REGULATION OF WORKER REPRODUCTION IN THE HONEY BEE (*APIS MELLIFERA* L.)

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ABSTRACT

Reproductive division of labour is a defining characteristic of eusocial insects. In honey bees, there is normally a single, highly fecund queen, responsible for producing all the brood in the colony. Workers are functionally sterile, developing their latent ovaries only upon queen loss. Workers cannot mate, and are only capable of laying unfertilised, male eggs. I investigated the effects of various chemical, genetic, and nutritional factors on the ovary development of honey bee workers.

I demonstrate that queen mandibular pheromone inhibits worker ovary development in caged queenless workers to the same degree as queen extracts. Four newly identified queen pheromone components did not inhibit ovary development alone, nor did they increase the efficacy of the other components.

Anarchistic bees are a line developed by recurrent selection in which workers commonly reproduce in queenright colonies. There was no difference between the ovary development of anarchistic or wild type workers in colonies headed by anarchistic or wild type queens, therefore queen type is not responsible for the phenomenon. Anarchistic workers perceive queen pheromones, and anarchistic queens produce an attractive blend, as I found no differences in the retinue response of either worker type to either queen type. There also was no difference in response to queen pheromones at a high dose. At lower doses,

however, wild type workers were more inhibited by queen pheromones than were anarchistic workers.

Both adult and larval diet influenced adult ovary development, but workers fed high quality diets as adults had higher levels of ovary development than those fed low quality diets as adults regardless of larval diet quality. Nutrition is likely responsible for the seasonal variation observed in ovary development.

Disruptive selection resulted in lines of bees with high or low levels of ovary development. High ovary development colonies collected far more pollen than their low line counterparts. Cross-fostering workers from the high line into the low line and vice versa demonstrated that there is an effect of both genotype and rearing environment. These results demonstrate the complex interactions between nutrition, pheromones, genetics, and environment that determine worker reproductive potential.

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CHAPTER 1: INTRODUCTION: REPRODUCTIVE CONFLICT IN SOCIAL INSECTS AND PROXIMATE FACTORS AFFECTING WORKER OVARY DEVELOPMENT

1.1 Introduction

Individuals of nearly all animal species interact in some manner with other individuals of the same species. In the social insects, these interactions are particularly complex and ongoing. Individuals in insect societies coordinate their efforts, and are specialised for different tasks including defence, brood care, food gathering, and reproduction. Social insects use this group organisation to meet ecological challenges that solitary animals face alone. Social insect species are found in the orders Hymenoptera, Coleoptera, Isoptera, and Thysanoptera, with the Hymenoptera containing the largest number of social species.

Social wasps, bees, and ants are among the most studied social organisms, particularly the honey bee (*Apis mellifera* L.). There is enormous variation in the complexity of insect societies, ranging from species with little or no caste differentiation to the higher termites with complex systems of social organisation and caste determination. As such, the social insects offer a unique opportunity to study the evolution of animal sociality, in different ecological contexts and along many different phylogenetic lineages.

In this thesis, I examine the proximate factors that inhibit reproduction in worker honey bees. This opening chapter introduces the ultimate reasons preventing or limiting worker reproduction, and discusses the proximate mechanisms by which worker ovary development is inhibited in social insect colonies. Beginning with the ultimate explanations as to why workers may forgo reproduction in social insects, the first section of this chapter discusses the effects of relatedness, indirect fitness, direct fitness, and colony level costs on the likelihood of attempted reproduction. The second section focuses on the proximate mechanisms by which worker reproduction is inhibited, including environmental, genetic, and social influences at the ovary development level. These proximate mechanisms are important for the ultimate explanations, as they may affect the genetic bases of these traits, potential evolutionary trajectories, and the behavioural options available to individual workers. Finally, this chapter discusses the levels of selection acting on worker ovary development, and conclusions that can be drawn from the literature.

The subsequent chapters of my thesis focus on the proximate pheromonal and nutritional factors that affect worker ovary development in honey bees. They examine the effect of queen pheromones on worker ovary development (Chapter 2), anarchistic honey bee queen pheromones (Chapter 3), the anarchistic worker response to queen and brood pheromones (Chapter 4), seasonal and nutritional effects on worker ovary development (Chapter 5), and the effects of a selection program for worker ovary development (Chapter 6).

1.2 Ultimate factors inhibiting worker reproduction

Insect societies are defined by reproductive division of labour. Individual colony members are not all equally fecund; rather than rearing their own offspring, some individuals help to rear the offspring of other colony members. Kin selection provides a powerful model for understanding the evolution of such cooperative behaviours (Hamilton 1964). Individuals within colonies are related, allowing for the evolution of members who forgo personal reproduction in favour of assisting reproductive kin. In eusocial insect colonies, it is normally only the 'queen' that reproduces, with all other female colony members directing their efforts towards maintaining the colony and raising the queen's brood. It is this reproductive skew that forms the basis of insect societies.

In haplodiploid insect societies with a single queen, workers are related to their own sons by 0.5, to the male offspring of super-sisters (sisters with common mother and father) by 0.375, to their brothers by 0.25, and to the male offspring of their half-sisters (sisters sharing only their mother) by 0.125 (Table 1). As a result, worker preference for colony male production is: *their own sons* > *the sons of their super-sisters* > *the sons of the queen* > *the sons of their half-sisters*. In societies with a singly mated queen, all workers are super-sisters. In these colonies, workers should favour both their own sons ($r = 0.5$) and their nephews ($r = 0.375$) to the queen's sons (their brothers, $r = 0.25$), creating the potential for conflict over male production within the colony.

In polyandrous species such as many bees and ants, this potential conflict between queen and worker is reduced because of the large number of times the queen mates. In these species, most workers are half-sisters. Polyandrous mating results in lower average relatedness between workers, and consequently between workers and the average worker-laid egg. Workers should therefore favour their own (r = 0.5) and queen-laid eggs (r = 0.25) to eggs laid by other workers (average r approaching 0.125). This preference should result in worker – worker conflict over male production, and ultimately worker destruction (policing) of eggs laid by other workers (Ratnieks 1988).

Table 1-1 The relatedness of a given worker to other colony members. Numbers are mean proportion of alleles shared in the two individuals (r).

Relatedness to:	**Queen** ♀	**Super-sister** ♀	**Half-sister** ♀	**Their own son** ♂	**Queen's son** ♂	**Super-sister's son** ♂	**Half-sister's son** ♂
Worker 'A' ♀	0.5	0.75	0.25	0.5	0.25	0.375	0.125

Worker policing, the consumption of worker-laid eggs by other workers, is costly to both the egg-layer and the colony as a whole. Colony efficiency and individual fitness would both be increased if, under circumstances of effective policing, workers did not attempt to reproduce. The inhibition of ovary development is an effective mechanism by which worker reproduction can be suppressed, and the need for policing diminished.

Two theories have been developed to explain the ultimate reasons why reproductive suppression occurs in social insects. These two theories are not mutually exclusive, and likely operate together to greater and lesser extents depending on the insect society and colony stage in question. First, the queen (or other dominant reproductive individual) may suppress the development of worker reproduction by direct physical aggression or pheromonal mechanisms (Fletcher and Ross 1985, Velthuis *et al.*1990). Second, Seeley (1985) and Keller and Nonacs (1993) proposed that rather than controlling worker reproduction, the queen honestly signals her presence and reproductive status. As a result, workers then act to maximise their inclusive fitness; raising siblings when the queen is present and attempting to rear their own offspring when she is not. Thus, selection has acted to increase worker reproductive self-restraint (via the suppression of ovarian development) in circumstances where policing is effective, and attempts to reproduce lead to a loss of colony efficiency (Ratnieks 1988, Hammond and Keller 2004). While the 'direct suppression' and 'honest signal' theories disagree on the ultimate cause of worker ovary inhibition, both place special emphasis on its role in social insect evolution.

Ratnieks (1993) postulated that the extent of attempted reproduction is a compromise between the probability of eggs being reared to adulthood and the cost to the colony of attempted reproduction and policing. Worker egg-laying would not be favoured if the probability of survival was low, or the cost to the colony was high. Worker egg-laying may not be costly to colony productivity at low levels, which could explain the observed presence of rare reproductive

workers in some queenright honey bee colonies (Ratnieks 1993). Cole (1986) determined that for the ant *Leptothorax allardycei*, the time workers spend caring for queen-laid brood is negatively correlated with the time they spend engaging in dominance interactions which suppress worker reproduction. He calculated that worker reproduction could spread through the population only if the cost of these dominance interactions to colony reproduction was below a threshold value (17-22% of total colony output for *L. allardycei*). However, the factors that contribute to the trade-off between the benefits and costs of attempted worker reproduction remain poorly understood for most taxa.

1.3 Proximate factors inhibiting worker ovary development

In the context of this thesis, 'ovary development' refers to the activation of the ovaries in adults, as opposed to the development of this organ in pre-adult life stages. For example, in honey bee (*Apis mellifera* L.) colonies with a mated and laying queen (queenright colonies), very few workers have ovaries that are developed enough to contain a mature egg. Ratnieks (1993) found that only one worker in 10 000 had a fully developed egg in her ovaries. He concluded that this low level of ovarian development indicated that the absence of worker-produced males is due largely to a lack of attempted reproduction, rather than effective policing of worker-laid eggs.

The lack of attempted worker reproduction in queenright colonies is a common feature in social insect species for the ultimate reasons described in the previous section. The following section describes the proximate influences on worker

ovary development in both queenright and queenless colonies. These factors are of evolutionary importance because they are the means by which selection can act on this trait. These factors affect the strategy sets available to individuals, potential evolutionary trajectories, and the genetic basis for reproductive traits that have ultimate evolutionary importance. This section of the introductory chapter will explore the ecological, and life history, genetic, and social factors that are proximate influences on worker ovary development in social insects. This includes the effects of nutrition, age, morphology, offspring relatedness, policing efficiency, trophic egg production, genetic differences between and within species, the presence of dominant individuals, colony size, trophallaxis, and interworker aggression.

1.3.1 Ecological and Life History Factors

Nutrition

In most social insect taxa, food quantity and quality play a pivotal role in both caste differentiation and fecundity within castes (see Hunt and Nalepa 1994 for a review). Proteins, essential to oogenesis, are consumed by reproductive individuals; sugars are retained by workers and provide energy for daily tasks (Wheeler 1994). A protein-rich diet promotes the development of ovaries, whereas a diet lacking in protein limits oogenesis. The ability of workers to develop their ovaries and lay eggs is therefore dependent on the amount and quality of the nourishment they receive both as larvae and as adults. Young workers are dependent on adult workers for food resources, and older colony members determine the amount of resources given to the developing brood. The

resources available to the colony determine the fecundity of queens and workers; the quantity and quality of food available to the developing brood, the quantity and quality of individuals available to act as nurses, and the food available to adult workers all influence fecundity.

Nourishment has been implicated in queen body size and fecundity in the social wasps (reviewed in O'Donnell 1998), ants (reviewed in Wheeler 1994), bumble bees (Free 1957, Vogt *et al.*, 1998), stingless bees (reviewed in Sommeijer and Bruijn 1994), and honey bees (reviewed in Winston 1987). For example, in the ant *Iridomyrmex humilis,* the queen is fed trophic (non-reproductive) eggs laid by the workers, and her oviposition rate is directly correlated with her trophic egg consumption (Bartels 1988). Supplemental nitrogen has been found to increase ovariole number in neotenic female termites, but not in the primary reproductives (Brent and Traniello 2002). These results suggest that the nutritional supplement may release the young neotenic females from nutritional limits to their fecundity, whereas it has no effect on adult primary reproductives.

In addition to queens, the fecundity of workers is also dependent on the amount and quality of the nourishment they receive. For example, Lin and Winston (1998) found that increases in the protein content of the diet of caged adult worker honey bees were matched by increases in the level of ovary development. Génissel *et al.* 2002 found that queenless workers of the bumble bee *Bombus terrestris* produced a large number of males when fed high protein *Prunus* pollen, fewer males with lower quality *Salix* pollen, and no offspring with

low protein *Taraxacum* pollen. Eberhard (1969) demonstrated that dominant worker wasps (*Polistes canadensis*), which lay more eggs than subordinate workers, gained better nutrition than subordinates by eating a greater share of available food and by eating eggs laid by subordinate individuals. In these wasps, ovarian development is determined by rank in a dominance hierarchy, which in turn determines access to food, rather than a simple relationship between ovarian development and nutrition (Wilson 1971, and references therein).

Worker Age

Age can also affect ovary development in adult workers, although the effect of age is poorly understood for most social taxa. Lin *et al.* (1999) reported that four and eight day old honey bees (*Apis mellifera mellifera*) developed ovaries faster than older or younger bees. In queenless groups of honey bees, Delaplane and Harbo (1987) found that there was no consistent difference between bees 11-15 days old and those greater than 54 days old in the number of drones produced per worker. In *A. m. capensis*, the age of workers at colony dequeening is inversely related to their subsequent ovarian development (Hepburn *et al.* 1991). Similarly, young ants more frequently reproduce following queen loss (Smeeton 1981, van Walsum *et al.*1998). However, age is not always inversely correlated with reproductive ability. Worker age is positively correlated with number of eggs laid in the Meliponinae species *Melipona subnitida* (where workers frequently contribute to colony reproduction) (Koedam *et al.* 1999).

Morphology

The morphological specialisations of social insects can greatly affect their reproductive potential. In social insect colonies, there often are individuals specialised for nest defence, foraging, brood care, and nest construction. Individuals face a trade-off between morphological specialisations for these work activities and their potential reproduction. For example, individuals specialising in foraging may lack the fat reserves required for egg production, or workers specialised in defence may spend valuable energy in growing armour rather than ovaries during development. Individuals specialised for reproduction, such as physogastric termite queens, may be so morphologically specialised they are little more than walking ovaries, unable to perform other tasks to maintain the colony.

Reproductive division of labour can evolve before worker morphological specialisation, or morphological differentiation can drive the evolution of division of labour. In eusocial thrips, Chapman *et al.* (2002) suggest that soldier morphology and behaviour evolved in the presence of substantial levels of soldier reproduction (the latter strategy), whereas reproductive division of labour evolved before morphological differentiation in the Hymenoptera (the former strategy). In eusocial Australian gall-inducing thrips, reproductive skew is negatively correlated with gall size (Wills *et al.* 2001). The authors suggest that the evolution of smaller galls limited the resources available to the offspring of soldier morph individuals, and was a major factor contributing to the evolution of an altruistic caste in gall-dwelling species.

The evolution of an obligately sterile worker caste in Ponerine ants is believed to have occurred through changes to worker morphology. Villet *et al.* (1991) studied seven species of Ponerine ant, and determined that workers were obligately sterile. In these species, workers entirely lack ovaries. The workers of these species are very small, suggesting that body size and the evolution of sterile workers may be linked in these genera. Obligate sterility provides an efficient mechanism whereby the queen can maintain reproductive dominance without incurring the losses of colony efficiency associated with policing. Ovary loss eliminates all potential direct reproduction in workers. Natural selection, therefore, should not favour this strategy unless either the increase in colony efficiency or the decrease in conflict offers a large fitness reward at the colony level.

Offspring Relatedness

The likelihood of attempted worker reproduction is ultimately the sum result of a series of costs and benefits to the individual worker. It is determined by the magnitude of the individual's loss of indirect fitness from attempted reproduction (through loss of colony efficiency and therefore fewer siblings reared) and the potential benefits gained by direct fitness (determined by the survival of eggs to adulthood and production offspring of their own and the relatedness value of these eggs to the egg-layer). If the benefits to direct fitness outweigh the costs to indirect fitness, the individual should attempt to reproduce when possible. The relatedness value of offspring to potential egg-layers (an aspect of direct fitness) has been shown to be of clear importance in social insects. Species in which

workers are able to lay offspring that are highly related to them are more likely to do so than species whose offspring are less related to them.

Relatedness has been implicated in soldier reproduction in the thrips *Kladothrips hamiltoni*. Female soldiers mate with their siblings, and this incest causes both the foundress and soldiers to be more related to soldier-produced female dispersers than any other colony members (Kranz *et al.* 1999). Approximately 60-80% of the dispersing second generation produced by the colony are the result of this soldier reproduction (Kranz *et al.* 1999). In other social insects, both this type of sibling mating and worker reproduction are rare (*e.g.* most Hymenoptera, Isoptera).

The potential benefit gained from worker reproduction is affected not only by relatedness, but also by offspring sex. Normally, honey bee workers can only lay male eggs, and worker reproduction is rare. Workers of the subspecies *A. m. capensis* (the Cape honey bee), however, can lay female eggs parthenogenetically, and colonies of this subspecies are able to requeen themselves from worker-laid female eggs (Hepburn *et al.* 1988). This pattern would potentially provide increased benefits to laying workers, as their female parthenogenetic offspring can themselves lay new females, and it is possible that they could become the colony's new queen. Therefore, Cape honey bee workers should be more likely to develop ovaries and begin egg-laying than other honey bee races, whose workers cannot lay female eggs. This argument is supported by the findings of Hepburn and Allsopp (1994), who found that after queen loss,

75% of *A.m. capensis* workers underwent some ovarian development within two weeks, and 12% of workers produced mature eggs. In contrast, only 30% of the workers of the geographically neighbouring race *A. m. scutellata* (whose workers can lay only male eggs) had developed ovaries, and only 1% produced mature eggs in the same time period.

Trophic Egg Production

The production of trophic eggs may be a strategy by which workers can maintain active ovaries. Many stingless bee and ant species produce these non-reproductive eggs which generally are used to feed the queen. However, the continued production of this egg type may facilitate subsequent 'switching' to the production of reproductively viable eggs when the colony loses its queen. Trophic egg production could ensure rapid ovary development, which would be beneficial when the opportunity to lay viable eggs arises (West-Eberhard 1981). In some cases, trophic egg production may allow 'cheaters' to lay both trophic and viable eggs (Crespi 1992). For example, in many stingless bee species, workers produce fully viable eggs that may either be consumed by the queen, or reared to maturity (Beig 1972, Zucchi 1993).

Policing Efficiency

The effectiveness of policing strategies is dependent on the ability to discriminate worker-laid from queen-laid eggs. This may contribute to the large number of workers that reproduce in many stingless bee species. Because only one size of cell is produced for both female and male eggs, it may be difficult for policing

individuals to distinguish between queen-laid female or male eggs, and worker-laid male eggs (Peters *et al.* 1999). Stingless bee queens generally mate singly, so all workers are full-sisters, and more highly related to each other than to the queen. Stingless bee workers should therefore prefer worker-laid eggs to queen-laid male eggs, whereas the queen prefers that she lays all eggs. Different sized cells would make it easier for the queen to distinguish worker-laid eggs from her own, and consume those laid by workers. Thus while the queen should prefer different cell sizes for drone and worker eggs, workers should prefer that they are reared in the same size cell (Peters *et al.* 1999). As workers are in control of cell construction, there is no difference in size between drone and worker cells in stingless bees.

In contrast, honey bee queens mate multiply, and workers are, on average, less related. Workers are more closely related to their brothers (queen-laid) than to their half-nephews (worker-laid), and so workers should agree to let the queen lay all male eggs in the colony. While stingless bee workers should prefer that males be produced by workers, honey bees should prefer that males be laid by the queen. This hypothesis is supported by the fact that unlike stingless bees, honey bees lay male eggs in morphologically distinct cells. Workers police and consume eggs laid by other workers, a behaviour aided by the different cell sizes. As a result, stingless bee workers frequently contribute to male production (Beig *et al* 1982, Sakagami 1987), while honey bee workers do not (Ratnieks 1988). Workers of the common wasp *Vespula vulgaris* have a policing

mechanism similar to that of the honey bee, where workers remove worker-laid eggs, and leave most queen-laid eggs (Foster and Ratnieks 2001).

1.3.2 Genetic Factors

Subspecies Differences

Genetic differences among species, subspecies, and patrilines influence the propensity of workers to develop mature ovaries. In queenless colonies, these differences contribute to determining the genetic composition of the drones produced subsequent to queen loss. Ruttner and Hess (1981) found differences in rapidity of ovarian development among seven subspecies of honey bee, including *A.m. scutellata*, *A.m. capensis*, and *A.m. mellifera*. Africanised workers develop their ovaries and begin to lay eggs more quickly than do European bees (Zillikens *et al.* 1998), and in mixed European/Africanised colonies many times more Africanised drones are produced (Hellmich *et al.* 1986). This difference is believed to have contributed to the spread of Africanised bees in South America (Zillikens *et al.* 1998)

Differences Within Single Species

Genetic differences among members of the same subspecies also affect ovarian development in the honey bee. Workers compete amongst themselves for reproduction in queenless colonies, and much of this competition occurs at the ovary development stage. In *A. m. mellifera*, differences in the paternity of workers affect rates of ovary development (Page and Robinson 1994, Martin *et al.* 2004), and workers that are able to lay eggs soon after queen loss are more

successful (Miller and Ratnieks 2001). However, differences in oviposition rate (and therefore ovary development) among different patrilines do not necessarily translate to biases in drone production (Robinson *et al.* 1990, Martin *et al.* 2004). In addition, some workers may be incapable of ovary development (Harris and Harbo 1991).

These within-species differences, when extreme, can lead to the evolution of 'cheaters' that reproduce even in queenright colonies. Montague and Oldroyd (1998) identified a rare behavioural phenotype of honey bee (*Apis mellifera mellifera*), 'anarchistic' colonies, in which workers commonly have developed ovaries in the queenright condition and contribute substantially to drone production. In one anarchistic colony, one subfamily (patriline) of workers accounted for 90% of the drone progeny; only 10% of drones were produced by all other subfamilies and the queen. Surprisingly, they found that a different subfamily dominated drone production when the colony was queenless. It is believed that selection for worker reproduction in this anarchistic line has reduced the effect of brood and queen pheromones on worker ovary inhibition (Oldroyd *et al.* 1999, Barron and Oldroyd 2001). Workers in the anarchistic colonies are able to evade policing by producing a queen-like pheromone to protect their eggs, which acts to reduce discrimination by policing workers (Oldroyd *et al.* 1994, Oldroyd and Ratnieks 2000). In these anarchistic bees, workers of a particular genotype are able to develop eggs and rear many drones to maturity. These anarchistic colonies do not survive well without assistance,

however, and it is likely that this trait would be maladaptive under natural conditions (Barron *et al.* 2001).

