
5	B 89	4	2			1	1																						
5	B 90		1			1																							
5	B 91		1			1																							
5	B 92		1		1																								
5	B 93	3	7						1																				
5	B 94	3	2			2																							
5	B 95		4																1										
5	B 96		8		1	1																							
5	B 97				1		1																						
5	B 98	2	18			1				1									1										
5	B 99	2	3			1							1																
5	B 100	1	1		2														1										
5	W 1	5	5		1	2	1												1										
5	W 2	1	7			1																							
5	W 3	1	3																										
5	W 4		4																1										
5	W 5		1																1										
5	W 6	1	1						1										1										
5	W 7	4	6		1		1										1		1										
5	W 8	1	4		2																								
5	W 9		2																	1									
5	W 10	1	6		4														1										
5	W 11	1		1	1														1	1									
5	W 12		3																										
5	W 13		1																										
5	W 14		3																										
5	W 15		1																										
5	W 16		4																										

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
5	W 17		1																										
5	W 19	2	5		1		1												1										
5	W 21	1																											
5	W 22		2																										
5	W 24	2	1																										
5	W 25	1	1																										
5	W 27	3	3			1		1																					
5	W 28	2	4		1	3	1		1																				
5	W 29	5	8																										
5	W 30		3						1	1									1										
5	W 31		1																										
5	W 33	2	1			1																							
5	W 34		1			1																							
5	W 35	2			2																								
5	W 36		1		1				1																				
5	W 37	1	2																										
5	W 38	2				2																							
5	W 39	1	2																										
5	W 40	4	3		1	1																							
5	W 41		3																	1									
5	W 42		4		1							1																	
5	W 43		1						1																				
5	W 44		5				2																						
5	W 45																		2										
5	W 46		3		1	1																							
5	W 47		3																										
5	W 48	8	4		1	3													1										
5	W 49	2	6		1																			1					
5	W 50	2																	1										
5	W 52		3																										
5	W 53	1	4						1																				
5	W 54	1	1			1							1																
5	W 57		1																										
5	W 58		4				1																						
5	W 59		3		1	2							1						1										
5	W 60		4																										
5	W 61	1																											
5	W 62																												
5	W 63	2	11			1																							
5	W 64		3			1																							
5	W 65	1	6			1			1										1										
5	W 66		2																										
5	W 67	1	1				1																						
5	W 68		4		1														1										
5	W 69	3	6																1										
5	W 70		1		1	1																							
5	W 71	4	8																										
5	W 72	2	1		1				1																				
5	W 73	5	1		1		1												2										
5	W 74	2	7		1														1										
5	W 75	2	7		1														1										
5	W 77	1	1																1							1			
5	W 78	1				2																							
5	W 79	2	1																										
5	W 80	2	4			1																		1					
5	W 81	1	1																1										

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
5	W 82		1			1			1																				
5	W 83		1																										
5	W 84		4				1												1										
5	W 85	4	7		1	2	1										1						1						
5	W 86	1	1																										
5	W 87		3			1																							
5	W 88		1		2																					1			
5	W 89	1	5		1	2	1		2											1									
5	W 90	2	5		1	1	1					1							2	2									
5	W 91	2	6		1	2			1										1										
5	W 94	4	10		1	1	1																						
5	W 95	1	2																					1					
5	W 96		5		1																								
5	W 97	4	4		1	1																							
5	W 98																												
5	W 99	3	2				1																						
5	W 100	2	3		1					1				1															
6	R 1	3	9																										
6	R 4		1																										
6	R 5	6	3		1		2												1										
6	R 6	2	1		1														1										
6	R 7		1																										
6	R 8	1	4		1	1			1																				
6	R 9	1	4			2																							
6	R 10		2																										
6	R 11	2	4																										
6	R 12	1	1		1																								
6	R 13						1																						
6	R 14	2	7																2										
6	R 15	1	7		4	1								2															
6	R 16	1	2			1			1																				
6	R 17		5																										
6	R 18	1	8																1										
6	R 19	4	1		1	1						1							1										
6	R 20		9		2																								
6	R 21	1	6			1																							
6	R 22		1																										
6	R 23	1	2	1								1																	
6	R 24																												
6	R 25	2	4						1																				
6	R 26	4	5		1																								
6	R 27	2	6						1																				
6	R 28	2	7			1			1									3	1										
6	R 29	3	2																										
6	R 30	1	3																										
6	R 31		1																										
6	R 32	2	3																										
6	R 33	2	6		1	4													1										
6	R 34		2			1																							
6	R 35		1																										
6	R 36	2	6			1													1	1									
6	R 37	2	2		1				1																				
6	R 38		5																										
6	R 39		9																										
6	R 40	1	4			1			1										1										
6	R 41	2	8			1													2										

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
6	R42		2																										
6	R43		4				1													1									
6	R44		2				2												1										
6	R45	1	1		2	1																							
6	R47	1	1		1																								
6	R48	3	1																1										
6	R49		2			2	1												3										
6	R51	1	1																										
6	R52	5	10																										
6	R54	7	7			2			1																				
6	R56	2	3		1	1																							
6	R57	4	2			1													1										
6	R59	1			1																								
6	R60	2																											
6	R62	1	3																										
6	R63	3	2																										
6	R64		9																										
6	R65				1														1						1				
6	R66	5	3																1										
6	R67																												
6	R68	1																											
6	R69	1		1																									
6	R70	1																											
6	R71				1																								
6	R72	2	1			2						1														1			
6	R74	2				1													1										
6	R76		2																2										
6	R77	1	4																										
6	R78	3	2																1										
6	R79	2	2		1	2			1								1												
6	R80	1	1																1										
6	R81	1	2																										
6	R82	1			1																								
6	R83	1	3																2										
6	R84		2		1	1	1		1																				
6	R85																												
6	R86		1																										
6	R87		2			1													1	1									
6	R88	1	5			1													1										
6	R89	3	6			1							1																
6	R90				1																								
6	R91	3	3		1	1																							
6	R92		4						1										1										
6	R93	3	9			1	1		1		1								1										
6	R94		3			1																							
6	R95		3			1													1										
6	R96		3																										
6	R97	1	7																										
6	R98	1				1	1					1							1										
6	R99																		1										
6	R100		1				1																						
6	Y1		2																										
6	Y2	1																						1					
6	Y3	4	3		2	1												1	1										
6	Y4	1	1																										
6	Y5	1	3			2	1												1										

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
6	Y 6	3	3			1										1		1											
6	Y 7	1																											
6	Y 8	1	4			1																		1					
6	Y 9		2		1				1										1										
6	Y 10		2																										
6	Y 12	4	7																										
6	Y 13		10		1				1											1									
6	Y 14		5			1													1										
6	Y 16																		1										
6	Y 17	3	11		1	1																							
6	Y 19																												
6	Y 20	1	3		1									1															
6	Y 21		2																										
6	Y 22	2	1		1					1					1									1					
6	Y 23	2	8		1																								
6	Y 24		6		1				1																				
6	Y 25	2																		1									
6	Y 26	1				1																							
6	Y 27	4	8		1	1			1									1	1										
6	Y 28	2	2																										
6	Y 29	1	1		2														1										
6	Y 30	11																											
6	Y 31		1																										1
6	Y 32		1																										
6	Y 33	1	9		1	2															1								
6	Y 34	1	3		1				1					1										1					
6	Y 37	1							1																				
6	Y 38	1	2																										
6	Y 39		1																										
6	Y 40	1	1																										
6	Y 41		1																										
6	Y 42	2	7		2		2																						
6	Y 43		3																										
6	Y 44	2	2		1	1																							
6	Y 45	1	4						1											1									
6	Y 46	1		1																									
6	Y 48					1																							
6	Y 49	2	2			1																							
6	Y 50		1		1															1									
6	Y 51		1																										
6	Y 52	1	2		1	1													1										
6	Y 53	1	5		1														1										
6	Y 54		3						2										1										
6	Y 55		2																										
6	Y 56	1	2																1										
6	Y 57	1																											
6	Y 58		3																										
6	Y 60		1																										
6	Y 61	4	5		1	2													1										
6	Y 62	1	1																										
6	Y 63	1	1																	1									
6	Y 65		1																										
6	Y 66		4																	1									
6	Y 67	2	2														1			1									
6	Y 68		6																										
6	Y 69	2	4				2												2										

Appendix 4.2 continues

Colony	Worker ID	1	2	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30	33
6	Y 70																												
6	Y 71		1					1																					
6	Y 72	3	3						1																				
6	Y 73			1																									
6	Y 74	2	6							1																			
6	Y 76					1																							
6	Y 77																												
6	Y 79		3							1																			
6	Y 80		1			1																							
6	Y 81	2																											
6	Y 82	1	4																										
6	Y 83	1	1																										
6	Y 86	1			1	1																							
6	Y 87													1															
6	Y 88	1				1																							
6	Y 91		4			1																							
6	Y 92	3	1			1													1										
6	Y 93	3	1																1										
6	Y 94	2	3	1		1													1										
6	Y 95	2	7																										
6	Y 96		1																										
6	Y 97		1																										
6	Y 98	1	5				1		1	1									1										
6	Y 99	2																											
6	Y 100								1																				

Appendix 4.3: Queen-worker distance [cm] of all observed labelled workers of both colonies before the merger (day 1-14).

Colony	worker ID	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	B 1								26.9	14.0	16.6		16.8		
5	B 2	20.0		32.0			27.1					20.6	28.3	20.6	
5	B 3														
5	B 4			69.5		10.0		50.0					15.0		
5	B 5							70.7			4.7	9.1		36.9	
5	B 7	20.6		67.1		11.2			55.0						
5	B 9	33.5		62.6				33.5	18.0	16.6					
5	B 10							31.6	25.0	25.5		10.0	11.2		
5	B 12	31.6		60.8			16.9	11.2							
5	B 13			65.2			30.0								
5	B 14					15.8		33.2							
5	B 15					11.2	30.0								
5	B 16	10.0													
5	B 17	11.2				18.0	20.0								
5	B 18	55.9				35.4		42.5	20.7	7.1		40.3		5.0	
5	B 19								10.2		42.8	10.0	65.2		
5	B 20							15.8							
5	B 21	11.2		55.9		35.4	0.0								
5	B 22	25.5					10.0	30.2	5.0	30.5	32.6	38.1	42.7	32.5	
5	B 23			38.1				96.2	25.5		8.5	11.2	30.2	18.2	
5	B 24	20.6													
5	B 25	31.6					7.1	23.3	45.8	21.8	50.2	42.5	14.1		
5	B 26						2.5	15.8							
5	B 28			60.4		36.1	8.1	50.2							

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	B 29	11.2				15.8									
5	B 30	41.2													
5	B 31	22.4		54.1			5.0	50.0							
5	B 32			25.5		10.0	14.1		12.1	44.4	35.2	45.3	40.3	32.0	
5	B 33						55.2								
5	B 34	38.1					15.8	48.3	28.0	20.6		10.0	42.1	52.2	
5	B 35	50.2		61.0				50.0		20.6	15.0		57.5		
5	B 36	50.2					42.0	50.0		24.6	39.4	5.0	41.2	20.6	
5	B 37	0.0		50.4		7.5		15.0			46.1				
5	B 38	29.2						57.0	28.0	20.6	11.2		30.0		
5	B 39												55.9		
5	B 40														
5	B 41			47.4			8.1	70.7	26.9		55.2	42.2			
5	B 42	11.2						41.2			10.0				
5	B 43									5.0				15.0	
5	B 44			49.2		21.2			13.5	47.4	38.1	53.2	47.4	51.0	
5	B 45			20.6			45.0			65.2					
5	B 46	50.2					55.2								
5	B 47									67.7	10.0	50.2			
5	B 48						27.6	36.4			18.2	65.0	45.3		
5	B 49						15.0		13.7	38.1	11.2	50.2	58.3	33.5	
5	B 50			58.3			9.1	14.1		70.2	37.8	22.8	23.9		
5	B 51			39.6		7.9	5.0	45.0	11.2	60.8		20.0			
5	B 53	30.0					10.0			42.4			67.7	32.0	
5	B 54								21.2	10.6	23.7	62.0	10.4	55.9	
5	B 55					5.0		15.8							
5	B 56	25.0							46.1	6.0	40.3		11.2	11.2	
5	B 57			49.2				45.6		37.2		25.7	50.0		
5	B 58														
5	B 59					30.4	31.6			25.5	55.2	55.2	5.0		
5	B 60						35.0	40.8							
5	B 61			70.2			25.0	55.0				55.0			
5	B 62			58.3		30.4		52.6		25.5	25.5				
5	B 63					15.8		36.4							
5	B 64					10.6	15.8	11.2		51.0	15.8		26.0	15.8	
5	B 65	22.4						40.3				41.2			
5	B 66	30.4					14.6	100.1	10.0	45.3	40.8	17.7	19.1	33.5	
5	B 67			71.6											
5	B 68	14.1													
5	B 69			61.0				30.4	35.4		5.0				
5	B 70						7.1	55.9							
5	B 71	0.0					2.5	42.7	57.0	55.2	55.9		10.0	63.0	
5	B 72	7.1		54.1		60.8						35.4			
5	B 73					31.6									
5	B 74			5.0				45.4		44.4	20.2		57.0		
5	B 75			26.9				14.1	20.2			33.3	14.1	71.6	
5	B 76					0.0	15.0	20.0							
5	B 77							24.6	30.0	65.2	22.3		53.7	70.7	
5	B 78	60.2		5.0					15.8	34.8		31.3	25.0	45.0	
5	B 79					45.6			18.0	10.0	32.2		26.2		
5	B 80					19.1		55.0						70.7	
5	B 81						10.0								
5	B 82	29.2		30.0		55.9	20.6	15.0		46.1	26.9	60.0	23.6		
5	B 84							40.3							
5	B 85	35.4		53.4			18.0	34.9	46.1	25.9	40.3	40.3	42.9	31.9	
5	B 86							36.4							

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	B 87	28.5					5.0	21.2		50.2		70.2			
5	B 88														
5	B 89			0.0		20.2	10.0						61.8		
5	B 90			60.2		55.2									
5	B 91							36.4							
5	B 92					15.0		5.0							
5	B 93			39.1		35.8		10.6		61.8	11.2			38.1	
5	B 94							45.7			14.1	58.0	14.1	40.3	
5	B 95					22.7									
5	B 96	14.1								20.6	25.5	51.0	51.0	52.3	
5	B 97							45.3		15.0					
5	B 98	10.0		25.5			15.8	43.4		14.7	34.0	52.4	30.4	24.6	
5	B 99	8.1					10.0						35.0		
5	B 100	33.5		29.2								20.6			
5	W 1	20.0		67.1		20.6		45.0		15.8		15.0	11.2	25.9	
5	W 2							41.5	26.9	51.0	15.4	20.6		35.4	
5	W 3			25.0		11.2	7.1								
5	W 4								25.5	27.3	11.2	55.2			
5	W 5						5.0								
5	W 6	25.0								15.7					
5	W 7			67.1			35.4	33.7		34.1	52.2	65.2	26.9	14.1	
5	W 8			58.5				9.1					55.6	37.2	
5	W 9														
5	W 10	20.0						5.0	21.2	11.2		47.4	20.6		
5	W 11							30.4					40.0		
5	W 12					25.8									
5	W 13						57.0								
5	W 14	33.5							31.6	68.0					
5	W 15													52.2	
5	W 16			53.9					30.4	51.0					
5	W 17						7.1								
5	W 19							34.3	30.8	33.4	25.0				
5	W 21	7.1													
5	W 22			58.5		11.2									
5	W 24			25.5								11.2			
5	W 25			40.3									20.6		
5	W 27			47.4								11.2	41.8	30.0	
5	W 28					33.0			11.2	25.8	25.0		33.2		
5	W 29							14.0	12.1	32.6		25.5	60.8	45.1	
5	W 30	25.5				7.1				5.0			40.3	25.0	
5	W 31						25.5								
5	W 33	20.3		25.0				14.1							
5	W 34														
5	W 35			56.3									25.0		
5	W 36			72.8			14.1								
5	W 37	25.5		69.6		11.2									
5	W 38			28.3			30.0	18.0							
5	W 39			35.4		55.2									
5	W 40	15.8		30.4		11.2				49.0	30.0		45.5		
5	W 41					10.6		18.0							
5	W 42			10.0					8.5	15.4					
5	W 43							15.8							
5	W 44					33.5	15.0			60.8		6.0			
5	W 45	0.0						45.3							
5	W 46			69.6			5.0								
5	W 47					13.7									

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	W 48	50.2		5.0					15.6	12.0		63.0	35.4	20.6	
5	W 49	5.0				25.0	13.5		47.4	20.0	41.2		51.0		
5	W 50	25.0						5.0							
5	W 52	30.0		69.6											
5	W 53			71.6				61.8		11.2			35.4		
5	W 54	8.5						43.1							
5	W 57							30.0							
5	W 58			12.5		48.7									
5	W 59	39.4					11.2	42.7		25.5					
5	W 60										47.6	18.0			
5	W 61	10.0													
5	W 62														
5	W 63							60.2		31.5	25.6		55.8	52.1	
5	W 64			58.3			15.0								
5	W 65	50.2		40.3			35.4	35.2	25.0			55.0	10.0	14.6	
5	W 66							41.2				60.8			
5	W 67							49.2			7.1				
5	W 68	10.0				11.2	36.4								
5	W 69							35.4	52.2	33.9		55.2	21.2	42.7	
5	W 70			30.2						21.2					
5	W 71						20.6			0.0	47.1	10.0	40.3	29.2	
5	W 72	50.5						12.7							
5	W 73						10.0		25.0	40.0		10.0	26.6	10.0	
5	W 74					43.6		91.2			29.6	50.1	29.5		
5	W 75								18.3	52.4	40.9	10.0			
5	W 77					10.6	7.1	93.9							
5	W 78			15.0											
5	W 79						7.1	29.2		70.2					
5	W 80	50.2		30.4			11.2	11.2			15.8	45.0			
5	W 81			31.6		50.2		35.0							
5	W 82						7.1		18.5						
5	W 83					11.2									
5	W 84			43.0		33.5			45.7	6.0					
5	W 85			30.7			35.0	18.2	31.0	33.5	30.1	22.4		5.0	
5	W 86									32.0					
5	W 87			32.0		46.1	18.0								
5	W 88	51.0						93.9							
5	W 89						22.8	34.7		36.1	27.5			55.5	
5	W 90						70.2		45.8	37.6	30.3		36.4	42.9	
5	W 91			62.6		15.8		30.1	26.9	20.0	55.2	63.3	50.8		
5	W 94	11.2				55.2		26.6	55.9	13.5	23.5	60.0	60.2	50.2	
5	W 95			61.0			41.2				7.1				
5	W 96					11.2			40.3		15.8	42.6			
5	W 97								31.0				12.5	57.5	
5	W 98														
5	W 99						35.0	64.8			11.2				
5	W 100	50.2		44.2			7.1	22.4				45.3			
6	R 1	5.0		26.8	15.0		45.3	30.4	20.3	43.0					
6	R 4							7.1							
6	R 5		46.1		46.1	0.0	31.6		52.7	31.6			20.6	67.1	5.0
6	R 6				36.4					36.4		7.1			18.0
6	R 7			22.4											
6	R 8	41.2			18.2	11.2	7.1				55.5				
6	R 9		51.0				42.8			22.4	50.0				
6	R 10				30.0										
6	R 11				29.2					11.2					

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6	R 12				5.0										
6	R 13														55.9
6	R 14	7.1	52.2		5.0		35.4		45.3	25.5					31.7
6	R 15		7.1	45.0		39.2	32.8					24.7		55.9	
6	R 16				27.6	22.4				41.5	7.1			92.2	
6	R 17					45.5			7.1					50.0	
6	R 18							40.2	45.3	5.0			18.0		
6	R 19			7.1	45.7		20.3					24.6			
6	R 20				10.0	29.7	33.0	18.0			22.4		15.8		
6	R 21		36.4		52.2						41.2		50.0	61.0	
6	R 22		15.8												
6	R 23	5.0		34.5									15.8		
6	R 24														
6	R 25			10.0						24.0					
6	R 26	40.0		0.0	35.0		11.2	10.0		11.2					
6	R 27					22.4	37.7	5.0			50.0			93.7	
6	R 28	44.7		46.1	18.0	7.1	10.6				3.3	53.1	42.5		
6	R 29	13.8										12.8		46.1	
6	R 30			50.0		42.7						45.3		75.2	
6	R 31		15.8												
6	R 32	45.3			15.8									40.0	31.6
6	R 33				11.2	28.7	13.8				7.1			22.4	30.4
6	R 34						26.9						55.9		
6	R 35							5.0							
6	R 36			22.4	15.0						32.9	60.0	66.0		50.6
6	R 37				17.7									27.5	35.4
6	R 38				30.4	15.8		7.1				11.2			
6	R 39						24.3				15.8		20.0	74.3	
6	R 40				16.9			5.0		40.3		25.5		43.0	
6	R 41	5.0			33.5	41.2	35.8			41.2					
6	R 42					42.7						55.2			
6	R 43						7.1	40.3			18.0	25.0		39.1	
6	R 44			30.0			45.0							40.0	
6	R 45	5.0	52.2	45.0	18.3										
6	R 47				20.6		30.1								
6	R 48			45.3	42.7				58.3				60.2	46.1	
6	R 49				20.6	5.0	19.7				10.0				
6	R 51		47.4		22.4										
6	R 52			45.0				7.1	40.0	11.2	50.0	18.0	44.3	94.2	
6	R 54	11.2		45.0	15.8	41.2	33.3	35.2	49.2	9.4				50.0	
6	R 56			40.0	26.0								32.5	70.0	25.5
6	R 57			5.0	15.8		40.3				18.0			33.0	
6	R 59				55.5										
6	R 60		51.0						20.6						
6	R 62			11.2							50.0		57.0		
6	R 63	30.4	0.0	10.0											21.8
6	R 64							15.0		5.0			35.4		69.5
6	R 65			42.6								65.2			
6	R 66	45.0			10.0	11.2	24.9		40.3			25.5		25.0	
6	R 67														
6	R 68			5.0											
6	R 69	50.2	14.1												
6	R 70								20.6						
6	R 71			5.0											
6	R 72		11.2		28.0		40.9	30.4							
6	R 74			50.0									13.9		

