Causes and consequences of genetic caste-bias in the eusocial Hymenoptera

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Author contributions are as follows: REM designed the experiments, carried out the experimental work, analysed the data and wrote the manuscript. CLF assisted with the genotyping of ants. WOHH supervised the work, assisted with the design of the experiments, the data analysis and drafting of the manuscript.

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Abstract

The eusocial Hymenoptera is one of the most successful groups to ever colonise the planet. They are defined by reproductive division of labour, whereby reproduction in a colony is dominated by one or a few queens, whilst all other individuals are more-or-less sterile. There has been much interest in understanding the mechanistic basis of caste determination, and particular interest in the evidence for genotypic variation in the propensity of individuals to become queens. However, the mechanisms underlying such genotypic variation in caste fate are as yet unknown. In this thesis, I examine potential causes and consequences of genetic caste-bias using the honey bee, *Apis mellifera*, and *Acromyrmex* leaf-cutting ants. In *A. mellifera*, individuals with queen-biased genotypes were shown to have a higher growth rate than those from genotypes with no caste-bias or a worker-bias. The variable response, in terms of growth rate, of genotypes to treatment with a juvenile hormone analogue suggested that variation in caste propensity could potentially arise from differential responses to environmentally induced physiological cues. In addition, an immune challenge by the fungal parasite *Ascosphaera apis* was found to affect the growth rate of *A. mellifera* larvae and caste determination at the colony level, suggesting that resistance to parasites may also be important in determining caste fate. In many species, workers have some control over caste determination, and I show that *Ac. octospinosus* workers were able to discriminate between nestmate and non-nestmate brood, even after larvae had been in contact with the fungus garden. That workers were able to discriminate between larvae within the nest suggests that they may be able to differentiate closely related kin during caste determination, despite homogenisation of fine scale cues. Finally, a positive relationship between patriline queen-worker skew and fluctuating asymmetry, an indicator of developmental stress, was found in *Ac. echinatior*, suggesting that subtle costs to caste-biasing strategies could explain the rarity of queen-biased genotypes. Overall this work shows that genotypic differences in the physiological response to environmental cues may underlie genetic caste-bias in the social insects. However, understanding the interaction between physiology, environment and genotype is essential if we are to understand the ultimate determinants of individual caste fate.

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1. General introduction

1.1 The eusocial Hymenoptera

The eusocial Hymenoptera (ants, some bees and some wasps) are arguably the most ecologically successful group of species when judged by phyletic longevity and its population-level causes: number of species, the occupation of unique adaptive zones, the sustainability of populations over time and breadth of geographic range (Wilson, 1987; Wilson, 1990). Eusociality is an advanced form of colonial existence, defined by overlapping generations, cooperative care of young and reproductive division of labour (Michener, 1969; Wilson, 1971; Wilson, 1975). The causal determinants of this extreme success all relate to group living, and include the regulation of conflicts, disease control, and cooperation in essential tasks such as obtaining resources, nest building and defence (Deneubourg & Goss, 1989; Schmid-Hempel, 1998; Beshers & Fewell, 2001; Hart & Ratnieks, 2005; Boomsma & Franks, 2006; Ratnieks *et al.*, 2006; Cremer *et al.*, 2007; Detrain & Deneubourg, 2008). The order Hymenoptera contains incredible variation in social structure, which ranges from solitary to highly eusocial, and in its ecology and life-history. Such variation, coupled with a truly global colonisation, makes the Hymenoptera a fascinating group in which to study social evolution and diversity.

Furthermore, a fundamental principle of social evolution is that societies are rife with actual and potential conflicts because each individual only cooperates to maximise its own inclusive fitness (Hamilton, 1964a). Understanding the evolution and maintenance of cooperation in the face of such conflicts remains one of the major unanswered questions in biology today (Pennisi, 2005). The eusocial insects are one of the pinnacles of social evolution, exhibiting perhaps the most extreme form of altruism observed in the animal kingdom, and thus represent an ideal group in which to study the causes, consequences and dynamics of social cooperation and conflict.