1.3.3 Social Factors

Presence of Dominant Individual

The presence of a reproductively active queen inhibits ovarian development in many social insects. The presence of a queen has been observed to restrict ovarian development in many diverse ant groups, including *Leptothorax, Myrmica, Plagiolepsis,* and *Formica*, although this may be achieved through a variety of physiological mechanisms (Wilson 1971, and references therein). Orphaned worker ants (*Diacamma* sp.) have higher levels of ovary development than non-orphaned workers (Kikuta and Tsuji 1999). In honey bees, the inhibitory effect of the queen is well described. Jay (1970) reported far higher proportions of laying workers in queenless European honey bee colonies than in queenright ones. When the colony is dequeened, a relatively large number of workers will develop ovaries, and begin egg-laying (Winston 1987). Worker ovary development is also stimulated during periods when the potential of queen loss is heightened. In honey bees, the proportion of workers with developed ovaries is greater in colonies soon before and after swarming, when there is a greater chance of queen loss (Kropácová and Haslbachová 1969, Winston 1987, van der Blom 1991). Colonies of the Japanese bee *Apis cerana* can produce laying workers or 'false queens' when dequeened (Sakagami 1958). Not only can false

queens lay eggs, but ovary development and oviposition decrease in other workers when a false queen is present.

In many species, it is pheromones that inhibit the development of worker ovaries in the presence of a dominant individual. In the *Diacamma* ants of Japan, gamergates (mated workers) produce a pheromone that inhibits worker ovary development, and workers lay eggs only in the absence of a gamergate (Tsuji *et al.* 1999). In the honey bee, the inhibition of worker ovaries is the result of a complex blend of pheromones produced by the queen and brood. In the absence of brood, the queen alone is not sufficient to completely inhibit worker ovarian development (Jay 1969). Pheromones produced by worker brood have a considerable inhibitory effect (Jay 1969, Trouiller *et al.* 1991, Mohammedi *et al.* 1998). Ovary development is highest when both the queen and brood are absent, lower when just the queen is present, and lowest with the queen and brood or just brood (Jay 1972). Larvae that are present, but separate from workers, are sufficient to inhibit development (Jay 1972). Laying workers also have an inhibitory effect on ovary development (Jay 1975), although comb availability does not (Jay and Jay 1976). Finally, Oldroyd *et al.* (1999) surmise that their selection programme for anarchistic workers may have reduced the production of brood pheromones, leading to worker reproduction in queenright colonies.

Such inhibitory pheromonal signals could either chemically suppress ovaries or, in situations where workers would gain more inclusive fitness by raising their sisters than by attempting to reproduce, simply signal that the queen and/or

brood are present and that ovary development would be maladaptive for the workers. In some species, pheromones may not act to exert physiological control *per se*, but rather act as an 'honest' fecundity signal (Alexander *et al.* 1991, Keller and Nonacs 1993). Although constrained by their conflicts with other workers and the queen, workers are necessarily in control of their response to chemical signals (Crespi 1992). In queens of the ant *Solenopsis invicta*, there is a positive correlation between pheromone production and fecundity (Fletcher and Blum 1983), providing evidence for the 'honest signal' hypothesis. In Cape honey bees, the highest concentration of laying workers occurs in queenless (5%), then virgin queenright (2.5%), then mated-queenright colonies (<1%) (Hepburn *et al.* 1991), providing further evidence for a negative correlation between worker reproduction and queen fecundity.

In the mole-rats, a group of small burrowing mammals, two species show socially inhibited fertility. The naked and Damaraland mole-rats (*Heterocephalus glaber* and *Cryptomys damarensis*) both fit the definition of eusociality as applied to social insects, with reproductive division of labour, morphologically distinct castes, and overlapping generations. In naked-mole rats, non-breeders of both sexes are reproductively suppressed by social contact with the breeding female. In females, ovulation is blocked, and males have reduced numbers and motility of sperm (Faulkes and Bennett 2001). Female ovulation is also blocked in the Damaraland mole-rat. The mechanism may be different than that employed by the naked mole-rats, suggesting that the suppression of ovulation may have evolved more than once in the social mole-rats. Convergent evolution between

highly eusocial species in two distantly related groups, the insects (e.g., eusocial bees and ants) and the mammals (mole-rats), suggests that the suppression of worker reproduction at the physiological level (*i.e.* ovary development rather than post-oviposition policing) is an important strategy in the evolution of reproductive skew and animal societies.

Colony Size

Alexander *et al.* (1991) first noted that small colonies are generally simple, and have little morphological differentiation between workers and reproductives. In contrast, colonies with large populations tended to be more socially complex, and have a higher degree of 'morphological skew'. Small colonies should demonstrate intense conflict over caste determination because as colony population size decreases, the likelihood of each worker becoming the dominant reproductive increases (Alexander *et al.* 1991, Bourke 1999). In large colonies, workers should be relatively compliant to manipulations of their caste (*i.e.* reproductive status) because they have a much lower chance of becoming the dominant individual (Bourke 1999).

Colony size affects not only the likelihood that a given worker will have opportunity to reproduce, but also the mechanisms available to a dominant individual whereby subordinate reproduction can be suppressed. Sakagami (1977, 1982) noted that in socially 'advanced' groups, behavioural control is replaced by pheromonal control where the queen does not exert behavioural dominance. Such aggressive behaviours would be made difficult if the queen

possesses morphological and behavioural specialisations. In addition, it would be difficult for a queen to physically dominate the many individuals that make up a large colony, whereas pheromones could potentially be employed to suppress reproduction in a large number of subordinates.

In the paper wasp *Polistes bellicosus* the dominant individual has decreased control of reproduction in large groups (Field *et al.* 1998). As physical aggression is the primary means of reproductive control in this species, this suggests that dominant individuals have difficulty controlling large numbers of subordinates in this manner. Species with large colonies such as some ants, honey bees, and Vespine wasps have inhibitory pheromones that can affect large numbers of individuals. These societies rely at least partially on these pheromonal signals (Jay 1970, Sakagami 1977, Fletcher and Ross 1985, Heinze *et al.*1997), whereas bee and ant species with smaller colonies typically rely on physical aggression and post-oviposition policing (Kukuk 1992, Monnin and Ratnieks 1999). Colonies of intermediate size could therefore be predicted to utilise both behavioural and simple pheromonal inhibitory mechanisms. This is the case for many bumble bee species, where queen control is believed to be the result of combined physical aggression and pheromonal inhibition (Roseler and Van Honk 1990). Colony size therefore affects both the likelihood of a worker attempting to reproduce (e.g. by length of dominance hierarchies, probability of inheriting the nest etc.) and manner of ovary inhibition by dominant individuals (e.g. pheromonal versus behavioural).

Worker-Worker Trophallaxis

Trophallaxis, the liquid food exchange among colony members, can greatly affect female fecundity. Trophallaxis between adult colony members is a common feature of many insect societies; among them many wasp, ant, bee, and termite species. Trophallactic exchanges involve not only the transfer of nutrients, but frequently include chemical signals as well. In the lower termites, trophallaxis involves the transfer of nutrients, the distribution of caste regulating pheromones, and the transfer of cellulose-digesting protozoans (Nalepa 1994). In this way, such exchanges affect ovary development directly through inhibitory pheromones, and indirectly through the exchange of nutrients. The presence of young increases the fecundity of primary and secondary reproductives in termites, presumably because the young assist with colony labour and provide trophallactic secretions to reproductives (Brent and Traniello 2001). Nutritionally augmented fecundity via trophallactic exchanges can be directed at specific individuals or groups of individuals in a colony. In termites, the positive effect of larvae on fecundity is greater for secondary females than primary because secondary reproductives have fewer stored resources and are more dependent on social assistance than primary termite females (Brent and Traniello 2001).

It has long been suspected that trophallactic exchange is also important in worker fecundity in the Hymenoptera. While Mayer *et al.* (1998) found no clear difference in ovarian development between the donors and recipients of trophallaxis among honey bee workers aged 25-45 days, Lin and Winston (1998) suggest that nurse bees could affect the ovarian development of other workers

via trophallaxis. They contend that nurse bees could affect the ovarian development of workers by altering the quantity and quality of nutrients exchanged in trophallaxis between workers. Trophallaxis is also known to play an important role in the dominance hierarchies of social wasps. Food is transferred via trophallaxis from subordinate or sub-dominant individuals to more dominant individuals, and dominant workers receive more food than those they dominate (Hunt 1994). Little is known, however, about how the selective transfer of nutrition through colonies may influence the development of worker ovaries. Nutrients could act as a proximate mechanism by which dominance hierarchies and nepotistic interactions differentially influence the reproductive capability of individual workers. Future work should focus on the flow of nutrients through social insect colonies to determine the effects of trophallaxis on fecundity.

Worker-Worker Aggression

Worker-worker aggression can also lead to ovary inhibition. In the ant *Leptothorax gredleri,* dominance is established by antennation and biting, and high-ranking individuals have greater ovarian development (Heinze and Oberstadt 1999). Heinze *et al.* (1997) further established that *Leptothorax* ants (10 species) rarely engaged in aggressive interactions when the queen was present, and did not lay large numbers of eggs. When queenless, however, dominance interactions became much more frequent, and high-ranking workers began laying eggs. Visscher and Dukas (1995) found that aggressive behaviour was directed towards workers with developed ovaries in the honey bee (but see Dampney *et al.* 2002). Bumble bees (e.g. *Bombus terrestris*) also have a colony

stage in which dominance behaviours by workers retard ovary development in other workers. There are two distinct stages of bumble bee colony growth and ovary inhibition: (1) before the 'competition phase', when the queen inhibits worker ovarian development, and (2) during the 'competition phase', when dominant workers inhibit the ovarian development of other workers (Bloch and Hefetz 1999). Reproductive hierarchies, where dominant workers suppress the ovary development of subordinate workers, are common in social insects. Dominance hierarchies also appear to influence ovarian development in *Polistes* wasps, with rank determining level of development (Wilson 1971).

1.4 Levels of Selection

Natural selection on worker ovary suppression acts at both the individual and colony level. However, selection at these two levels may not always act in concert, and may, in many instances, act in opposition. At the individual level, workers are most closely related to their own offspring, yet they normally forgo reproduction in favour of rearing siblings (some of whom may have $r > 0.5$). It may be in the worker's interest to not develop ovaries, but to aid the dominant individual. This would depend on the inclusive fitness benefits a worker gains by reproducing (which are determined by the probability of eggs surviving, policing effectiveness, egg acceptability, and egg sex) relative to the cost to her inclusive fitness of giving up aid to the dominant individual (Visscher 1989).

At the colony level, workers with developed ovaries are subject to aggression, and eggs are removed via policing. Both aggression and policing reduce colony

efficiency thereby increasing the cost of reproduction to the reproducing worker. These behaviours also reduce the fitness gains of the queen and other workers, who would benefit more if the workers never developed ovaries. At the population level, laying workers can contribute substantially to the drone population during mating periods (Page and Erickson 1988, Moritz *et al.* 1998). Queens therefore would have a substantial opportunity to mate with the offspring of laying workers, who could pass on traits responsible for worker laying. It may be that the ovary development observed in queenright colonies is the result of developmental noise, or of genetic variation in the sensitivity to ovary-inhibiting signals. In the latter case, the observed levels of variation in natural colonies would be the result of selection favouring workers who are able to reproduce when queenless (promoting ovary development), and selection against colonies with workers that reproduce when queenright (promoting ovary inhibition).

1.5 Conclusions

Despite the variation in mechanisms of ovary inhibition among taxa, several common patterns emerge. In many primitively social groups, inhibition is often controlled in part behaviourally, and is incomplete (*Polistes* wasps, halictine bees). In highly social groups, inhibition is largely controlled pheromonally, and is nearly complete (e.g., honey bees, termites, many ants) (Sakagami 1982). The method by which the dominant individual suppresses the ovaries of workers depends on the ecology, physiology, phylogeny, and social structure of the society in question. Colony size, temperature, nutrition, number of queen

matings, queen reproductive status, and colony development stage all play a role. Nevertheless, some degree of worker ovary inhibition is a universal characteristic of eusocial insects. Thus the inhibition of worker ovaries represents an efficient stage at which worker reproduction can be manipulated, without loss of colony efficiency, whether due to queen control or honest signalling.

Differences exist among taxa with respect to the mechanisms of inhibition, and conditions required for ovarian development in workers. The important questions left to be examined concern the adaptive value of this variation. That is, what are the effects of ecological, genetic, and social factors on the inclusive fitness of workers who attempt to reproduce, and those who do not? Although ovary inhibition is a common mechanism by which dominant individuals suppress the reproduction of subordinates, it is not clear what the outcome of its loss would be. For example, what would be the result to colony efficiency if there were no ovary inhibition, just worker policing? Other important unresolved questions include the development of 'cheaters' (what causes some individuals to develop ovaries and attempt to reproduce when others do not?), and the contribution of laying workers to the population genetics of various species.

1.6 Objectives

This thesis seeks to examine the proximate mechanisms by which worker reproduction is inhibited in the honey bee (*Apis mellifera* L.). The objective of this work is to provide a greater understanding of the proximate and ultimate factors contributing to reproductive skew, and ultimately the evolution of insect

societies. The chapters of this thesis examine the following aspects of honey bee worker ovary development: the effect of queen pheromones on worker honey bee ovary development (Chapter 2), anarchistic honey bee queen pheromones (Chapter 3), the anarchistic worker response to queen and brood pheromones (Chapter 4), seasonal and nutritional effects on worker ovary development (Chapter 5), and the effects of a selection program for worker ovary development, as well as the relationship between ovary development and pollen foraging traits (Chapter 6).

CHAPTER 2: THE EFFECT OF QUEEN PHEROMONES ON WORKER HONEY BEE OVARY DEVELOPMENT[1]

2.1 Abstract

I examined the effects of synthetic honey bee queen mandibular pheromone (QMP), four newly identified queen retinue pheromone components, and queen extracts on the ovary development of caged worker bees. The newly identified compounds did not inhibit worker ovary development alone, nor did they improve the efficacy of QMP when applied in combination. QMP was as effective as whole queen extracts at ovary regulation. Caged workers in the QMP and queen extract treatments had more developed ovaries than did workers remaining in queenright colonies. I conclude that QMP is responsible for the ovary-regulating pheromonal capability of queens from European-derived *Apis mellifera* subspecies, although other factors are required for complete inhibition.

2.2 Introduction

Division of labour is a defining characteristic of social insect colonies, and includes the partitioning of reproduction to one or a few individuals. However, in most social insects the less-reproductive castes are capable of reproducing when the dominant individual is removed. When this occurs, previously mated

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individuals can attain reproductive status and lay fertilised eggs, or unmated individuals can lay unfertilised eggs. Queen dominance is maintained via antagonistic physical interactions in 'primitively' social groups and through pheromones in more complex insect societies. Determining the proximate factors by which dominant individuals regulate the reproduction of subordinates is essential to understanding the evolution of insect societies. In this study I report results that address a long-standing controversy in honey bee biology, the nature of the queen-produced compounds that inhibit worker ovary development.

Workers in the highly eusocial honey bee *Apis mellifera* L. cannot mate, and except for one African subspecies (*A. m. capensis*) can lay only haploid male eggs. Worker ovaries are undeveloped in the presence of a laying queen, and worker reproduction in most European honey bee populations queenright colonies is rare (Visscher 1989, but see Oldroyd *et al.* 1994). In the absence of a queen, some workers' ovaries develop, and they begin to lay drone (male) eggs.

The most critical influences on worker ovary development are the presence of a queen and / or her brood (de Groot and Voogd 1954, Butler and Fairey 1963, Jay 1968). Two esters produced by worker brood, ethyl palmitate (EP) and methyl linoleate (MLN), are active in regulating ovary development (Mohammedi *et al.* 1998). Queen-produced substances also limit ovarian development in workers, although the identity of the active compounds produced by the queen has been unclear.

(E)-9-oxodec-2-enoic acid (9ODA), one component of the queen mandibular gland pheromone (QMP), was first suggested to be major ovary-regulating primer pheromone produced by the queen (Butler and Fairey 1963), but studies of its effects and those of the more complete five-component blend (Slessor *et al.* 1988) have had ambiguous and contradictory results. Partial inhibition of worker ovary development by queen heads or mandibular gland components has been demonstrated to varying degrees (de Groot and Voogd 1954, Verheijen-Voogd 1959, Butler 1959, Butler and Fairey 1963, Velthuis 1970a, Lin 1999). However, Willis *et al.* (1990) found no effect of QMP at even high doses in queenless colonies. In addition, a queen abdomen alone or a queen without mandibular glands can inhibit worker ovary development (Velthuis and van Es 1964, Velthuis 1970b).

Extracts of whole body washes of queens have produced the most complete inhibition (Butler 1957, Verheijen-Voogd 1959), suggesting a second queen source of inhibitory pheromones (Winston and Slessor 1998). Tergal gland secretions may regulate worker ovary development in the African subspecies *A. m. capensis* and *A. m. scutellata* (Wossler and Crewe 1999b), which also could be the case in European subspecies. Other queen-produced pheromones also may be involved in the regulation of worker ovary development.

The queen mandibular pheromone is composed of five constituent compounds: (*E*)-9-oxodec-2-enoic acid (9-ODA), both enantiomers of (*E*)-9-hydroxydec-2-enoic acid (9-HDA), methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-

methoxyphenylethanol (HVA) (Slessor *et al.* 1988). The mandibular glands of a mated, laying queen contain on average 200 μg 9-ODA, 80 μg 9-HDA (85% (R)-(-)), 20 μg HOB, and 2 μg HVA (Pankiw *et al.*, 1996). This quantity is referred to as one queen equivalent (Qeq), and is the approximate amount produced by a single queen in 24 hours (Naumann *et al.* 1991).

Keeling *et al.* (2003) recently identified four additional queen-produced compounds that function synergistically with QMP in attracting workers to form a retinue around the queen. These compounds are inactive alone, but greatly increase retinue activity when combined with QMP. They also may be active in worker ovary regulation. The compounds include:

1) Methyl oleate (methyl (*Z*)-octadec-9-enoate) (MO), found throughout the bodies of both mated and virgin queens, although the location of biosynthesis is unknown.

2) Coniferyl alcohol ((*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-ol) (CA), a photo-sensitive compound found in the mandibular glands of mated/laying but not virgin queens.

3) Hexadecan-1-ol (PA), found in the Dufour's gland and cephalic labial gland of mated/laying queens, and only in the abdomen of virgin queens.

4) Linolenic acid ((*Z*9,*Z*12,*Z*15)-octadeca-9,12,15-trienoic acid) (LEA), found primarily in the thorax and abdomen of mated and virgin queens, but also detected in queen heads.

The objectives of the study were to 1) examine the four queen compounds newly identified by Keeling *et al.* (2003) for their potential function in regulating worker ovary development, and 2) resolve the role of QMP in worker ovary inhibition.

2.3 Materials and Methods

Experiment 1

Bees used in this study were of the mixed European descent that is common in North America, closest to the European subspecies *A. m. ligustica*. Frames containing pupae were taken from 25 colonies in the Simon Fraser University apiaries 4-7 June 2001, and incubated overnight. The following morning four cohorts of 30 newly emerged workers from each colony were put into cages, with each cage containing workers from only one colony (total of 4 treatments x 25 colonies = 100 cages). Each cage was assigned one of four pheromone treatments: (1) solvent blank (diethyl ether) containing no pheromone (**Control**), (2) the 5-component synthetic queen mandibular pheromone (**QMP**, Phero-Tech Inc., Delta, B.C.), (3) three of the newly identified compounds, MO+CA+PA (**3**), or (4) QMP+MO+CA+PA (**QMP+3**). The treatments with the new compounds contained 2.49 μg/Qeq MO, 0.14 μg/Qeq CA, and 0.39 μg/Qeq PA, all well within the typical range found in a mated, laying queen (Keeling *et al.* 2003). Only three of the four new compounds were used in this experiment, since the fourth had

not yet been identified. All treatments were presented as 0.1 queen equivalent (Qeq) per day, applied to a glass slide in 10 μl of diethyl ether. One queen equivalent of pheromone is the amount extractable from a single queen at a given time, by homogenisation in a solvent. It is difficult to determine precisely the amount received by an individual worker in a colony, but the dose of 0.1 Qeq was designed to best approximate the higher side of the total received naturally by workers over the course of 24 hours.

Caged workers were kept at 34°C in the dark, and fed a 1:1 mixture of honey and royal jelly by volume, and water *ad libitum,* optimal conditions for worker ovary development (Lin 1999). The pheromone treatments were labelled so that the identity of treatments was not known until after all the bees in the experiment had been dissected. Red light was used during daily food and pheromone applications to minimise the degradation of pheromone compounds, some of which are photosensitive. After 14 days the worker bees were frozen, and stored in sealed Petri dishes to avoid desiccation.

Experiment 2

Newly emerged worker honey bees were collected as per Experiment 1 from 6 colonies between 18-19 July 2001. Cages of 30 bees were assigned to each of 5 treatments (total of 5 treatments x 6 colonies = 30 cages),: (1) Solvent blank (**Control**), (2) QMP as in experiment 1, (3) QMP+MO+CA+PA+LEA (**QMP+4**), (4) whole queen extract blend (**WQE**), or (5) in queenright natal colony (**QR**). The treatments with the new compounds contained 0.75 μg/Qeq MO, 0.28 μg/Qeq

CA, 1.62 μg/Qeq PA, and 100 μg/Qeq LEA. While experiment 1 used amounts of MO, CA, and PA typical to mated / laying queens, the quantities of the four new compounds used in experiment 2 were matched to the quantities found in the whole queen extract (based on GCMS analysis of trimethylsilyl derivatives using undec-10-enoic acid as the internal standard) (Keeling *et al.* 2003)). Whole queen extracts were prepared from 16 mated laying queens removed from their colonies, immediately placed on dry ice, and frozen at -80°C until extraction. The 16 queens were individually homogenised and extracted repeatedly in diethyl ether (total 1mL/Qeq), then pooled together (Keeling *et al.* 2003). Pheromone treatments were applied as in experiment 1, at 0.1 Qeq/day in 100 μl diethyl ether. Caged worker bees again were fed a 1:1 mixture of honey and royal jelly by volume (but diluted with 10% water to prevent the food from becoming overly viscous), and had free access to water.