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6	R 76								30.0				42.7	76.3	
6	R 77				5.0								55.9	15.8	43.0
6	R 78			55.2	15.0		23.9		45.3						
6	R 79			46.1		37.6	10.0		35.4	16.9		31.6	36.4		
6	R 80		46.1	50.0											
6	R 81			55.9								65.8			
6	R 82			30.6											
6	R 83			11.2			21.2		47.8						
6	R 84			14.1	15.0	5.0					18.0				
6	R 85														
6	R 86					18.0									
6	R 87					5.0			20.6	23.1			60.4		
6	R 88		14.1		21.2	35.4	41.2	7.1	45.3						
6	R 89			25.6						22.7	18.0	24.7	20.0	39.1	
6	R 90									11.2					
6	R 91			37.3	57.0	27.6		5.0							
6	R 92			7.5									39.1	85.0	
6	R 93		51.6		15.8	41.2	52.2	11.2			45.0	13.5		53.9	41.8
6	R 94	0.0					42.7				36.4				
6	R 95	46.1	52.2				7.1							79.1	
6	R 96												28.3	25.5	
6	R 97						50.2		14.1	17.5	15.8		60.2		
6	R 98	30.4			61.8	30.4		5.0			5.0				
6	R 99														
6	R 100	40.3		50.0											
6	Y 1						25.5				42.7				
6	Y 2		52.2	50.0											
6	Y 3	11.4		15.0		11.2			35.2	44.7	5.0			60.0	
6	Y 4							7.1					14.1		
6	Y 5				15.8	33.7	35.9							65.0	
6	Y 6			5.0		10.4		5.0		35.4					
6	Y 7				7.1										
6	Y 8		51.0	35.0								35.4		55.0	
6	Y 9		18.0					5.0					41.0		7.1
6	Y 10												65.8		
6	Y 12					18.3	40.0	5.0	50.2	10.0	40.3		41.1	87.9	
6	Y 13				20.2		35.0			41.2	49.2	67.6	35.4	82.9	
6	Y 14					18.9	5.0	11.2					46.6		
6	Y 16			15.0											
6	Y 17	41.7		35.4	18.0	14.1	39.1				22.4	9.1	43.0	60.4	
6	Y 19														
6	Y 20											31.6	34.0	53.2	35.4
6	Y 21				13.7										
6	Y 22		25.3		5.0	15.8		10.0							
6	Y 23	26.2		5.0	11.2	25.5	41.2				52.8	45.0	60.0		
6	Y 24						42.7	45.3				25.0	70.2	57.0	
6	Y 25			45.1	22.4										
6	Y 26			51.2											
6	Y 27		11.2	2.5			30.4	5.0	5.0	7.5	23.8	20.0	33.5		
6	Y 28		31.6	10.6									40.3		
6	Y 29						51.9		37.9						
6	Y 30				15.8	7.1	31.6					50.0	54.3	57.0	
6	Y 31								55.0	83.2					
6	Y 32					46.1									
6	Y 33	42.8	14.1		17.8		6.5	7.1		30.4	40.3				
6	Y 34						11.2	35.4	45.0	47.2		30.4	11.2		

Appendix 4.3 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6	Y 37			5.0											
6	Y 38	40.3		7.1				31.6							
6	Y 39		7.1												
6	Y 40	45.3	10.0												
6	Y 41					5.0									
6	Y 42				10.0	40.3	11.2		60.0	20.6	93.4	60.0		40.3	
6	Y 43	0.0		5.0								20.6			
6	Y 44		42.7					11.2	45.3	33.4					
6	Y 45				0.0	20.0	55.2	15.8							
6	Y 46	7.5													
6	Y 48														
6	Y 49				38.0						36.1				
6	Y 50			50.0								50.2		53.2	
6	Y 51			5.0											
6	Y 52					20.6		36.4		23.2			5.0		
6	Y 53				46.1	36.4		31.6			10.6		28.3		
6	Y 54		13.1		10.6		37.8								
6	Y 55								5.0	7.1					
6	Y 56			6.8											
6	Y 57		52.2												
6	Y 58			5.0										69.5	
6	Y 60														
6	Y 61		42.7	45.0			10.6	35.4		63.0	50.2				
6	Y 62		5.0											69.6	
6	Y 63	40.0		15.0										50.0	
6	Y 65												35.4		
6	Y 66				15.8								60.2	41.2	22.4
6	Y 67	40.3				8.5								57.7	
6	Y 68				46.1						44.0				7.1
6	Y 69			50.2						11.2		20.0	36.4		34.5
6	Y 70														
6	Y 71				7.1									80.2	
6	Y 72					15.8				25.0		36.4			
6	Y 73			50.0											
6	Y 74					46.1	48.5						62.3	56.2	
6	Y 76			0.0											
6	Y 77														
6	Y 79						58.2								
6	Y 80		0.0												
6	Y 81			7.1	15.0										
6	Y 82							10.0	10.0						
6	Y 83										38.1			36.4	
6	Y 86						47.1								
6	Y 87		14.1												
6	Y 88	40.3		7.1											
6	Y 91					36.4	34.0			0.0		15.0			
6	Y 92			50.0	26.8	10.0									20.0
6	Y 93			35.3										28.3	62.6
6	Y 94	15.8		55.2	21.2	10.0					8.1	40.3			
6	Y 95				10.0	20.6		10.0		33.4			7.1		35.4
6	Y 96	40.3													
6	Y 97														35.4
6	Y 98			45.3	11.2	14.0	49.9					53.9		68.0	
6	Y 99		53.7												
6	Y 100	40.3													

Appendix 4.4: The individually performed tasks of every observed labelled worker after the merger (only observed behaviours after the merger are shown).

Colony	worker ID	Task																								
		1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
5	B 1																									
5	B 2		13									1														
5	B 3		2																							
5	B 4																									
5	B 5	6	20																							
5	B 7																									
5	B 9																									
5	B 10																									
5	B 12	1																								
5	B 13																									
5	B 14																									
5	B 15	1																								
5	B 16																									
5	B 17	4	17				1				1															
5	B 18		1																							
5	B 19																									
5	B 20																									
5	B 21		1																							
5	B 22		16				1																			
5	B 23	1	8																							1
5	B 24																									
5	B 25	1	1			1			1																	
5	B 26		2																							
5	B 28	2																								
5	B 29		2																							
5	B 30																									
5	B 31	1																						2		
5	B 32		11																							
5	B 33																	1								
5	B 34	2	8	1																						
5	B 35	1	13														1									
5	B 36		10					1																		
5	B 37	2	3														1									
5	B 38		15			1	2					1														
5	B 39	1																								
5	B 40																									
5	B 41	2	8		1		1	1							1	21	1									
5	B 42		1																							
5	B 43																									
5	B 44		7																							1
5	B 45																									
5	B 46																									
5	B 47																									
5	B 48		2																							
5	B 49		6																							1
5	B 50																									
5	B 51	3	11			1																				1
5	B 53	2				1																				1

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
5	B 55																									
5	B 56		2																							
5	B 57																									
5	B 58																									
5	B 59	1																								
5	B 60	1																								
5	B 61		2																							
5	B 62																									
5	B 63																									
5	B 64																									
5	B 65		1																							
5	B 66				4				1								1	1								
5	B 67																									
5	B 68																									
5	B 69																									
5	B 70		1																							
5	B 71																									
5	B 72																									
5	B 73																									
5	B 74		8				1																			
5	B 75	3	4				1									1						1				
5	B 76																									
5	B 77	3	11							1																
5	B 78	3	6			1																				
5	B 79	2	15			1											1									
5	B 80																									
5	B 81																									
5	B 82																									
5	B 84																									
5	B 85	1	20				1																			1
5	B 86																									
5	B 87	3	3																							
5	B 88		1																							1
5	B 89		3																							
5	B 90																									
5	B 91		1																							
5	B 92																									
5	B 93	2	7					1					1													
5	B 94	1	5			1			1																	
5	B 95		4																							
5	B 96		11																							1
5	B 97	1	6			2	1																			
5	B 98	2	6																							
5	B 99	4	5														1									
5	B 100																									
5	W 1	2	13				1										1									
5	W 2		14																							
5	W 3	1	19																							
5	W 4	1																								

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
5	W 5																									
5	W 6		1																							
5	W 7																									
5	W 8	1	18																							
5	W 9		2																							
5	W 10		2																							
5	W 11		2																							
5	W 12																									
5	W 13																									
5	W 14		1																							
5	W 15		2																							
5	W 16	1	9		1																					
5	W 17		3																							
5	W 19	3	12										1											1		
5	W 21	1	2			1	1													1						
5	W 22																									
5	W 24		1																							
5	W 25																									
5	W 27	2	8			1																				
5	W 28	1	4																							
5	W 29	1	3									1							1							
5	W 30	1																								
5	W 31		2																							
5	W 33																									
5	W 34																									
5	W 35		1																							
5	W 36																									
5	W 37																									
5	W 38	1	9																							
5	W 39		1																							
5	W 40	4											1											1		
5	W 41		1																							
5	W 42																									
5	W 43																									
5	W 44	1	9																							
5	W 45	1	8																							
5	W 46																									
5	W 47																									
5	W 48	5	7				1	1									1									
5	W 49		6						2		1															
5	W 50	2	12																							1
5	W 52																									
5	W 53	1	9																							
5	W 54																									
5	W 57																									
5	W 58		1																							
5	W 59																									
5	W 60	4	8				2		1																	
5	W 61	3	15																							
5	W 62		1																							

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
5	W 63	4	14																							
5	W 64		2																							
5	W 65	2	10				1																			
5	W 66																									
5	W 67						1																			
5	W 68	2	6		1												1									
5	W 69	2	17											1			1	1								
5	W 70					1																				
5	W 71		12				1	1																		
5	W 72																									
5	W 73		1																							
5	W 74	1																	1							
5	W 75	3	3				1																			
5	W 77	3	10																							
5	W 78	1	14								1															
5	W 79																									
5	W 80							1																		
5	W 81																									
5	W 82	1						1																		
5	W 83																									
5	W 84						1																			
5	W 85		4																						2	
5	W 86																									
5	W 87																									
5	W 88																									
5	W 89	1	19				1																			
5	W 90	3	3	1																						
5	W 91	1																								
5	W 94		12					1										1								1
5	W 95		1																							
5	W 96	1	12				1				1															
5	W 97	1	13			1			1																	
5	W 98		2																							
5	W 99	1	17				1										1									
5	W 100	1	3			1		1									1						1			
6	R 1	7	1			2	2																			
6	R 4																									
6	R 5	6	9			1		1																		
6	R 6	2	11				1																			
6	R 7																									
6	R 8		9														1									2
6	R 9		2																							
6	R 10	2	13						1																	
6	R 11																									
6	R 12	4	6														1									
6	R 13					1																				
6	R 14	2	6														1									
6	R 15	6	12																		1					
6	R 16	1	14				1																			
6	R 17	2	11																							

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
6	R 18	3	19								1	1														
6	R 19	1	4					1			1															
6	R 20	3	13				1		1								1									
6	R 21	3	10																							
6	R 22		1																							
6	R 23	2	14																							
6	R 24	1																								
6	R 25	3	11														1									
6	R 26					1																				
6	R 27	1	10				1	1									1	1								
6	R 28	3	9			1					1															1
6	R 29	2	3																							
6	R 30	1	16				2																			1
6	R 31	3	12															1								1
6	R 32	6	10																							
6	R 33		11																							
6	R 34	5	1								1						1									
6	R 35																									
6	R 36	5	8				1										1									1
6	R 37	4	3			1																				
6	R 38	2	2				1	2									1		1							
6	R 39		6																							
6	R 40	1	7														1									
6	R 41																1									
6	R 42																									
6	R 43	4	14				1																			
6	R 44		14				1										1									
6	R 45		1																							
6	R 47																1									
6	R 48	1	6																							
6	R 49																									
6	R 51		1																							
6	R 52	3	11				1		1																	
6	R 54	1	5																							
6	R 56	3	13					1																		1
6	R 57		7				1																			
6	R 59	2	7																							
6	R 60	1	6																							
6	R 62	6	9	1																						1
6	R 63	1	9					1																		
6	R 64	2	24																							
6	R 65																									
6	R 66	2	6			1																				
6	R 67		1																							
6	R 68		1																							
6	R 69																									
6	R 70		2																							
6	R 71		2																							
6	R 72		1																							
6	R 74		4														2									

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
6	R 76		5			1												1								
6	R 77	1	8																							1
6	R 78																									
6	R 79	2	2		1																					
6	R 80	1	1																							
6	R 81																									
6	R 82		2																							
6	R 83	1	1																2							
6	R 84	1						1			1							1								
6	R 85	1				1																				
6	R 86																									
6	R 87		10																							
6	R 88	3	4																							
6	R 89	3	20					1	1																	
6	R 90																									
6	R 91		1																							
6	R 92	1	7																							
6	R 93		17									1					1									
6	R 94		1																							
6	R 95	2	8						1																	
6	R 96	1	17																							1
6	R 97		5															1								1
6	R 98	1	8														1									
6	R 99																									
6	R 100																									
6	Y 1		1																							
6	Y 2		1																							
6	Y 3	1	8																							
6	Y 4		9					2				1														
6	Y 5	1	13																							
6	Y 6		2																							
6	Y 7																									
6	Y 8	4	4														1									
6	Y 9	4	15			1	1	1									1									
6	Y 10	2	9				2																			
6	Y 12		6																							
6	Y 13	1	5				2																			
6	Y 14	3	8					1																		
6	Y 16	5	9																							
6	Y 17	2	8					1										1						1		1
6	Y 19		2																							
6	Y 20		12								1															
6	Y 21																									
6	Y 22																									
6	Y 23	11																								1
6	Y 24	1	13					2																		
6	Y 25		1																							
6	Y 26		1																							
6	Y 27	1	17														1									
6	Y 28																									

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
6	Y 29		4																							
6	Y 30	2	9																							
6	Y 31																									
6	Y 32																									
6	Y 33		1																							
6	Y 34	2	2																							
6	Y 37																									
6	Y 38																									
6	Y 39		3																							
6	Y 40		1																							
6	Y 41		1																							
6	Y 42	2	6																							
6	Y 43																									
6	Y 44																									
6	Y 45	1	1																							
6	Y 46																									
6	Y 48																									
6	Y 49		1				1																			
6	Y 50	1	8																							
6	Y 51																									
6	Y 52	2	4								1															
6	Y 53	2	12																							
6	Y 54																									
6	Y 55																									
6	Y 56																									1
6	Y 57	1	5																							
6	Y 58	1	8				1																			
6	Y 60	1	3																							
6	Y 61		2				1																			
6	Y 62	2	18						1																	
6	Y 63	2	8				2										1									
6	Y 65		3			1			1									1								
6	Y 66		14																							
6	Y 67		7														1									1
6	Y 68	5	4					2			1															
6	Y 69	1	11			1		1																		
6	Y 70		1																							
6	Y 71	1	5																							
6	Y 72	2	5					1																		
6	Y 73	2	12						1																	
6	Y 74		12				1																			
6	Y 76					1																				
6	Y 77		2																							
6	Y 79	3	13														1									
6	Y 80	1																								
6	Y 81																									
6	Y 82	1																								
6	Y 83		5																							
6	Y 86		1																							
6	Y 87																									

Appendix 4.4 continues

Colony	worker ID	1	2	3	4	5	6	7	8	9	10	12	13	14	16	18	20	21	24	25	26	27	29	30	31	32
6	Y 88																									
6	Y 91	2	5							1																
6	Y 92		2																							
6	Y 93	5	9										1				1									
6	Y 94	2	11					1	1										1							
6	Y 95	4	5														1									
6	Y 96		1																							
6	Y 97							1									1									
6	Y 98		8																							
6	Y 99		1																							
6	Y 100																									

Appendix 4.5: Queen-worker distance of all observed labelled workers of both colonies after the merger (day 15-25).

Colony	worker ID	Day										
		15	16	17	18	19	20	21	22	23	24	25
5	B 1											
5	B 2	41.8	15.0	53.9			55.9	65.2	31.1	20.0		51.5
5	B 3	22.0				28.3	15.8					
5	B 4											
5	B 5	37.5	28.0	11.2		25.5	66.5	37.5	24.3	44.9	23.1	76.2
5	B 7											
5	B 9											
5	B 10											
5	B 12	86.3				86.3						
5	B 13											
5	B 14											
5	B 15	30.0				30.0						
5	B 16											
5	B 17	62.5			80.6	56.1	29.2	72.8	66.4	63.2	66.2	65.7
5	B 18											
5	B 19											
5	B 20											
5	B 21					97.1						
5	B 22	58.4				88.6	71.9	60.2	49.7		41.2	38.5
5	B 23	57.5			83.8		56.4		50.0	61.8		35.4
5	B 24											
5	B 25											
5	B 26	42.4							42.4			
5	B 28	14.6	14.6									
5	B 29	21.2	14.1									28.3
5	B 30											
5	B 31	56.7		53.2								60.2
5	B 32	49.4		25.5			65.8		31.6	75.0	49.2	
5	B 33	36.4										36.4
5	B 34	38.1		20.6	50.2			11.2	31.6	69.6	45.3	
5	B 35	49.8		22.4	67.1			52.0	78.3		40.3	39.0
5	B 36	54.3	40.0			70.7			32.0	61.8		66.8
5	B 37	32.4	20.6			48.2					28.3	

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
5	B 38	46.8	18.0	39.1	85.0		57.1	25.5	62.2	44.7	43.1	
5	B 39	83.8								83.8		
5	B 40											
5	B 41	41.3		53.2	47.2		31.6		31.6		58.2	26.3
5	B 42	29.2						29.2				
5	B 43											
5	B 44	43.7			32.0		37.9	35.4	46.1			67.1
5	B 45											
5	B 46											
5	B 47											
5	B 48	25.0							25.0			
5	B 49	42.3	22.4		55.0		33.5					58.3
5	B 50											
5	B 51	46.7				47.0	60.9	38.1	62.5	18.0		53.9
5	B 53	40.0	40.0									
5	B 54											
5	B 55											
5	B 56	7.1									7.1	
5	B 57											
5	B 58											
5	B 59											
5	B 60	45.3					45.3					
5	B 61	69.6							62.6			76.5
5	B 62											
5	B 63											
5	B 64											
5	B 65	57.0		57.0								
5	B 66	50.6		58.5	69.5	54.3	44.7	53.2	53.2			21.2
5	B 67											
5	B 68											
5	B 69											
5	B 70	21.2										21.2
5	B 71											
5	B 72											
5	B 73											
5	B 74	25.6			33.5		28.8		24.1	15.8		
5	B 75	55.8					53.9				43.7	69.7
5	B 76											
5	B 77	50.6	21.2			55.9	28.2		50.9	79.9	45.1	72.8
5	B 78	32.9	15.8	51.5	65.0	40.3		25.0			11.2	21.2
5	B 79	47.8		26.9		55.2	61.8	25.0	65.7	55.9	63.3	28.3
5	B 80											
5	B 81											
5	B 82											
5	B 84											
5	B 85	54.3	15.8	40.6		77.8	39.0	60.2	75.1	60.8		65.1
5	B 86											
5	B 87	37.3	40.3						35.2	36.4		
5	B 88	51.2					22.4	80.0				
5	B 89	81.4							81.4			

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
5	B 90											
5	B 91											
5	B 92											
5	B 93	41.4	29.6	53.2		40.0			36.6		47.5	
5	B 94	29.7		36.1			5.0	36.4	41.2		10.0	49.2
5	B 95	41.8							31.6	81.4	12.5	
5	B 96	48.7					42.5	40.0	69.6	41.2	50.3	
5	B 97	35.9	26.9		58.5	60.5	15.8	18.0				35.4
5	B 98	41.5	25.5	53.2		35.0	60.0			25.5		50.1
5	B 99	47.1	20.6		61.0					46.1	60.8	
5	B 100											
6	R 1	31.6		16.1	47.8	33.5	32.3	50.0	10.0			
6	R 4											
6	R 5	45.9		57.0	49.2	29.6	39.1	53.9	30.7	41.2	45.3	66.7
6	R 6	32.3	18.0	31.6		35.0	71.6		20.0	42.7	25.2	14.1
6	R 7											
6	R 8	36.9				48.0	46.1	53.2	22.8		23.1	28.3
6	R 9											
6	R 10	51.5	51.0	35.0	60.4		79.1	58.5	64.3	14.1	63.2	38.2
6	R 11											
6	R 12	42.7			57.0	31.6		53.2	40.3	33.5	40.3	
6	R 13	42.7			42.7							
6	R 14	51.4	25.0			38.1	38.1	80.6	66.7	60.0		
6	R 15	38.8	39.2	43.0	40.3	50.0		51.7	26.3	37.2		22.9
6	R 16	65.7	23.7		86.3	60.4		96.2	79.3		48.3	
6	R 17	61.7		42.4	42.7	85.0	54.1		76.3	57.0	60.5	75.2
6	R 18	67.4			15.8	63.2	70.2	102.2	77.0	95.3	40.2	75.4
6	R 19	36.1	18.0		11.2			69.5		38.3		43.7
6	R 20	67.8	49.7		105.9	62.0	68.1	51.5	69.6			
6	R 21	34.1	25.5	38.3			38.3		29.2		32.6	41.0
6	R 22	18.0		18.0								
6	R 23	46.8			60.8	71.8	65.2	15.0	33.5	63.2		18.0
6	R 24	32.0	32.0									
6	R 25	42.7		31.6		45.3	40.0	50.0	46.5			43.0
6	R 26	25.0	25.0									
6	R 27	57.8	63.9		80.0	74.3	45.3		31.6	80.2		29.2
6	R 28	38.5	41.5	7.1	25.0	48.4			38.8	70.5		
6	R 29	27.7					30.4				27.1	25.5
6	R 30	67.4	37.5			65.8	70.7	57.0	75.0	95.1	66.2	71.6
6	R 31	39.6	29.7		40.0	40.0	54.1	55.2	15.8	40.0	42.8	38.4
6	R 32	31.7	55.9	37.8	11.2	14.1	14.1		59.9	40.3	20.6	31.6
6	R 33	41.9		18.0	91.0				50.0	27.4	42.5	22.4
6	R 34	38.4	51.0	48.6				30.4	30.4			31.6
6	R 35											
6	R 36	52.4	49.0			85.4	47.3	30.4				49.7
6	R 37	41.4				36.1			70.2			18.0
6	R 38	29.9	43.0					7.1	50.0	42.5		7.1
6	R 39	49.6				50.0	65.1		25.0			58.3
6	R 40	45.8	18.0	61.0		66.7			42.4			40.7
6	R 41	22.4	22.4									