1.2 Conditions for the evolution of eusociality in the Hymenoptera

Reproductive division of labour is perhaps the defining trait of eusociality (Crespi & Yanega, 1995). Within the colony, females are divided into reproductive queens and usually sterile workers. In many species, these castes are morphologically distinct, often markedly so; queens tend to be large and specialised for reproduction, whereas workers can be adapted for specific non-reproductive tasks (Wilson, 1971; Oster & Wilson, 1978). Such extreme altruism can be hard to understand in evolutionary terms. The haplodiploid sex determination system of the Hymenoptera is such that full-sisters have an unusually high relatedness (Figure 1.1). Theoretically, workers can achieve higher inclusive fitness by staying in the nest and aiding the survival of their reproductive sisters than they would were they to raise their own offspring, depending on the sex ratio of males and reproductive females produced by the colony (Wilson, 1971; Wilson, 1975; Trivers & Hare, 1976; Oster & Wilson, 1978). However, the independent origins of eusocial behaviour both within and outside the Hymenoptera, as well as the low relatedness of workers to their brothers, means that haplodiploidy alone cannot account for the evolution of Hymenopteran eusociality (Hamilton, 1972; Bourke & Franks, 1995; Queller & Strassmann, 1998).

Nonetheless, this should not be interpreted to mean that relatedness is not a condition required for altruistic actions within social groups and, indeed, haplodiploidy may predispose a species to eusociality under certain conditions (Bourke & Franks, 1995; Linksvayer & Wade, 2005; Fromhage & Kokko, 2011). The principles of kin selection theory state that an altruistic act can evolve if the benefit to the recipient in terms of reproductive output, multiplied by the relatedness between actor and recipient, is greater than the cost to the actor (Hamilton, 1963; Hamilton, 1964a; Hamilton, 1964b). Consequently, relatedness is thought to be an essential condition for the evolution of eusociality, even if it is not directly causal. In the eusocial Hymenoptera, workers increase colony productivity, and the close relatedness of colony members means that their inclusive fitness is greater than that which could be gained through a solitary lifestyle (Queller & Strassmann, 1998; Foster *et al.*, 2006; Lehmann & Keller, 2006; Boomsma, 2007; Crozier, 2008). Unequivocal evidence for the role of kin-selection (inclusive fitness) theory in the evolution of eusociality comes from phylogenetic analyses showing that monandry was the ancestral state

when eusociality arose in each of the nine eusocial Hymenopteran lineages, that is, the evolution of eusociality in the Hymenoptera was always proceeded by, or coincided with, high relatedness (Hughes *et al.*, 2008a; Boomsma *et al.*, 2011).