Since sufficient emerging workers to complete the queenright treatment were not available from many colonies, pupae were removed again from each of these colonies one week after the initial brood removal. Newly emerged workers were marked using plastic tags, and placed back into their natal colony. In experiment 2, both caged workers and those in colonies were collected after 10 days. Concurrent research indicated no difference in ovarian development between workers collected at 10 and 14 days, the mean scores of treatments applied in both experiments 1 and 2 were similar, and workers with fully developed eggs were found after 10 days.

Ovary Dissections

For each experiment, 5 bees from each cage were randomly chosen, dissected, and the mean level of ovary development determined. Ovaries were classified using a 5-point scale (as per Pernal and Currie 2000) as: (0) resting, with small ovarioles close together; (1) oogenesis beginning, cells slightly swollen; (2) slightly developed, eggs distinguishable from trophocytes, egg volume not exceeding that of the nutritive follicle; (3) moderately developed, with egg volume exceeding that of the nutritive follicle; and (4) fully developed, with at least one fully elongated 'sausage-shaped' egg, and only a remnant of the nutritive follicle remaining. Each bee was assigned a single score according to the level of her most developed ovariole.

Statistical Analyses

The mean ovary development scores per colony were analysed using analysis of variance (ANOVA). While the data satisfied the assumption of normality, unequal variances were detected in the first experiment. As ANOVA is robust with respect to heterogeneity of variances when sample sizes are equal (Zar 1984), it was conducted on both experiments. Tukey's honestly significant difference (HSD) test was used to compare means among the treatments. All statistical analyses were conducted using JMPIN version 4.0.3 (SAS Institute, Inc.).

2.4 Results

Experiment 1

There was a significant difference in ovary development among treatments in experiment 1 ($F(3,96)=48.24$, $p<0.0001$). Workers exposed to the treatments containing the 5-component synthetic queen mandibular pheromone (QMP and QMP+3) had significantly lower mean ovary scores than did the workers given the treatments not containing QMP (Control and 3) (Figure 2-1).

Experiment 2

There was no difference in ovary scores between the QMP, QMP+4, and queen extract treatments, although the control and queenright treatments differed from all other groups ($F(4,25) =27.67$, $p<0.0001$). The workers in the control treatment had the highest mean ovary scores, the QMP, QMP+4, and queen extract treatments were intermediate, and workers in queenright colonies had the lowest ovary development scores (Figure 2-2).

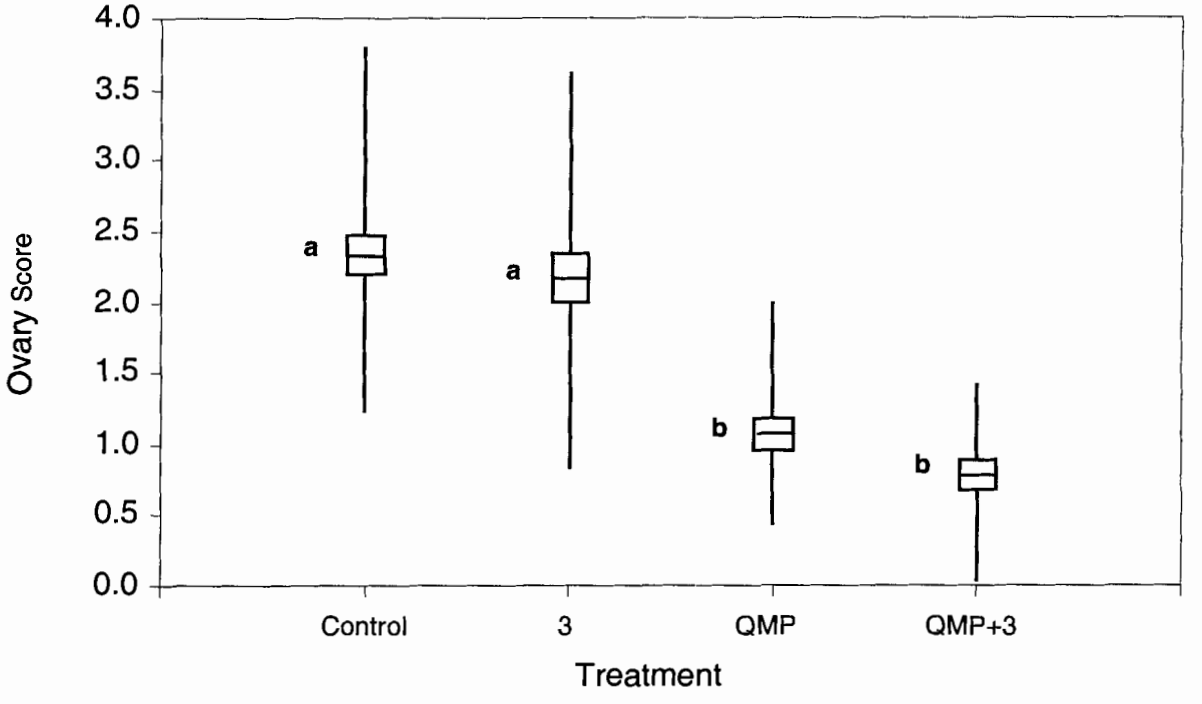

Figure 2-1 Worker ovary score by treatment in pheromone experiment 1. Shows mean ± 2 S.E., and range. Control, diethyl ether blank; 3, methyl oleate (MO), coniferyl alcohol (CA), and hexadecan-1-ol (PA); QMP, 5-component queen mandibular pheromone; QMP + 3, QMP, MO,CA, and PA. Workers exposed to treatments containing QMP (QMP and QMP+3) had lower levels of ovary development than those exposed to treatments without QMP (Control and 3), as denoted by lowercase letters.

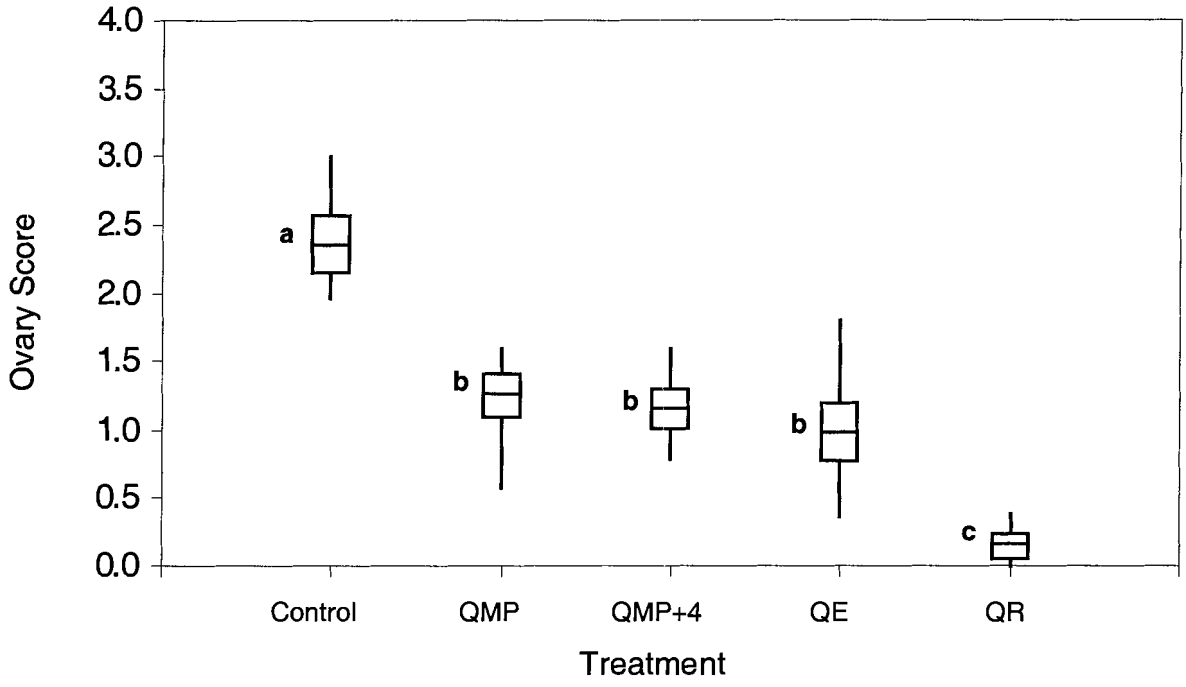

Figure 2-2 Worker ovary score by treatment in pheromone experiment 2. Shows mean ± 2S.E., and range. Control, diethyl ether blank; QMP, 5-component queen mandibular pheromone; QMP + 4, QMP, methyl oleate, coniferyl alcohol, hexadecan-1-ol, and linolenic acid; QE, whole queen extract; QR, in queenright colony. Lowercase letters denote significantly different groups.

2.5 Discussion

The results of this study clearly demonstrate that the five-component queen mandibular pheromone inhibits ovary development in worker bees. These results agree with the findings of Butler and Fairey (1963) and Lin (1999), but disagree with those of Willis *et al.* (1990). A queen abdomen or a queen with mandibular glands removed can inhibit worker ovary development (Verheijen-Voogd 1959, Velthuis and van Es 1964, Velthuis 1970b, Kaatz *et al.* 1992), and this finding has been used to argue that queen pheromones in addition to QMP are involved in regulating worker ovaries. Tergal gland secretions may inhibit worker ovary development in African subspecies (Wossler and Crewe 1999a), however, I found no significant difference between the QMP and queen extract treatments. Thus, there appear to be no additive or synergistic effects of QMP with other queen compounds to inhibit ovary development in European honey bees.

Although previous studies have found that full queen body washes inhibit worker ovary development more than mandibular gland extracts alone (de Groot and Voogd 1954, Butler 1957, Verheijen-Voogd 1959, van Erp 1960), the disagreement of previous studies with the present experiment may reflect imperfect dissections, insufficient or variable dosages, or inadequate sample sizes of previous studies. The use of synthetic mandibular pheromone in this study allowed control of both the dose and the relative proportions of each of the constituent compounds in the treatment. While Willis *et al.* (1990) found no effect of synthetic QMP on worker ovary development, the use of caged bees in the

present study enabled greater control over worker age, nutrition, and the distribution of the treatment pheromones. I found a large range in the mean level of ovary development among the colonies used in this study (Figures 2-1 and 2-2), suggesting that previous studies also may have failed to detect an effect of QMP due to low sample size and large variation among colonies.

Workers that remained in their natal colony had less developed ovaries than did workers in all caged treatments, including queen extract. This difference is likely due to a combination of factors, including the larger group size (Lin *et al.* 1999), exposure to ovary-inhibiting larval esters (Mohammedi *et al.* 1998), and possibly poorer nutrition of bees in colonies. The caged workers in this study were fed 45 or 50% royal jelly, a diet stimulating greater ovary development than the pollen and honey diet found in queenright colonies (Lin and Winston 1998).

Esters produced by worker larvae also regulate ovary development (Mohammedi *et al.* 1998, Lin 1999). Mohammedi *et al.* (1998) reported that ethyl palmitate and methyl linolenate both inhibited ovary development when fed to workers (effective EP dose 600leq/bee/day x 90 ng/leq = 54 000 ng). It is interesting to note that Keeling *et al.* (2003) found ethyl palmitate in extracts of both virgin and mated queens (means of 331±97 and 323±74 ng/Qeq, respectively), thus these esters would have been present in the queen extract treatment. At these doses, low compared to those used by Mohammedi *et al.* (1998), EP did not increase the effectiveness of a whole queen extract in ovary inhibition compared to QMP alone. While the regulation of worker honey bee ovary development may seem

overly complex (involving both queen and brood), it is desirable to have redundancy built into pheromone-based signalling systems. In the case of the honey bee, queen pheromones that regulate ovary development would be essential when no brood was present in the colony, such as during natural periods of dearth or queen replacement.

Little is known about genetic variation in worker ovary development or the response of workers to ovary-inhibiting pheromones. The results of this study have demonstrated the large amount of variability among colonies, even within a single apiary. It is clear that QMP plays an important role in regulating worker reproduction in honey bee colonies, but further investigation into the respective roles of queen and brood pheromones as well as genetically-based differences between colonies would provide a more complete understanding of this important process.

CHAPTER 3: ANARCHISTIC HONEY BEE QUEEN PHEROMONES[2]

3.1 Abstract

Anarchistic honey bees are a line developed by recurrent selection in which workers frequently lay eggs. In unselected colonies, workers refrain from reproduction in response to pheromonal signals that indicate the presence of brood and a queen. I show that queen type (anarchistic or wild type) has no effect on rates of ovary development of anarchistic or wild type workers. Anarchistic larvae do not inhibit worker ovary development to the same degree as wild type larvae, however all colonies in this experiment contained only wild type larvae. Anarchistic workers had greater rates of ovary development than wild type workers in colonies headed by either queen type. I therefore conclude that there must be differences in the transmission or reception of queen pheromones, or worker sensitivity to these compounds. These results clearly demonstrate that anarchy is a complex syndrome, not simply the result of reduced pheromone production by anarchist queens and larvae.

[2] A version of this chapter was previously published as: Hoover SER, Oldroyd BP, Wossler TC, Winston ML (2005) Anarchistic queen honey bees have normal queen mandibular pheromones. Insectes Soc 52(1):6-10. Reproduced here with kind permission of Birkhäuser Publishing Ltd. Basel, Switzerland.

3.2 Introduction

Unmated honey bee (*Apis mellifera*) workers can lay unfertilized eggs that give rise to fully functional adult males. When broodless or queenless, many adult workers develop their ovaries and commence laying eggs (Page and Erickson 1988). However, in colonies with both a laying queen and brood, only about one bee in 10,000 has fully formed eggs in her ovaries (Ratnieks 1993). Pheromonal signals produced by brood (Arnold *et al.* 1994) and queens (Hoover *et al.* 2003) mediate this functional sterility.

The vast majority of workers do not have developed ovaries when these signals are present. In theory, workers can maximise their reproductive success by rearing sons of the queen to which they are more closely related than the sons of half-sister workers (Hamilton 1964). Furthermore, workers effectively enforce functional sterility among their nest mates by eating most or all worker-laid eggs ('policing') (Ratnieks 1988, Ratnieks and Visscher 1989, Ratnieks 1995, Visscher 1996, Oldroyd and Ratnieks 2000). This policing mechanism is so efficient that virtually no worker-laid adult males can be detected by genetic analyses (Visscher 1989, Halling *et al.* 2001, Oldroyd *et al.* 200a), despite the fact that up to 6% of unfertilised eggs in a colony may be laid by workers (Visscher 1996). However, recent evidence suggests that rather than being 'policed,' worker-laid eggs may be consumed by other workers more frequently because of their inherently reduced viability compared to queen-laid eggs (Pirk *et al.*, 2004)

Thus, although workers are more related to their own eggs than those of the queen in polyandrous bees, the low probability of their eggs being reared favours self-restraint in worker reproduction (Seeley,1985, Keller and Nonacs 1993).

Despite the fact that natural selection appears to favour functional worker sterility in honey bee colonies with brood and queen, occasional colonies are found in which worker reproduction is common (Oldroyd *et al.* 1994, Montague and Oldroyd 1998, Châline *et al.* 2002). By recurrent selection from one such colony, Oldroyd *et al.* have developed an 'anarchistic' line of honey bees. Workers of this strain are unusual in many respects. Foremost is that 3 - 30% of workers have developed ovaries (Oldroyd *et al.* 1999, Oldroyd and Osborne 1999, Barron *et al.* 2001), even in the presence of a queen and brood. These bees provide an experimental resource for examining the proximate mechanisms by which worker fertility is regulated in wild type bees.

Queens signal workers using a variety of chemicals secreted from their mandibular (Slessor *et al.*1988, Keeling *et al.* 2003), tergal (Wossler and Crewe 1999a, 1999b, 1999c) and other glands (Keeling *et al.* 2003). The mandibular glands in particular have substantial effects on workers (Pankiw *et al.* 1995, Pettis *et al.* 1995a, 1995b, Melathopoulos *et al.* 1996, Brockmann *et al.* 1998, Huang *et al.* 1998, Pankiw *et al.* 1998, Pettis *et al.* 1998). Pheromones produced by the queen's mandibular glands attract workers (Kaminski *et al.* 1990, Pankiw *et al.* 1994, Pankiw *et al.* 1995), inhibit queen cell production (Pettis *et al.* 1995a, Melathopoulos *et al.* 1996), increase nectar foraging (Pettis *et al.* 1995b, Pankiw

et al. 1998), delay the age at first foraging and juvenile hormone secretion (Pankiw *et al.* 1998), and inhibit ovary development (Butler and Fairey 1963, Hoover *et al.* 2003). Worker-produced queen mandibular pheromone (QMP) also is involved in the establishment of dominance hierarchies among queenless workers (Moritz *et al.* 2000). Individuals that produce the greatest amount of QMP are more likely to attract courts of non-reproductive workers (Plettner *et al.* 1993).

Anarchistic colonies possess unusual characteristics in addition to widespread worker reproduction, including the frequent construction of supersedure queen cells (Barron *et al.* 2001). Queen mandibular gland secretions, particularly (E)-9-keto-2-decenoic acid, inhibit the construction of queen cells (Pettis *et al.* 1995a). I therefore speculated that queen cell construction and other unusual phenotypes of anarchistic bees might be a consequence of reduced production of queen mandibular pheromones by anarchistic queens and that these deficient pheromonal signals might contribute to the anarchistic syndrome.

To determine if anarchistic queens produce a deficient queen signal, and what the effects of this might be on levels of ovary development, I determined the effects of queen genotype (anarchistic or wild type) on the proportion of workers with developed ovaries, independently of brood type. Oldroyd *et al.* (2001b) showed that the brood of anarchistic colonies fails to inhibit ovary development in both wild type and anarchistic workers, independently of queen genotype. The current experiment is the reciprocal: it tests the effect of queen genotype (wild

type or anarchist) on the proportion of each type of worker with developed ovaries, while holding brood genotype constant.

3.3 Materials and methods

In December 2000, anarchistic (n = 10) and wild type (n = 14) queens were reared in the same colony by grafting day-old larvae from appropriate mother queens. A second batch of queens were produced in January 2003 (n = 3 of each genotype). For both sets of queens, mature pupae were transferred into individual mating nuclei and allowed to mate naturally. Although the offspring of the anarchistic queens were not purely of the anarchistic genotype due to the queens mating with wild type drones, the queens themselves should have expressed all the attributes of anarchistic queens.

Following Oldroyd *et al.* (2001b), two-chamber colonies of wild type bees (3 in 2000 and 3 in 2003) were dequeened and then divided into pairs of single-chamber colonies. Members of each pair were approximately equalised for population size (n ≈ 10,000 adult workers), food reserves and brood. The split colonies were furnished with a cage made of queen excluder material. Each cage was supplied with two empty brood combs. I introduced one of the wild type queens into the queen-excluder cage of one of each of the pairs of colonies, and one of the anarchistic queens into the cage of the other colony of each pair. Queens were restricted to these two frames in order to ensure that all queen-laid brood was found and removed from the colonies, and the only brood present was the wild type brood I supplied. The frames outside of the cages were also

examined for worker-laid brood. Workers had free access to the combs and queen inside the cages.

Five days later, after these six queens were established and laying, I added an equal number (approximately 100) of unrelated, marked, day-old workers from a total of four anarchistic and four wild type colonies to each of the experimental colonies. Each experimental colony received workers from one anarchist and one wild type colony. On the day the marked bees were introduced, and every third day thereafter, I removed the combs with new eggs from the colonies and replaced them with empty ones. Additionally, each colony was given a comb of wild type eggs and larvae. Thus, the marked workers were never exposed to the larvae of the queens in the colony, only to wild type larvae. Therefore, the only difference between the paired colonies was the genotype of the queen and her eggs (anarchist versus wild type).

After 14 days, the marked workers were retrieved from colonies and kept frozen prior to dissection. Because I did not kill the colonies prior to removing the marked workers, many workers were outside the colony during the collection period and therefore retrieval rates were low (Figures 3-1 and 3-2). Workers were dissected, and their ovaries scored as containing or not containing eggs of any size (Oldroyd *et al.* 2001b).

3.4 Results

Of the 497 wild type workers retrieved, only five (1%) had developed ovaries (Figure 3-1a). In contrast, 35 of the 517 (7%) anarchistic workers retrieved had developed ovaries (Figure 3-1b). There was no effect of queen type on rate of wild type worker ovary development (Fisher's exact test of no association between queen genotype and worker ovary development, P=0.685; Figure 3-1a). There also was no significant difference in the ovary development of anarchistic workers between queen genotypes (Fisher's exact test, P=0.133; Figure 3-1b), although in all colony pairs except one the anarchists had higher rates of ovary development in colonies headed by anarchist queens. As predicted, a greater proportion of the anarchistic workers had developed ovaries than did wild type workers in colonies headed by both anarchistic (Fisher's exact test, P<0.001) and wild type queens (Fisher's exact test, P<0.012).