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
6	R 42											
6	R 43	32.2	42.5		38.2	15.4		52.9	34.0		28.0	14.1
6	R 44	52.3	38.1	25.0		28.0		39.1	80.6	82.5	51.5	73.9
6	R 45	15.8								15.8		
6	R 47	75.2				75.2						
6	R 48	54.4			87.3	70.2				35.0	25.2	
6	R 49											
6	R 51											
6	R 52	55.7	55.9		55.9		28.5	70.0	60.4		63.2	
6	R 54	36.8			46.1	32.0	32.7		36.4			
6	R 56	50.1		25.5	50.2	72.9	54.1	47.4	58.3		61.9	30.4
6	R 57	29.6							31.6	42.7	10.6	33.5
6	R 59	38.3		15.8	23.8		80.8				33.0	
6	R 60	56.9		47.4		76.2	72.5		55.2		33.0	
6	R 62	38.9	43.0	53.2		36.8	27.0		35.4	40.3		36.4
6	R 63	57.9				54.3	35.4	70.0	51.9	70.2		65.9
6	R 64	63.3		25.0		93.4	68.4	55.2	67.3	58.5	60.1	78.2
6	R 65											
6	R 66	42.0	15.8				51.0	35.0	49.9	46.4	53.9	
6	R 67	35.0				35.0						
6	R 68											
6	R 69											
6	R 70	45.2	25.5			65.0						
6	R 71	67.7				67.7						
6	R 72	20.6							20.6			
6	R 74	69.5	62.6						76.3			
6	R 76	38.2		33.5	40.3			57.0			21.8	
6	R 77	46.8	47.4	50.0		58.3			23.9	54.3		
6	R 78											
6	R 79	46.2	43.0			50.2					45.3	
6	R 80	57.8		46.1							69.5	
6	R 81											
6	R 82	49.3				58.3			40.3			
6	R 83	45.7	36.4		55.0							
6	R 84	30.9	29.2	32.7								
6	R 85	27.7	15.0	40.3								
6	R 86											
6	R 87	45.2	25.0			53.9		58.3		43.7		
6	R 88	40.6					50.0		51.8			20.0
6	R 89	53.8	21.2		65.9	58.3	50.6	77.3	65.3	53.6		38.6
6	R 90											
6	R 91	31.6										31.6
6	R 92	36.8			15.8	66.1	55.9				25.5	20.6
6	R 93	37.9	30.0		25.5	25.5	7.1	70.7	42.4	65.0	30.1	45.1
6	R 94											
6	R 95	40.0	24.1	25.0				61.8	48.0			41.2
6	R 96	56.4		60.2	80.6	58.0	56.4	35.9	47.2			
6	R 97	36.5			42.5	35.2	42.7					25.5
6	R 98	38.5		26.9			68.0		7.1	30.4	60.2	
6	R 99											

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
6	R 100											
5	W 1	45.9		56.6		57.0			45.9		40.2	30.0
5	W 2	55.5			33.7	42.4	72.8	66.9	60.9	73.6	35.4	58.4
5	W 3	59.8		26.2	57.0	63.0	69.3	90.1	61.6		57.7	53.2
5	W 4	80.0								80.0		
5	W 5											
5	W 6											
5	W 7											
5	W 8	53.3	30.4	54.1		53.4	44.7		57.1	61.5	52.8	72.6
5	W 9	90.1						90.1				
5	W 10	22.4										22.4
5	W 11	30.4									30.4	
5	W 12											
5	W 13											
5	W 14	65.8				65.8						
5	W 15											
5	W 16	34.7			26.5	26.9	57.0		58.3			5.0
5	W 17	46.2				70.0	22.4					
5	W 19	59.4			58.3		64.7	60.4	67.7	46.1		
5	W 21	30.5		35.4	30.4	40.3		15.8				
5	W 22											
5	W 24	63.2							63.2			
5	W 25											
5	W 27	37.0	10.0			53.9	23.3		26.9		52.0	55.9
5	W 28	82.5						82.5				
5	W 29	49.2			65.7					50.0		32.0
5	W 30											
5	W 31											
5	W 33											
5	W 34											
5	W 35	35.4									35.4	
5	W 36											
5	W 37											
5	W 38	45.6					55.2	33.5	36.5	57.0		
5	W 39	61.8										61.8
5	W 40	25.5	28.6								32.0	16.0
5	W 41	60.0					60.0					
5	W 42											
5	W 43											
5	W 44	53.2	36.4		91.8	62.4				22.4		
5	W 45	48.4		45.0		41.3	26.9	61.0	78.3			38.1
5	W 46											
5	W 47											
5	W 48	55.5				74.7	65.2			47.6		34.5
5	W 49	41.1	20.0		82.8		49.5	69.6	9.1			15.8
5	W 50	51.4				20.6	61.8	79.1		50.2		45.3
5	W 52											
5	W 53	41.3	30.0			57.0	72.8	18.0		65.2	16.9	28.9
5	W 54											
5	W 55	35.4										35.4

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
5	W 57											
5	W 58	73.8				73.8						
5	W 59											
5	W 60	37.9		32.5	44.9	45.0	49.7		30.4	40.3		22.4
5	W 61	47.9	23.8	50.1	56.2	40.3	46.4			52.6		65.6
5	W 62	33.5				33.5						
5	W 63	42.9	20.5		80.1		46.1	53.9	50.0	15.0	55.0	22.4
5	W 64	34.9			58.5			11.2				
5	W 65	45.4		36.1	49.2	39.1	58.5	58.3	38.1			38.8
5	W 66											
5	W 67	31.6				31.6						
5	W 68	30.8	20.6	54.0		35.4			46.1	8.1	11.2	40.3
5	W 69	61.9	33.5			25.0	70.2	90.6	77.6	76.5	46.8	74.6
5	W 70	0.0	0.0									
5	W 71	28.7		30.4		51.0			26.3	7.1		
5	W 72											
5	W 73	45.3			45.3							
5	W 74											
5	W 75	36.7				65.0				5.0	40.0	
5	W 77	32.0	10.0	63.2	36.1	41.8		15.8	30.0		20.3	39.1
5	W 78	42.2	18.0	21.2	45.3	32.4	38.7		47.2	79.9	27.4	69.6
5	W 79											
5	W 80											
5	W 81											
5	W 82	8.1										8.1
5	W 83											
5	W 84	35.4					35.4					
5	W 85	35.9		7.1			63.6	55.0				18.0
5	W 86											
5	W 87											
5	W 88											
5	W 89	66.0	20.6	37.8		97.1	63.9	105.9	81.0	55.0		66.7
5	W 90	27.1		25.5				35.4	32.6	15.0		
5	W 91	31.6			31.6							
5	W 94	43.3	5.0		80.6	37.0	31.0		38.8		53.4	56.8
5	W 95											
5	W 96	54.0	5.0				42.4	95.5	59.6	52.2		69.3
5	W 97	51.0			51.0	63.7	76.2	29.5	40.3	45.6		
5	W 98	52.1								65.2		39.1
5	W 99	40.4		45.3		38.9	38.7	35.4	38.1		21.2	65.0
5	W 100	38.8			50.0	22.4	26.2	60.2		35.4		
6	Y 1	25.5						25.5				
6	Y 2	27.5!				67.5!						
6	Y 3	39.5	14.1			40.3			53.1	50.2		
6	Y 4	42.0	20.6	24.7				39.1	72.1	75.2		20.6
6	Y 5	42.1	25.8	29.2	86.3		28.3	30.0	36.4	40.3		60.4
6	Y 6	30.0										30.0
6	Y 7											
6	Y 8	29.8	20.0			0.0	52.0					47.2
6	Y 9	32.6			8.1	53.0	39.2	20.6	48.9		34.0	24.3

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
6	Y 10	49.0	14.1			61.4	35.0	55.0	35.0	93.4		
6	Y 12	32.0					51.0		20.0			25.0
6	Y 13	43.5	22.5			30.4		40.3		80.8		
6	Y 14	44.5	25.0			34.8	40.3		46.1			76.2
6	Y 16	44.9			70.2	25.0		52.4		30.5	45.3	46.1
6	Y 17	34.8		30.4			61.9	35.4		21.5		24.8
6	Y 19	63.2		63.2								
6	Y 20	42.9		63.2		22.4	65.2	47.2	40.4		25.5	36.5
6	Y 21											
6	Y 22											
6	Y 23	33.3			75.4	27.8				14.1	21.5	27.5
6	Y 24	64.4	14.6				77.4	101.2	55.9	72.8		
6	Y 25											
6	Y 26	40.3				40.3						
6	Y 27	53.0		25.0	60.2		40.3	67.3	30.4	100.1	61.5	39.4
6	Y 28											
6	Y 29	28.5					32.0				25.0	
6	Y 30	53.2				70.0			41.2		49.2	52.2
6	Y 31											
6	Y 32											
6	Y 33											
6	Y 34	25.0			25.0							
6	Y 37											
6	Y 38											
6	Y 39	50.4				40.3	60.4					
6	Y 40											
6	Y 41											
6	Y 42	35.8			57.0				32.3			18.0
6	Y 43											
6	Y 44											
6	Y 45	36.7	36.7									
6	Y 46											
6	Y 48											
6	Y 49	28.3	28.3									
6	Y 50	66.7							40.3	90.1	53.0	83.3
6	Y 51											
6	Y 52	48.3			83.8	20.0				41.2		
6	Y 53	38.1	14.1	15.8	83.8		56.3	11.2	31.6		45.1	47.2
6	Y 54											
6	Y 55											
6	Y 56	46.1					46.1					
6	Y 57	37.3					40.3		34.3			
6	Y 58	44.9							28.3	61.0	30.4	59.8
6	Y 60	57.2					55.9					58.5
6	Y 61	8.1						8.1				
6	Y 62	53.7	21.7	25.5	60.4	70.0	71.9		43.0	51.8	52.7	86.1
6	Y 63	35.0	20.0	57.0		70.6	65.2			7.5	14.1	10.4
6	Y 65	19.8					36.4	17.7	20.0		5.0	
6	Y 66	31.1		20.6				35.4	20.6	66.7	7.5	35.8
6	Y 67	54.7		63.2	55.2	61.8	42.7		39.1		60.2	60.8

Appendix 4.5 continues

Colony	worker ID	15	16	17	18	19	20	21	22	23	24	25
6	Y 68	30.2		30.0	14.1	44.9	57.6			5.0		29.4
6	Y 69	44.4			39.1	68.0	28.3	50.7	43.0			37.2
6	Y 70	67.1			67.1							
6	Y 71	34.1				20.6			30.4			51.4
6	Y 72	38.2	32.7		46.1				33.7		40.3	
6	Y 73	52.3			50.0	77.2		40.3	65.0	63.7	22.4	47.7
6	Y 74	57.8		72.1		64.3	40.3	68.8	25.1			76.2
6	Y 76											
6	Y 77	71.6										71.6
6	Y 79	42.8	15.9			63.7	35.4		30.3	50.0		61.7
6	Y 80	96.6				96.6						
6	Y 81											
6	Y 82											
6	Y 83	45.2		14.1	76.3							
6	Y 86	20.0	20.0									
6	Y 87											
6	Y 88											
6	Y 91	24.6		20.6			27.2			30.4		20.0
6	Y 92	55.9					55.9					
6	Y 93	64.4				76.4	60.9	99.2	65.8		21.2	62.7
6	Y 94	41.1	15.3		55.2		43.7	38.1	32.0			62.5
6	Y 95	52.9	15.0		90.6			38.1		68.0		
6	Y 96	25.0							25.0			
6	Y 97	49.0				61.8			36.1			
6	Y 98	32.5	0.0			58.2			30.4			41.2
6	Y 99	35.0						35.0				

Chapter 5
Egg laying and egg removal by workers are positively correlated in queenright Cape honeybee colonies (*Apis mellifera capensis* Esch.)

Abstract Queenright Cape honeybee colonies exhibit considerable egg laying by workers. However, recent findings suggest that they can show efficient removal of worker-laid eggs, which is subject to environmental variation. This variation leads to periods of both low and high egg removal, so one potential mechanism of how workers could achieve successful reproduction would be laying eggs in periods of low egg removal to potentially increase the survivability of their eggs. If laying workers use this strategy one would expect a negative correlation between egg laying and egg removal. Here we study whether egg laying by workers is correlated to the level of egg removal in the Cape honeybee. Egg removal rates for queen (N=240) and worker-laid (N=240) eggs and egg laying by workers were investigated in three queenright test colonies. Worker-laid eggs were removed significantly faster than queen-laid eggs, confirming that queenright colonies of *A. m. capensis* are able to police worker-laid eggs. Significant differences in egg laying by workers were found between the test colonies, with a total of 57 worker-laid eggs were observed in the test colonies. The data show that egg removal and egg laying by workers are positively correlated, indicating that egg removal and egg laying are co-dependent in Cape honeybees and that workers do not use periods with low egg removal to increase the survival of their eggs. The data rather suggest that egg removal could be triggered by the number of worker-laid eggs in the colonies. Alternatively, but not mutually exclusive, periods with low egg removal may have disadvantages for the survival of worker derived offspring. A combination of the intercolonial variation for the number of eggs laid by workers, their egg removal rates, and the ability of workers to remove worker-laid eggs may explain the phenotypic variation for successful worker reproduction in queenright Cape honeybee colonies.

5.1. Introduction

With few exceptions (Oldroyd et al. 1994), worker-laid eggs are removed by other workers in queenright colonies of the European subspecies of *Apis mellifera* (Ratnieks & Visscher 1989). This seems to be based on relatedness grounds (Ratnieks 2000), because laying workers usually produce male offspring (Free 1987). However, laying workers of the Cape honeybee (*A. m. capensis*) produce female offspring (Onions 1912; Neumann et al. 2000b), leading to predictions that egg removal is either not expressed at all, or is less expressed in this subspecies (Greeff 1996). Nevertheless, queenright Cape honeybee colonies exhibit worker policing (Chapter 2), indicating that removal of worker-laid eggs can also be based on colony efficiency grounds.

Brood above the queen excluder is more frequently observed in queenright colonies of Cape honeybees (Pettey 1922; Tribe & Allsopp 2001; personal observations) than in other subspecies of *A. mellifera* (Visscher 1996). It has been shown that such brood is actually worker-derived (Moritz et al. 1999), indicating successful worker reproduction despite the presence of a queen and egg removal. Indeed, thousands of queenright colonies of the neighbouring subspecies *A. m. scutellata* were taken over by laying *A. m. capensis* workers (Allsopp & Crewe 1993), showing that *A. m. capensis* workers are facultative social parasites (Hepburn & Allsopp 1994; Neumann et al. 2001). These observations strongly indicate that laying workers of *A. m. capensis* are able to evade worker policing, but what potential strategies, if any, could these laying workers use to increase the survival of their eggs?

Worker policing is subject to environmental variation within colonies of *A. m. capensis*, thus leading to periods of low egg removal rates under unfavourable weather conditions (Chapter 3). Because worker policing is only exercised against eggs (Ratnieks & Visscher 1989), worker-laid eggs need only survive three days after oviposition, which might fit well in a time window of low egg removal. So one potential strategy, which we designate as hypothesis 1 could be that laying workers are able to evaluate periods of low egg removal and lay their eggs during this time window. In this case one would expect a negative correlation between worker egg laying and the removal of worker-laid eggs by other workers, because workers should lay more eggs when there is less egg removal by other workers and *vice versa*.

But, the same environmental factors which affect egg removal behaviour, may also reduce egg-laying activity (hypothesis 2). In this particular case one would expect a positive correlation between worker egg laying and removal of worker-laid eggs by other workers, because laying worker activity and egg removal behaviour are affected in the same way. Alternatively a third hypothesis emerges, but not necessarily mutually exclusive of hypothesis 2,

that laying workers may not be able to evaluate periods of low egg removal periods and egg removal is simply triggered by the number of worker-laid eggs present in the colony, also leading to a positive correlation between egg laying and egg removal. The more or less eggs laid by workers, the more or less that have to be more removed by other workers. A final and fourth hypothesis is that, egg laying and egg removal may be completely independent of one another. In this particular case no correlation would be expected. Here we test these four hypotheses by evaluating egg laying and removal of worker-laid eggs by workers in queenright Cape honeybee colonies.

5.2. METHODS

Two queenless and four queenright *A. m. capensis* colonies were obtained from Port Elizabeth and placed in a test apiary in Grahamstown, South Africa. All colonies were unrelated to avoid any bias derived from nestmate recognition on egg removal behaviour (Pirk et al. 2001a, Chapter 8), and housed in 10-frame standard Langstroth hives with two brood boxes. The colonies were given two days to settle to avert absconding (Hepburn et al. 1999). Then, three test combs (**A, B, C**) were placed in the brood nest of each of three queenright test colonies two days before the experiments began to avoid any potential impact of the introduced comb (Breed et al. 1995) on egg removal behaviour. The queens of the three test colonies were placed in small wooden cages [8cm × 4cm × 2.5cm] with gauze mesh [mesh width = 2 mm], to allow feeding by workers but preventing the queens from egg laying (Fig. 5.1). These cages were attached in a frame of empty comb (**B**) and returned to the middle of the brood nest (Fig. 5.2). Test frame **A** was sandwiched between two brood frames according to standard methods for evaluating egg removal rates (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000). Another empty test frame (**C**) was also placed in each of the colonies to evaluate egg laying by workers (Fig. 5.2).

From the next morning onwards worker-laid eggs in each of the test colonies were counted once daily at 09:00 after removing the **C** test frames on four sequential days. During counting, sheets of transparent films were placed over the **C** combs and the position of each egg was recorded by using a reference grid to avoid double counting and to count only eggs laid by workers within a 24 hour period. Simultaneously on these four days, the **A** test combs were used to evaluate the level of egg removal for queen and worker-laid eggs (Ratnieks & Visscher 1989; Ratnieks 1993).

The level of egg removal was measured as the proportion of eggs removed from the total number of eggs that were transferred expressed as a percentage. One queenright and two queenless colonies were used as egg sources. Twenty queen and 20 worker-laid eggs were transferred daily on each of the **A** test combs, which were then again sandwiched between two brood frames of the test colonies (Fig. 5.2). After 24 hours the **A** test combs were briefly removed, all remaining eggs were counted and then removed before transferring a new set of eggs onto the comb. It is assumed that egg removal and egg laying could equally occur on both **A** and **C** combs**,** so that any difference in the numbers of egg between the combs would a be systematic error. The **B** combs were not moved in any way during the experiment to keep the disturbance of the colony and the queen to a minimum.

Mann Whitney U-tests were used to compare the level of egg removal of queen and worker-laid eggs on the **A** test combs after 24 hours. A χ^2-test was performed to test the difference in the number of worker-laid eggs between the colonies. The same test was used to compare the level of egg removal of worker-laid eggs between the three colonies. Both tests were performed to investigate possible intercolonial variation. A Spearman rank order correlation was calculated for the number of worker-laid eggs and the level of egg removal of worker-laid eggs in the test cells.

5.3. RESULTS

On the **A** test combs a total of 240 queen-laid and 240 worker-laid eggs were tested for egg removal rates on a daily basis (Table 5.1). Worker-laid eggs were removed significantly faster than queen-laid eggs (Mann-Whitney U-test: $Z= 2.22$, $P<0.026$). There were no differences in the levels of egg removal of worker-laid eggs between the colonies ($\chi^2 =1.37$; $df = 2$, n.s.).

A total of 57 eggs laid by workers were recovered from the **C** combs in the test colonies (Table 5.1). Significant differences were found between the colonies in the number of worker-laid eggs ($\chi^2 = 30.74$; $df = 2$, $P<0.0001$). The number of worker-laid eggs that were laid and the level of egg removal of worker-laid eggs were significantly positively correlated (spearman Rank Order Correlation: $r_s= 0.6$, $P< 0.039$).

5.4. DISCUSSION

The data confirm earlier findings that worker honeybees in queenright colonies of *A. m. capensis* are able to recognise and remove worker-laid eggs (Chapter 2). Moreover, this study shows a significant colony variation in egg laying by workers but not for egg removal behaviour. The data also show that egg removal and egg laying by workers are significantly positively correlated in the Cape honeybee, indicating that egg removal and egg laying are co-dependent.

The standard method for evaluating egg removal behaviour in queenright honeybee colonies uses non-nestmate queen and worker-laid eggs (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000). Thus, nestmate recognition for eggs (Visscher 1986) affects egg removal estimates by overestimating the removal rates of alien worker-laid eggs compared to native worker-laid eggs (Chapter 8). Although alien worker-laid eggs (transferred into the colony) were compared with native worker-laid eggs (laid in the colony) in this study, this would simply result in a systematic error that is the same for all colonies and would not affect the correlation between egg removal and egg laying.

The positive correlation between the number of worker-laid eggs and egg removal rates clearly indicates that Cape honeybee laying workers do not specifically lay eggs during periods of low egg removal rates. That this constitutes a strategy for workers to achieve successful reproduction in the presence of a queen can thus be rejected. Periods of low egg removal rates may be disadvantageous for the survival of worker derived brood, e.g. through reduced brood care of recently hatched larvae by nurse bees (Blaschon & Crailsheim 2001). Alternatively, workers are unable to discriminate between periods of low and high egg removal, so that the level of egg removal may be triggered by the number of eggs found in the combs. Both explanations would lead to a statistically positive correlation between egg laying and egg removal.