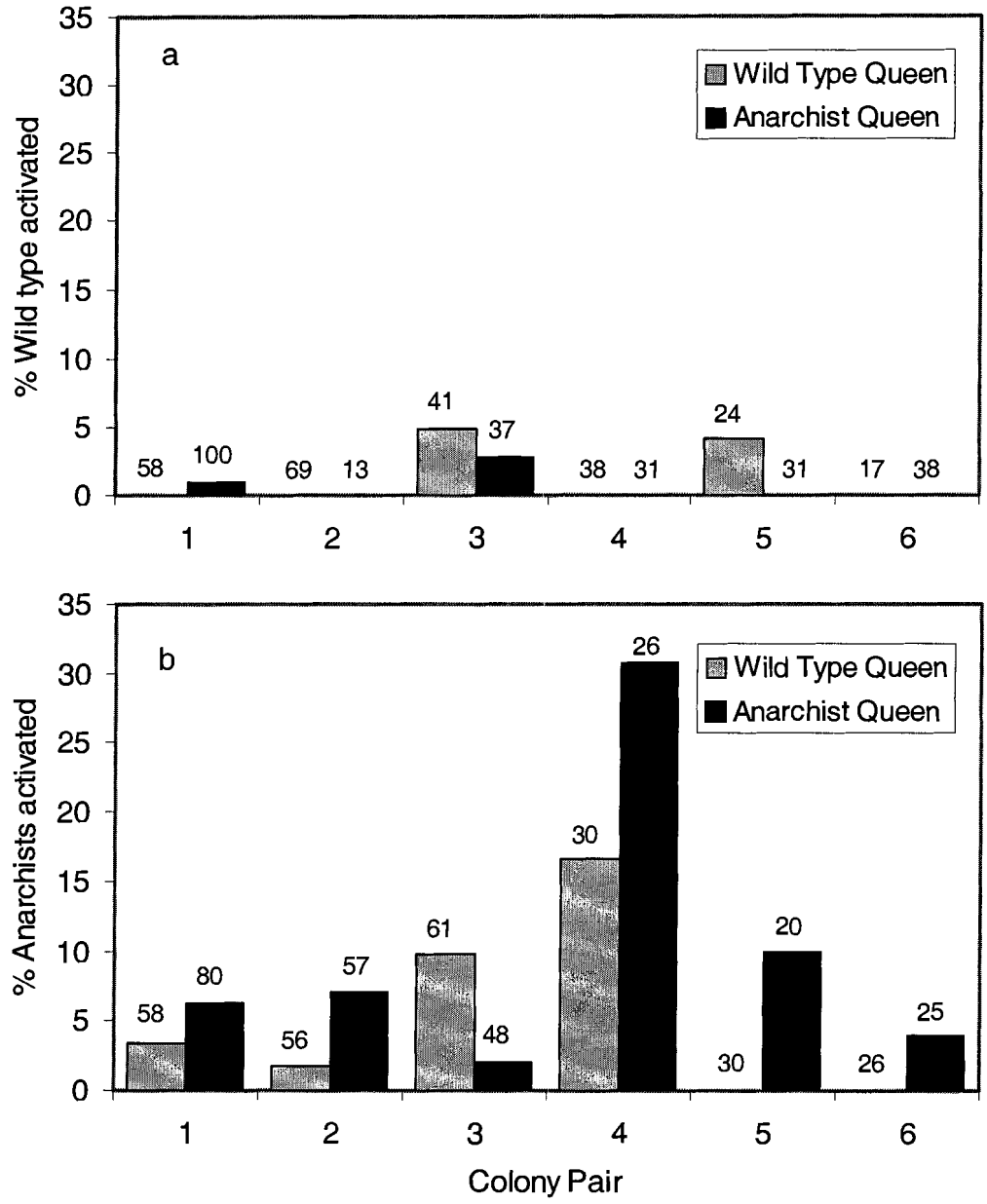

Figure 3-1 Proportion of (a) wild type and (b) anarchist workers with developed ovaries in colonies.

Workers were matured in colonies headed by anarchistic or wild type queens (black or grey bars, respectively). Numbers above bars are the sample size of bees recovered from that colony split.

3.5 Discussion

This study has shown that the mandibular gland secretions of anarchistic queens are similar to those of wild-type queens (Hoover *et al.* 2005a). Although there was a trend towards increased anarchist ovary activation in colonies headed by anarchist queens, queen genotype (anarchistic or wild type) had no significant effect on the rate of ovary development of unrelated workers. This may be due in part to the low sample sizes used in this study, which were a result of the rarity of the anarchistic colonies themselves. Nevertheless, anarchistic workers had higher rates of ovary development than wild-type workers. Because I show no differences between anarchist and wild type queens, I suggest that the differences observed in worker ovary development between anarchists and wild types arise through at least two pathways: 1) signals arising from the brood are reduced in anarchists (Oldroyd *et al.* 2001b), and 2) worker responses to queen and brood pheromones differ between the two worker types. This is in contrast to the hypothesis that differences in ovary development were due to differences in signals arising from wild type and anarchist queen mandibular glands.

Anarchistic workers had higher proportions of development than did wild type workers after exposure to either queen genotype. This further demonstrates that workers of the anarchistic line respond differently than wild type workers to the signals that normally inhibit ovary development. Anarchist ovary development was similar to that typically observed when anarchistic workers are reared in queenright wild-type colonies (Barron and Oldroyd 2001).

Queen mandibular pheromones inhibit the production of queen cells in queenless colonies (Pettis *et al.* 1995a, Melathopoulos *et al.* 1996), but anarchistic colonies frequently build supercedure cells in the presence of their queen (Barron *et al.* 2001). As there is no difference between anarchist and wild type queen pheromones, the supercedure cell construction may be due to disrupted signals from the brood or reduced worker response to inhibitory queen pheromones, rather than from decreased queen mandibular pheromone production (Hoover *et al.* 2005a).

When large numbers of workers start laying eggs in worker cells of anarchistic colonies, there is very little queen-laid brood present. Under these circumstances, queens become isolated to a small area of a single comb where they appear to be neglected (Benjamin P Oldroyd, personal observations, Barron *et al.* 2001b). I had suspected that this was due to inadequate production of queen mandibular pheromones, but this proved not to be the case. While *my* data do not exclude the possibility that anarchistic queens produce inadequate signals involving other queen pheromones (Keeling *et al.* 2003), the queen mandibular gland components appear to be normal. In addition, queen signals may interact with signals from the brood, and I may have found different results in colonies with anarchistic brood. Finally, the responses of anarchistic and wild type workers to similar queen pheromone blends differs, as evidenced by the higher rates of anarchistic ovary development in this study.

Many factors could potentially contribute to produce the observed anarchistic traits. The isolation and neglect of anarchistic queens by their workers suggests that the retinue attraction pathway may be interrupted. If anarchist workers are not attracted to their queen, they may be receiving a lower dose of the ovary-inhibiting queen pheromones. In this way, the reduced retinue attraction could lead to increased ovary development. Anarchistic workers could also have an increased threshold dose at which queen pheromones inhibit their ovary development.

The difference between anarchistic and wild type workers may lie in the transmission of, reception of, or sensitivity to queen pheromones. Most likely, the anarchistic syndrome is the result of a number of different factors, including decreased inhibition by anarchistic brood, that all contribute to the observed phenotype. Future studies of the anarchistic bees should endeavour to isolate the mechanism by which anarchists are able to develop their ovaries despite the presence of a queen.

CHAPTER 4: RETINUE ATTRACTION AND OVARY DEVELOPMENT: RESPONSES OF WILD TYPE AND ANARCHISTIC HONEY BEES (*APIS MELLIFERA*) TO QUEEN AND BROOD PHEROMONES[3]

4.1 Abstract

In most social insect colonies, workers do not attempt to lay eggs in the presence of a queen. However, in the honey bee (*Apis mellifera*), a rare phenotype occurs in which workers develop their ovaries and lay large numbers of male eggs despite the presence of a fecund queen. I examined the proximate mechanisms by which this 'anarchistic' behaviour is expressed. I tested the effects of brood and queen pheromones on retinue attraction and worker ovary development using caged worker bees. I found no difference between the anarchistic and wild type queen pheromones in the retinue response elicited in either wild type or anarchistic workers. Further, I found that anarchistic queens produce a pheromone blend that is as effective at inhibiting ovary development as the wild type queen pheromone. However, anarchistic workers are less inhibited by queen pheromones than their wild type counterparts, in a dose-dependent manner. These results show that the anarchistic phenomenon is not due to changes in the production of queen pheromones, but rather is due in part to a shift in the worker response to these queen-produced signals. In addition, I

[3] A version of this chapter was previously published as: Hoover SER, Winston ML, Oldroyd BP (2005) Retinue attraction and ovary activation: responses of wild type and anarchistic honey bees (*Apis mellifera*) to queen pheromones. Behavioral Ecology and Sociobiology (*published online 09/05*). Reproduced here with kind permission of Springer Science and Business Media.

demonstrate the dose-dependent nature of the effect of queen pheromones on honey bee worker ovary development.

4.2 Introduction

Reproductive division of labour is one of the primary characteristics that define eusocial insect societies. In many Hymenopteran colonies, it is normally only the queen that reproduces. Workers in these societies do not normally mate, and can therefore produce only male offspring, which develop from unfertilised eggs. All workers normally direct their assistance toward maintaining the colony, and raising the queen's brood. In the polyandrous honey bee *Apis mellifera*, the queen is normally the sole female reproductive, responsible for the production of both male and female offspring. Less than 1% of males produced by queenright honey bee colonies are the result of worker reproduction (Visscher 1989). Should a colony lose its queen, however, workers are capable of ovary development and egg-laying behaviour.

A clear example of genetically-determined 'cheating' behaviour has been demonstrated in the 'anarchistic' honey bees (*Apis mellifera* L.) observed in both Australia (Oldroyd *et al.* 1994, Montague and Oldroyd 1998) and the UK (Châline *et al.* 2002). In these rare 'anarchistic' colonies, workers frequently lay male eggs despite the presence of a fecund queen, and many of the eggs are reared to maturity. Oldroyd and colleagues have now bred a line of honeybees in which worker ovary development and egg-laying behaviour is common (Barron *et al.* 2001). Hereafter I use the term 'anarchistic' to describe bees of this line, whether

or not they actually have developed ovaries. The anarchic phenomenon necessarily involves two separate steps. First, workers must be able to develop their ovaries despite the presence of inhibitory pheromones, and second they must lay eggs that escape worker policing.

Prior to egg laying, anarchists must be capable of activating their ovaries despite the presence of a queen and her brood, both of which produce pheromones that normally inhibit worker ovary development (Arnold *et al.* 1994, Mohammedi *et al.* 1998, Hoover *et al.* 2003). Anarchistic workers are capable of activating their ovaries in queenright colonies (Oldroyd *et al.* 1999, Barron and Oldroyd 2001), and a number of factors appear to be responsible for this unusual phenomenon. First, anarchistic larvae are believed to produce less of the inhibitory compounds, or a less inhibitory blend (Oldroyd *et al.* 2001b). Second, anarchists are believed to have a higher threshold for these inhibitory pheromones, allowing many anarchist workers to develop their ovaries even in queenright wild type colonies (Barron and Oldroyd 2001, Oldroyd *et al.* 2001b, Hoover *et al.* 2005a). Anarchistic workers escape egg policing in two ways; they are both less discriminatory against worker-laid eggs, and anarchistic workers lay eggs of greater acceptability to other workers (Oldroyd and Ratnieks 2000).

My investigation of the proximate mechanisms leading to the anarchistic syndrome was threefold. First, I compared the retinue attraction of anarchistic and wild type workers to queen-produced pheromones. Queen honey bees produce a 9-component pheromone blend that is highly attractive to workers

(Kaminski *et al.* 1990, Keeling *et al.* 2003). Workers attracted by this pheromone antennate and groom the queen, forming a 'retinue' around her (Kaminski *et al.* 1990), thereby coming in contact with components of the queen pheromone that inhibit ovary development (Hoover *et al.* 2003). Pankiw *et al.* (1994, 1995) found genetic variation in the retinue response of bees to queen pheromone in wild type workers. I hypothesised that anarchistic workers might be less attracted to the retinue pheromones, come into contact with the queen less frequently, and thus avoid ovary inhibition where wild type workers do not. I further hypothesised that anarchistic queens might produce a less attractive pheromone blend than wild type queens, thus reducing the level of inhibition experienced by workers in colonies headed by anarchistic queens. This type of queen avoidance has been observed in Cape honey bees (*Apis mellifera capensis*), where drifted workers (bees originating from other colonies that have 'drifted' into the colony in question) are found on average to be more distant from the queen within the colony than their non-drifted counterparts, and is thought to be a predisposition for social parasitism (Neumann *et al.* 2003).

Secondly, using caged workers, I examined the effects of brood pheromones, anarchist queen pheromones, and wild type queen pheromones on both anarchist and wild type worker ovary development. I hypothesised that these pheromones would inhibit anarchistic worker ovary development less than that of their wild type counterparts. Finally, I examined the effect of queen pheromone dose on worker ovary development. In this case, I predicted that rates of ovary development would decline as the dose of pheromone was increased, but that

the rate of decline would be lower in workers of the anarchistic line than wild type workers. I hypothesised that anarchistic workers would require a higher dose of queen pheromone to inhibit ovary development at a level comparable to wild type workers.

4.3 Methods and Materials

The honey bees used in this study were *Apis mellifera mellifera*, reared using standard beekeeping techniques at the Universities of Sydney and Western Sydney, Australia. Anarchistic colonies were the product of many generations of selection for worker reproduction, and identified as queenright colonies with workers laying and rearing a large number of worker-laid drones.

Preparation of queen extracts

Mated, laying queens were removed from colonies at the University of Western Sydney (December 2002), and mailed frozen on dry ice to Simon Fraser University for extraction. Each anarchist (AN) (n=15) and wild type (WT) (n=15) queen was homogenised and repeatedly extracted in distilled diethyl ether. Each body segment was homogenised individually, to give a combined total extract of 2000 μl per queen. Queen extracts were used as pheromone treatments in the retinue bioassays, and the ovary development experiments (a and b). Individual extracts were combined to give a blend of 15 queens per genotype (AN versus WT). The synthetic queen retinue pheromone (QRP) consists of the 5-component queen mandibular pheromone (QMP, produced by Phero Tech Inc.) plus methyl oleate (3.8 μg/Queen equivalent (Qeq)), coniferyl alcohol (0.15

μg/Qeq), hexadecane-1-ol (1.1 μg/Qeq), and linolenic acid (22 μg/Qeq) (Keeling *et al.* 2003).

Retinue Bioassays

Pseudoqueen retinue bioassays (Kaminski *et al.* 1990, Keeling *et al.* 2003) were conducted at the University of Sydney (February 2003) to compare the attraction of anarchistic and wild type workers to various pheromone treatments. Treatments included: a solvent (diethyl ether) control (Control), synthetic 9-component queen pheromone (QRP) (Keeling *et al.* 2003), the anarchist (Q-AN), and wild type queen extracts (Q-WT).

To perform the assay I caught fifteen nurse-aged worker bees taken from brood combs containing larvae. I introduced these workers into Petri dishes that had been modified to add a hole in the side to allow the assay workers and lure to be introduced easily (Kaminski *et al.* 1990). Test treatments were applied to Pasteur pipette lures fashioned to be the approximate size of a queen, with a dimple to contain the test fluid (Kaminski *et al.* 1990). Lures were spotted with 0.01 queen equivalents of treatment, and bioassays were conducted under red light to minimise the degradation of light-sensitive components. The bioassay began after the evaporation of the solvent from the lure with the insertion of the treated lure into a hole in the side of each dish. The number of bees contacting the lure was counted every 30 seconds for 5 minutes (as per Kaminski *et al.* 1990, Keeling *et al.* 2003), and the sum of these counts was recorded as the score for that dish. Five anarchist and 5 wild type colonies were assayed 10

times per colony, for a total of 100 individual retinue bioassays per pheromone treatment.

Ovary Development

Ovary development experiments were conducted as per Hoover *et al.* (2003). For each experiment, workers from all test colonies were emerged overnight in incubators. The workers were placed in groups of 30 in cages such that each colony was represented by one cage per treatment per experiment. Queen pheromone treatments were applied to a glass slide in diethyl ether, which was allowed to evaporate before the slide was presented to the bees. Caged workers were kept at 34°C in the dark, and fed a 1:1 mixture of honey and royal jelly by volume (diluted 10% with water) and water *ad libitum*. After 10 days the worker bees were frozen and stored at -80°C prior to dissection. After dissection, each ovary was assigned a score from 0 (completely resting) to 4 (mature egg present) according to the methods of Pernal and Currie (2000), and Hoover at al. (2003). A bee with an ovary score of 3 or 4 was considered to have 'developed' ovaries.

Brood Pheromone experiment

The brood pheromone experiment was conducted in February 2003, and included four anarchist and four wild type colonies. The brood pheromone consists of a blend of 10 components normally present in honey bee larvae, including: methyl palmitate, methyl oleate, methyl stearate, methyl linoleate, ethyl palmitate, ethyl oleate, ethyl stearate, ethyl linoleate, and ethyl linolenate

(LeConte *et al.* 1990). *My* brood blend included 9 of these esters in the proportions observed in Le Conte *et al.* (1990). Ethyl stearate was not included, but is not believed to be necessary for ovary inhibition (Mohammedi *et al.* 1998). The brood pheromones were delivered to the cages mixed with their food. Four pheromone treatments were applied: solvent blank (diethyl ether) (Control), low dose of brood pheromone (Low) (20 Larval equivalents (Leq)/bee/day), high dose of brood pheromone (High) (200 Leq/bee/day), and QRP + low brood pheromone dose (Queen + Low) (0.1 Qeq/day + 20 Leq/bee/day).

Queen Pheromone experiment

The queen pheromone experiment was conducted in February 2003, and included 4 anarchist and 5 wild type colonies. I placed microscope slides treated with the appropriate pheromone in the test cages, replacing them daily: solvent blank (diethyl ether) (Control), anarchist queen extract (Q-AN) (0.1 Qeq/day), wild type queen extract (Q-WT) (0.1 Qeq/day), and synthetic 9-component queen pheromone (QRP) (0.1 Qeq/day).

Queen Pheromone Dose experiment

The queen pheromone dose experiment was conducted in November 2003, and included six anarchist and six wild type colonies. Four queen pheromone treatments were applied as above: solvent blank (diethyl ether) (Control), QMP (5-component blend, Phero Tech Inc.) (0.001 Qeq/day) (0.001), QMP (0.01 Qeq/day) (0.01), and QMP (0.1 Qeq/day) (0.1).

Statistical Analyses

Statistical analyses consisted of 2-way analyses of variance (ANOVAs) on colony mean ovary / retinue scores. Such a design allowed for analysis of both pheromone and bee type (AN versus WT) effects. Prior to analysis, data were log x+1 transformed to better meet the assumptions of the ANOVA model (Zar, 1984). Because I predicted *a priori* that anarchists should have higher levels of ovary development and lower retinue attraction scores than wild type workers, 1-tailed tests were performed when comparing mean anarchist and wild type scores. Where significant results were obtained, Tukey's HSD was used to detect differences among groups.

4.4 Results

Retinue Bioassays

There was no effect of bee type (AN versus WT) on the retinue response ($F_{1, 32}$ = 1.02, $p_{1\text{-tailed}}$ = 0.16, Figure 4-1). However, the queen pheromone treatments elicited significantly more contacts from the workers than did the control treatment ($\bar{x}_{AN}$ = 3.7 ±1.0, $\bar{x}_{WT}$ = 2.2 ±0.97), with no difference between the synthetic QRP ($\bar{x}_{AN}$ = 13.8 ±2.1, $\bar{x}_{WT}$ = 20.4 ±6.2), anarchist ($\bar{x}_{AN}$ = 20.0 ±5.3, $\bar{x}_{WT}$ = 12.4 ±2.9) or wild type ($\bar{x}_{AN}$ = 16.4 ±2.2, $\bar{x}_{WT}$ = 13.0 ±2.4) queen pheromones ($F_{3,32}$ = 14.60, $p < 0.0001$). I conclude that anarchist workers are as attracted to queen pheromones as wild type workers, and that anarchist queens are equally attractive to both anarchistic and wild type workers.

Ovary Development - Brood Pheromone

There was a significant effect of pheromone treatment ($F_{3,24} = 7.58$, $p = 0.001$) but not bee genotype ($F_{1,24} = 1.69$ $p_{1\text{-tailed}} = 0.10$) on the mean ovary scores (Figure 4-2). Whereas the brood pheromones had no clear effect, the Queen + Low treatment inhibited ovary development of both anarchistic and wild type bees ($\bar{x}_{AN} = 0.55 \pm 0.15$, $\bar{x}_{WT} = 0.42 \pm 0.23$), and accounted for the treatment effect. Similar trends were observed for % of workers with developed ovaries (those scoring 3 or 4) (Figure 4-2).

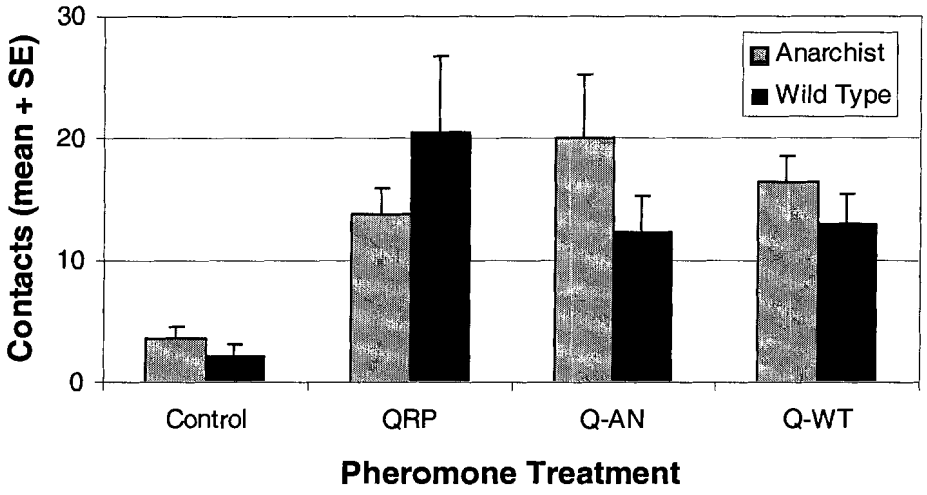

Figure 4-1 Effect of queen pheromones on worker retinue attraction for both anarchistic and wild type workers.

Pheromone treatments included a solvent control (diethyl ether), synthetic queen retinue pheromone (QRP), anarchist queen extract (Q-AN), and wild type queen extract (Q-WT). Ten bioassays were conducted per colony per treatment, with the mean of these used in the statistical analysis.

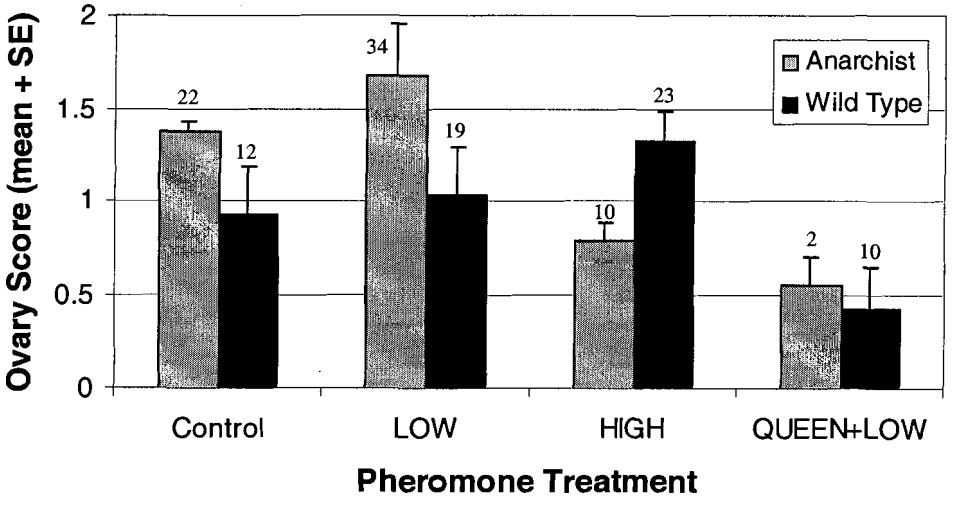

Figure 4-2 Effect of queen and brood pheromones on worker ovary development for both anarchistic and wild type workers.

Pheromone treatments included a solvent control (diethyl ether), a low dose of brood esters, a high dose of brood esters, and synthetic queen mandibular pheromone (QMP, 5-component) + the low dose of brood esters. Numbers above bars are percent of individuals with 'developed' ovaries (those scoring '3' or higher, with eggs that are fully developed or nearly so (larger than their associated nutritive cells)).

Ovary Development - Queen Pheromone

The queen pheromone treatments inhibited worker ovary development ($F_{3,28}$ = 13.70, $p < 0.0001$) compared to the control ($\bar{x}_{AN} = 0.79 \pm 0.21$, $\bar{x}_{WT} = 1.13 \pm 0.42$), with no difference between the anarchist queen ($\bar{x}_{AN} = 0.11 \pm 0.04$, $\bar{x}_{WT} = 0.06 \pm 0.01$), wild type queen ($\bar{x}_{AN} = 0.15 \pm 0.07$, $\bar{x}_{WT} = 0.09 \pm 0.05$), or QRP pheromones ($\bar{x}_{AN} = 0.34 \pm 0.10$, $\bar{x}_{WT} = 0.12 \pm 0.06$), (Figure 4-3). I conclude therefore, that anarchist queens produce a pheromone blend that inhibits ovary development. Although anarchist workers had higher mean ovary scores than wild type workers when exposed to any of the queen pheromone treatments, no effect of bee type on ovary development was detected ($F_{1,28} = 0.01$, $p_{1\text{-tailed}} = 0.48$). Again, similar trends were observed for mean % developed as for mean ovary score (see Figure 4-3).

Ovary Development - Queen Pheromone Dose

Very few bees, either anarchist or wild type, had developed ovaries (score 3 or 4) at any dose of QMP. The mean ovary score of anarchists exposed to the highest pheromone dose was 0.6, whereas the wild type workers were more inhibited, with a mean score of 0.3 (Figure 4-4). There was a significant effect of both pheromone treatment ($F_{3,40} = 30.41$, $p < 0.0001$) and of bee type on mean ovary development score, with anarchists more developed than wild type workers ($F_{1,40} = 5.99$, $p_{1\text{-tailed}} = 0.009$) at the two higher doses (0.01 and 0.1 Qeq).

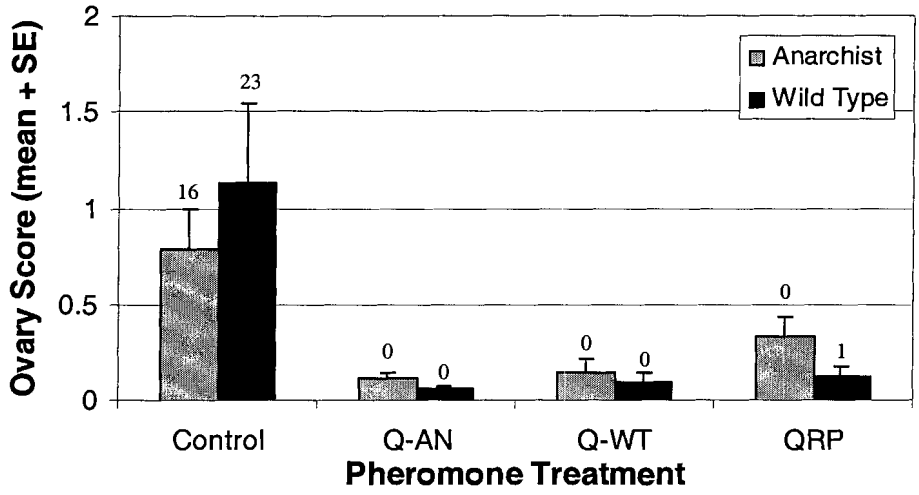

Figure 4-3 Effect of queen pheromones on worker ovary development for both anarchistic and wild type workers.

Pheromone treatments included a solvent control (diethyl ether), anarchist queen extract (Q-AN), wild type queen extract (Q-WT), and synthetic queen retinue pheromone (QRP). Numbers above error bars are percent of individuals with 'developed' ovaries (those scoring '3' or higher, with eggs that are fully developed or nearly so (larger than their associated nutritive cells)).

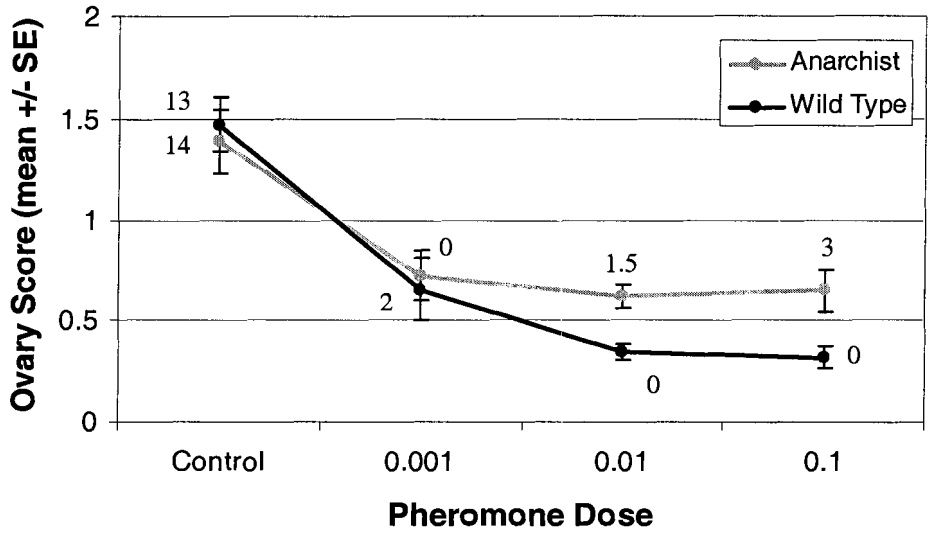

Figure 4-4 Effect of queen pheromone (QMP) dose on worker ovary development for both anarchistic and wild type workers.

Numbers beside error bars are percent of individuals with 'developed' ovaries (those scoring '3' or higher, with eggs that are fully developed or nearly so (larger than their associated nutritive cells)).

4.5 Discussion

Retinue Bioassays

Anarchist queens produce a pheromone blend that is equally attractive to both anarchist and wild type workers, as are a synthetic 9-component blend and extracts of wild type queens. This finding is in agreement with Hoover *et al.* (2005a), who detected no difference between anarchist and wild type queens in their analysis of the five components from the queen mandibular pheromone. Further, the retinue bioassays clearly demonstrate that anarchist workers can perceive, and are attracted to, queen-produced pheromones. Previous observations indicating that anarchist queens are neglected (Barron *et al.* 2001) are therefore not due to a simple lack of worker attraction to the queen. Any effect of queen type observed as part of the anarchistic syndrome can not be attributed to workers simply ignoring their queen due to lack of attraction to retinue pheromones.

Ovary Development - Brood Pheromone

In colony experiments, anarchists appear to have a lower ovary development threshold than do wild type workers for brood pheromones. Anarchist workers frequently develop their ovaries in the presence of wild type brood, whereas wild type workers do not (Oldroyd *et al.* 2001b). However, no difference was detected between the bee types in *my* cage experiments. This may be due to the fact that I found no effect of brood pheromone in the absence of queen pheromone.

Previous studies have demonstrated an inhibitory effect of the brood esters on ovary development (Arnold *et al.* 1994, Mohammedi *et al.* 1998), and it is uncertain why no clear effect of brood pheromone was observed at either of the doses used in this experiment (20 or 200 larval equivalents / bee / day). This may have been due to the method by which the pheromone was administered in *this* study (mixed in food), although brood esters mixed in food have previously been effective (Mohammedi *et al.* 1998). Alternatively, the lack of effect may be due to the absence of ethyl stearate. This explanation is also unsatisfactory, as ethyl palmitate and methyl linolenate alone have demonstrated inhibitory effects when administered in a honey / icing sugar mix (Mohammedi *et al.* 1998).

Other methodological differences could have led to this disagreement of results. While Mohammedi *et al.* (1998) reported differences for 14-day-old workers, none were detected in workers aged 7 days. This study analysed workers at 10 days of age, which may have been too young to observe an effect of brood pheromone. Differences in the method of ovary score classification also could lead to differing results, although this does not fully explain the observed lack of inhibition by brood pheromones. It is possible that the overall level of worker ovary development in this study was too low to observe the effect due to the drought conditions in New South Wales at the time, and the analysis of relatively young workers.

Ovary Development - Queen Pheromone

These results demonstrate that queen pheromones are inhibitory to ovary development in anarchist workers, despite their having increased rates of ovary development in queenright colonies compared to wild type workers (Montague and Oldroyd 1998, Barron and Oldroyd 2001, Barron *et al.* 2001, Hoover *et al.* 2005a). Further, anarchist queens also produce an inhibitory blend. A previous study found no effect of queen genotype (WT or AN) on worker ovaries using live queens in small colonies, and no difference between anarchist and wild type queen pheromone blends (Hoover *et al.* 2005a). Combined with the current experiment, these studies confirm that the anarchistic syndrome is not due to any failing of the anarchistic queens, as both in colonies and cages queen type did not affect rates of ovary development of either anarchistic or wild type workers.

Ovary Development - Queen Pheromone Dose

While the queen pheromone of wild types and anarchists are identical (Hoover *et al.* 2005a), anarchistic workers respond to ovary inhibiting pheromones differently than do wild type workers (Montague and Oldroyd 1998, Barron and Oldroyd 2001). Wild type workers are increasingly inhibited by higher doses of pheromones, and are more inhibited at a given dose. While both bee types were inhibited equally at the lowest dose, anarchists had significantly higher levels of ovary development than wild type workers at the two higher doses. Both bee types appear to reach an 'inhibitory plateau' at a certain dose, beyond which increases in the dose are not matched by further ovary inhibition. Anarchists appear to reach this plateau at a lower dose than wild type workers. Further, the

minimum mean ovary score achieved by the high pheromone doses was higher for anarchistic (0.6) than for the wild type (0.3) workers.

It is difficult to determine the actual dose of pheromones received by individual workers in a honey bee colony, and the appropriate dose for studying ovary inhibition remains unclear. Honey bee pheromones can have distinct effects at different doses. For example, the retinue attraction of workers to queen pheromones at low doses (Kaminski *et al.* 1990) is not observed at high doses. Figure 4-4 demonstrates not only that the inhibitory effect of QMP is dose-dependent, but also that the dose response curve can vary substantially even within the same species.

Recent work by Amdam *et al.* (2004) has demonstrated that there is a clear link between pollen foraging behaviour and worker reproduction. The low mean levels of ovary development observed in both the brood and queen pheromone experiments, even in the control treatment, are likely due in part to the severe drought conditions in New South Wales at the time of these experiments. The drought conditions would likely reduce the amount of protein available to developing larvae in the form of pollen, in turn affecting their ability to develop mature ovaries as adults (Pernal and Currie 2000). Further, anarchistic worker reproduction is most prevalent in the spring (Montague and Oldroyd 1998, Barron *et al.* 2001), whereas these experiments were conducted in the late summer. The dose experiment was conducted the following spring, and levels of ovary development in the control treatment were comparatively higher for both

anarchist and wild type bees. This may explain why there was no significant difference between anarchists and wild types in the late summer queen pheromone experiment, yet a difference was detected at the same dose in the spring queen pheromone dose experiment. While, as predicted, anarchists had higher mean ovary development scores than did the wild types in the presence of all three queen pheromones (Q-AN, Q-WT, and QRP) this result was not significant in the late summer experiment. The following spring, in the dose experiment, a significant effect was observed using the 5-component queen mandibular pheromone. Anarchy is a complex syndrome that requires a number of conditions be met for its full expression, including good larval nutrition.

Worker reproduction in social insects is a multifaceted phenomenon; the emergent behaviour observed is the result of many contributing factors. Worker reproduction is influenced by the probability of success of laying (due to prevalence and efficiency of policing, and egg viability), costs to indirect fitness caused by cheating (due to loss of colony efficiency), social constraints (workers with developed ovaries may be physically attacked (Visscher and Dukas 1995, but see Dampney *et al.* 2002), or physical constraints (such as physiology, age, or nutrition). Anarchistic workers are different from wild type in that they have an increased reproductive success rate (they are less discriminatory police, and lay eggs of greater acceptability (Oldroyd and Ratnieks 2000)), they have reduced indirect fitness (colonies do poorly (Barron *et al.* 2001), likely due to reduced work rates (Dampney *et al.* 2004)), and their ability to exhibit the anarchistic phenomenon is dependent on environmental conditions (as evidenced by the

reduced rates of ovary development during drought conditions). This phenomenon appears to be mediated by the brood and queen pheromone threshold for ovary development.

Hamilton (1972) predicted that genetically 'selfish' traits would arise through a 'raised threshold for response' to inhibitory queen pheromones. I provide direct evidence that anarchistic honey bees are an example of just such a system of cheaters. The response of anarchistic workers to queen pheromones clearly differs from wild type, enabling them to increase their direct fitness by rearing their sons. Whether the anarchistic syndrome is an example of escape from queen control, or simply demonstrates one end of the natural variation in the response to an honest signal of the queen's fecundity (Seeley 1985, Keller and Nonacs 1993) is not clear. What is clear, however, is that the anarchistic syndrome is a complex phenomenon that requires a number of separate conditions be met for its full expression.

CHAPTER 5: WORKER HONEY BEE OVARY DEVELOPMENT: SEASONAL VARIATION AND THE INFLUENCE OF LARVAL AND ADULT NUTRITION[4]

5.1 Abstract

I examined the effect of larval and adult nutrition on worker honey bee (*Apis mellifera* L.) ovary development. Workers were fed high or low pollen diets as larvae, and high or low protein diets as adults. Workers fed low protein diets at both life stages had the lowest levels of ovary development, followed by those fed high protein diets as larvae and low quality diets as adults, then those fed diets poor in protein as larvae but high as adults. Workers fed high protein diets at both life stages had the highest levels of ovary development. The increases in ovary development due to improved dietary protein in the larval and adult life stages were additive. Adult diet also had an effect on body mass, whereas larval diet did not. The results demonstrate that both carryover of larval reserves and nutrients acquired in the adult life stage are important to ovary development in worker honey bees. Carryover from larval development, however, appears to be less important to adult fecundity than is adult nutrition. Seasonal trends in worker ovary development and mass were examined throughout the brood rearing season. Worker ovary development was lowest in spring, highest in mid-summer, and intermediate in fall.

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5.2 Introduction

Egg production by female insects is typically a nutrient-limited process, initiated only if enough resources are available to supply eggs with sufficient protein, lipids, vitamins and minerals for development (Wheeler 1996). In holometabolous insects, this nourishment can be acquired in larval or adult life stages, or both. Other necessary activities such as flight, foraging, and dispersal compete with ovary development for energy and protein, creating a trade-off between fecundity and other life-history traits (Wheeler 1996). This is particularly true in social insects, as significant amounts of nutrients are channelled into brood care. In social insects, these trade-offs may occur among individuals within a single colony, as well as within a single individual. Social insects are defined by caste differentiation and the unequal partitioning of reproduction, which has evolved partially through the unequal distribution of nourishment to individual members of the society (Hunt and Nalepa 1994).

Larval nutrition and adult fecundity are strongly linked in social insects, and are related by two mechanisms: 1) through nutrient reserves carried forward from larval development and available for adult reproduction, and 2) through effects on adult body size (Hunt and Nalepa 1994). As adults, there is a trade-off between fecundity and the ability to work; activities such as nursing and foraging deplete nutrient reserves that could otherwise contribute to ovary development and oogenesis (Hunt and Nalepa 1994, Wheeler 1996). Recent work has suggested that there may be a third mechanism linking nutrition to adult behaviour and

fecundity (Amdam *et al.* 2005, Toth and Robinson 2005). Developmental processes in larvae are affected by nutrition, and could potentially lead to ovaries that are more or less likely to be developed in the adult life stage. Nutrition could, through such processes, produce workers 'predisposed' for ovary development. Such workers would essentially be 'primed' for ovary development, in addition to effects of nutrient stores or body size.

In the honey bee (*Apis mellifera* L.), workers can normally only lay unfertilised male eggs, and generally do so only when the colony is queenless. Workers have 3-26 egg-producing ovarioles per ovary, compared to a queen's 150-180 (Snodgrass 1956, Sakagami and Akahira 1958). The degree of a worker's ovary development upon queen loss is dependent on a number of factors she experiences as an adult, including aggression from other workers (van der Blom 1991, Visscher and Dukas 1995, but see Mayer *et al.* 1998 and Dampney *et al.* 2002), temperature (Lin and Winston 1998), pheromones from the queen (Butler and Fairey 1963, Hoover *et al.* 2003) and her brood (Jay 1972, Mohammedi *et al.* 1998), and trophallactic interactions with other workers (Korst and Velthuis 1982, but see Mayer *et al.* 1998). Social interactions such as aggression and trophallaxis can indirectly, through nutritional means, affect the degree of ovary development. For example, van der Blom (1991) found that workers directed aggression towards bees with developed ovaries. Attacked workers frequently lost food through trophallaxis to 'bystanders'. This loss of food would then likely diminish a worker's capacity to further develop her ovaries.

Both larval and adult nutrition also directly affect honey bee ovary development. As larvae, workers are progressively fed high-protein 'brood food' produced by the mandibular and hypopharyngeal glands of young adult 'nurse' bees, as well as some pollen and honey (Winston 1987). To rear a worker from egg to pupation requires 125-145 mg pollen (Alfonsus 1933, Rosov 1944), primarily fed to the larva as brood food. Underfeeding of larvae results in dwarf bees, and the size and weight of emerging workers is affected by both the age and relative number of nurse bees available to feed developing larvae (Jay 1964). Larval nutrition also has been implicated in determining the number of ovarioles in adult ovaries (Rhein 1933). Finally, the developmental pathway of honey bee eggs is not genetically determined. The differentiation of queens and workers is achieved through nutrition – it is the quantity and quality of the food received as larvae that distinguishes the development of the two castes. Intermediate feeding of female larvae results in intercastes.

As adults, pollen intake also is crucial to newly pupated bees, which are still undergoing organ and glandular development and the growth of fat bodies (Winston 1987). For example, adult drones developing in colonies with low pollen stores take longer to reach sexual maturity, or do not mature (Free and Williams 1975). A worker diet low in pollen during the first few days of adult life results in poor glandular development, and decreased longevity (Maurizio 1950, Haydak 1970). Worker nutrition is also associated with division of labour between house bees and foragers (Toth and Robinson 2004). The link between dietary protein and ovary development in adult workers has been well-documented

(Maurizio 1950, Pain 1963, Jay 1975, Harris and Harbo 1990, Jay and Jay 1993, Lin and Winston 1998, Pernal and Currie 2000). A protein-rich diet promotes the development of queenless worker ovaries, whereas a diet lacking in protein restricts oogenesis. Lin and Winston (1998) found that increasing the protein content of the diet of caged adult worker bees resulted in matching increases in the level of ovary development observed.

It is this relationship between nutrition and fecundity that is believed to be responsible for seasonal variation in worker ovary development. Seasonal trends in the level of worker ovary development have been reported previously from the Netherlands (Velthuis 1970) and the Czech Republic (in queenright colonies, Kropáčová and Haslbachová 1969), but no such trend has been found in the number of ovarioles in each ovary (Levin and Haydak 1951). As temperature also affects worker ovaries (Lin and Winston 1998), this factor also could contribute to the seasonal effect.

In this study, I examined the effects of both larval and adult nutrition on worker honey bee ovary development and body mass. I predicted that both larval and adult nutrition would affect ovary development, although the relative contribution of nutrition at each of the two life stages was previously undetermined. Unlike previous studies, this experiment considers the combined effects of adult and larval diet. The amount of food resources available to honey bee colonies varies throughout the year with the availability of plants in bloom. If nutrition affects

worker ovary development in honey bee colonies, there should be a strong correlation between the season (due to both available forage and stored resources) and ovary development. I assessed the ovary development of caged queenless workers taken from SFU apiaries throughout the months in which the colonies were rearing brood (the brood-rearing season).

5.3 Methods and Materials

General Methods

I used the honey bee *Apis mellifera* of mixed European descent that is common in North America, and most closely resembles the European subspecies *A. m. ligustica*. All experiments and surveys were conducted in the Fraser Valley, or on the Simon Fraser University Campus in Burnaby, British Columbia, Canada.

Ovary Assessments

Frames containing sealed brood were removed from a colony and the bees were allowed to emerge overnight in a warm (33°C) room. The following morning, the newly emerged worker bees were caged in groups of 30. They were fed a mixture of royal jelly and honey (1:1) diluted by 10% with water. Both food and water were available *ad libitum* to all of the cages, which were kept at 34°C in the dark for 10 days. The bees were then frozen until dissection.

Both ovaries were examined in each bee, and the most developed ovary was scored using a modified Velthuis (1970) scale (as per Pernal and Currie 2000, Hoover *et al.* 2003). The ovaries were scored as 0: ovaries completely resting

and thread-like, small ovarioles not easily separated; 1: ovaries slightly swollen, but egg cells cannot be distinguished from nutritive cells; 2: ovarioles slightly 'bumpy', egg and nutritive cells can be distinguished, nutritive cells larger than egg cells; 3: ovarioles 'bumpy', egg cells larger than nutritive cells; or 4: at least one ovariole contains a fully mature ovum. Ovaries scoring a 3 or 4 were considered 'developed'.

Seasonal Survey

The ovary development of queenless caged worker bees was evaluated, as described above, from April to November, the most active period for brood rearing in 2002 and 2003 (see Table 5-1 for sampling dates and number of colonies). One cage of bees was taken from each colony sampled at each sampling date, and the mean ovary development score of all the workers in that cage was used in subsequent statistical analyses.