However, some *A. m. capensis* colonies show worker-laid brood above the queen excluder, while others at the same apiary did not (Pettey 1922; Tribe & Allsopp 2001; personal observations). This is rather difficult to explain by environmental variation alone (Chapter 3) and suggests that intracolonial factors are highly important. What potential factors could explain successful worker reproduction in queenright Cape honeybee colonies? One possible factor could be that some laying workers may evade policing by laying eggs, which have low removal rates, a mechanism similar to anarchistic honeybee workers (Oldroyd & Ratnieks 2000). Indeed, highly virulent *A. m. capensis* workers which invade colonies of the

neighbouring subspecies *A. m. scutellata* (Hepburn & Allsopp 1994) show a lower level of egg removal than laying workers of *A. m. scutellata* (Martin et al. 2002). Moreover, there is considerable variation for egg removal rates among *A. m. capensis* laying worker populations (Chapter 7). Alternatively, but not mutually exclusive, the number of worker-laid eggs may simply exceed egg removal capacity of the colonies.

Indeed, the egg laying rates in the test colonies were considerably higher than non-anarchistic colonies of the European subspecies of *A. mellifera* (12 worker-laid eggs in 3 months; Visscher 1996), and comparable to a half-anarchistic European colony (about 6 worker-laid eggs per day; Ratnieks et al. 2002). This supports earlier findings that *A. m. capensis* workers may lay considerable numbers of eggs per day (Velthuis et al. 1990), and further suggests that the high variation for egg laying as observed among the test colonies (see Table 5.1 and also Hepburn et al. 1991) may be an important factor for the survival of worker-derived brood. These results are also consistent with earlier findings of high intercolonial variation for ovarial development in queenright Cape honeybee colonies (Hepburn et al. 1991). Finally, the efficiency of egg removal may vary between *A. m. capensis* colonies. Whereas we could not find such variation for egg removal, some colonies of *A. m. capensis* may show no egg removal behaviour at all (Beekman, personal communication).

Our data show that the occurrence of worker-derived brood above the excluder is unlikely to result from a strategy of laying workers to evade periods of high egg removal. We rather conclude that a combination of intercolonial variation in the number of eggs laid by workers, their egg removal rates and the ability of policing workers to remove worker-laid eggs may explain the phenotypic variation for successful worker reproduction in queenright Cape honeybee colonies.

Table 5.1: Numbers of worker-laid eggs and number of eggs remaining or removed after 24 hours in the queenright *A. m. capensis* test colonies on four sequential days. Means and standard deviations are shown.

Colony	Day	Test comb **A**		Test comb **C**
		Worker eggs remaining (Removal rates [%])	Queen eggs remaining (Removal rates [%])	Worker-laid eggs
1	1	0 (100)	6 (70)	14
	2	3 (85)	6 (70)	23
	3	4 (80)	3 (85)	0
	4	6 (70)	9 (55)	0
Mean ± sd		3.25 ± 2.5 (83.75 ± 12.5)	6 ± 2.45 (70 ± 7.5)	9.25 ± 11.3
2	1	0 (100)	6 (70)	10
	2	0 (100)	4 (80)	5
	3	3 (85)	3 (85)	1
	4	5 (75)	6 (70)	1
Mean ± sd		2 ± 2.45 (90 ± 12.25)	4.75 ± 2.65 (76.25 ± 7.5)	4.25 ± 4.27
3	1	2 (90)	6 (70)	0
	2	3 (85)	6 (70)	2
	3	8 (60)	10 (50)	1
	4	7 (55)	9 (55)	0
Mean ± sd		5.0 ± 2.9 (75 ± 17.56)	8.75 ± 2.06 (56.75 ± 10.3)	0.75 ± 0.95

Fig. 5.1: The queen cage of frame B. The B frame was sandwiched between two brood frames in the bottom box next to empty combs A and C (Fig. 5.2), so that the queen was still present in the brood nest and workers had partial access to the queen.

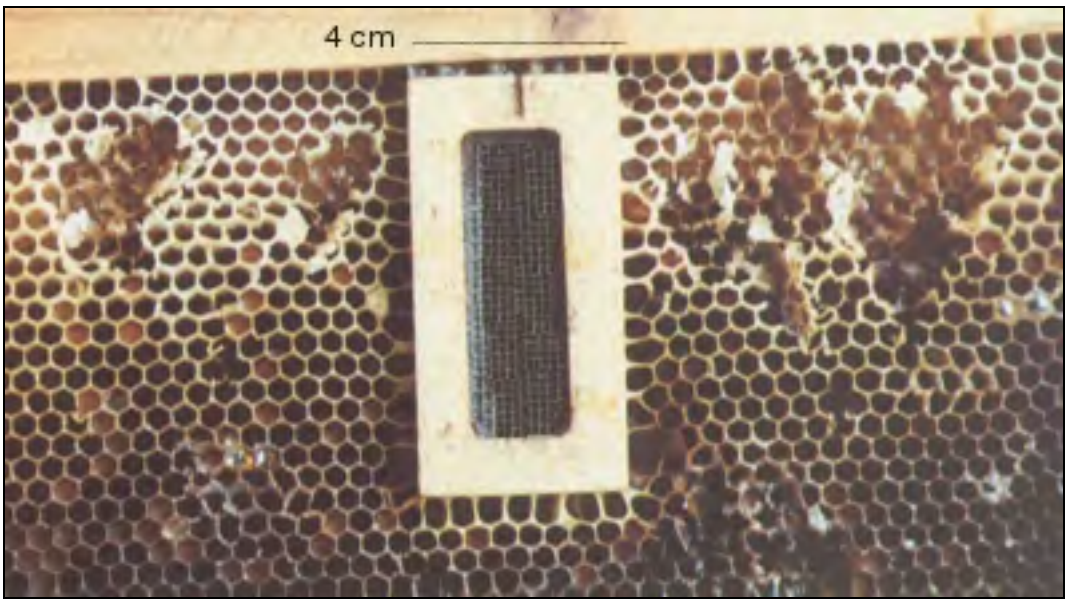
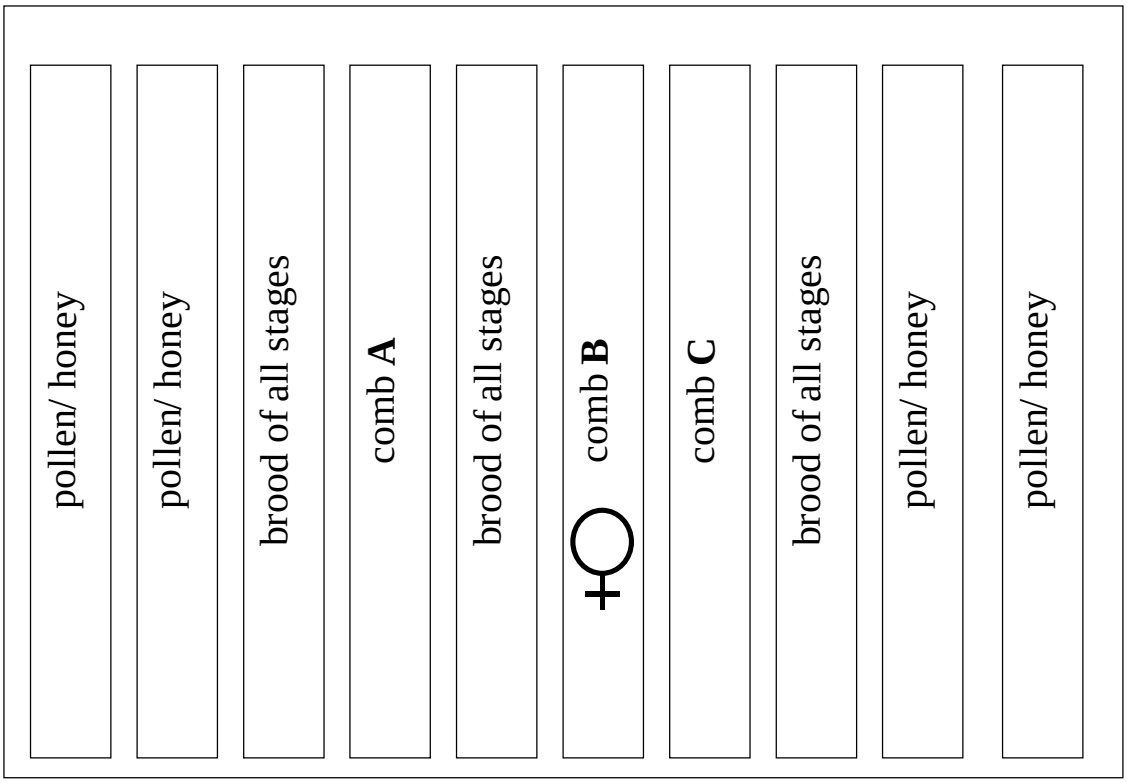


Fig. 5.2: A schematic drawing of the arrangement of the test combs (A, B, C) in the bottom boxes of the three test colonies. The combs in the top box were empty or contained honey and/or pollen.



Chapter 6
Queen evasion by Cape honeybee laying workers (*Apis mellifera capensis* Esch.)

Summary Laying workers of the Cape honeybee (*Apis mellifera capensis*) are facultative social parasites. In queenright host colonies, such workers have to evade worker policing (removal of their eggs by other workers) to successfully reproduce. Because queenright colonies of other subspecies (e.g. *A. m. scutellata*) are highly susceptible hosts, social parasitic workers probably use behavioural tactics to evade policing. The number of worker-laid eggs and egg removal rates for queen and worker-laid eggs were recorded in the top and bottom boxes in each of three queenright *A. m. capensis* and *A. m. scutellata* colonies. All queens were caged in the bottom boxes. A total of 71 eggs were laid by workers in the *A. m. capensis* colonies but none in the *A. m. scutellata* colonies. Moreover, *A. m. capensis* workers laid significantly more eggs in the top boxes (N = 67) than in the bottom boxes (N = 4) of the queenright colonies ($P<0.0001$). The egg removal data show that both subspecies are able to police worker-laid eggs, because more queen-laid eggs remained than worker-laid eggs in the bottom boxes. However, fewer *A. m. capensis* worker-laid eggs remained in the bottom boxes than in the top boxes. Moreover, whereas *A. m. capensis* also shows egg removal behaviour of worker-laid eggs in the top boxes, no significant differences between the removal rates of worker and queen-laid eggs were found in the top boxes of the *A. m. scutellata* colonies. Our results indicate that *A. m. capensis* laying workers actively evade the queen. Because worker policing is reduced in areas further away from the queen, this evasion behaviour constitutes a tactic for social parasitic workers to achieve successful reproduction in queenright host colonies.

6.1. Introduction

Social parasitism is widespread in social insects (Wilson 1971; Wcisclo 1981; Hölldobler & Wilson 1990; Schmid-Hempel 1998). Mated gynes seek and invade host colonies and start reproducing at the expense of a host. A variety of different behavioural strategies and tactics can be used by both host and parasite to achieve or counter successful parasite reproduction (Wilson 1971; Wcisclo 1981; Hölldobler & Wilson 1990; Schmid-Hempel 1998). Unmated workers of many species of eusocial Hymenoptera may reproduce parthenogenetically (Crozier & Pamilo 1996). Workers usually produce haploid offspring, developing into males (arrhenotoky, Crozier 1975), but in a few species (Wenseleers & Billen 2000) workers' offspring are diploid females (thelytoky, Crozier & Pamilo 1996).

Thelytoky appears to predispose a taxon for the evolution of aggressive worker reproductive traits (Greeff 1996) and consequently for social parasitism by workers (Neumann et al. 2001). Indeed, both thelytoky and facultative social parasitism occur in laying workers of the Cape honeybee, *Apis mellifera capensis* (Onions 1912; Hepburn & Crewe 1990; Hepburn & Allsopp 1994; Neumann et al. 2001; Neumann & Hepburn 2002). Workers of the Cape honeybee show a rather unique set of queen-like traits related to worker reproduction such as high fecundity, longevity, high pheromonal and ovarial development (Neumann & Hepburn 2002). Many more Cape honeybee workers show the phenotype of both high ovarial and pheromonal development than in other honeybee subspecies (Velthuis 1970; Tribe 1981; Hepburn 1992b). Such workers have been termed pseudoqueens (Velthuis et al. 1990) and are able to evoke retinue behaviour in other workers and to suppress the rearing of replacement queens (Anderson 1968).

In the context of social parasitism by laying *A. m. capensis* workers what are the potential defence strategies of honeybee host colonies? In queenright honeybee colonies, worker-laid eggs are eliminated by worker policing (Ratnieks 1988; Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996). The workers possibly distinguish queen-laid and worker-laid eggs by means of a queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995) derived from the Dufour´s gland (Katzav-Gozansky et al. 2000). However recent results indicate that the Dufour´s gland does not secret any kind of egg-marking pheromone (Katzav-Gozansky et al. 2001). Because both *A. m. capensis* and *A. m. scutellata* are able to police their own and the eggs of the other subspecies eggs (Neumann et al. 2002), the removal of worker-laid eggs appears to constitute an important resistance mechanism against social parasitism by laying workers for queenright colonies.

However, the high incidence of successfully parasitized queenright *A. m. scutellata* colonies (Allsopp & Crewe 1993) strongly indicates that laying *A. m. capensis* workers are somehow able to evade worker policing. Moreover, the occurrence of brood above a queen excluder is also common in queenright Cape honeybee colonies (Pettey 1922; Tribe & Allsopp 2001; personal observations) and up to 1/3 of the offspring in such colonies can be worker-derived (Moritz et al. 1999). Thus, it is very likely that laying workers of *A. m. capensis* have evolved strategies to avoid worker policing in queenright colonies.

Egg removal rates of worker-laid eggs are one case in point. Recently it has been shown that eggs laid by *A. m. capensis* workers invading *A. m. scutellata* in its native range have lower removal rates compared to eggs laid by *A. m. scutellata* host workers (Martin et al. 2002). This is intermediate between *A. m. capensis* queen and worker-laid eggs from the native range of the Cape bee (Neumann et al. 2002). Several observations indicate that laying workers may also show behavioural tactics to evade worker policing.

In contrast to *A. m. scutellata* workers, workers of *A. m. capensis* evade queens (Moritz et al. 2001) and such workers have a more queen-like pheromonal bouquet (Moritz et al. 2002). Because brood is commonly found above the queen excluder (personal observations), the distance of the queen may not only affect the spatial distribution and pheromonal and ovarial development of workers (Moritz et al. 2000a; Moritz et al. 2000b Moritz et al. 2001), but also worker policing and egg-laying behaviour. Queen substance is dispersed by messenger bees in the colony (Velthuis 1972; Seeley 1979), but in large colonies or where the queen is restricted to a certain area of the hive (e.g. when using queen excluders in commercial beekeeping practice) workers may raise emergency queens (Butler 1960a; Lensky & Slabezki 1981; Swart et al. 2001), strongly indicating that the queen signal is weakened in those areas. This behaviour is used in commercial beekeeping to raise new queens before splitting a colony (Swart et al. 2001). Given that the removal of worker-laid eggs is also affected by distance from the queen, queen evasion by *A. m. capensis* laying workers may constitute a behavioural tactic to achieve successful reproduction in queenright colonies. Here we investigate egg laying and egg removal by workers in queenright *A. m. capensis* and *A. m. scutellata* colonies to test for spatial differences in egg laying and egg removal by workers.

6.2. Materials and methods

Four queenright colonies and one queenless colony of *A. m. capensis* were obtained from its native range (Port Elizabeth) and placed at a test apiary in Grahamstown, South Africa. Likewise, four queenright colonies and one queenless colony of *A. m. scutellata* were used in another apiary nearby Pretoria, South Africa (cf. Hepburn & Radloff 1998 for a review on the biology and distribution of the two subspecies). All colonies were housed in two 10 frame standard Langstroth hives and given two days to settle down to avoid absconding (Hepburn et al. 1999).

For both subspecies, the queens of the three queenright colonies were caged in containers (8cm x 4cm x 2.5 cm) with gauze on two sides, so that the workers had access to the queen (Chapter 6). No queen excluder were used in the discriminator colonies. The cages were placed in a comb containing brood of all a stages (Chapter 6). Empty test combs for evaluating egg removal rates (**A**) and worker egg-laying (**B**) were placed into the top and bottom boxes of each of the three discriminator colonies two days before the experiments started to avoid any potential influence deriving from the comb (Breed et al. 1995).

One queenright and one queenless *A. m. capensis* colony were used as egg sources for the three queenright *A. m. capensis* discriminator colonies. Likewise, one queenright and one queenless *A. m. scutellata* colony were used as egg sources for the three queenright *A. m. scutellata* discriminator colonies. Eggs derived from queenright colonies were assumed to be laid by the queen and eggs from the queenless colonies were assumed to be laid by workers. Furthermore, multiple eggs per cell were regarded as laid by workers were an single egg per cell on the bottom of the cell were counted as laid by the queen. Egg removal rates for queen and worker-laid eggs were evaluated according to standard protocols (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) with the following modifications. On 4 sequential days, 20 eggs of each egg source colony were transferred using special forceps (Taber 1961) on each test comb **A.** Two of the **A** test combs were placed in each of the three discriminator *A. m. capensis* and *A. m. scutellata* colonies. One **A** test comb and the frame with the caged queen were sandwiched between two brood frames, containing brood of all stages, and placed in the brood box. The second one **A** test comb was also sandwiched between two brood frames and placed in the honey super. Two **B** test combs were also placed in each discriminator colony, next to the sandwiched **A** test combs.

The top and the bottom boxes were supplied with the same number of brood frames (eggs, unsealed and sealed brood) so that the brood nest had the same size in both boxes. All

other frames contained honey and/or pollen. After 24 hours all **A** and **B** test combs were briefly removed and all remaining eggs on the **A** test combs were counted. The **B** test combs were screened for the occurrence of worker-laid eggs to determine the egg laying rate and all visible eggs and their positions on the combs were recorded using transparent sheets to avoid double counting of eggs. This procedure was repeated on 5 and 3 sequential days for *A. m. capensis* and *A. m. scutellata* discriminator colonies respectively. Two of the three *A. m. scutellata* absconded during the experiment, so that only data for the first three days were available. Mann-Whitney U tests were performed to test for differences between the number of remaining queen and worker-laid eggs on the **A** test combs. A χ^2-test was used to compare the number of worker-laid eggs on the **B** test combs in the top and bottom hive boxes. All statistical analyses were performed using Statistica©. The relevant raw data is shown in Appendix 6.

6.3. Results

After 24 hours significantly fewer worker-laid eggs remained on the **A** test combs after 24 hours than queen-laid eggs both in the bottom and in the top boxes (Table 6.1) in the *A. m. capensis* discriminator colonies. In the *A. m. scutellata* discriminator colonies significantly fewer worker-laid eggs remained on the **A** test combs in the bottom box but not in the top box after 24 hours (Table 6.1). Moreover, in the *A. m. capensis* discriminator colonies significant fewer worker-laid eggs remained in the bottom than in the top box (Table 6.1). But there were no significant differences in the removal rates of worker-laid eggs between the top and bottom boxes in the *A. m. scutellata* discriminator colonies.

A total number of 71 worker-laid eggs appeared on the **B** test combs in the queenright *A. m. capensis* discriminator colonies. Significantly different numbers of eggs were found in the top and in the bottom boxes (top boxes: N = 67 eggs, bottom boxes: N = 4 eggs; χ2-test: $\chi 2 = 33.79$, df= 14, $P < 0.002$). No worker-laid eggs were found on the **B** test combs in the queenright *A. m. scutellata* discriminator colonies.

6.4. Discussion

The data show that *A. m. capensis* workers preferentially oviposit in areas away from the queen in queenright colonies, where the removal of worker-laid eggs is reduced. Thus, our data are a clear indication that queen evasion constitutes a behavioural tactic of Cape honeybee laying workers as well as of social parasitic Cape honeybee workers to achieve successful reproduction in queenright host colonies despite worker policing. The data suggest that the distance of workers from the queen not only affects their pheromonal and ovarial development (Moritz et al. 2001) but also plays an important role for both egg laying by workers and for the removal of such eggs by other workers.

Our results support earlier findings (Chapter 2) that both *A. m. capensis* and *A. m. scutellata* are, in principle, able to police worker-laid eggs because significantly fewer worker-laid eggs remained in the bottom boxes after 24 hours. However, whereas worker policing also occurred in the top boxes of the *A. m. capensis* colonies, this was not the case in *A. m. scutellata*. This suggests that worker policing in the African honeybee *A. m. scutellata* is not always effective due to less expressed egg removal behaviour or lower removal rates of worker-laid eggs. This is supported by earlier findings of high numbers of remaining worker-laid eggs (Chapter 2) and field observations that many more queenright colonies of both *A. m. capensis* and *A. m. scutellata* show worker derived brood above the queen excluder (Pettey 1922; Tribe & Allsopp 2001; McGregor, personal communication; personal observations) than colonies of European honeybee subspecies. Moreover, the number of remaining *A. m. capensis* worker-laid eggs was significantly lower in the bottom box than in the top box of the queenright *A. m. capensis* colonies. These findings indicate that the distance from the queen has an impact on egg removal behaviour of workers and thus on the survival chances of worker-laid eggs. The data also suggest that evaluating the removal of worker-laid eggs above the queen excluder might actually underestimate the true removal rates in a colonies' brood nest. What are potential reasons for this spatial effect?

Visscher (1996) suggested that normal queen pheromone transmission may be disrupted in an experimental set up using caged queens. Restricting the queen from entering certain parts of the hive such as by using queen excluders (as it is typical in commercial beekeeping) or cages (this study) may indeed interrupt normal queen pheromone transfer, especially if messenger bees (Velthuis 1972; Seeley 1979) are not fully effective or not as effective as the queen herself. Our experimental design probably amplified such an interruption, but the basic effects should be the same. In queenless colonies, worker policing

eventually vanishes (Miller & Ratnieks 2001). So the physical distance from the queen as a source of pheromones affects the efficiency of worker policing behaviour.

Because worker policing is reduced away from the queen (e.g. above the queen excluder), worker derived offspring in such areas clearly has greater survival chances. Thus, one might expect laying workers to take advantage of this behavioural strategy. Indeed, oviposition by *A. m. capensis* workers was not random. The data clearly show that *A. m. capensis* laying workers preferentially lay their eggs further away from the queen. It appears as if queen evasion by laying *A. m. capensis* workers is highly adaptive for social parasitism of queenright host colonies. Moreover, while *A. m. capensis* workers laid 71 eggs during the 5 day period in the three test colonies, not a single worker-laid egg was found in the *A. m. scutellata* colonies. This clearly shows that *A. m. capensis* laying workers are not sufficiently suppressed and may readily produce large numbers of eggs despite the presence of a queen.

Our data agree well with what is known from field observations with respect to the social parasitism of *A. m. scutellata* host colonies by *A. m. capensis* laying workers (Allsopp & Crewe 1993; Allsopp 1995; Magnuson 1995). Brood of social parasitic Cape honeybee workers initially appears at the outermost frames, then closer to the actual brood nest and finally the brood nest of the host queen is flanked by brood frames with *A. m. capensis* worker-laid brood (Allsopp 1995). When brood hatches outside of the brood nest, brood pheromones probably attract young nurse bees to the recently hatched larvae (Le Conte et al. 1995). Our results suggests a possible explanation why colonies of this subspecies are so prone to invasions by *A. m. capensis* laying workers (Allsopp & Crewe 1993, Hepburn & Allsopp 1994). Because as indicated in this study for the worker policing is less well expressed in the top box of host colonies or sometimes is not fully effective, like for the *A. m. scutellata* colonies, so that the preferential oviposition in those areas clearly enhances the survival chances of laying worker offspring. Indeed, when the use of queen excluders is omitted, it seems as if takes considerably longer for social parasitic workers to take over host colonies (A. Schehle, personal communications). Therefore, we recommend omitting the usage of queen excluders by beekeepers in endangered areas. We conclude that queen evasion by laying workers is an important factor for laying workers to achieve successful reproduction in the presence of the queen and for the spread of social parasitic Cape honeybees in regions of other honeybee subspecies.

Table 6.1: The mean ± sd of the numbers of queen-laid and worker-laid eggs remaining on the **A** test combs in the honey super and brood boxes of the queenright discriminator colonies after 24 hours. Only the data of the first three days of the *A. m. capensis* discriminator colonies are analysed because data for the *A. m. scutellata* discriminator colonies were only available for the first three days (see methods). The results of the Mann–Whitney U tests comparing the different groups of eggs are shown. A group is defined by the type of egg and the position of the eggs, so queen laid eggs in the top box belong to a different group than queen laid eggs in the bottom box. (Bonferroni adjustments to the levels of significance were used: $P < 0.025$, n.s.= not significant)

Subspecies	Egg type	Position	mean ±sd	Egg removal trend	Egg type	Position	mean ±sd	Z-values	P-values
A. m. scutellata	Queen-laid	honey super	5.22±4.21	slower than	Worker-laid	honey super	4.22±3.00	0.13	n.s.
	Queen-laid	brood nest	5.89±2.37	slower than	Worker-laid	brood nest	2.56±1.67	2.78	*P<0.01*
	Queen-laid	brood nest	5.89±2.37	slower than	Queen-laid	honey super	5.22±4.21	-0.93	n.s.
	Worker-laid	honey super	4.22±3.00	slower than	Worker-laid	brood nest	2.56±1.67	1.37	n.s.
A. m. capensis	Queen-laid	honey super	6.89±1.96	slower than	Worker-laid	honey super	3.22±3.30	2.30	*P=0.022*
	Queen-laid	brood nest	5.11±1.05	slower than	Worker-laid	brood nest	0.44±0.73	3.58	*P<0.001*
	Queen-laid	honey super	6.89±1.96	slower than	Queen-laid	brood nest	5.11±1.05	1.03	n.s.
	Worker-laid	honey super	3.22±3.30	slower than	Worker-laid	brood nest	0.44±0.73	2.29	*P=0.022*

Appendix 6

Appendix 6.1: Number of remaining queen and worker-laid eggs after 2 and 24 hours in the honey super and the brood nest in the queenright *A. m. capensis* discriminator colonies. (note: data for 2 hours are not discussed in the Chapter 6)

Experimental day	Discriminator colony	Position	type of eggs	0 hours	2 hours	24 hours
1	*capensis* 2	honey super	queen	20	8	6
1	*capensis* 2	brood nest	queen	20	8	5
1	*capensis* 2	honey super	worker	20	6	2
1	*capensis* 2	brood nest	worker	20	1	0
1	*capensis* 3	honey super	queen	20	7	6
1	*capensis* 3	brood nest	queen	20	10	5
1	*capensis* 3	honey super	worker	20	3	0
1	*capensis* 3	brood nest	worker	20	7	0
1	*capensis* 4	honey super	queen	20	10	6
1	*capensis* 4	brood nest	queen	20	9	6
1	*capensis* 4	honey super	worker	20	4	3
1	*capensis* 4	brood nest	worker	20	6	0
2	*capensis* 2	honey super	queen	20	8	6
2	*capensis* 2	brood nest	queen	20	6	6
2	*capensis* 2	honey super	worker	20	3	0
2	*capensis* 2	brood nest	worker	20	3	0
2	*capensis* 3	honey super	queen	20	10	9
2	*capensis* 3	brood nest	queen	20	8	3
2	*capensis* 3	honey super	worker	20	5	1
2	*capensis* 3	brood nest	worker	20	5	0
2	*capensis* 4	honey super	queen	20	9	6
2	*capensis* 4	brood nest	queen	20	7	6
2	*capensis* 4	honey super	worker	20	7	7
2	*capensis* 4	brood nest	worker	20	3	0
3	*capensis* 2	honey super	queen	20	5	4
3	*capensis* 2	brood nest	queen	20	4	4
3	*capensis* 2	honey super	worker	20	3	3
3	*capensis* 2	brood nest	worker	20	2	1
3	*capensis* 3	honey super	queen	20	8	9
3	*capensis* 3	brood nest	queen	20	10	6
3	*capensis* 3	honey super	worker	20	3	3
3	*capensis* 3	brood nest	worker	20	1	1
3	*capensis* 4	honey super	queen	20	10	10
3	*capensis* 4	brood nest	queen	20	8	5
3	*capensis* 4	honey super	worker	20	6	10
3	*capensis* 4	brood nest	worker	20	2	2
4	*capensis* 2	honey super	queen	20	8	3
4	*capensis* 2	brood nest	queen	20	7	0
4	*capensis* 2	honey super	worker	20	6	2
4	*capensis* 2	brood nest	worker	20	1	2
4	*capensis* 3	honey super	queen	20	9	6
4	*capensis* 3	brood nest	queen	20	10	3
4	*capensis* 3	honey super	worker	20	6	2
4	*capensis* 3	brood nest	worker	20	7	2

Appendix 6.1 continues

Experimental Day	Discriminator colony	Position	type of eggs	0 hours	2 hours	24 hours
4	*capensis* 4	honey super	queen	20	10	5
4	*capensis* 4	brood nest	queen	20	3	3
4	*capensis* 4	honey super	worker	20	10	4
4	*capensis* 4	brood nest	worker	20	0	2
5	*capensis* 2	honey super	queen	20	9	6
5	*capensis* 2	brood nest	queen	20	10	0
5	*capensis* 2	honey super	worker	20	9	9
5	*capensis* 2	brood nest	worker	20	4	1
5	*capensis* 3	honey super	queen	20	10	3
5	*capensis* 3	brood nest	queen	20	7	1
5	*capensis* 3	honey super	worker	20	8	2
5	*capensis* 3	brood nest	worker	20	0	1
5	*capensis* 4	honey super	queen	20	10	3
5	*capensis* 4	brood nest	queen	20	7	2
5	*capensis* 4	honey super	worker	20	8	3
5	*capensis* 4	brood nest	worker	20	0	2

Appendix 6.2: Number of remaining queen and worker-laid eggs after 24 hours in the honey super and the brood nest in the queenright *A. m. scutellata* discriminator colonies.

Experimental day	Discriminator colony	Position	type of eggs	0 hours	24 hours
1	*scutellata* 1	brood nest	queen	20	6
1	*scutellata* 1	honey super	queen	20	6
1	*scutellata* 1	brood nest	worker	20	2
1	*scutellata* 1	honey super	worker	20	7
1	*scutellata* 2	brood nest	queen	20	6
1	*scutellata* 2	honey super	queen	20	2
1	*scutellata* 2	brood nest	worker	20	2
1	*scutellata* 2	honey super	worker	20	4
1	*scutellata* 3	brood nest	queen	20	9
1	*scutellata* 3	honey super	queen	20	6
1	*scutellata* 3	brood nest	worker	20	0
1	*scutellata* 3	honey super	worker	20	9
2	*scutellata* 1	brood nest	queen	20	4
2	*scutellata* 1	honey super	queen	20	3
2	*scutellata* 1	brood nest	worker	20	3
2	*scutellata* 1	honey super	worker	20	3
2	*scutellata* 2	brood nest	queen	20	3
2	*scutellata* 2	honey super	queen	20	2
2	*scutellata* 2	brood nest	worker	20	5
2	*scutellata* 2	honey super	worker	20	0
2	*scutellata* 3	brood nest	queen	20	9
2	*scutellata* 3	honey super	queen	20	12
2	*scutellata* 3	brood nest	worker	20	5
2	*scutellata* 3	honey super	worker	20	0
3	*scutellata* 1	brood nest	queen	20	8

Appendix 6.2 continues

Experimental day	Discriminator colony	Position	type of eggs	0 hours	24 hours
3	*scutellata* 1	honey super	queen	20	1
3	*scutellata* 1	brood nest	worker	20	2
3	*scutellata* 1	honey super	worker	20	5
3	*scutellata* 2	brood nest	queen	20	5
3	*scutellata* 2	honey super	queen	20	3
3	*scutellata* 2	brood nest	worker	20	3
3	*scutellata* 2	honey super	worker	20	6
3	*scutellata* 3	brood nest	queen	20	3
3	*scutellata* 3	honey super	queen	20	12
3	*scutellata* 3	brood nest	worker	20	1
3	*scutellata* 3	honey super	worker	20	4

Chapter 7
Short-sighted selection in a social parasitic honeybee strain

Social parasitism by self-replicating, female-producing workers occurs in the Cape honeybee *Apis mellifera capensis* (Hepburn & Radloff 1998; Beekman et al. 2000; Neumann et al. 2001) and colonies of other honeybee subspecies are highly susceptible to infestation (Hepburn & Allsopp 1994; Beekman et al. 2000; Neumann et al. 2001). We found high within-host virulence but low transmission rates in a strain of social parasitic *A. m. capensis* workers invading the neighbouring subspecies *A. m. scutellata*. In contrast, parasitic workers from the native range of *A. m. capensis* showed low within-host virulence but high transmission rates. To our knowledge this is the first empirical support for the short-sighted selection hypothesis for parasite virulence (Schmid-Hempel 1998), which predicts that winners of within-host competition are poorer at transmission to new hosts.

Honeybees, *Apis mellifera*, are eusocial insects with a well-developed reproductive division of labour between the workers and the queen. A suite of queen pheromones suppresses worker ovary activation (Free 1987). Nevertheless, in rare anarchistic honeybee colonies of European subspecies workers may successfully reproduce despite the presence of a queen (Oldroyd et al. 1994). Worker reproduction in colonies with a queen (queenright) is common in the Cape honeybee *A. m. capensis* (Pettey 1922; Moritz et al. 1999; Tribe & Allsopp 2001), native to the Cape region of South Africa (Hepburn & Radloff 1998). Some laying *A. m. capensis* workers may develop into pseudoqueens with high ovarial development and a queen-like pheromonal bouquet (Hepburn & Radloff 1998).

It has long been established (Onions 1912) that these pseudoqueens may parasitize colonies of other honeybee subspecies including the neighbouring *A. m. scutellata* (Hepburn & Radloff 1998; Neumann et al. 2001). After successful transmission (Neumann et al. 2001) these pseudoqueens initiate oviposition despite the presence of the host queen (Hepburn & Radloff 1998). Since worker-laid eggs are usually removed by other workers in queenright colonies (worker policing, Ratnieks & Visscher 1989), these pseudoqueens are somehow able to evade this host defence mechanism. In Cape honeybee colonies worker policing seems to be less efficient (Moritz et al. 1999), indicating that pseudoqueens eggs may have lower removal rates (Chapter 5) and/or lay their eggs further away from the queen (Chapter 6). The hatched larvae of the parasitic workers are preferentially fed by the host colony workers

(Beekman et al. 2000) until the host colony shows the "dwindling colony syndrome" (Hepburn & Allsopp 1994) and rejects its own queen. Since migratory beekeepers moved *A. m. capensis* repeatedly into *A. m. scutellata* populations in the highveld of South Africa (Allsopp 1995) parasitic workers have spread widely and invaded a large number of host colonies (Hepburn & Allsopp 1994). The resulting "*capensis*" calamity for South African beekeepers (Allsopp 1992) suggests that these invasive parasitic workers are highly virulent.

This sets the conditions for the short-sighted selection hypothesis for parasite virulence, which predicts that winners of within-host competition (resulting from multiple infestations) are poorer at transmission to new hosts (Schmid-Hempel 1998). Empirical evidence is lacking but it has been suggested to occur in HIV, polio and bacterial meningitis (Schmid-Hempel 1998). We tested this hypothesis by comparing the within-host virulence, the transmission capacity and the genotypic composition of social parasitic workers from populations occurring in the natural distribution area of the Cape honeybee (Port Elizabeth, Eastern Cape Province), with a parasitic population that had recently invaded *A. m. scutellata* in its native range (Gauteng Province, formerly Transvaal).

Worker policing was investigated for *A. m. capensis* queen-laid eggs and for worker-laid eggs of the two different populations to evaluate within-host virulence of the parasitic workers. Six queenright colonies and four laying worker colonies of *A. m. capensis* were obtained from Port Elizabeth. Four queenless *A. m. scutellata* colonies (C1-C4) heavily infested with *A. m. capensis* laying workers were obtained from two distant apiaries (Heilbron and Graskop; ~390 km apart) in Gauteng and placed at the same test apiary at Rhodes University (Grahamstown). Egg removal rates were determined by transferring queen and worker-laid eggs from source colonies of each population into queenright *A. m. capensis* discriminator colonies (The relevant raw data is shown in Appendix 7.1). The mean numbers of remaining eggs were significantly different between worker-laid eggs from Gauteng and worker and queen-laid eggs from Port Elizabeth (Table 7.2). Worker-laid eggs from Gauteng showed intermediate removal rates, significantly different from both queen and worker-laid eggs from Port Elizabeth. The removal rates for worker-laid eggs from Port Elizabeth were three times higher compared to worker-laid eggs from Gauteng, showing that the parasitic workers from Gauteng had a much higher virulence.

Transmission capacity was evaluated by placing a un-infested *A. m. scutellata* test colony from Gauteng in the same apiary after the queenright *A. m. capensis* colonies had been removed (Fig. 7.1). The test colony was separated by at least 10m and dense vegetation from all other colonies to prevent accidental drifting (Neumann et al. 2000a) which may mask

active host finding of the high and low virulent strain of the social parasite (Neumann et al. 2001). After four weeks, the test colony showed all signs of the "dwindling" colony syndrome, including both the loss of the queen and the presence of worker-laid eggs. Three weeks after colony C5 lost its queen, sealed diploid laying worker offspring of all colonies present at the test apiary were genotyped at four DNA microsatellites (Estoup et al. 1994; Table 7.1). Queen genotypes of the queenless *A. m. capensis* colonies were inferred from the laying worker offspring. At least 4 *A. m. capensis* laying workers from more than one of the Port Elizabeth colonies appeared in the test colony, 11 laying workers from external colonies not located in the apiary; and not a single one from the high virulent strain (Gauteng, Fig. 7.1). This strongly indicates that the high virulent strain from Gauteng has a significantly reduced transmission capacity ($\chi^2 = 12.4$, df = 2, $P<0.003$).

The Gauteng population is virtually genetically uniform, even though derived from two distant localities (Heilbron and Graskop; Nei's genetic identity = 0.99; Nei 1987), while the Port Elizabeth population is genotypically diverse (Table 7.1) and genetically different from the samples collected in Gauteng (Nei's genetic identity: Port Elizabeth /Heilbron = 0.20; Port Elizabeth /Graskop = 0.22). Since recombination via crossing-over is virtually lacking in female-producing parthenogenesis of *A. m. capensis* laying workers (Moritz & Haberl 1994; one crossing-over event which equals less than 1% recombination rate in this data set), this strongly indicates that the Gauteng population originated from a single laying *A. m. capensis* worker. The level of egg removal of queen and worker-laid eggs from Port Elizabeth and worker-laid eggs from Gauteng after 24 hours were determined (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) by transferring 270 eggs (N = 10 each trial) from three different source colonies of each group (queen-laid eggs from Port Elizabeth, worker-laid eggs from Gauteng and from Port Elizabeth) into test cells in three queenright *A. m. capensis* discriminator colonies from Port Elizabeth on 3 sequential days. There were significant differences among the three tested groups (ANOVA, df = (2,41) $F=22.37$, $p<0.0001$, Table 7.2). All three groups passed tests of normality (Kolmogorov-Smirnov $d = 0.3336$, $P > 0.05$) and tests of homogeneity of the variances (Levene's $F < 3.50$, df= (2,42), $P > 0.01$) and hence the univariate ANOVA procedure outcomes could be considered as reliable.

Only a parasitic worker with the highest within-host virulence can successfully out-compete others (Moritz et al. 1996). Because queenless colonies do not frequently re-queen from laying worker offspring (Hepburn & Radloff 1998) and recombination events are rare (Moritz & Haberl 1994), selection among strains of social parasitic worker was further

enhanced. This may explain why the parasitic laying worker strain from Gauteng showed significantly higher virulence than parasitic workers from Port Elizabeth due to the oviposition of eggs with low removal rates; a mechanism similar to anarchistic honeybee workers (Oldroyd et al. 1994; Oldroyd & Ratnieks 2000). The data indicate that severe intra-host competition in the region of *A. m. scutellata* resulted in a single parasitic *A. m. capensis* strain with high virulence, although multiple introductions occurred (>200 *A. m. capensis* colonies were brought to the region of *A. m. scutellata*, Allsopp 1995). The reduced dispersal capacity in this high virulent strain is to our knowledge the first empirical evidence for the "short-sighted selection hypothesis" for parasite virulence (Schmid-Hempel 1998). Beekeepers facilitate the spread of the parasitic workers, enhancing the "*capensis*" calamity and offering an opportunity for short-sighted selection and the evolution of a social parasite in real time.

Fig. 7.1: Experimental design and results of the transmission test apiary. The number of dispersed matrilines between the colonies of the different populations and the test colony are shown. (White rectangles = colonies with *A. m. capensis* laying workers; shaded colony = *A. m. scutellata* test colony; grey irregulars = vegetation; dashed lines = dispersed matrilines among *A. m. capensis* colonies; solid lines = dispersed matrilines into the test colony).

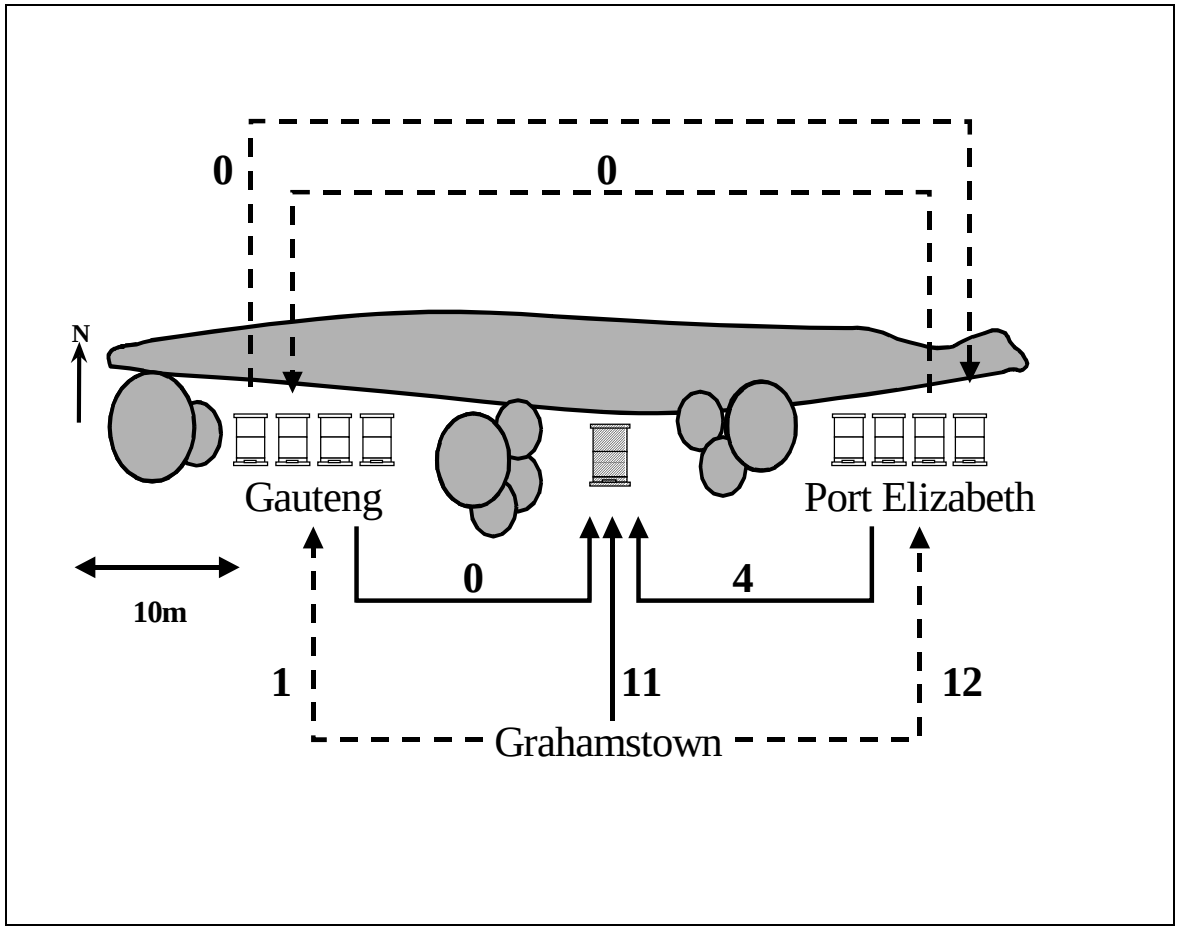


Table 7.1: Worker genotypes of the sample from the Gauteng population (Graskop and Heilbron) and the test colony for four DNA Microsatellites. (Alleles in base pairs, N = number of workers with the same genotype, nd = not detected).