Table 5-1 Number of colonies sampled and date workers were caged for ovary development seasonal survey conducted on caged queenless worker bees

Date	April 30 2002	July 2 2002	September 26 2002	May 18 2003	June 28 2003	August 14 2003	October 1 2003
Number of Colonies	59	27	11	25	20	24	19

In 2003, from June to September, I also assessed the amount of pollen and brood in the colonies rearing the workers used in the survey. These assessments were conducted three weeks prior to removing the emerging brood,

to determine the amount of food resources available to the colonies for brood rearing, relative to the amount of brood they were rearing. This allowed us to generate a pollen: brood ratio, which would indicate the nutritional status of each colony at the time the experimental bees were being reared as larvae. The total comb area containing larvae in uncapped cells and the total area containing stored pollen were measured for each colony. All 2003 workers were also weighed prior to dissection.

To detect seasonal differences, the data were analysed using Analysis of Variance (ANOVA) on the mean ovary score. In 2003, the pollen to brood ratio and mean worker mass per colony were also analysed using ANOVA, with Fisher's Least Significant Difference post hoc test to distinguish between groups. A chi-squared test was used to test the proportion of bees with each development score per sampling date. Linear regression was used to detect relationships between mean level of ovary development and mean worker mass per colony for each sampling date.

Feeding Experiment

The feeding experiment was conducted on 30 small colonies in May 2004, each with a laying queen, one frame of sealed brood, two frames of honey, two empty brood frames, and five frames covered with adult workers. To begin the experiment, all comb containing uncapped larvae, eggs, or pollen was removed from the colonies, and replaced with empty comb. Colonies were randomly assigned to one of two feeding treatments: (1) a high pollen diet, consisting of

400g of field collected pollen sprinkled into one side of a frame (comb), or (2) a low pollen diet, in which the colony received an empty comb. Both treatments received a water-feeder frame to ensure the colonies had adequate access to water. The treatment frames were placed in the colonies early in the morning before any bees were flying. After the combs were all in place, the entrances to all of the colonies were screened using wire mesh screen held in place with tacks. This wire mesh prevented any bees from leaving, while still allowing workers to fan and ventilate the colony.

The colonies remained closed for nine days, during which time the colony queens were laying, but no workers could forage. On the tenth day, the screens were removed from the colony entrances, and the bees were allowed to take cleansing flights to defecate and forage at will. By this time, the cells with eggs laid just after the colonies were closed up were capped, so that the brood would have been reared for their entire larval development with only the amount of pollen available from the experimental treatments.

The experimental brood (that which developed from eggs - larvae – pupae during the treatment period) began to emerge 12 days after the screens were removed. The brood was allowed to emerge overnight and caged as described above. Workers from each colony were put in groups of 30 into two cages. One cage received a low protein diet consisting of 75% honey and 25% water, while the other received a high quality mix of royal jelly and honey (1:1) diluted by 10% with water. In this way, four feeding treatments were created: 1) low pollen diet

as larvae and low protein diet as adults (LL), 2) low pollen diet as larvae but high protein diet as adults (LH), 3) high pollen diet as larvae but low protein as adults (HL), and 4) high pollen as larvae and high protein as adults (HH). As above, these workers were kept for 10 days at 34^0C, and then frozen prior to being weighed and dissected.

The mean ovary score and mean worker body mass per cage of 30 bees were analysed using a 1x4 ANOVA, and differences between the treatments were identified using a Fisher's Least Significant Difference post hoc test. Linear regression was used to detect relationships between colony mean ovary development and mean worker mass. To determine if the effects of diet treatments were additive, less than additive, or synergistic, I compared the sum of the single effects of improved larval and adult diets with the combined effects of improved diet at both life stages using a Wilcoxon test ($\alpha = 0.05$). For this I added the effect of randomly paired samples of corresponding treatments (e.g. changes in ovary development caused by high protein larval diet (HL) plus those caused by high protein adult diet (LH) plus the baseline level of ovary development (LL). The computed sums of single effects (= LL+ (HL-LL) + (LH-LL)) were compared with the corresponding data from randomly chosen samples from the treatment containing both improved diets (combined effects) (HH). If the sum of the single diet effects was less than the combined diet effect, the combined effect would be considered synergistic; if not significantly different the combined effect was considered additive. If the sum of the single effects was

greater than the combined effect, the combined effect would be considered less than additive.

5.4 Results

Seasonal Survey

Mean ovary scores were higher in 2002 than in 2003 ($F(1,178) = 4.15$, $p = 0.04$), and there was a significant difference among the mean scores throughout the brood rearing season ($F(6,178) = 45.99$, $p<0.001$, Figure 5-1). Ovary development was lowest in the spring and early summer months (April 2002 , May 2003, June 2003), highest mid-summer (July 2002), then moderate in the late summer and fall (August 2003, September 2002, October 2003).

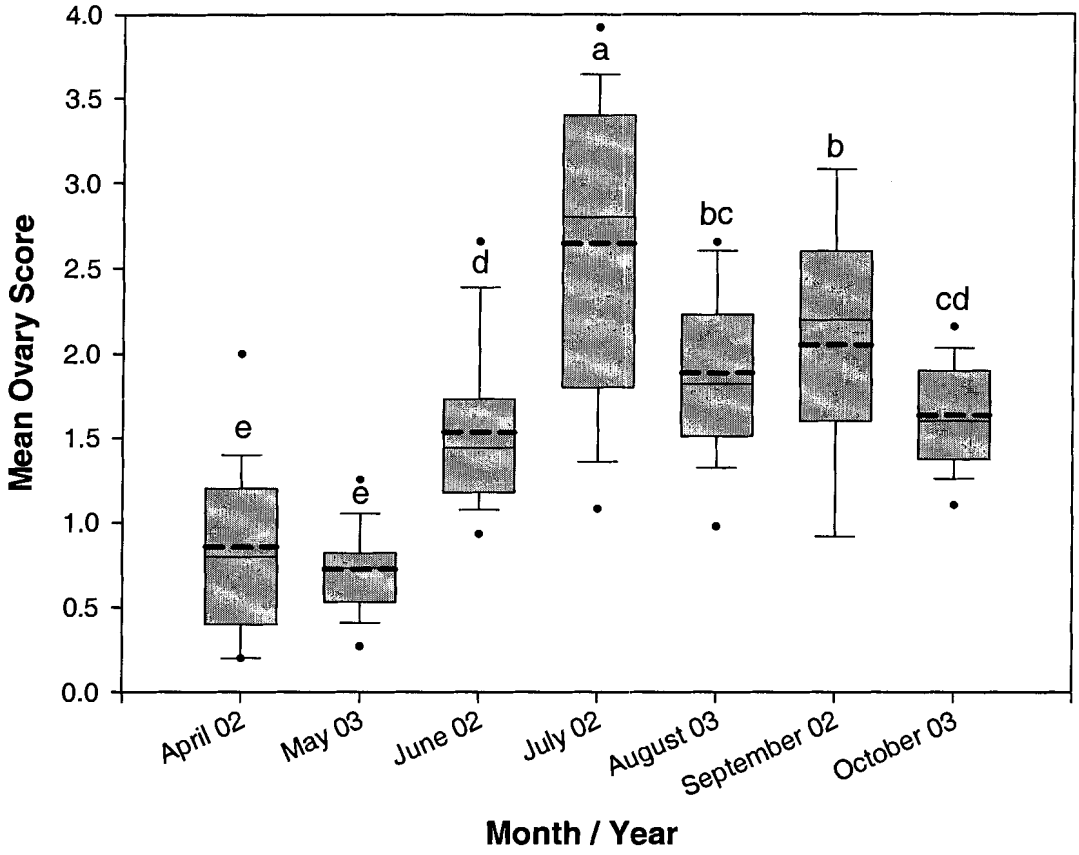

Figure 5-1 Mean ovary development of caged, queenless honey bee workers surveyed in 2002 and 2003.

Significant differences among samples are indicated by different letters (LSD, α = 0.05). Boxes encompass 25th-75th percentiles, whiskers are 5th and 95th percentiles. Dashed lines inside boxes are means, solid lines are medians. Circles outside the whiskers are outliers.

In 2003, bee mass, ovary development, and the ratio of pollen to uncapped brood in the colony all were lowest early in the year, increasing as the season progressed (Figure 5-2). Worker mass at age 10 days was lowest in May ($\bar{x}$=0.110g), then higher from June on (F (3, 84) = 5.54, p = 0.002) ($\bar{x}_{June}$ =0.119g, $\bar{x}_{August}$=0.117g, $\bar{x}_{October}$=0.124g). There was a significant positive relationship between worker mass and ovary score in all 2003 samples except May, when there was very little ovary development (June R^2 = 0.27, p = 0.009, August R^2 = 0.18, p = 0.026, October R^2 = 0.26, p = 0.004). The ratio of the area of comb occupied by pollen to that occupied by brood increased from mid-summer through to the fall (F (2, 75) = 6.77, p = 0.002). In the June assessment, the ratio was less than one ($\bar{x}$ = 0.86 ± 0.12) indicating that there was a greater area occupied by brood than by pollen, whereas in both August and October there was more pollen than brood ($\bar{x}_{August}$=2.86 ± 0.53, $\bar{x}_{October}$=5.52 ± 1.44).

There were differences throughout the season in ovary development not only in the mean ovary score per colony, but also in the proportion of workers with ovaries at each developmental stage (χ^2 (12) = 774.5, p = 0.001, Figure 5-3). The vast majority of workers in May had either score 0 (completely resting) or 1(slightly swollen) ovaries, whereas workers in June and October generally had score 1 or 2 (eggs and nutritive cells distinguishable) ovaries. No score 3 ovaries were found until June, and ovaries containing fully mature eggs (score 4) were only found in August. At this sampling date, many workers had score 1, 2, and 3

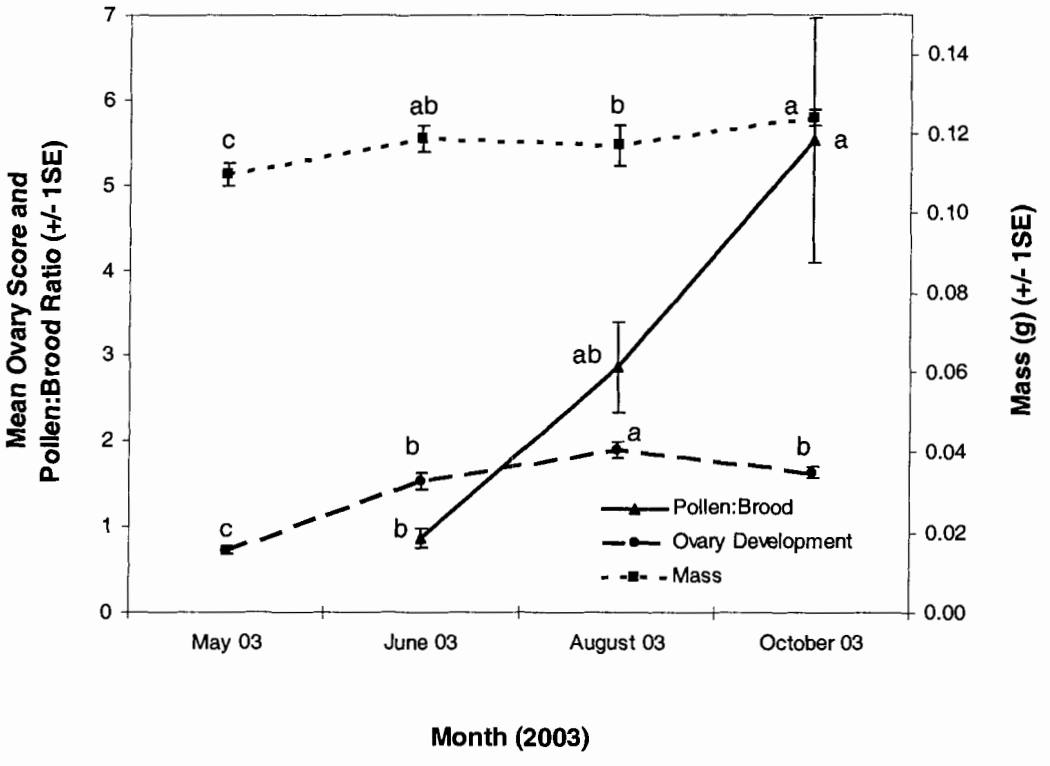

Figure 5-2 Mean worker ovary development of honey bee colonies surveyed in 2003.

Ovary development (dashed line), mean worker mass (dotted line) and pollen:brood ratio (solid line), by sampling date. For each parameter, significant differences among sample dates are indicated by different letters (LSD, α = 0.05).

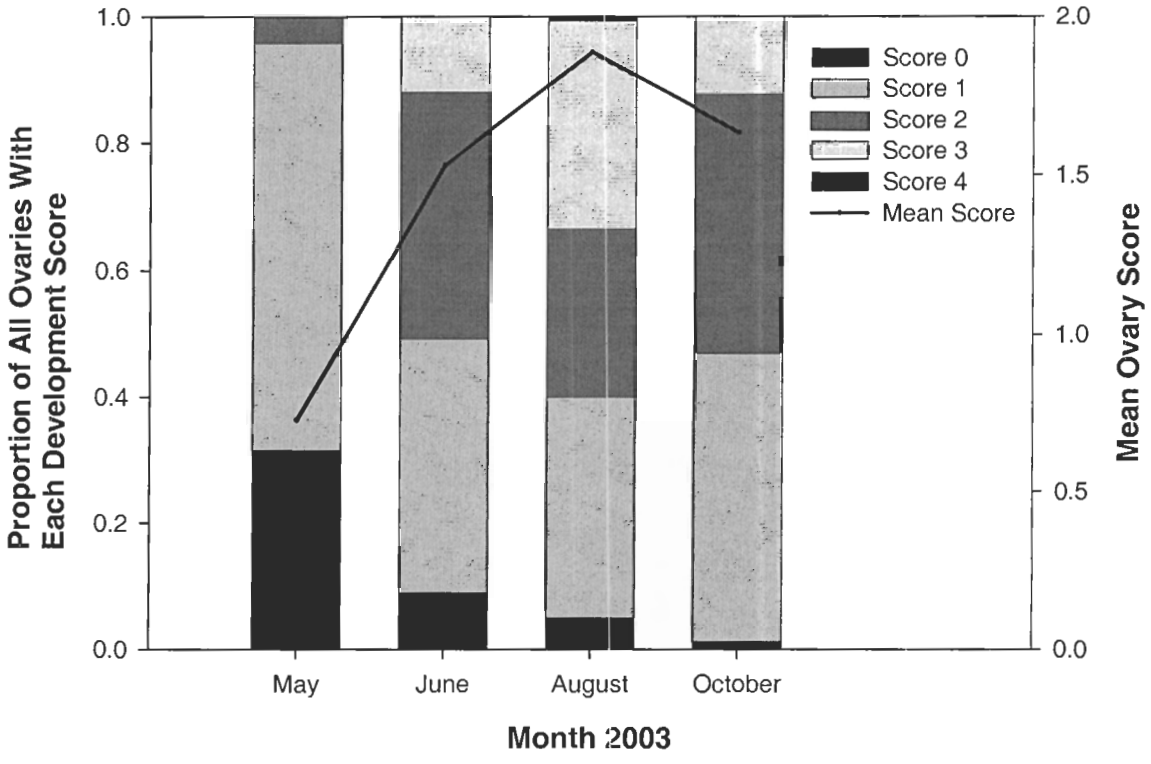

Figure 5-3 Proportion of all workers with each ovary development score for 2003.

Ovary development samples at each sampling date are represented by bars, and the solid line represents mean ovary development.

ovaries, with a few having 0 and 4. While the mean score in 2003 was highest in August, the lowest proportion of workers with completely resting (0) ovaries was found in October, having decreased throughout the season.

Feeding Experiment

Both adult and larval diet affected ovary development ($F(3, 44) = 25.11$, $p = 0.001$, Figure 5-4). The workers given protein-poor diets at both the larval and adult life stages had the lowest level of ovary development, followed by those fed high protein diets as larvae but poor quality diets as adults, and poor quality diets as larvae but high protein diets as adults. Workers fed high quality protein-rich diets as both larvae and adults had the highest levels of ovary development. In the two low protein adult diet treatments (LL and HL) no bees had 'developed' ovaries (those scoring a 3 or 4), whereas 2.8% and 6.9% of those in the high protein adult diets had developed ovaries (LH and HH, respectively).

As there was no difference between the sum of the single diet effects and the effect of the combined diet, the effects of larval and adult diet were considered additive ($p = 0.594$, see arrows, Figure 5-4). One worker in the HH treatment was found to have more than 50 ovarioles in each ovary, but the other workers from this colony did not have an unusual number of ovarioles, nor was the worker queen-like in any other morphological characteristic. The workers fed the protein-rich adult diet weighed more at 10 days old than those fed the low protein adult diet, regardless of larval dietary protein levels ($F(3,22) = 4.78$, $p = 0.01$)

(Figure 5-5). The difference between the mean mass of workers fed the highest quality diet (HH, $\bar{x}$ = 0.083g) and lowest quality diet (LL, $\bar{x}$ = 0.067g) was 0.017g, however workers fed the HL treatment had the lowest masses of all treatments ($\bar{x}$=0.064g). There was a significant positive overall (among treatment) relationship between mass and ovary development (R^2=0.23, p = 0.014), but this relationship was not significant within any of the treatments (p>0.05).

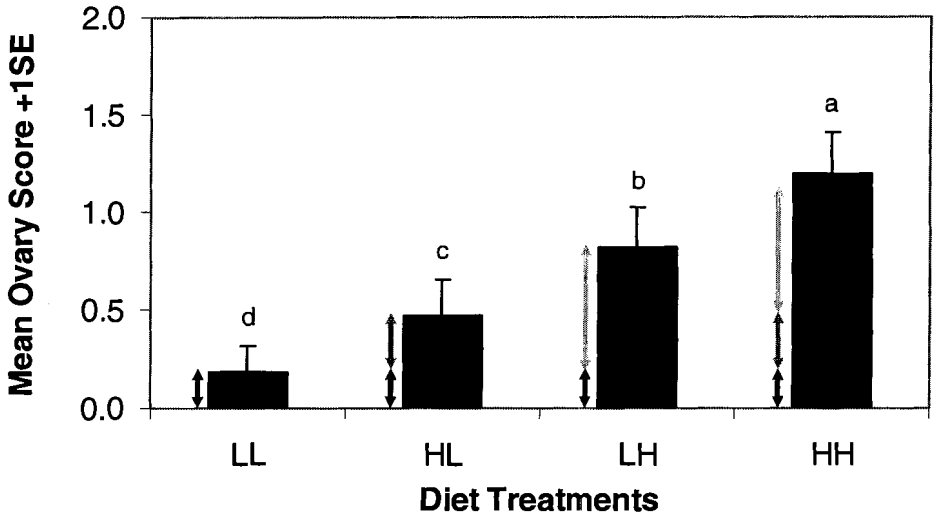

Figure 5-4 Mean ovary development of worker bees given one of four diet combinations.

Treatments were: low pollen as larvae and low protein as adults (LL), high pollen as larvae but low protein as adults (HL), low pollen as larvae but high protein as adults (LH), and high pollen as larvae and high protein as adults (HH). Significant differences among samples are indicated by different letters (LSD, α = 0.05). Arrows represent the magnitude of each diet / life stage combination alone. Black arrows represent the basal level of ovary development observed when workers are fed protein poor diets as both larvae and as adults, dark grey arrows represent the increase in ovary development observed in workers fed high protein diets as larvae, and light grey arrows represent the increase in ovary development observed in workers fed high protein adult diets. The effects of enhanced adult and larval diet on worker ovary development were additive, as visually demonstrated by stacking all the arrows.

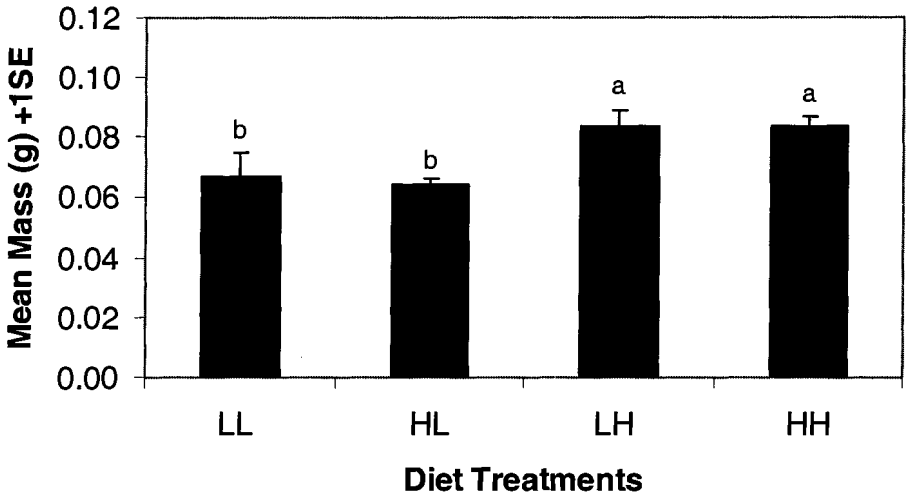

Figure 5-5 Mean mass of worker bees given one of four different diet combinations.

Low pollen as larvae and low protein as adults (LL), high pollen as larvae but low protein as adults (HL), low pollen as larvae but high protein as adults (LH), and high pollen as larvae and high protein as adults (HH). Significant differences among samples are indicated by different letters (LSD, $\alpha = 0.05$).

5.5 Discussion

The results of this study show that both larval and adult worker nutrition greatly affect body mass and ovary development. In the seasonal survey, the ovary development of queenless caged workers was highest from mid-summer through the fall. This trend is likely caused both by the seasonal availability and quality of pollen, and by the quantity and quality of nurse bees available to feed developing brood. Bees emerging in spring would have been developing before the bulk of the plants flowered in this region, and these bees had lower masses and levels of ovary development (Figure 5-2). In contrast, bees emerging in mid-summer would have developed when many local plants were in flower to provide ample pollen. As expected, these workers had the highest levels of ovary development. However, the availability of nurse bees likely also varies seasonally, and it is probable that a small number of nurse bees would be less effective at provisioning larvae than a large number of nurse bees with equivalent pollen stores. Further, workers developing in the early spring would have been fed by old bees that had survived through the winter, compared to those reared later in the year, which would have had a large population of high quality nurse bees. Both of these factors, together with pollen availability, likely contributed to the observed seasonal trend in ovary development.