Population	Colony / apiary	Different genotypes	A107		A24		A28		A43		N
Gauteng	1/ Graskop	1	176	181	90	94	131	131	121	142	19
		2	158	169	90	90	124	127	nd	nd	1
Gauteng	2/ Graskop	1	176	181	90	94	131	131	121	142	15
		2	176	181	90	94	131	131	121	121	3
Gauteng	3/ Heilbron	1	176	181	90	94	131	131	121	142	18
Gauteng	4/ Heilbron	1	176	181	90	94	131	131	121	142	15
Port Elizabeth	5/ PE	1	137	137	94	100	127	131	132	132	1
		2	137	137	96	100	127	134	132	132	1
		3	137	141	90	100	127	127	132	142	1
		4	137	171	90	100	124	124	126	132	1
		5	137	171	90	100	124	124	132	132	1
		6	137	171	90	100	127	127	126	142	3
		7	137	171	90	100	134	134	132	132	2
		8	137	171	98	100	127	131	132	132	1
		9	141	141	90	100	127	134	126	132	1
		10	141	171	90	100	124	124	142	142	1
		11	169	171	90	100	124	127	126	142	1
		12	169	171	92	98	127	127	132	132	1
		13	169	171	92	98	134	134	142	142	1
		14	169	171	98	100	127	131	132	142	1
		15	169	171	100	100	127	131	132	132	1

Table 7.2: The mean ± sd of the numbers of queen-laid and worker-laid eggs from Port Elizabeth and of worker-laid eggs from Gauteng remaining in the three queenright *A. m. capensis* discriminator colonies after 24 hours. The results of the Newman-Keuls Post-hoc comparison are shown.

Type of egg	Mean±sd	Trend	Type of egg	Mean±sd	Newman-Keuls test P-Value
Queen-laid	4.2±1.52	Slower than	Worker-laid from Gauteng	2.07±1.71	0.0004
Queen-laid	4.2±1.52	Slower than	Worker-laid from PE	0.73±1.16	0.0002
Worker-laid from Gauteng	2.07±1.71	Slower than	Worker-laid from PE	0.73±1.16	0.014

Appendix 7

Appendix 7.1: Number of remaining queen and worker-laid eggs from the Port Elizabeth (PE) population after 24 hours in the queenright *A. m. capensis* discriminator colonies and the number of remaining worker-laid eggs from Gauteng population after 24 hours.

Experimental day	Discriminator colony	Type of egg	0 hour	24 hours
1	C1	Queen PE	10	3
1	C1	Worker PE	10	0
1	C1	Worker (Gauteng)	10	0
1	C2	Queen PE	10	4
1	C2	Worker PE	10	0
1	C2	Worker (Gauteng)	10	1
1	C3	Queen PE	10	6
1	C3	Worker PE	10	2
1	C3	Worker (Gauteng)	10	4
2	C1	Queen PE	10	5
2	C1	Worker PE	10	1
2	C1	Worker (Gauteng)	10	1
2	C2	Queen PE	10	6
2	C2	Worker PE	10	0
2	C2	Worker (Gauteng)	10	4
2	C3	Queen PE	10	6
2	C3	Worker PE	10	0
2	C3	Worker (Gauteng)	10	1
3	C1	Queen PE	10	3
3	C1	Worker PE	10	0
3	C1	Worker (Gauteng)	10	1
3	C2	Queen PE	10	5
3	C2	Worker PE	10	2
3	C2	Worker (Gauteng)	10	4
3	C3	Queen PE	10	5
3	C3	Worker PE	10	1
3	C3	Worker (Gauteng)	10	1
4	C1	Queen PE	10	6
4	C1	Worker PE	10	0
4	C1	Worker (Gauteng)	10	5
4	C2	Queen PE	10	4
4	C2	Worker PE	10	0
4	C2	Worker (Gauteng)	10	4
4	C3	Queen PE	10	3
4	C3	Worker PE	10	0
4	C3	Worker (Gauteng)	10	3
5	C1	Queen PE	10	1
5	C1	Worker PE	10	0
5	C1	Worker (Gauteng)	10	0
5	C2	Queen PE	10	3
5	C2	Worker PE	10	1
5	C2	Worker (Gauteng)	10	1
5	C3	Queen PE	10	3
5	C3	Worker PE	10	4
5	C3	Worker (Gauteng)	10	1

Chapter 8
Worker policing and nestmate recognition of eggs in the honeybee (*Apis mellifera* L.)

Summary It has been shown that honeybees are able to recognise their own queen and to discriminate between own and alien eggs. Moreover, current evidence suggests that honeybee workers are able to discriminate between queen-laid and worker-laid eggs by means of a postulated queen-produced egg-marking pheromone. Nevertheless, the effect of nestmate recognition of eggs has only been shown in the context of queen rearing and not egg removal behaviour. The levels of egg removal were evaluated in three queenless *Apis mellifera capensis* and three queenright *Apis mellifera scutellata* discriminator colonies of native queen-laid, native worker-laid, alien queen-laid and alien worker-laid eggs using standard policing methods. The results show that policing occurs in queenless *A. m. capensis* colonies six days after dequeening and in the presence of mass egg laying by workers. This is surprising since it has been assumed that worker policing should collapse as soon as workers start to produce eggs in high numbers. Furthermore, both subspecies showed that alien eggs were removed faster than native ones. Native worker-laid eggs were removed significantly slower than alien queen-laid eggs in *A. m. capensis* test colonies, which shows that nestmate recognition cues override any postulated caste specific queen egg-marking pheromones. Moreover, for the first time the effect of treatment, nestmate recognition and caste can be quantified. 37.2% in *A. m. scutellata* and less than 37.2% in *A. m. capensis* of the egg removal can be explained because of the treatment effects. 33.3% (*A. m. scutellata*) and 60.6% (*A. m. capensis*) are caused by the effect of nestmate recognition of eggs and only 16.7% (*A. m. scutellata*) and 25% (*A. m. capensis*) are caused because of caste. The data show that using aliens eggs to evaluate policing behaviour is overestimating the actual level of egg removal in honeybees.

8.1. Introduction

Nestmate recognition in social insects is fundamental for colony integrity (Crozier & Pamilo 1996). Workers of *Apis mellifera* are able to recognise their own queen (Butler 1960b) and to distinguish alien and native queen-laid eggs (Visscher 1986). In honeybee populations where natural mergers of colonies are common (Hepburn & Whiffler 1988; Chapter 4) and intraspecific parasitism occurs (Allsopp & Crewe 1993; Hepburn & Allsopp 1994; Neumann et al. 2001) the recognition of alien (unrelated) eggs is important to prevent alien worker or queen reproduction and maintain colony integrity.

After the merging of two or more colonies only one queen usually survives (Hepburn & Whiffler 1988; Kigatiira 1988; Chapter 4) so that one part of the new colony is unrelated to the queen and therefore the inclusive fitness of these worker is zero. This seems puzzling from an evolutionary perspective, because workers which are not related to the queen can not indirectly pass any genes onto the next generation. The benefits of a merger could be on a population level assuring that larger colonies have a higher probability of survival by lowering their *pro rata* costs (Hepburn & Radloff 1998). An opportunity for the workers, which are not related to the queen, to pass their genes onto future generations would be to lay eggs. Nevertheless, the other half of the workers are not related in any way to the laying worker offspring and therefore should develop retaliatory actions.

One retaliatory behaviour to prevent reproduction by unrelated workers would be the removal of these eggs, but workers have to be able to discriminate between native (related) and alien (unrelated) eggs as indicated in European honeybees (Visscher 1986). This ability of nestmate recognition for eggs gains importance when parasitic honeybees, like Cape honeybee laying workers in South Africa, occur in the population (Neumann & Hepburn 2002). It has been shown that a proportion of Cape honeybee workers actively disperse into other colonies (Neumann et al. 2001) which cannot be explained by drifting (Rauschmayer 1928). Nevertheless all these workers are able to gain direct fitness via parthenogenetic reproduction and can compete in the production of sexuals, so this reproduction of alien workers would reduce the inclusive fitness of the native workers.

The worker policing theory (Ratnieks 1988) predicts that the loss of inclusive fitness of workers due to native laying worker reproduction favours the diffusion of a "policing gene". Therefore worker policing behaviour should be favoured in species where alien worker reproduction occurs and therefore workers have to be able to discriminate between alien and native eggs. To recognise alien worker-laid eggs it is necessary that eggs contain cues which can be used for nestmate recognition in addition to any postulated queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995).

Queen-laid and worker-laid eggs are distinguished by means of a queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995). The queen pheromone bouquet is different when compared to a worker's and unique in a colony (Pain 1955), so a caste specific queen-produced egg marking pheromone (Ratnieks 1992; Ratnieks 1995) would also be unique in the colony. If cues derived from such queen-marking pheromone are more important than nestmate recognition cues, we would expect that alien queen-laid eggs would be removed slower than native worker-laid eggs. However, if the colony specific components are more important than a postulated queen-produced egg-marking pheromone then the opposite is expected. Moreover, to evaluate the actual level of removal of queen and worker-laid eggs it is necessary to know what proportions of the eggs are removed, because of nestmate recognition and what proportions are due to the different treatment effects from workers towards queen and worker-laid eggs. Differences in the removal rates have usually been interpreted as caused by cues derived from caste differences between workers and queens (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000; among others) since the treatment of the eggs are the same.

Modified standard policing experiments (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) were performed to test the effect of nestmate recognition of eggs, treatment and caste specific components on egg removal behaviour in *A. m. capensis* and *A. m. scutellata*.

8.2. Materials and methods

8.2.1. General methods

All colonies used for the experiments were unrelated and derived from wild swarms caught in the natural distribution areas of *A. m. capensis* (Port Elizabeth) and *A. m. scutellata* (Pretoria). (cf. Hepburn & Radloff 1998 and Hepburn et al. 1998 for a detailed review of the biology and natural distribution of the two subspecies; Fig. 1.2) The colonies were housed in two 10-frame standard Langstroth hives and given two days to settle to prevent absconding (Hepburn et al. 1999). Egg removal rates were evaluated using standard methods (Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) with the following modifications. The test combs were placed into the colonies two days before the experiment began to avoid any potential influence deriving from the comb (Breed et al. 1995).

8.2.2. Experiment 1, A. m. scutellata

Four queenright and one queenless colonies were placed in an apiary near Pretoria, South Africa. The queens of the three test colonies were caged in small wooden containers ([8cm × 4cm × 2.5cm]; Fig. 5.1), ensuring sufficient nursing by workers but preventing the queens from laying eggs. One day before caging the queens, an empty comb was placed in the brood nest to provide enough native queen-laid eggs for the three-day experiment. For the experimental period all test eggs were stored on glass plates inside a Styrofoam box provided with moist paper towels to prevent dehydration (Ratnieks, personal communication), which was placed in an incubator at 35°C before the eggs were transferred into the test combs. One queenright and one queenless colony were used as egg sources for the three queenright test colonies. Twenty male worker-laid eggs from the queenless source colony; 20 alien male queen-laid eggs from the queenright source colony and 20 native male queen-laid eggs from the discriminator colony were transferred on the test combs using special forceps (Taber 1961). One test comb was then placed into the brood nest of each discriminator colony to avoid any spatial effect on egg removal rates (Chapter 6). All types of eggs (alien worker-laid, alien queen-laid and native queen-laid eggs) were used from the first experimental day onwards. The test frame and the frame with the caged queen were sandwiched between

two brood frames in the brood nest of the bottom hive box. On three sequential days the test combs were briefly removed after 24 hours and the remaining eggs counted. Native worker-laid eggs did not appear during the experiment in the three test colonies.

8.2.3. Experiment 2, A. m. capensis

Four queenright and one queenless colonies were placed in a test apiary in Grahamstown, South Africa. The three test colonies were dequeened the day before the experiment started. One queenright and one queenless colony were used as egg sources for the three queenless test colonies. Twenty alien female diploid queen and worker-laid eggs from the one queenless and one queenright source colony, and 20 native female worker-laid eggs were transferred on the test combs using special forceps (Taber 1961), which were placed into the brood nest of each discriminator colony to avoid any spatial effect on egg removal rates (Chapter 6). In the test colonies alien worker and queen-laid eggs were used from the first of the experiment onwards. Native worker-laid eggs were used from the fourth experimental day onwards, when mass egg laying by workers provided enough eggs for the experiment. The test comb was sandwiched in between two brood frames (Ratnieks 1993) in the brood nest in the bottom box of the test colonies. On six sequential days the test combs were briefly removed after 24 hours and the remaining eggs counted.

8.2.4. Data analysis

ANOVAs and Newmans-Keuls post hoc comparisons were performed to test for significant differences in the level of egg removal among the three different types of eggs per experiment. The same tests were used to test for differences among the test colonies in the removal of alien queen and worker-laid eggs. χ^2- tests were used to test for differences in the daily removal rates of alien queen-laid and alien worker-laid eggs to investigate a possible breakdown of policing in the queenless test colonies of *A. m. capensis*. The statistical package Statistica© was used to conduct the statistical analyses. The relevant raw data is shown in Appendix 8.

8.3. Results

8.3.1. Experiment 1

A total of 180 of each of the three types of eggs were transferred to evaluate egg removal behaviour. All three variables (three different type of eggs) passed tests of normality (Kolmogorov-Smirnov $d = 0.1891$, $P > 0.05$) and tests of homogeneity of the variances (Levene's $F < 7.62$, $df = (2,24)$, $P > 0.01$ hence the ANOVA procedure outcomes could be considered as reliable. There were highly significant differences in the removal rates among the three types of eggs (ANOVA, df= (2,24) F=18.66, p<0.001; Table 8.1). However, there were no significant differences among the colonies in the removal of alien queen-laid (ANOVA, df= (2,6) F= 0.67, n.s.) or alien worker-laid eggs (ANOVA df= (2,6) F= 0.45, n.s.). Alien queen-laid eggs were removed not significantly slower than alien worker-laid eggs (Table 8.1, Fig. 8.1). However, alien worker-laid eggs were removed significantly faster than native queen-laid eggs. Moreover, alien queen-laid eggs were removed significantly faster than native queen-laid eggs (Table 8.1, Fig. 8.1).

8.3.2. Experiment 2

The level of egg removal for 360 alien queen-laid, 360 alien worker-laid and 180 native worker-laid eggs was evaluated. The ANOVA procedure outcomes could be considered as reliable because all three variables (all three type of eggs) passed tests of normality (Kolmogorov-Smirnov $d = 0.1473$, $P > 0.05$) and tests of homogeneity of the variances (Levene's $F < 4.39$, $df = (2,24)$, $P > 0.01$). The ANOVA revealed no significant influence of the test colonies on the removal of alien queen-laid (ANOVA, df = (2,15) F= 1.13, n.s.) or alien worker-laid eggs (ANOVA, df = (2,15) F= 0.57, n.s.). Furthermore, the ANOVA analysis revealed that the type of eggs had a significant effect on egg removal behaviour (ANOVA, df= (2,24) F= 19.02, P<0.001; Table 8.1). Alien queen-laid eggs were removed at a significantly slower rate than alien worker-laid eggs (Table 8.1, Fig. 8.2); and native worker-laid eggs were removed significantly slower than alien worker-laid eggs. Moreover, native worker-laid eggs were removed significantly slower than alien queen-laid eggs (Table 8.1, Fig. 8.2). The three queenless test colonies showed egg removal behaviour throughout the experiment, despite the

presence of mass egg laying by workers (χ^2-test: day 1, df =2, χ^2=7.25; P<0.03; day 6, df=2, χ^2=6.45, P<0.04; Fig. 8.3).

8.4. Discussion

Our results show that colony specific rather than caste specific components dominate egg removal behaviour because native *A. m. capensis* worker-laid eggs were removed slower than alien queen-laid eggs. Moreover, both subspecies show a nestmate recognition ability for eggs, because native eggs were always removed at a significantly slower rate than their alien counterparts. The queenless *A. m. capensis* test colonies showed egg removal behaviour despite the presence of mass egg laying by workers. Finally, for the first time we are able to separate the effect of treatment, nestmate recognition and caste on egg removal behaviour.

In contrast to findings in queenless European honeybees, where policing behaviour breaks down as soon as mass egg laying by workers occur (Miller & Ratnieks 2001), egg removal behaviour is still expressed in queenless Cape honeybee colonies despite the presence of mass egg laying by workers (Fig. 8.3). Cape honeybees can raise a new queen from worker-laid female eggs (Allsopp & Hepburn 1997; Hepburn & Radloff 1998), these colonies are not hopeless when queenless as the European colonies are after the lost of the queen. A queenless colony could become queenright after a period of queenlessness in which policing would again be favoured (Chapter 2), so it would be an evolutionary adaptation for *A. m. capensis* colonies not to lose the ability to discriminate between worker-laid and queen-laid eggs during queenlessness.

The data support earlier findings; that *A. m. capensis* is able to police worker-laid eggs (Chapter 2) because alien worker-laid eggs were removed significantly slower than alien queen-laid eggs in the three queenless *A. m. capensis* test colonies supporting. However, the three *A. m. scutellata* test colonies showed no significant difference in the removal of queen and worker-laid eggs (Table 8.1, Fig. 8.1). Earlier data (Chapter 2) indicates that more worker-derived eggs remain than in European honeybees (Ratnieks & Visscher 1989). Indeed there is a variation among *A. m. scutellata* colonies for brood above the excluder (Tribe & Allsopp 2001; Mc Gregor, personal communication) indicating that policing behaviour is variable. Moreover, the variability for egg removal

behaviour could be an explanation for the susceptibility of *A. m. scutellata* colonies (Neumann et al. 2001) to infections by laying workers of the Cape honeybee. Similarly, the African honeybee life history, with absconding and subsequent merging (Herman 1922; Walter 1939; Silberrad 1976; Hepburn and Whiffler 1988; Kigatiira 1988; Chapter 4) results in a higher risk of queen loss. So, a reduced egg removal behaviour could be adaptive in allowing colonies to have an earlier start at worker reproduction after queen loss.

Nevertheless several studies show policing in arrhenotokous honeybees (Ratnieks & Visscher 1989) and especially in *A. m. scutellata* (Chapter 2). Our results support the results of (Visscher 1986) and indicate that workers are able to detect the origin of male eggs. Furthermore in this study it is shown that workers are able to detect the origin of female eggs that, together with earlier results (Visscher 1986), indicate that the sex of the eggs does not influence nestmate recognition ability. The data show that colony specific cues are more important than a postulated egg marking pheromone of the queen (Ratnieks 1992; Ratnieks 1995), because alien queen-laid eggs were removed faster than worker-laid eggs. What can the reasons be to suggest that colony specific components are more strongly expressed and more important than a postulated queen marking pheromone?

Natural colony mergers are common in African honeybees (Hepburn & Whiffler 1988; Hepburn & Radloff 1998; Chapter 4), so as an adaptation to the merging of unrelated swarms it would be important for the colony to recognise their own eggs. After the merger, one half of the workers are not related to the new queen, so on a colony level these workers can compete for the reproduction of sexual to get their genes into the next generation. However, for the other one half group of workers, which are related to the new queen, this worker reproduction would mean a decrease of their inclusive fitness and therefore prevent such unrelated worker reproduction. Moreover, in populations with parasitic laying worker traits (Beekman et al. 2000; Neumann et al. 2001) the ability to recognise between alien and native eggs supports colony integrity and enables workers to prevent reproduction by non-nestmates. We therefore conclude that nestmate recognition of eggs is adaptive in African honeybees and the results indicate that nestmate recognition of eggs occurs in European honeybees as well (Visscher 1986).

However, another study found no nestmate recognition ability in anarchistic honeybee colonies (Oldroyd & Ratnieks 2000). At first glance this seems in contrast to our results, but on closer inspection these results (Oldroyd & Ratnieks 2000) may actually support our findings. The trait of the anarchistic bees has basically two features: 1. Low or non-policing of the colony; and 2. A high numbers of laying workers that lay eggs with low removal rates (Oldroyd & Ratnieks 2000). Naturally occurring anarchistic colonies were bred for the first of the two mentioned features (Oldroyd & Osborne 1999) which resulted in a higher relatedness. So in anarchistic bees the nestmate recognition ability may be masked because of the features of this trait. Moreover anarchistic eggs were removed at a slower rate than foreign queen-laid eggs (Experiment 2, Oldroyd & Ratnieks 2000). In this experiment 50% of the anarchistic eggs were native to the discriminator colony, indicating and supporting our results that colony specific nestmate recognition cues on the eggs are more important than caste specific once.

Moreover, for the first time it is possible to quantify the effect of treatment, caste and colony components. The effect of treatment causes 37.2% removal of queen-laid eggs in experiment 1, assuming that native queen-laid eggs were only removed because of the treatment. Experiment 2 suggests that the effect of the treatment in *A. m. capensis* is smaller than in *A. m. scutellata* (Table 8.1). By comparing the level of egg removal of the two different queen-laid eggs in *A. m. scutellata* and the level of removal of the two types of worker-laid eggs in *A. m. capensis*, 33.3% and 60.6% respectively can be assigned to the effect of nestmate recognition. Finally the effect of the caste is only 16.7% and 25% in experiment 1 and 2 respectively. The results of Visscher (1986) suggest that the ability for nestmate recognition of eggs is not a specific behaviour of African honeybees, therefore the use of alien eggs in policing experiments result in a systematic overestimation of the levels of removal and does not represent real levels of egg removal. Furthermore, because of the small proportion of egg removal which can be explained by caste differences, it would be possible that similar nestmate recognition cues of eggs masked differences in the egg removal behaviour. Therefore, we conclude that the use of only native laid eggs prevent an overestimation and evaluated the actual differences in the removal behaviour of workers towards queen and worker-laid eggs.

Table 8.1: Total number and percentage of remaining eggs after 24 hours for native and alien queen and worker-laid eggs in the test colonies of *A. m. scutellata* and *A. m. capensis*. The means and standard deviations and the results of the Newmans-Keuls post hoc comparison are shown (N = trials, QL = queen-laid, WL = worker-laid, significant P-values are in bold).

	Subspecies	Egg source	Mean±sd of remaining eggs (remaining eggs as a %)	Trend	Egg source	Mean±sd of remaining eggs (remaining eggs as a %)	Newmans-Keuls; P-value	N
Experiment1	*A.m. scutellata*	Alien QL eggs	5.89±2.37 (29.5±11.9)	Slower	Alien WL eggs	2.56±0.56 (12.8±2.8)	0.06	9
		Alien QL eggs	5.89±2.37 (29.5±11.9)	Faster	Native QL eggs	12.78±5.56 (63.9±27.8)	**0.001**	9
		Alien WL eggs	2.56±0.56 (12.8±2.8)	Faster	Native QL eggs	12.78±5.56 (63.9±27.8)	**0.0002**	9
Experiment2	*A. m. capensis*	Alien QL eggs	7.56±2.92 (37.8±14.6)	Slower	Alien WL eggs	2.56±2.83 (12.8±14.2)	**0.018**	9
		Alien QL eggs	7.56±2.92 (37.8±14.6)	Faster	Native WL eggs	14.67±6.00 (73.4±30.0)	**0.002**	9
		Alien WL eggs	2.56±2.83 (12.8±14.2)	Faster	Native WL eggs	14.67±6.00 (73.4±30.0)	**0.0002**	9

Fig. 8.1: Mean number and SD of remaining eggs after 24 hours in the three *A. m. scutellata* discriminator colonies. Different letters indicate significant differences between the groups (Tab. 8.1).

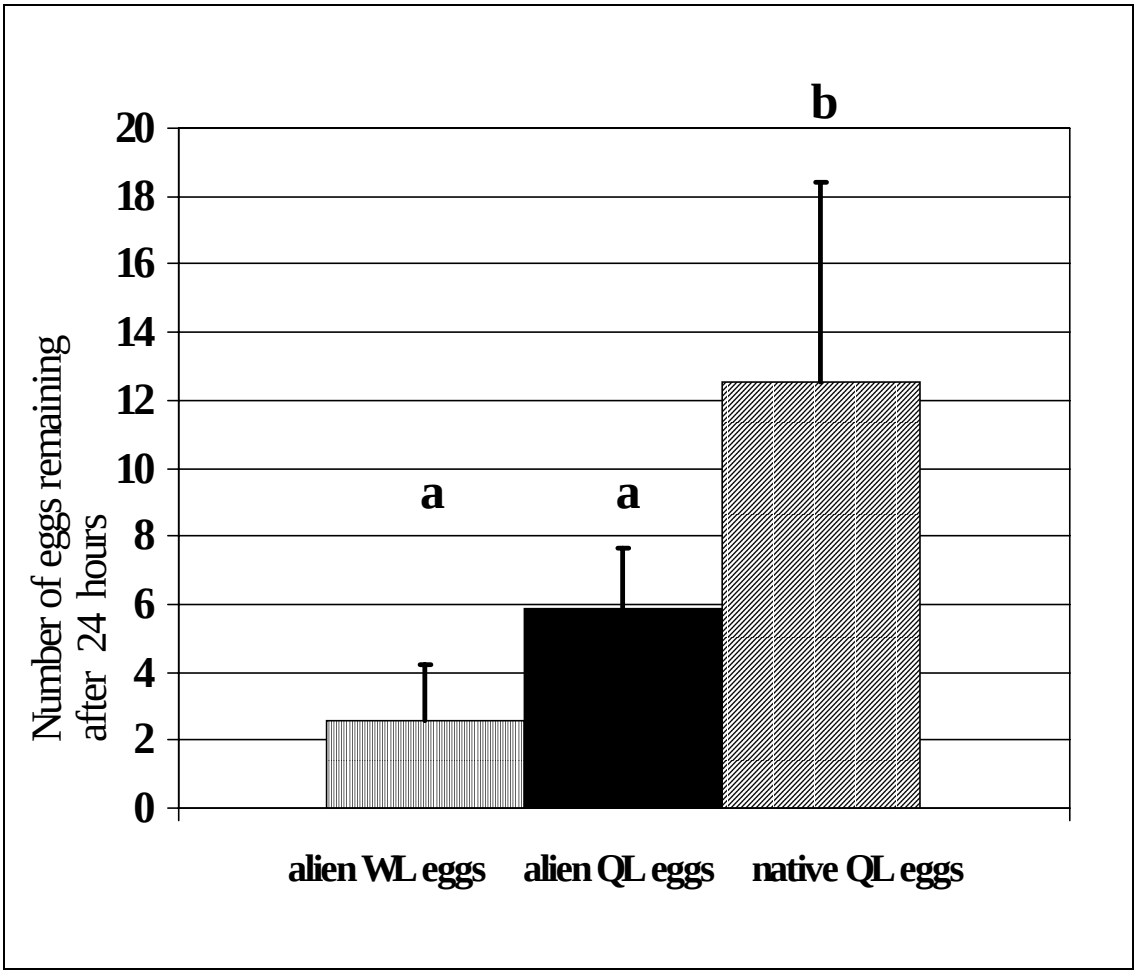


Fig. 8.2: Mean number and SD of remaining eggs after 24 hours in the three *A. m. capensis* discriminator colonies. Different letters indicate significant differences between the groups (Tab. 8.1).

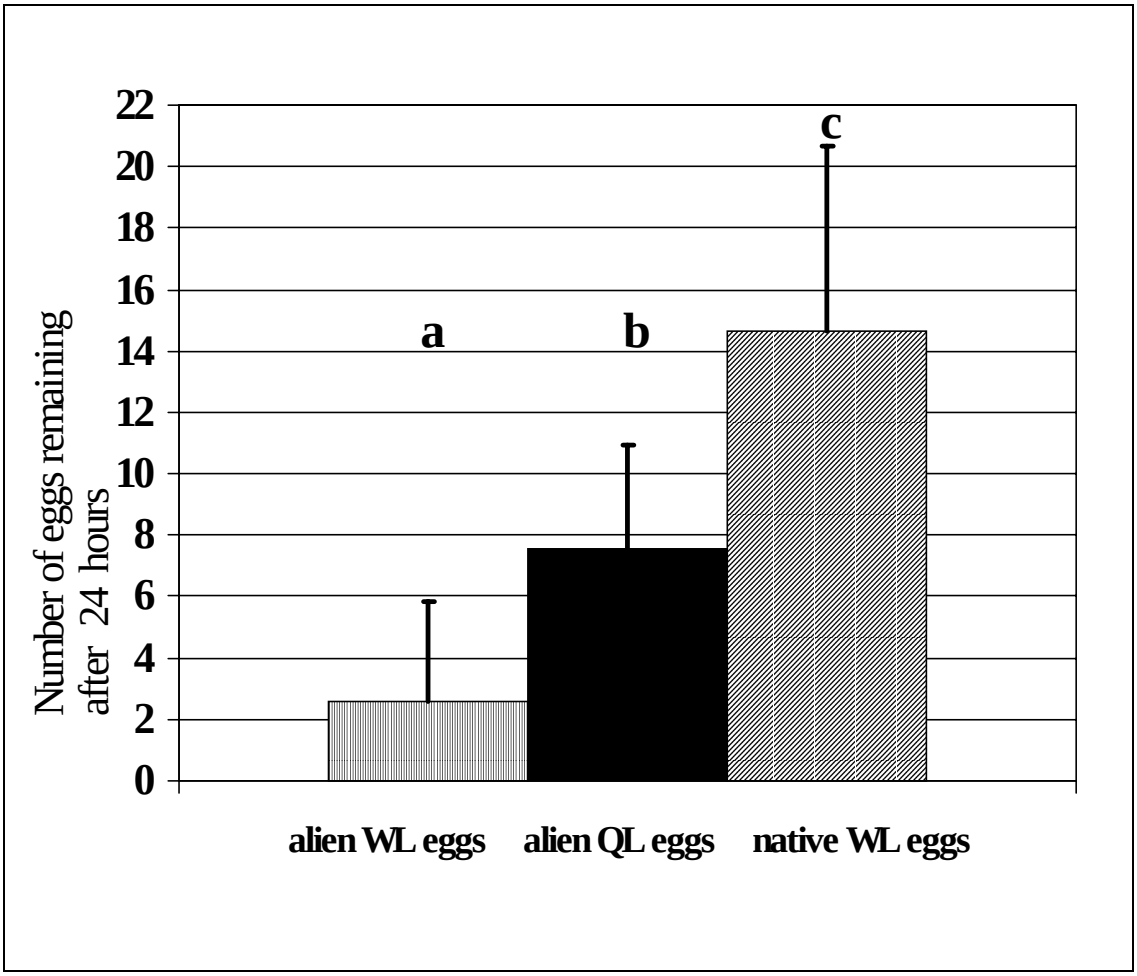
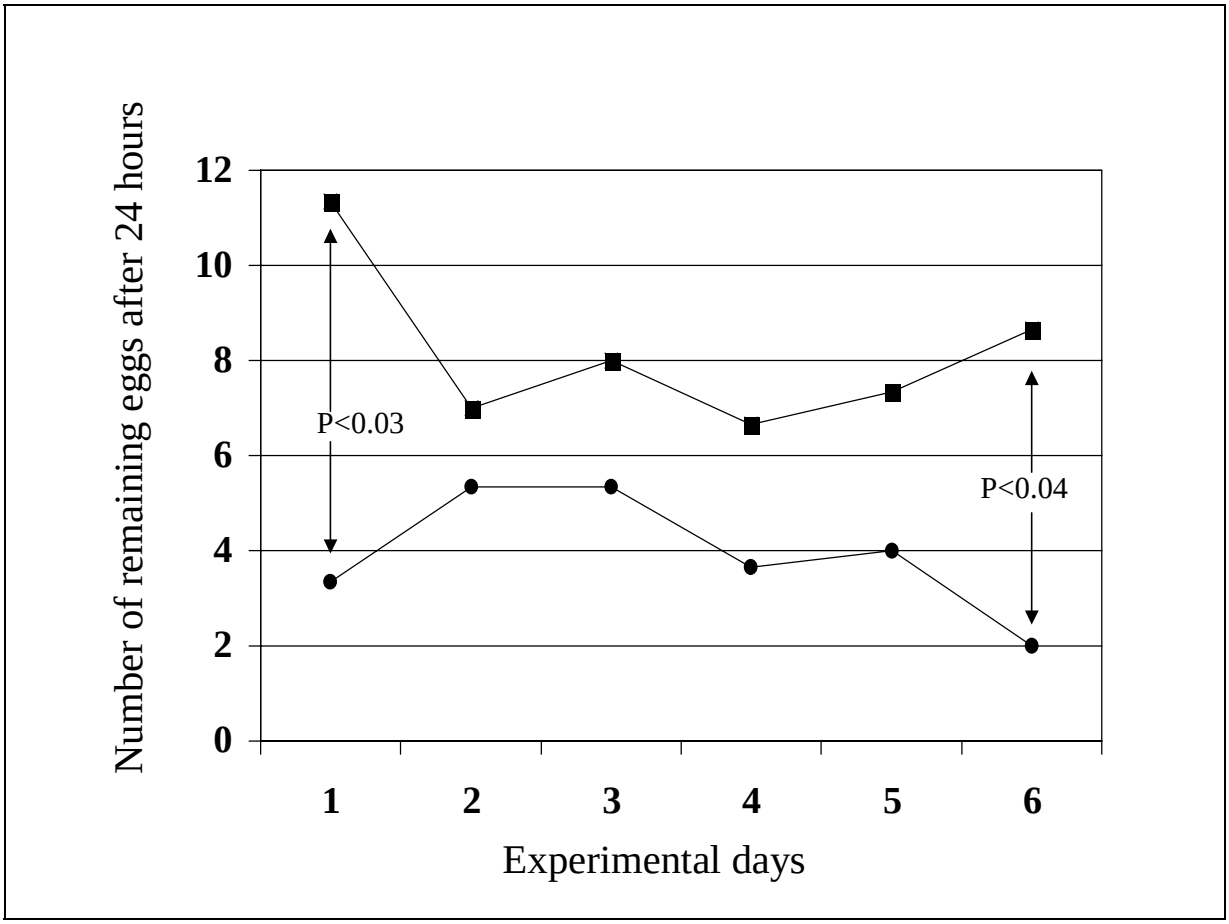


Fig. 8.3: Average number of remaining alien queen (squares) and alien worker-laid eggs (circle) in three queenless *A. m. capensis* test colonies. The queen was removed from these colonies on day 0. The results of the χ^2-tests are shown for day 1 and 6.



Appendix 8

Appendix 8.1: Number of remaining alien queen and worker-laid eggs and native worker-laid eggs after 24 hours in the three queenless *A. m. capensis* discriminator colonies.

Experimental day	Discriminator colony	Type of egg	0 hours	24 hours
1	*capensis* 2	Alien queen	20	13
1	*capensis* 2	Alien worker	20	10
1	*capensis* 3	Alien queen	20	14
1	*capensis* 3	Alien worker	20	3
1	*capensis* 4	Alien queen	20	7
1	*capensis* 4	Alien worker	20	0
2	*capensis* 2	Alien queen	20	4
2	*capensis* 2	Alien worker	20	5
2	*capensis* 3	Alien queen	20	10
2	*capensis* 3	Alien worker	20	11
2	*capensis* 4	Alien queen	20	2
2	*capensis* 4	Alien worker	20	3
3	*capensis* 2	Alien queen	20	4
3	*capensis* 2	Alien worker	20	3
3	*capensis* 3	Alien queen	20	2
3	*capensis* 3	Alien worker	20	2
3	*capensis* 4	Alien queen	20	8
3	*capensis* 4	Alien worker	20	7
4	*capensis* 2	Alien queen	20	12
4	*capensis* 2	Alien worker	20	0
4	*capensis* 2	Native worker	20	20
4	*capensis* 3	Alien queen	20	4
4	*capensis* 3	Alien worker	20	0
4	*capensis* 3	Native worker	20	3
4	*capensis* 4	Alien queen	20	4
4	*capensis* 4	Alien worker	20	3
4	*capensis* 4	Native worker	20	20
5	*capensis* 2	Alien queen	20	10
5	*capensis* 2	Alien worker	20	8
5	*capensis* 2	Native worker	20	18
5	*capensis* 3	Alien queen	20	6
5	*capensis* 3	Alien worker	20	0
5	*capensis* 3	Native worker	20	10
5	*capensis* 4	Alien queen	20	6
5	*capensis* 4	Alien worker	20	5
5	*capensis* 4	Native worker	20	18
6	*capensis* 2	Alien queen	20	11
6	*capensis* 2	Alien worker	20	3
6	*capensis* 2	Native worker	20	19
6	*capensis* 3	Alien queen	20	7
6	*capensis* 3	Alien worker	20	0
6	*capensis* 3	Native worker	20	15
6	*capensis* 4	Alien queen	20	8
6	*capensis* 4	Alien worker	20	4
6	*capensis* 4	Native worker	20	9

Appendix 8.2: Number of remaining alien queen and worker-laid eggs and native queen-laid eggs after 24 hours in the three queenright *A. m. scutellata* discriminator colonies.

Experimental day	Discriminator colony	Type of egg	0 hours	24 hours
1	*scutellata* 1	Native queen	20	14
1	*scutellata* 1	Alien queen	20	6
1	*scutellata* 1	Alien worker	20	2
1	*scutellata* 2	Native queen	20	7
1	*scutellata* 2	Alien queen	20	6
1	*scutellata* 2	Alien worker	20	2
1	*scutellata* 3	Native queen	20	9
1	*scutellata* 3	Alien queen	20	9
1	*scutellata* 3	Alien worker	20	0
2	*scutellata* 1	Native queen	20	15
2	*scutellata* 1	Alien queen	20	4
2	*scutellata* 1	Alien worker	20	3
2	*scutellata* 2	Native queen	20	2
2	*scutellata* 2	Alien queen	20	3
2	*scutellata* 2	Alien worker	20	5
2	*scutellata* 3	Native queen	20	15
2	*scutellata* 3	Alien queen	20	9
2	*scutellata* 3	Alien worker	20	5
3	*scutellata* 1	Native queen	20	17
3	*scutellata* 1	Alien queen	20	8
3	*scutellata* 1	Alien worker	20	2
3	*scutellata* 2	Native queen	20	18
3	*scutellata* 2	Alien queen	20	5
3	*scutellata* 2	Alien worker	20	3
3	*scutellata* 3	Native queen	20	18
3	*scutellata* 3	Alien queen	20	3
3	*scutellata* 3	Alien worker	20	1

Chapter 9
General discussion

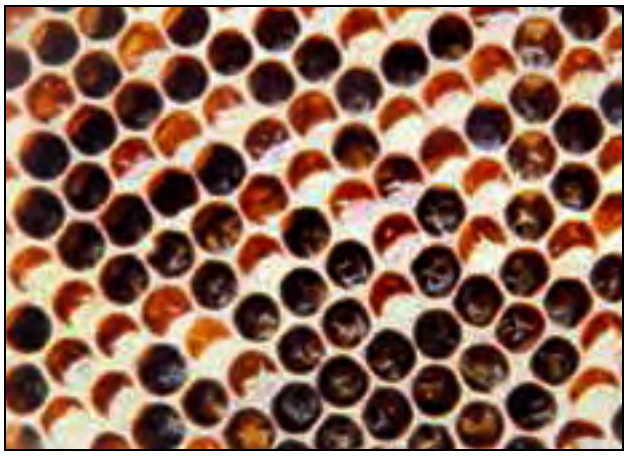
A major result of this study shows that in queenright colonies of both *A. m. scutellata* and more especially *A. m. capensis*, policing of worker-laid eggs of their own kind (cf. Chapters 2,3,5,6,7,8) and of each other (cf. Chapter 2) occurs. That *A. m. capensis* shows worker policing behaviour is in contrast to earlier theoretical predictions (Greeff 1996) and empirical findings (Moritz et al. 1999). However, the empirical findings (Moritz et al. 1999) only showed that brood can be worker-derived but not that worker policing is lacking (Fig. 9.2). Such brood could just be those eggs which escaped worker policing. The theoretical predictions were based on relatedness among *A. m. capensis* workers, because if workers reproduce thelytokously there are no costs involved in worker reproduction in terms of relatedness (Greeff 1996). Nevertheless, worker reproduction could be costly in terms of colony efficiency (Cole 1986; Ratnieks 1988; Crozier & Pamilo 1996).

It has been shown that the typical brood pattern in a honeybee colony is based on a self-organisation process and that the commonly observed pattern only develops if certain conditions are fulfilled (Camazine 1991). One of these conditions is that the queen lays only one egg per cell and this cell is in close proximity to cells already containing eggs or brood. It is common beekeeping practice to judge from the conditions of the brood nest if a healthy queen (one egg/cell) or laying workers (multiple eggs/cell) are laying in the colony. So it is safe to assume that worker oviposition behaviour is different to that of a queen because multiple eggs only occur in cells if laid by workers (Gary 2000; Tribe & Allsopp 2001; Fig. 9.1).

A gene for worker reproduction can invade a population of social insect species if the costs involving worker policing exceed a certain threshold (Crozier & Pamilo 1996). The same can happen with a policing gene if the costs derived from worker policing behaviour are low enough (Crozier & Pamilo 1996). The costs derived from worker policing in honeybees are probably small because workers check cells regularly. The costs involved in worker policing are probably much lower compared to the costs that would be derived from unhindered worker reproduction. The intensity of policing should be dependent on a trade off between the cost of policing and the cost of worker reproduction to overall colony efficiency and reproduction. What possible costs are involved in unhindered worker reproduction?

The queen normally lays one egg per cell (Ratnieks 1990), but laying worker activity however is characterised by multiple eggs per cell (Tribe & Allsopp 2001; Fig. 9.1) and workers may lay eggs in cells which already contain an egg (Tribe & Allsopp 2001). During the oviposition of these additional eggs into cells already containing an egg (Gary 2000), the pre-existing egg may be destroyed by being knocked over or crushed by the worker's abdomen. Moreover, only one larva can be reared to adulthood in a single cell and additional larvae are eaten by workers leading to costs associated with cannibalism (Elgar & Crespi 1992).

Fig. 9.1: Multiple eggs per cell in an *A. m. capensis* colony.



Furthermore, when many workers lay eggs in the same cells it may simply take longer for any cell to yield a worker (Chapter 2). In the case of queen cells, a delay could severely affect the overall colony performance and lower the fitness of the whole colony. Because workers tend to lay eggs especially in queen cells (Tribe & Allsopp 2001) it is self-evident that a delay in raising new queens during the swarming period may cause the late departure of a reproductive swarm or prevent swarming altogether.

Moreover, the overall colony performance may be affected by a high number of laying workers (Ratnieks 1988; Hillesheim et al. 1989), since such workers do not participate in hive tasks as much as subordinate workers (Moritz & Hillesheim 1985; Hillesheim et al. 1989). In this case policing behaviour would not be selected on a individual level, but rather on a colony level. Colonies with a high level of laying worker activity would be outperformed by other colonies with no or low laying worker activity in terms of reproductive swarming and survival, so that worker policing behaviour could be selectively favoured over evolutionary time.