While fewer individuals had completely resting ovaries in October, workers with fully mature eggs were only found in the August sample in 2003. If these workers were to lay eggs, the resultant drones would not be fully sexually mature

for another six weeks, well into October. Because the peak swarming period occurs mid-spring (Winston 1987), the majority of new queens would have already mated. The timing of swarming is relevant not only because it is a source of virgin queens, but also because it is a major cause of queen loss both in the new colony, and the original one. While colonies are likely to go queenless in the spring, these results show that very few workers would be able to develop their ovaries and rear drones at this time. There is a second, smaller peak of colony swarming in the fall (Winston 1987), and the drones laid mid-summer may be able to mate with the virgin queens taking over established colonies at this time. Queens can also be lost due to disease or accident at any time of the year.

Kropáčová and Haslbachová (1969) and Pain (1955) found seasonal variation in ovary development in queenright colonies, and found the highest levels in the spring. In contrast, Velthuis (1970) found the highest levels of ovary development in queenless bees from June through August, in colonies surveyed over a six year period. Velthuis' (1970) results agree with my own survey of queenless bees, and he also attributed the pattern to seasonal fluctuations in pollen availability. It is interesting to note that the timing of maximal ovary development of queenright bees coincides with period of peak swarm issuance, whereas the timing of maximal ovary development in queenless bees appears to be more dependent on nutritional factors.

The results of the feeding experiment clearly link larval nutrition to adult fecundity, however, carryover of larval reserves to the adult life stage appears to be less important to adult fecundity than adult nutrition. Workers fed well as adults had higher development scores than those fed poorly as adults, regardless of larval diet. The effect was additive, with the result of good diet in both life stages roughly equal to the increases caused by adding together the effects of good diet in either the larval or adult life stages alone. The two treatments with high protein adult diets resulted in higher ovary scores than the two high protein larval diets. This may be partially the result of the particular diets used in this study, but this result suggests the importance of adult diet to the physiological development of worker bees. It further demonstrates that while larval nutrition also influences ovary development, they do not rely on nutritional carry-over or 'physiological priming' from larval development to develop their ovaries as adults. Workers fed poorly as larvae are still able to have high levels of ovary development, if given access to a large quantity of high quality food as adults.

The high quality adult diet also resulted in heavier workers at 10 days old than low quality adult diet. While both the high and low protein adult diets were fed *ad libitum*, I did not measure the workers' consumption of the food, and workers given the high protein treatment could have eaten more than workers given the low quality diet. Because the adult diet had an effect, adult worker mass is not determined exclusively by larval diet. In fact, in this study, I saw no effect of larval

diet on adult mass at age 10 days. During development, the larvae were fed by nurse bees, who can adjust the quality and quantity of food given to each larva. In colonies with food shortages, honey bee nurses can adjust the nutrition they give to developing larvae by depleting their own nutrient reserves, or by cannibalizing other larvae. Thus the actions of the nurse bees could have mitigated any potential effects of larval pollen environment on adult worker mass. However, as I only measured adult mass at 10 days old, it is possible that an effect of larval diet could have been compensated for by adult feeding during those 10 days.

Pernal and Currie (2000) also found an effect of adult dietary protein on worker ovary development. They used larvae reared in open colonies, then fed the adults diets varying in pollen quality. Bees reared in open colonies then fed no pollen as adults had a mean ovary score of ~0.2, roughly equivalent to the LL treatment. Because my colonies were closed during the development period of the test workers, all the adult workers were forced to remain inside the colony. It is possible that this induced an increase in nursing activity, as older workers were unable to forage. Physical contact with foragers inhibits the behavioural maturation of young workers, delaying the transition from nurse to forager (Leoncini *et al.* 2004). This could have led to increased ovary development scores in this study if it increased the number of nurse bees available to feed brood compared to open colonies. Lin and Winston (1998) also found an effect of adult diet on ovary development. Their value for workers fed honey alone was ~0.5, and for 40% and 60% royal jelly were ~1.7 and 2.2, respectively. The mean

scores for workers fed honey alone are similar to ours, but are higher for those fed royal jelly. These results may also differ from ours due to seasonal effects on ovary development.

Larval nutrition is the proximate mechanism by which the African honey bee *A.m. capensis* is a social parasite of *A. m. scutellata*. When reared by workers of other subspecies, *capensis* workers receive more, higher quality food (Calis *et al.* 2002). This results in a whole suite of morphological and physiological changes in the workers, making them more queen-like (Calis *et al.* 2002). Anarchistic worker larvae (*A. mellifera* of mixed European descent), while able to lay in queenright colonies, are not fed more as larvae than wild type workers, and are not worker – queen intermediates (Beekman and Oldroyd 2003). Beekman and Oldroyd (2003) found that anarchists are poor nurses, and feed both wild type and anarchist brood less than they would be fed by wild type nurses. Anarchists, unlike Cape bees, therefore gather the resources for ovary development as adults; rather than directing the nutrients they receive towards nursing the brood, anarchists direct it toward developing their own ovaries. The feeding experiment demonstrates the importance of adult nutrition to ovary development in honey bees, and combined with the results of Beekman and Oldroyd (2003) clearly demonstrates the potential for exploitation by 'cheater' individuals.

There are two pathways by which holometabolous insects can sequester the resources required for ovary development. They may either carry these resources forward from their larval stage, or gain them as adults. In honey bees,

because caste differentiation is mediated via larval nutrition, the result of larval carry-over may be worker – queen intermediates, as in the Cape bees. The result of acquiring these nutrients as adults is ovary development without any other queen-like characteristics, as in the anarchistic bees.

There are many consequences of pollen shortage for honey bee colonies. Colonies with little stored pollen increase their foraging effort by individual changes in foraging rate and load size, as well as by recruiting more foragers (Fewell and Winston 1992, Fewell and Bertram 1999). During times of pollen dearth, when little food is available to nurse bees, they cannibalise young larvae. This behaviour has a two-fold benefit to the adults in the colony; they reduce the number of brood to feed, leaving only older larvae that have a short remaining developmental time, and they gain a source of protein that can be used to feed the older larvae (reviewed in Moritz 1994, Schmickl and Crailsheim 2001). The colonies given no pollen in the feeding experiment had relatively little brood, whereas the colonies given 400mL field-caught pollen reared more typical amounts of brood. I believe that this is likely due to worker cannibalism of the queen-laid eggs and young larvae rather than lack of egg-laying by the queen. This cannibalism would also have served to moderate the effect of low pollen availability on the remaining larvae.

The poor quality adult diets in the feeding experiment resulted in lighter workers. The average weight of a honey bee worker is 81 to 140 mg (Winston 1987). The bees in the feeding experiment ranged from an average of 64 mg in the HL

treatment to 83 mg in the highest quality nutrition treatment (HH). In the seasonal survey, the worker weights range from an average of 110 to 124 mg. Even early in the spring, when worker weights were at their lowest, they were higher than those from the high quality diet treatment in the feeding experiment. As the adults in the high quality treatment were caged and fed in the same manner as the survey workers, this difference must be the result of their larval environment. I did not, however, find any difference in worker weight between the high and low larval pollen treatments in the feeding experiment. It would seem likely then, that either the nurses in the 2004 feeding experiment were of poor quality or quantity relative to those in the 2003 survey of large colonies, or closing the colonies in the feeding experiment had a detrimental effect on worker weight.

Taken together, the results of these studies demonstrate that both nutrient reserves carried forward from the larval stage and nutrients acquired as adults are important to worker ovary development in honey bees. The implication of these results is that even given equal genetic propensity, some individuals will be more capable of becoming laying workers based purely on the circumstances of their larval and adult environment. Depending on time of year, colony state, and the method of food distribution, workers in social insect colonies will have varying ability to develop their ovaries should the colony lose its queen. Workers could potentially act nepotistically in queenless colonies, directing nutrition to more related colony members who could then develop their ovaries and lay male eggs.

Future experiments should focus on the influence of nurse quality and quantity on subsequent worker ovary development in honey bees, as well as the differences between ovary development in queenright and queenless colonies.

CHAPTER 6: DISRUPTIVE SELECTION ON WORKER STERILITY IN THE HONEY BEE

6.1 Abstract

Disruptive selection for worker sterility in the honey bee (*Apis mellifera* L.) resulted in the production of lines of bees with high or low levels of worker ovary development when queenless. By the third generation of selection, the high line had six times the mean ovary development score of the low line. Colony pollen income was strongly and positively correlated with ovary development, and the amount of pollen available to nurse bees appeared to affect larval nutrition, and consequently adult worker ovary development. Unselected colonies were intermediate to the high and low lines in both ovary development and pollen income. Both genotype and larval rearing environment affected worker ovary development, as cross-fostering the high and low lines resulted in workers with intermediate mean ovary scores. Thus, there was both a direct genetic effect on worker ovary development and an indirect genetic effect on the rearing environments within the colonies, mediated by pollen income influencing larval nutrition. These results provide further support for the recent suggestion that the suite of traits governing foraging is associated with worker reproduction in a complex manner.

6.2 Introduction

Workers in honey bee colonies with a mated queen present (queenright) are functionally sterile, and worker reproduction is rare (Jay 1968, Ratnieks 1993). However, workers will develop their ovaries and lay eggs should a colony lose its queen and be unable to rear a replacement. Worker honey bees cannot mate, and normally only lay unfertilised male eggs. These worker-laid drones can greatly increase the fitness of an otherwise sterile worker by mating with new queens from other colonies. Not all workers are able to reproduce, however, and workers compete amongst themselves for ovary development and drone production (Miller and Ratnieks 2001). In this chapter I present the results of a disruptive selection program for high and low levels of worker ovary development in queenless worker honey bees, both in cages and small colonies, as well as the results of cross-fostering these lines, in order to explore the proximate and ultimate factors mediating these aspects of worker reproduction.

Many honey bee behaviours have a demonstrated genetic component, including the retinue response of workers to a queen (Pankiw *et al.* 2000), defensive behaviours (Boch and Rothenbuler 1974, Breed and Rogers 1991), foraging and pollen-hoarding (Danka *et al.* 1987, Calderone and Page 1988, Frumhoff and Baker 1988, Page and Fondrk 1995), hive cleaning (Rothenbuler 1964, Robinson and Page 1988), tendency to swarm (Winston 1980), grooming (Frumhoff and Baker 1988, Kolmes 1989), and response to alarm pheromone (Collins 1979), among others. Selective breeding of honey bee lines for specific behaviours has been a fruitful avenue of research. Selection for high and low responding lines

of bees for pollen foraging (Page and Fondrk 1995) and worker response to queen pheromones (Pankiw *et al.* 2002) have yielded numerous important discoveries about foraging and communication in social insects (Pankiw and Page 2001, Pankiw *et al.* 2002, Keeling *et al.* 2003, Amdam *et al.* 2004). As reproductive division of labour is a defining feature of eusocial insect societies, selecting for lines of bees with high and low levels of worker reproduction (ovary development) may yield new insights into the evolution of such altruism.

In addition to genotype, rearing environment can influence the phenotype of honey bee workers in several ways that impact fitness at both the individual and colony level. For example, the nutrition workers receive as larvae affects their ability to develop their ovaries and lay eggs later in life (Beekman *et al.* 2000, Hoover *et al.* 2005b). Workers reared in colonies with little stored pollen have lower levels of ovary development as adults than workers reared in colonies with a surplus of pollen (Hoover *et al.* 2005b). The Cape honey bee *Apis mellifera capensis* is a social parasite of *A. m. scutellata* that uses differential larval feeding to gain reproductive advantage over its hosts (Beekman *et al.* 2000, Calis *et al.* 2002). Rearing environment also can affect individual behaviours such as nectar foraging, which in turn affect the honey reserves and therefore overall fitness of a colony. In a study of colonies selectively bred to hoard pollen, workers had higher response thresholds to sucrose solutions of varying concentrations when reared in high pollen-hoarding colonies than low pollen-hoarding colonies (Pankiw *et al.* 2002).

The rearing environment can itself be considered a colony-level phenotype, influenced by both the genetic composition of colony members, and the external environmental conditions experienced by the colony (Pankiw *et al.* 2002). In this way, the rearing environment can be an indirect genetic determinant of individual phenotype (Pankiw *et al.* 2002). Genetic differences between the two selected honey bee lines could influence the larval rearing environments within these colonies and subsequently the phenotypes of workers reared under these conditions.

I cross-fostered worker bees from the high and low ovary development lines to determine the effect of rearing environment on worker ovary development. I also measured the amount of pollen collected by the selected lines and unselected colonies, as protein availability has a large impact on worker ovary development (Hoover *et al.* 2005b).

6.3 Methods and Materials

Ovary Development Assay

To assess the ovary development of queenless caged workers, frames of comb containing pupae were taken from colonies in the Simon Fraser University apiaries, and incubated overnight. The following morning, 30 newly emerged workers from each colony were put into cages, with each cage containing workers from only one colony. Caged workers were kept at 32°C in the dark, were daily fed a 1:1 mixture of honey and royal jelly by volume diluted by 10% with water, and had free access to water. After 10 days the worker bees were

frozen, and stored in sealed Petri dishes to avoid desiccation prior to dissection. Both ovaries from each bee were dissected, and the most developed ovary was scored using a modified Velthuis (1970) scale (as per Pernal and Currie 2000, Hoover *et al.* 2003). The ovaries were scored as 0: ovaries completely resting and thread-like, 1: ovaries slightly swollen, but egg cells cannot be distinguished from nutritive cells; 2: egg and nutritive cells can be distinguished, nutritive cells larger than egg cells; 3: egg cells larger than nutritive cells; or 4: at least one ovariole contains a fully mature ovum.

Disruptive Selection Programme

A total of 59 Simon Fraser University honey bee colonies in the Fraser Valley, British Columbia were assayed in this manner in April 2002 to determine the variability in worker ovary development. Of these colonies, the 17 highest and 10 lowest scoring were again surveyed in July 2002. From these, 'high' and 'low' breeder colonies (generation 0) were selected as those demonstrating a seasonally consistent high or low response in the ovary development assay. These colonies all were headed by naturally mated queens derived from stock acquired from a number of commercial bee breeders in British Columbia, Canada, had been subject to standard beekeeping management practices, and were not a part of any other experimental programs. The four lowest scoring colonies and eight highest scoring colonies were used in an isolated mating program at Roberts Bank, BC. As only one isolated mating area was available, the low line was brought in and allowed to mate, then removed before high line colonies were brought in. In each case, half the colonies identified by the survey

were used as queen sources, and half were used as drone sources, to ensure that queens were not mating with their brothers. For the low line, the two colonies with the highest drone population were used as drone sources, and queens were reared from the other two colonies. For the high line, four colonies were randomly chosen to be drone sources, and four queen sources. The resultant mated, laying generation 1 queens were subsequently introduced to well-established colonies in Burnaby, BC.

In early June 2003, the eight high and three low colonies (generation 1) were surveyed to determine their response in the ovary development assay. The three highest and lowest scoring colonies were subsequently used as queen and drone sources for the next generation high and low lines, respectively. Instrumental insemination was used to mate queens from each of the three high and low source colonies to drones from the other two high or low source colonies, such that no queen was mated to her brother. These generation 2 queens were then introduced to established colonies. The following summer (early July 2004), these colonies were surveyed for ovary development and again the three highest and lowest scoring colonies were used as queen and drone sources for instrumental insemination of generation 3 queens.

Once laying, the resultant queens (generation 3) were introduced into established colonies in a single apiary, where they were allowed to rear brood and overwinter according to normal beekeeping practices in the region. The following summer, all workers in the colony would have been laid by the selected

queen. Instrumentally inseminated queens generally have poor longevity, and only one queen of each of the selected lines survived the winter with enough remaining sperm to rear a high proportion of worker brood the following summer (2005). All subsequent surveys and experiments were performed on these selected colonies, together with unselected (wild type) colonies. The ovary development of queenless caged workers from the high and low colonies, and three unselected colonies from the same apiary were surveyed in June 2005.

Queenless Colonies

Three small nucleus colonies were split from each of the high, unselected, and low colonies. Each of these colonies remained queenless, but was given a single frame each of pollen and honey, and three frames covered in worker bees. The mean worker ovary development score of these colonies was measured 10 days after they were made-up to compare the mean score of workers in cages and those in small colonies, a more natural setting for worker reproduction to occur.

Pollen Income

The pollen income of the high and low line colonies and the three unselected colonies was measured over the seven day period prior to the laying of the eggs used in caged worker ovary assessments. Each colony was fitted with a pollen trap under the bottom of the hive, consisting of layers of wire mesh that acted to scrape the pollen off the legs of incoming foragers. The total amount of pollen collected was divided by the number of days the trap was in place (7), and the

number of full boxes (supers) housing the colony (a measure of relative colony size) to give an estimate of the daily amount of pollen coming into the colony, scaled by colony size (gm/super/day).

Cross-fostering

In May 2005, I exposed workers from the high and low ovary development lines to different larval rearing environments (Figure 6-1). By transferring comb containing eggs, high and low line larvae were reared in both high and low line colonies from the egg to pupal stage. In this way four experimental treatments were created: high line workers reared in a high colony (H:H), high line workers reared in a low colony (H:L), low line workers reared in a high colony (L:H), and low line workers reared in a low colony (L:L). Frames of pupating bees were removed from their rearing colony prior to worker emergence, and placed in emergence cages in an incubator. A total of 30 newly emerged high line, low line, or wild type workers were then put into smaller cages, and their ovary development after 10 days was assessed as described above. In this experiment, the null hypotheses are that genotype and rearing environment will have no effect on the ovary development of individual workers. The alternate hypotheses are that workers reared in the high line colony, and those that were genetically high will have higher mean ovary scores than their counterparts that are reared in the low line or are genetically low.

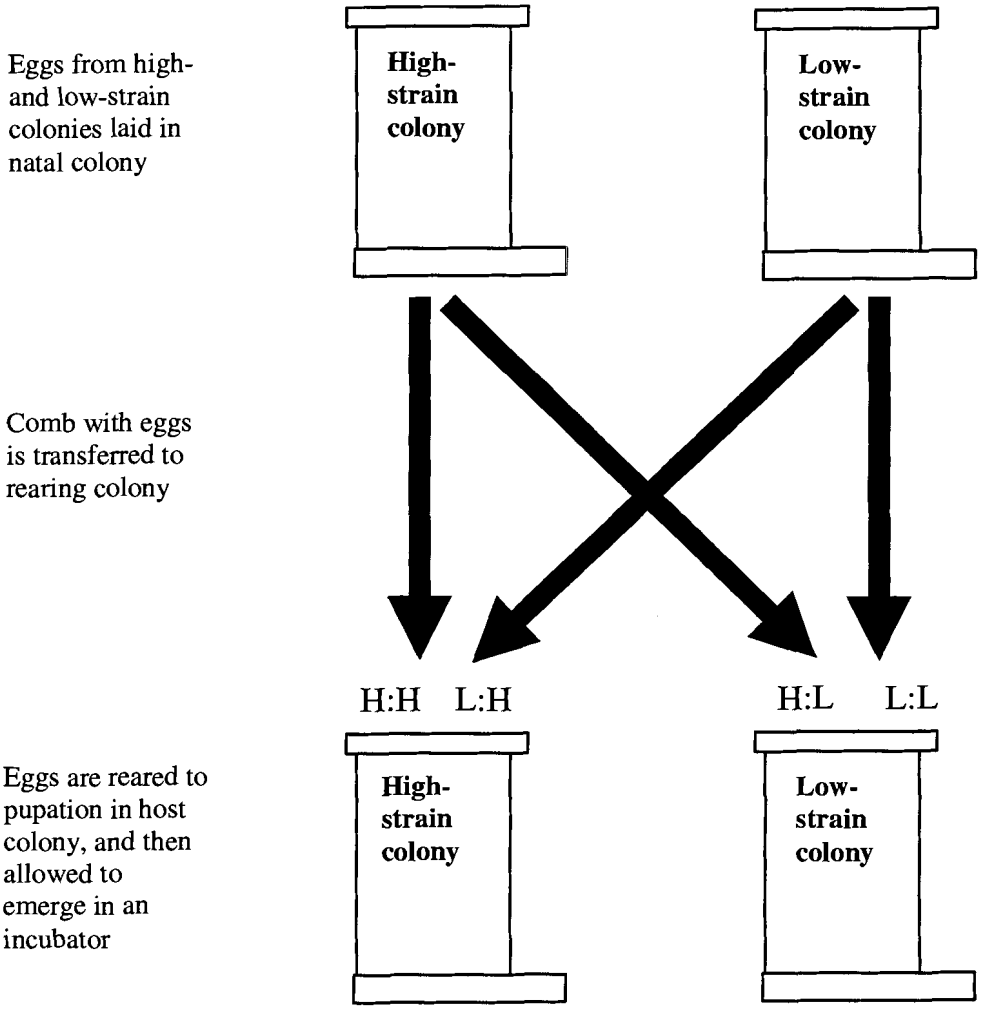

Figure 6-1 Experimental design of cross-fostering experiment.

Combs containing worker eggs from high and low line colonies were transferred to both high and low line colonies, where they were reared until pupation. Combs containing the worker pupae were then removed from the colonies, and the bees were allowed to emerge in an incubator. The four experimental treatments produced are (natal colony: host rearing colony) 1) high:high (HH), 2) low:high (LH), 3) high:low (HL), and 4) low:low (LL).

Statistical Analyses

Disruptive Selection Programme, Queenless Colonies, and Pollen Income

In the parental generation and the first two selected generations, the ovary development scores of high and low line workers were compared using t-tests on colony mean ovary scores. In 2005 there was only one colony remaining in each line, and the ovary development data were not normally distributed. Comparisons of 2005 data were made using individual ovary development scores with a Kruskal-Wallis test (χ^2 approximation).

Colony pollen income and mean ovary development scores were compared using linear regression. The Kruskal-Wallis test was also used to compare between the selected lines and the unselected colonies both in the caged workers and the queenless nucleus colonies.

Cross-fostering

Because the data were non-normal, non-parametric tests were used to analyse the results of the cross-fostering experiment. The effects of genotype and larval rearing environment on worker ovary development were analysed using the Kruskal-Wallis test. As I had *a priori* expectations that workers reared in the high line colony, and those that were genetically high would have higher mean ovary scores than their low line counterparts, I used one-tailed tests to determine if there were effects of rearing environment or genotype. The interaction of these two factors was analysed using analysis of variance (ANOVA), as it is robust to departures from normality (Zar 1984).