The occurrence of worker policing behaviour in *A. m. capensis* shows that in general this behaviour is not only favoured because of relatedness benefits (Ratnieks 1988), but also because of benefits derived from colony efficiency grounds. Since the benefits derived from

relatedness grounds are lacking in *A. m. capensis*, because of the thelytokous mode of worker reproduction (Greeff 1996), worker policing in *A. m. capensis* is therefore evolutionarily adaptive because of benefits derived from grounds of colony efficiency. This should result in a more fragile cost/benefit ratio for worker policing than in honeybee subspecies with an arrhenotokous mode of worker reproduction where benefits are derived from both colony efficiency and relatedness grounds.

Indeed, the high variation in successful worker reproduction among queenright *A. m. capensis* colonies indicates that sometimes the costs are higher than the benefits and sometimes vice versa. Furthermore, it is shown that unfavourable weather conditions can raise the costs of worker policing so that the costs exceed the benefits, which then results in a decrease of worker policing behaviour (Chapter 3). These costs could be increased by unfavourable weather conditions because it forces foragers to stay in the colony (Ribbands 1953) or, especially during rain, causes a shift in the nursing behaviour of house bees away from eggs and young larvae towards the care of older larvae (Blaschon & Crailsheim 2001). Lower temperatures cause an increase in brood warming activity (Seeley 1985) and this behaviour is mainly performed by house bees (Lindauer 1952). Because unfavourable weather conditions keep the foraging force within the colony, one might expect the level of worker policing to increase if workers of every age cohort (Lindauer 1952) participate equally in the removal of worker-laid eggs. However, the results (Chapter 3) suggest that not all workers participate in egg removal and that egg removal behaviour is mainly performed by house bees (Lindauer 1952).

These potential mechanisms, which may explain the observed decrease in worker policing, should be the same for all honeybee colonies. Moreover, the costs of worker policing and the benefits derived on colony efficiency grounds should be the same in thelytokous *A. m. capensis* as well as subspecies with arrhenotokous worker reproduction, so one may expect that environmental effects less readily affect the latter. This seems plausible, because benefits derived from relatedness grounds in arrhenotokous subspecies may be higher than the costs of worker policing, regardless of changing environmental conditions. Indeed, preliminary results indicate that changing environmental conditions do not affect egg removal behaviour in the same way (Appendix 3.1). However, studies which compare environmental effects on egg removal behaviour in arrhenotokous and thelytokous honeybee subspecies should be conducted under precisely the same environmental conditions. Because the experiments were conducted in two different apiaries (owing to national quarantine regulation, Government Notice R159 under the Agricultural Pests Act 1983, Johannsmeier

2001), and despite the results of this study (Appendix 3.1) the questions of if and how environmental changes affect egg removal behaviour in arrhenotokous honeybees are still to be answered.

On a priori grounds, potential reproductive conflict after a merger (Herman 1922; Walter 1939; Hepburn & Whiffler 1988; Hepburn 1993; Hepburn & Radloff 1998) is higher compared to potential reproductive conflict in a colony where all workers are the offspring of one queen (Fig. 1.4), because in the new unit workers which are not related to the queen have an inclusive fitness of zero if they do not themselves reproduce (Fig. 1.4). The results in Chapter 4 are based on the observation of a natural merger of two colonies. However, the observations are based on over 4000 individual behaviours and since it was a serendipity that two colonies containing labelled workers merged, it is highly probable that the results reflect common behavioural changes during the process of a natural colony merger. One aim of this study was to investigate how long it took for two unrelated colonies to become one social unit. Taking the queen-worker distance as an indicator of the process of social integration, the differences between the two cohorts vanish after 24 hours (Chapter 4).

However the merging of two unrelated colonies seems puzzling from an evolutionary perspective. One part of the new colony is unrelated to the queen so that the inclusive fitness of these workers is zero. One reason why that behaviour is evolutionarily adaptive could be because in the new unit the *pro rata* survival costs are probably reduced (Hepburn & Radloff 1998). The pollen income of colonies at the same apiary are substantially different (Synge 1947; Johannsmeier 1981) so it would be as if one compared the pollen income of a colony from Johannesburg with a colony from Berlin. Thus, a merger could have the advantage that the new unit derives pollen from a wider variety of different plants and so is less affected by changing flowering patterns.

Moreover, because if these colonies are unrelated or less related, as a consequence of a merger the genetic diversity within the new unit increases. Since the observed task shifts differed in the two cohorts, workers may respond to different behavioural threshold and task specialisations (Moritz & Page 1999). Possibly a higher number of more efficient genetic specialists (Fuchs & Moritz 1999) may also reduce *pro rata* costs in the new colony. Indeed, the task shifts differed in the two cohorts, indicating that workers responded differently to the range of tasks (Table 2.1, Appendix 4.2, 4.4) and because all marked bees were from one age-cohort these differences can be only partially explained by an age-related division of labour (Lindauer 1952). Alternatively, but not mutually exclusive explanation may be, because nearby colonies can be related to each other (Oldroyd et al 1995), that all worker gain

inclusive fitness during a merger because they are related to each other. However, since these observed colonies were only in close proximity because of observation hive experiments it seems that an explanation why natural colony merger are evolutionary adaptive may also be found in skew theory (Keller & Reeve 1998, Dugatkin & Reeve 2000).

All these factors may explain why natural mergers of honeybee colonies are evolutionarily adaptive in African honeybees. Taking the African life history into account with absconding and a higher predation risk than for European honeybee colonies (Seeley 1985; Hepburn & Radloff 1998), to abandon an old nest site and merge with an other colony may increase the survival of this new colony. However, workers which are not related to the queen gain no inclusive fitness, so for them it makes no difference whether they merge and survive or whether their colony dwindles, unless they are able to reproduce pathogenetically in the new unit. The common occurrence of brood above the excluder (Tribe & Allsopp 2001) suggests that there is a chance for such workers to reproduce. It may be that a lower expression of egg removal behaviour in African honeybees (more worker-laid eggs remained in the discriminator colonies than in other studies with European honeybees; i.e. Ratnieks & Visscher 1989; Ratnieks 1993; Oldroyd & Ratnieks 2000) is an adaptation to natural colony mergers because workers which are not related to the queen acquire a greater chance to reproduce and making the merger beneficial for both parts of the original two colonies.

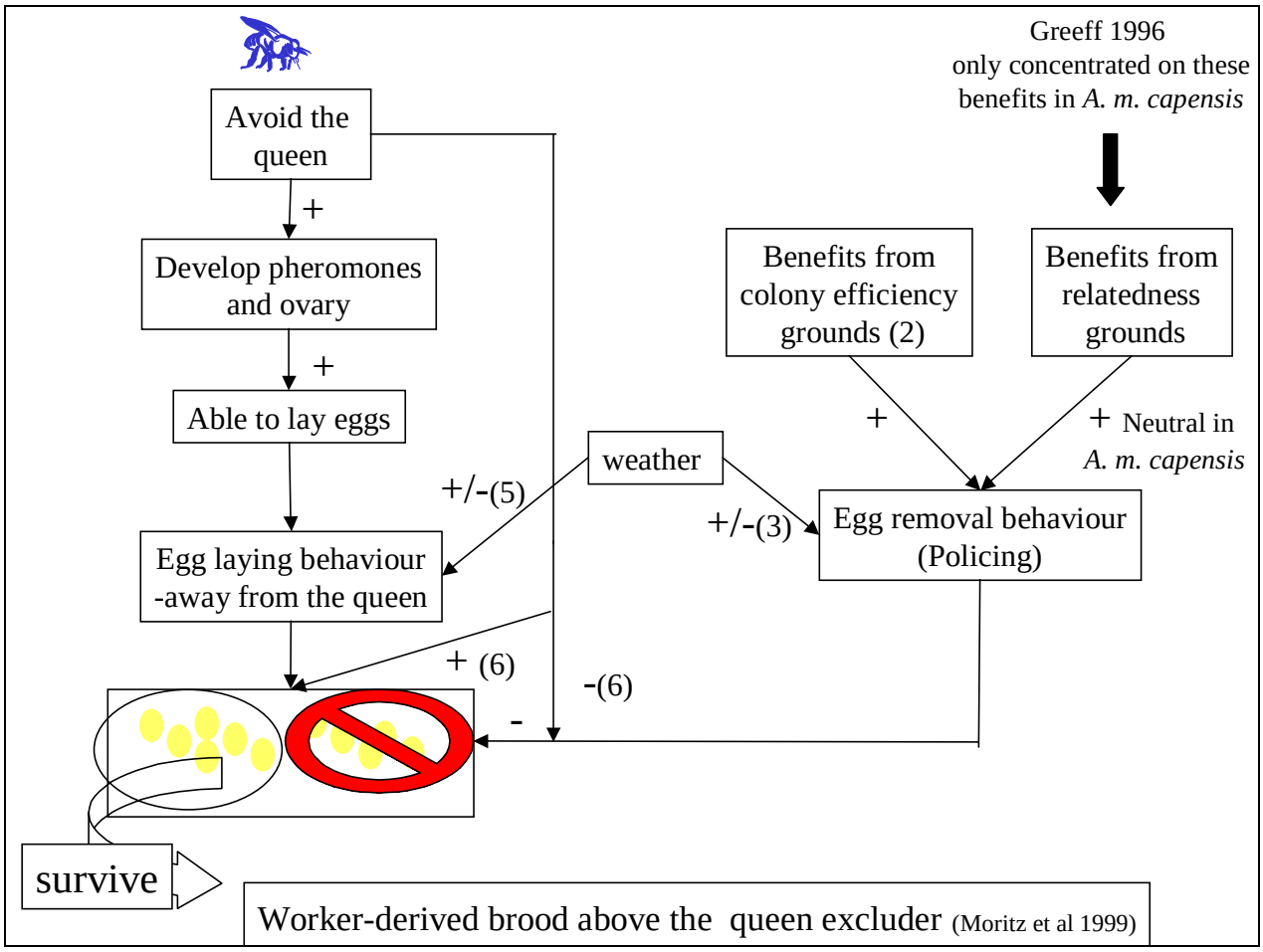
Nevertheless, there is still potential reproductive conflict among these workers so, in order to reproduce successfully laying workers have to avoid worker policing (Ratnieks 1988, Chapter 2) and the inhibitory pheromonal effects of the queen's pheromones on their own ovarial and pheromonal development (de Groot & Voogt 1954). Chapters 5 and 6 investigated potential behavioural strategies of laying workers to increase the survival chances of their eggs by escaping egg removal behaviour by other workers and the influence of the queen. Egg removal behaviour is variable and is affected by changing environmental conditions (Chapter 3). The results described in Chapter 5 show that workers do not lay their eggs during periods of low egg removal. So this is not a strategy of laying workers to achieve successful reproduction in the presence of the queen. Because unfavourable weather conditions, especially rain, are associated with reduced brood care of younger larvae (Blaschon & Crailsheim 2001), it may be disadvantageous for the survival of worker-derived brood. Alternatively, if workers are unable to discriminate between periods of low and high egg removal, the level of egg removal would be triggered by the number of eggs found in the cells.

The typical brood pattern in honeybee colonies emerges spontaneously from dynamic interactions among the processes of depositing and removing brood, pollen and honey without a plan specifying the spatial relationship (Camazine 1991). This self-organisation process is self-repairing (Camazine 1991): if the cell contents do not fit into the pattern, they are removed and replaced with the "right" contents, e.g. if there is honey in a cell where pollen should be. Oviposition behaviour of workers is different from that of a queen (Perepelova 1928) as indicated by a scattered brood pattern caused by laying worker activity (Hastings 1989; Tribe & Allsopp 2001).

The typical pattern on a comb emerges under certain conditions, one of which is that the queen lays eggs in cells next to others already containing brood or eggs (Camazine 1991). If a high number of worker-derived eggs are placed in the "wrong" cells, more are removed because this self-organisation process is self-repairing (Camazine 1991). The number of pseudoqueens in *A. m. capensis* colonies (Anderson 1963; Ruttner & Hesse 1981; Hepburn & Allsopp 1994) and the egg-laying rate is considerably higher than in European honeybees colonies (12 worker-laid eggs in 3 months; Visscher 1996). An alternative explanation for worker-derived brood above the excluder (Pettey 1922; Tribe & Allsopp 2001; personal observations) may be that the number of worker-laid eggs simply exceeds the egg removal capacity of the colonies. Furthermore, some pseudoqueens brood nests are indistinguishable from those of queens (Tribe 1981; personal observations) and this may also reduce egg-removal behaviour.

An alternative explanation, which does not preclude the previous explanation, for worker-derived brood in the presence of a queen is suggested by the results given in Chapter 6. It is known that some *A. m. capensis* workers evade the queen (Moritz et al. 2001) thereby reducing the pheromonal inhibitory effect on their own pheromonal and ovarial development (Moritz et al. 2000a; Moritz et al. 2001; Moritz et al. 2002; Fig. 9.2). Staying away from the queen enables workers to develop either or both their pheromones and ovaries. The avoidance of the queen also has the advantage that egg removal behaviour of worker-laid eggs by other workers is reduced (Chapter 6, Fig. 9.2).

Fig. 9.2: A schematic drawing of factors and their interactions with egg removal behaviour (policing) and egg-laying behaviour, which explains the phenomenon of worker-derived brood in queenright honeybee colonies (+ = increase or positive effect, - = decrease or negative effect, +/- = effect varies, numbers in brackets are the corresponding Chapter numbers).



Thus workers can achieve successful reproduction if they stay away from the queen, to enable them to develop their ovaries and queen-like pheromones and egg removal behaviour by other workers is reduced. However, some *A. m. capensis* colonies show worker-derived brood above the queen excluder, while others do not (Pettey 1922; Tribe 1981; Tribe & Allsopp 2001). This variation cannot be explained by environmental variation alone (Chapter 3), because colonies in any apiary probably experience the same environmental conditions. This suggests that variations in intracolonial factors cause the phenomenon that in some colonies brood occurs above the excluder.

The variation in the number of pseudoqueens present in a colony (Hepburn & Radloff 1998) may affect the amount of worker-laid eggs in the colony. The number of worker-laid eggs in the test colonies (Chapter 5) were considerably higher than in non-anarchistic colonies of European subspecies of *A. mellifera* (12 worker-laid eggs in 3 months; Visscher 1996), and comparable to a half-anarchistic European colony (about 6 worker-laid eggs per day; Ratnieks

et al. 2002). This supports earlier findings that *A. m. capensis* workers may lay a considerable number of eggs per day (Velthuis et al. 1990), and further suggests that the high variation in egg laying as observed among the test colonies (see Table 5.1 and also Hepburn et al. 1991) may be an important factor for the survival of worker-derived brood.

Another reason for the variation in the occurrence of worker-derived brood may be that some laying workers lay eggs which have low removal rates, a mechanism similar to anarchistic honeybee workers (Oldroyd & Ratnieks 2000) and others not. Indeed, highly virulent *A. m. capensis* workers which invade colonies of the neighbouring subspecies *A. m. scutellata* (Hepburn & Allsopp 1994) suffer a lower level of egg removal than laying workers of *A. m. scutellata* (Martin et al. 2002). Moreover, there is considerable variation for egg removal rates among *A. m. capensis* laying worker populations (Chapter 8) and also high intercolonial variation for ovarial development of workers in queenright Cape honeybee colonies (Hepburn et al. 1991; Hepburn & Crewe 1991).

Furthermore, the results in Chapter 6 not only explain the phenomenon of worker-derived brood above the excluder in queenright Cape honey colonies (Pettey 1922; Tribe & Allsopp 2001), but also why colonies of the neighbouring subspecies (see Heburn & Radloff 1998, 2002 for a review of the boundaries and distribution of both subspecies) are so prone to infestation by Cape honeybee laying workers (Allsopp & Crewe 1993). Because of this behavioural strategy of Cape honeybee laying workers (Chapter 6) and the lack of retaliatory behaviour (worker policing in the honey super) in *A. m. scutellata*, it is easy for a *A. m. capensis* worker to produce offspring. Likewise thelytoky appears to predispose a taxon for the evolution of aggressive worker reproduction (Greeff 1996; Greeff 1997), the offspring reared of such workers also develops into laying workers (Neumann & Hepburn 2002).

The recent transfer of a high number of *A. m. capensis* colonies into the native distribution area of *A. m. scutellata* (Allsopp 1995) inevitably set-up conditions for reproductive conflict between colonies of the two subspecies of *A. mellifera*. Moreover, besides the usurpation of numerous colonies of *A. m. scutellata* by *A. m. capensis* (Hepburn & Allsopp 1994) it also led to reproductive conflict among these parasitic Cape honeybee workers. Since the *A. m. capensis* population in northern South Africa (formerly Transvaal) is genetically uniform (Solignac et al. 2001), possibly the whole population originated from a single *A. m. capensis* laying worker (Kryger 2002) and because crossing over is rare during automictic parthenogenesis (Slobodchikoff & Daly 1971; Moritz & Haberl 1994), this strain is the result of reproductive competition among different genotypes.

The multiple infestations due to migratory beekeepers (Lundie 1954; Johannsmeier 1983; Allsopp 1995) combined with a series of traits of *A. m. capensis* that reflect important behavioural, physiological and genetic pre-adaptations for intraspecific social parasitism (Neumann & Hepburn 2002), sets the conditions for reproductive competition among different genotypes. The short-sighted selection hypothesis for parasite virulence predicts that winners of within-host competition (resulting from multiple infestations) are poorer at transmission to new hosts (Schmid-Hempel 1998). Indeed, this is the first empirical support for the short-sighted selection hypothesis for parasite virulence (Schmid-Hempel 1998), which predicts that winners of within-host competition are poorer at transmission to new hosts.

Only the parasitic worker with the highest within-host virulence can successfully out-compete others (Moritz et al. 1996) and because queenless colonies do not frequently re-queen from laying worker offspring (Hepburn & Radloff 1998) and recombination events are rare in thelytokous parthenogenesis (Moritz & Haberl 1994), selection among strains of social parasitic worker was further enhanced. This may explain why the parasitic laying workers of the *A. m. capensis* (Solignac et al. 2001) from the Gauteng (formerly Transvaal) area showed significantly higher virulence than parasitic laying workers from the natural distribution area of *A. m. capensis* (oviposition of eggs with low removal rates; a mechanism similar to anarchistic honeybee workers, Oldroyd & Ratnieks 2000). The severe intra-host competition in the region of *A. m. scutellata* resulted in a single parasitic, genetically identical strain with high virulence, although multiple introductions occurred (>200 *A. m. capensis* colonies were brought to the region of *A. m. scutellata*, Allsopp 1995). Beekeepers facilitate the spread of the parasitic workers, enhancing the "*capensis*" calamity and offering an opportunity for short-sighted selection and the evolution of a social parasite in real time.

In general the ability to recognise eggs of nestmates and non-nestmates would not only be a host defence mechanism against parasitic *A. m. capensis* laying worker (Allsopp & Crewe 1993; Hepburn & Allsopp 1994; Neumann et al. 2001; Neumann & Hepburn 2002)., it would also maintain colony integrity (Crozier & Pamilo 1996) and prevent undesirable reproduction by unrelated workers, regardless of how these worker entered the colony: merger (Chapter 4), drifting (Rauschmayer 1928; Free 1958; Jay 1966) or dispersing (Neumann et al. 2001). It is probable that eggs carry cues which may enable workers to discriminate between eggs laid by nestmates (native workers) and non-nestmates (alien workers) because the Dufour´s gland produces hydrocarbons which may play a role in nestmate recognition (Breed 1998) and the gland opens into the dorsal vaginal wall (Billen 1987).

The results given in Chapter 8 show that workers discriminate between alien and native eggs. Furthermore, the results indicate that only a small proportion of worker-laid eggs that are removed is caused by a postulated, queen-produced egg-marking pheromone (Ratnieks 1992; Ratnieks 1995). Moreover, the results also suggest that the usage of alien eggs to evaluate egg removal behaviour overestimates the actual removal rates because nestmate recognition of eggs has a stronger effect on egg removal behaviour than the postulated egg-marking pheromone. Since these results are consistent with findings in the context of queen rearing in European honeybees (Visscher 1986) it is safe to assume that nestmate recognition of eggs occurs in both African and European subspecies of *Apis mellifera*. However, another study (Oldroyd & Ratnieks 2000) found no effect of nestmate recognition of eggs on egg removal behaviour. That could be due to the fact that they used worker-derived eggs from anarchistic honeybee colonies (Oldroyd et al. 1994). Because nestmate recognition cues are both genetically and environmentally derived to varying extents (Wilson 1971; Jutsum et al. 1979; Breed 1983; Crozier 1988; Pirk et al. 2001b) and because the anarchistic honeybee colonies were especially inbred for certain features (Oldroyd & Osborne 1999), it could be that genetically derived cues were similar in that study (Oldroyd & Ratnieks 2000) and so may have masked the effect of nest recognition of eggs on egg removal behaviour.

Finally, I would like to briefly summarise the results. Firstly, benefits derived from colony efficiency grounds are able to support the expression of worker policing behaviour in a population of honeybees despite the absence of relatedness benefits. Since *A. m. capensis* and *A. m. scutellata* are able to police worker-laid eggs of the other subspecies, this indicates that the same underlying mechanisms are used for worker policing in both subspecies. Therefore, it is my opinion, that it is difficult to separate why worker-laid eggs are removed. It may be because workers are less related (relatedness grounds) or the eggs were just wrongly placed (colony efficiency grounds).

I think the term “worker policing” is biologically prejudicial and should not be used in this context because it presupposes and implies the reasons for egg removal behaviour rather than describing what actually happens, which is egg removal. Secondly, egg removal behaviour is affected by changing environmental conditions and workers are able to discriminate between eggs laid by nestmates (native eggs) and non-nestmates (alien eggs). Thirdly, after the natural merger of two colonies it takes 24 hours until the new colony acts as a socially integrated unit. Fourthly, not only the inhibitory effect of the queen pheromones on the pheromonal and ovarial development of workers declines with distance, but egg removal

behaviour is also affected by the distance from the queen. Lastly, the results from the parasitic Cape honeybee laying worker population in northern South Africa give support for the “short-sighted selection hypothesis” for parasite virulence.

Chapter 10

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