6.4 Results

Disruptive Selection Programme

The selection programme resulted in lines of bees with significantly different levels of ovary development. Colonies selected in 2002 to parent the first generation of high and low ovary development lines had mean scores of $\bar{x}_{high}$=3.57 ± 0.10 and $\bar{x}_{Low}$=1.65 ± 0.22 (Figure 6-2) and the data were normally distributed. The high parent stock had significantly higher mean ovary scores than did the low parent stock ($t(9) = 9.81$, $p = 0.0001$). The selected lines did not differ in their ovary development scores in generation 1 (2003, $t(9) = 0.97$, $p = 0.36$). This may be at last partially due to the rainy spring and early summer experienced in Vancouver in 2003. The high line possessed higher scores than the low line in generation 2 (2004, $t(11) = 4.63$, $p = 0.0007$), and in 2005 (generation 3) ovary development of workers from the high colony was significantly higher than those from the low colony ($\chi^2(1) = 12.07$, $p = 0.0005$), Figure 6-2). In this generation, the high line had six times the mean ovary score of the low line. While environmental variation between years makes it difficult to compare absolute values of ovary development between generations, the general trend is for decreasing ovary scores in the low line, with relatively little change in the high line scores after the first selected generation. In 2005, laying workers were observed in a queenright colony belonging to the high line. This condition persisted for several months, and worker-laid drones were reared to maturity.

Comparisons of ovary development of caged workers from generation 3 (2005) selected lines to unselected wild type workers revealed a significant effect of worker type on ovary scores ($\chi 2=11.57$, $p = 0.003$, Figure 6-3), with unselected colonies intermediate between the high and low line colonies.

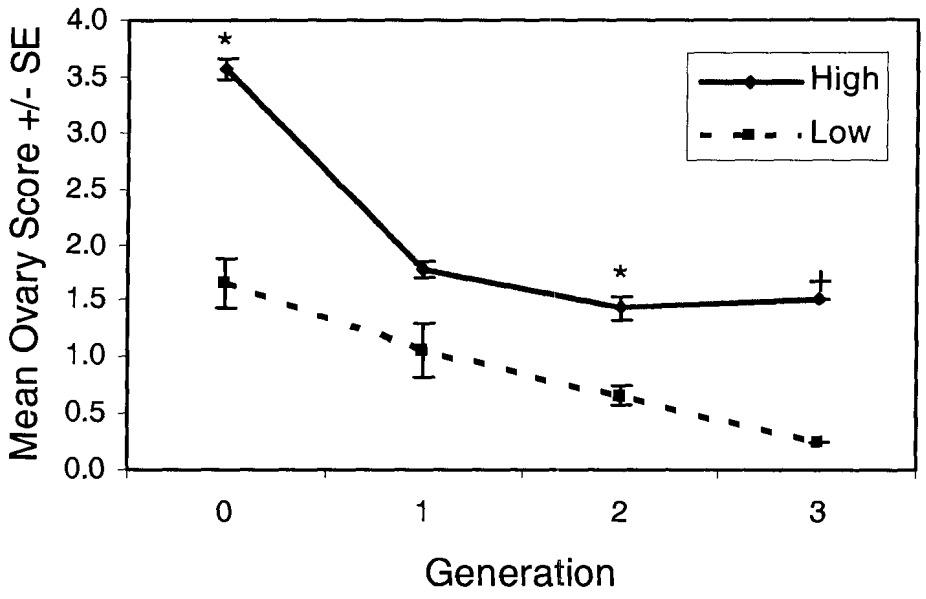

Figure 6-2 Mean ovary development scores of the initial parent colonies and three generations of the selected high and low lines.

Asterisks represent significant differences between high and low lines. Plus indicates significant differences between individual high and low colonies (n= 1 high and low in 2005).

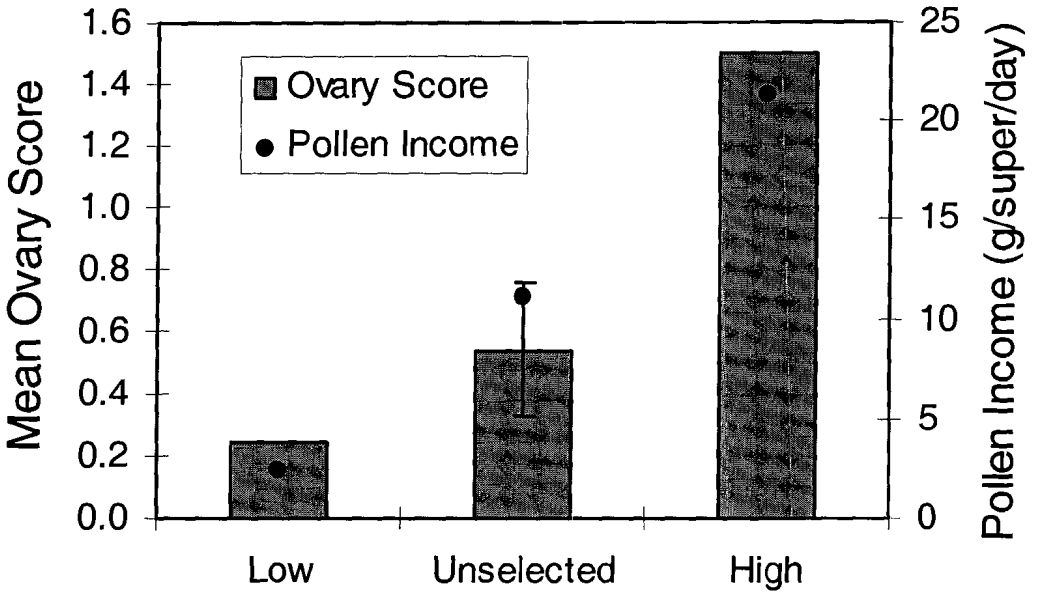

Figure 6-3 Mean ovary development of caged workers from selected lines. Low (n = 1), unselected (n = 3), and high (n = 1) colonies in June 2005. Dots are mean daily pollen income, scaled by colony size. Error bars are 1SE.

Queenless Colonies

There was a significant difference between the lines in the ovary development of queenless worker bees in small colonies (χ^2 (2) = 22.78, $p < 0.0001$), with the high line workers ($\bar{x}$ = 2.18 ± 0.58) being more developed than either the unselected workers ($\bar{x}$ = 1.05 ± 0.23) or the low line workers ($\bar{x}$ = 0.81 ± 0.20) (Tukey's HSD). These results show the same trend as that of the caged workers, although the magnitudes of all the mean ovary scores are increased. This may be due to a seasonal trend towards increasing ovary development levels over the summer (Hoover *et al.* 2005b), as the workers from the small colonies were collected later in the season than the caged workers.

Pollen Income

There was a significant relationship between colony pollen income and caged worker ovary development ($F = 35.04$, $p = 0.01$, $r^2 = 0.92$, Figure 6-3), with the high line colony having both the highest ovary development scores and the highest pollen income, and vice versa for the low line colony.

Cross-fostering

In the cross-fostering experiment, high line workers had higher ovary development than did low line workers regardless of larval rearing environment. However, both genotype (line) and larval rearing environment significantly affected ovary development scores (genotype: $\chi^2 = 11.63$, $p_{1\text{-tailed}} = 0.0003$; rearing environment: $\chi^2 = 2.90$, $p_{1\text{-tailed}} = 0.04$; Figure 6-4). There was no

interaction between genotype (line) and rearing environment ($F(1) = 15.95$, $p = 0.42$).

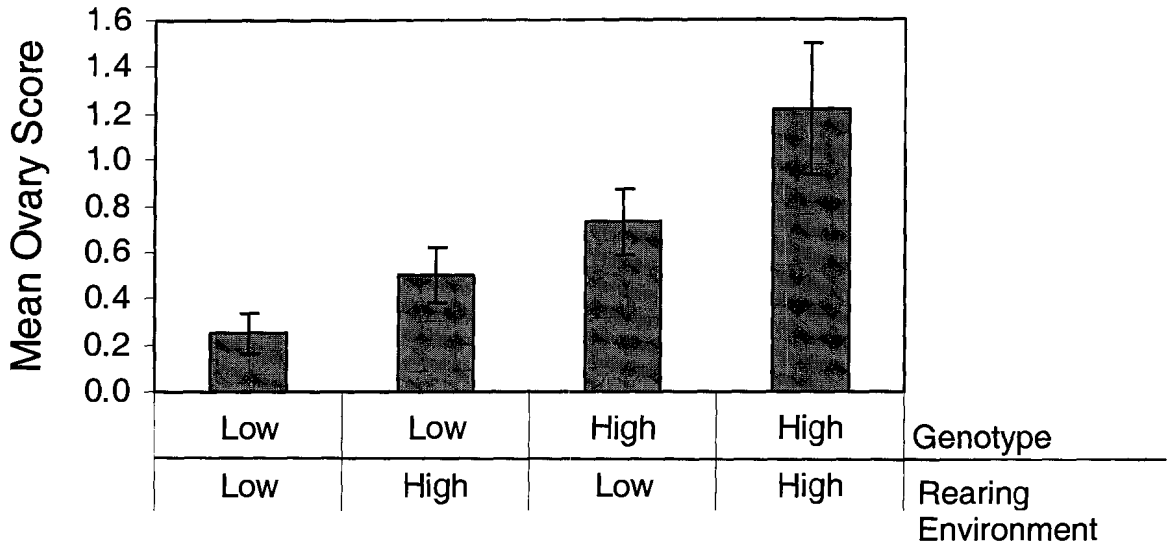

Figure 6-4 Mean ovary scores of queenless caged workers from selected lines reared either in their natal colony or cross-fostered into the other selected line. Genotype = selected line acting as egg source colony, rearing environment = selected line rearing workers from egg to pupal stage (host colony). Error bars are 1SE, replicates are cages from the same colony.

6.5 Discussion

Disruptive Selection Programme

I was successful at selecting lines of honey bees with high and low levels of ovary development, providing a valuable tool in understanding the evolution of worker sterility in honey bees. The high and low lines give us some indication of the potential variation between colonies in the level of worker reproduction when queenless, and they can be compared to determine potential fitness benefits and costs of worker reproduction. Further, these lines show differences in the amount they forage, and should be compared to lines selected for high and low pollen foraging (Page and Fondrk 1995) to help elucidate the link between reproduction and foraging in social insects.

The initial survey revealed a large amount of variation in ovary development of caged workers from colonies in the Simon Fraser University apiaries, suggesting that there may be a wide range in the propensity of workers in unselected colonies to develop their ovaries upon queen loss, and subsequently rear worker-laid drones. The first selected generation of the high line had much lower scores than the parental generation. This phenomenon is frequently observed in selection programmes as favourable genotypes may be disrupted by selection and recombination (Harlt 1988, Pankiw *et al.* 2000).

Previous studies have demonstrated the importance of both larval and adult nutrition to worker ovary development (Pernal and Currie 2000, Beekman *et al.* 2000, Hoover *et al.* 2005b In this study, the ovary development of both the selected lines and the unselected colonies was strongly correlated with the amount of pollen being brought into the colony at a time just prior to the period in which the experimental workers were larvae. In terms of both ovary development, and pollen collection, the unselected colonies (wild type) were intermediate between the high and low lines. Consequently, the selection program may have acted to produce lines of bees with high and low levels of pollen foraging.

Cross-fostering

Cross-fostering the selected lines demonstrated that there is both a genetic component to ovary development in individuals, as well as an effect of colony rearing environment, which in itself is likely partially genetically determined. This type of colony level genetic effect on rearing environment has previously been observed in lines of bees selected for high and low levels of pollen hoarding (Pankiw *et al.* 2002). Colonies in these selected lines produced rearing environments that affected the adult weight and sucrose response threshold of other workers reared in these colonies (Pankiw *et al.* 2002).

There is a high degree of correlation between colony pollen income and worker ovary development, with three potential mechanisms for this relationship. First, the selection programme may have acted to select for differences in the amount of pollen the high and low colonies collected, and this difference was translated

into differences in the nutrition of the larvae reared in those colonies. This in turn led to differences in their ovary development later in life. Second, the ovary development traits selected for and the pollen foraging traits may not have been causally linked, but rather were linked in some other manner (e.g. due to neighbouring positions on a chromosome). Third, development of ovary development genes and pollen foraging genes may be in some manner linked due to the evolution of honey bees from a solitary ancestor (the 'reproductive ground plan', as suggested by Amdam *et al.* 2004). The selection programme may have selected for workers with different hormonal profiles that pleiotropically affected both reproductive and foraging traits.

The results of the cross-fostering experiment strongly suggest that the first scenario is likely responsible for part of the difference observed between the two selected lines. Workers that were cross-fostered in the opposite line showed intermediate levels of ovary development, clearly demonstrating an effect of rearing environment. As the amount of pollen in the colony rearing the larvae has been demonstrated to affect their ovary development as adults (Beekman *et al.* 2000, Hoover *et al.* 2005b) it is likely that nutrition was responsible for this effect. The selection program may therefore have selected for colonies with rearing environments conducive to worker ovary development through increased pollen foraging, a genetic trait of the colony creating differing rearing environments that in turn affect worker phenotype. The second scenario, in which foraging and reproductive traits are linked by chromosomal position, can not be ruled out by these experiments, and also could play a role.

The third scenario, in which a common pleiotropic gene network controls both foraging behaviour and reproduction in worker honey bees, also deserves further study. Amdam *et al.* (2004) found that lines of worker bees selected to have high levels of pollen foraging also had high levels of vitellogenin, a yolk precursor protein, compared to lines selected for low levels of pollen foraging. They attributed this difference to the hormonal dynamics of the juvenile hormone action cascade. While they selected for foraging traits, and found associated reproductive traits, I selected for worker reproductive traits, and found associated pollen foraging traits. These two lines of evidence combine to give strong evidence that worker reproduction and foraging are linked in some manner, likely through pleiotropic hormonal effects. Future research should focus on these associations in honey bees and other social insects, both at the level of individual behaviour and the evolution of insect societies.

CHAPTER 7: SUMMARY

This study examined some of the factors affecting worker ovary development in honey bee colonies, and has yielded insights into the evolution of reproductive division of labour in social insects. Honey bee worker reproduction is affected by a complex network of environmental, colony, and individual level factors, summarised in Figure 7-1 (factors 1-13, parameters A-D).

Changes in any of these factors affect the ultimate profitability of ovary development to individual workers. For example, in the Cape honey bee (*Apis mellifera capensis*), workers are able to lay female eggs without mating. This increases both the value of the offspring (factor 12) (who can herself reproduce, and potentially develop into a queen), and her relatedness (11), thus increasing the benefit to the laying worker (C). Anarchistic honey bees can lay only male eggs, yet they frequently do so in queenright colonies. Workers lay eggs that are more acceptable to policing workers (2), are themselves less discriminatory police (3), and have a reduced response to inhibitory queen pheromones (6). These factors affect both their ability to develop their ovaries (D), as well as the probability of worker-laid eggs surviving (A). However, these colonies do poorly without intervention from beekeepers, likely because of reduced colony efficiency (4), which increases the cost to the colony of having laying workers (B).

This thesis also examined some of the factors that affect the ability of workers to develop ovaries (D). Specifically, I examined the worker response to queen pheromones (6), and how nutrition (9) and genetics (10) affect worker ovary development. Queen pheromones inhibit worker ovary development (factor 6). This response is largely affected by the five-component queen mandibular gland pheromone (QMP) (Chapter 2), and adding additional queen-produced substances did not increase the effect at the dose tested. Workers in queenright colonies have lower levels of ovary development than those given QMP in cages, most likely due to the presence of brood pheromone, social interactions with nest mates, decreased protein in their diet, and worker role as nurses feeding developing brood in colonies.

In wild type colonies, workers generally refrain from reproduction in response to pheromonal signals that indicate the presence of a queen and her brood. However, workers frequently lay eggs in a few lines of honey bees, anarchistic bees. I found (Chapter 3) that queen type (anarchistic or wild type) had no effect on rates of ovary development of either anarchistic or wild type workers. Anarchistic workers had higher rates of ovary development than wild type workers in colonies headed by either queen type. Therefore, there must be differences in the transmission or reception of queen pheromones, or worker sensitivity to these compounds.

Chapter 4 examined the response of anarchistic workers to queen and brood pheromones, testing the effects of brood and queen pheromones on retinue attraction and worker ovary development. The results demonstrated that there is no difference between the anarchistic and wild type queen pheromones in the retinue response elicited in either wild type or anarchistic workers. Further, anarchistic queens produce a pheromone blend that is as effective at inhibiting ovary development as the wild type queen pheromone. However, anarchistic workers are less inhibited by queen pheromones than their wild type counterparts, in a dose-dependent manner. When combined, these results reveal that the anarchistic phenomenon is not due to changes in the production of queen pheromones, but rather is due in part to a shift in the worker response to these queen-produced signals. These results are the first demonstration of the dose-dependent nature of the effect of queen pheromones on honey bee worker ovary development.

In Chapter 5 I examined the effect of larval and adult nutrition on worker honey bee ovary development, clearly demonstrating the importance of both larval and adult nutrition to worker reproduction. Adult diet had a larger effect than larval diet, and increases in ovary development due to improved nutrition in the larval and adult life stages were additive. There also were strong seasonal trends in ovary development in caged queenless workers. Worker ovary development was lowest in spring, highest mid-summer, and intermediate in the fall. This trend was likely due to the seasonal availability of pollen, as the feeding experiment clearly indicated the importance of protein availability to worker development.

Disruptive selection for worker reproduction resulted in the production of lines of bees with high or low levels of worker ovary development (Chapter 6). Colony pollen income was correlated strongly with ovary development. Unselected colonies were intermediate to the high and low lines in both ovary development and pollen income. Cross-fostering these selected lines revealed that both genotype and larval rearing environment affected worker ovary development, as cross-fostered workers had intermediate levels of ovary development. The selection program produced both a genetic effect on worker ovary development directly, and a genetic effect on the rearing environments the colonies produced. These genetic effects on rearing environment likely were mediated by pollen income that affected larval nutritional status.

The social insects are a large and diverse group, exhibiting broad variation in their mechanisms of ovary inhibition. However, the conceptual model in figure 7-1 provides a theoretical framework for worker reproduction in all social groups. Changes to any of the described factors affect the profitability of attempted reproduction, and future research should focus on locating instances of convergence of inhibition mechanisms in divergent groups, and relating these to social and ecological correlates.

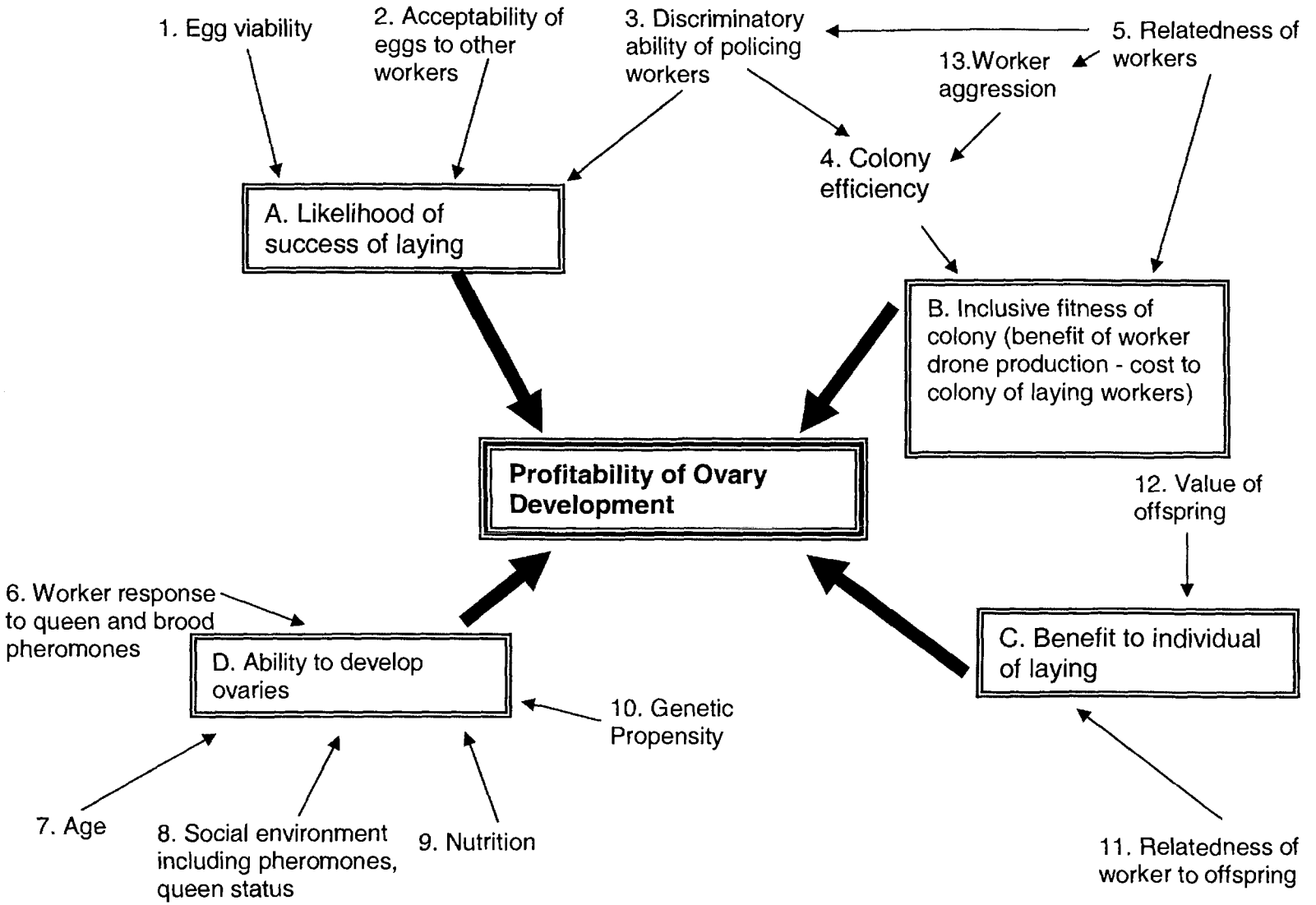

Figure 7-1 Potential mechanisms of worker ovary inhibition

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