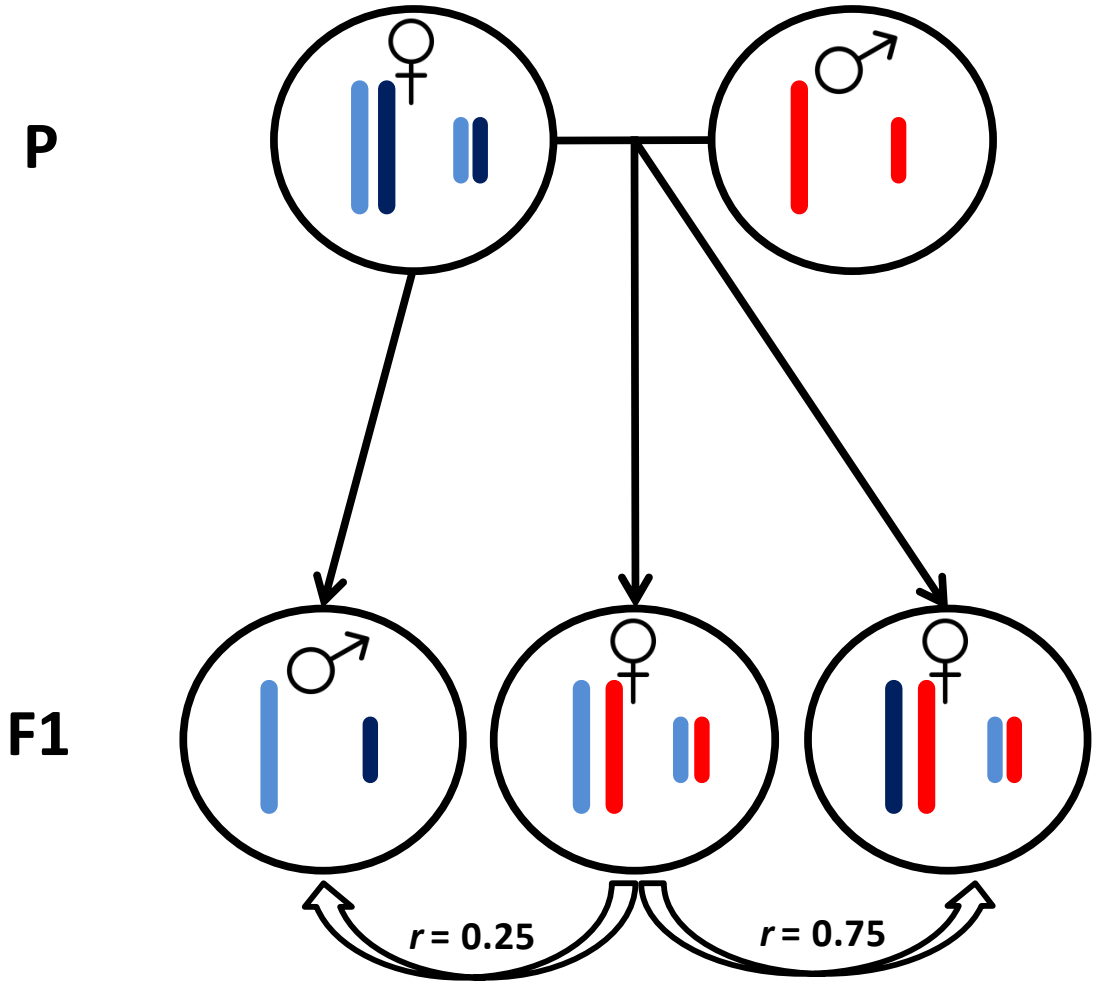

Figure 1.1 A schematic diagram showing the relationship between a singly mated Hymenopteran queen and her male and female offspring. Relatedness values (r) correspond to those between an F1 female, her diploid sister, and haploid brother.

Equally, the resolution of sex ratio conflict also provides evidence for the role of kin selection in social evolution. Relatedness asymmetries within Hymenopteran

societies mean that in monandrous colonies, workers favour greater investment in females than males. This is in direct conflict with the queen, who favours an equal sex ratio (Trivers & Hare, 1976; Nonacs, 1986; Pamilo, 1991). As mating frequency increases, this relatedness asymmetry decreases and so conflict over sex ratio should be less in colonies where the queen is multiply mated (Boomsma & Grafen, 1991). A correlation between relatedness asymmetry and the sex ratio of adult males and females has been shown in a number of species, and it seems that sex ratios are often under worker control (Ward, 1983; Mueller, 1991; Chan & Bourke, 1994; Queller & Strassmann, 1998; Sundström & Boomsma, 2001). In the ant *Formica exsecta*, for example, workers in singly mated colonies manipulate the sex ratio of brood so that it is female-biased, whereas multiply mated colonies specialise in male production (Sundström *et al.*, 1996). Here, the queen controls the primary sex ratio, but workers are subsequently able to manipulate it to their own advantage, thus increasing the fitness gained from each unit of investment and hence increasing their inclusive fitness (Boomsma & Grafen, 1991). Notably, however, evidence for worker manipulation of the sex ratio is not consistent across all species studied (Fjerdingstad *et al.*, 2002) and in some cases, queens have at least some control over the sex ratio (Pamilo & Seppä, 1994; Aron *et al.*, 1995; Helms, 1999; Sundström & Boomsma, 2001).

1.3 Polyandry in the eusocial Hymenoptera

The mating strategy of eusocial Hymenopteran queens ranges from monandry to extreme polyandry (Strassmann, 2001; Sumner *et al.*, 2004b; Tarpy *et al.*, 2004b). Multiple mating by queens could be viewed as maladaptive; it increases risk of predation and sexually transmitted diseases, is energetically costly and lowers relatedness among siblings, and hence worker inclusive fitness (Sherman *et al.*, 1988; Crozier & Fjerdingstad, 2001). Nonetheless, polyandry is taxonomically widespread in the eusocial Hymenoptera (Hughes *et al.*, 2008a) and consequently, it must also have associated benefits.

Most explanations for the evolution of polyandry are based around the fact that this strategy increases intracolonial genetic diversity (Keller & Reeve, 1994; Jennions &

Petrie, 2000; Crozier & Fjerdingstad, 2001; Hughes *et al.*, 2008b). Increased genetic diversity is associated with a number of benefits: it increases parasite resistance (Liersch & Schmid-Hempel, 1998; Schmid-Hempel, 1998; Baer & Schmid-Hempel, 1999; Schmid-Hempel & Crozier, 1999; Tarpy, 2003; Hughes & Boomsma, 2004); it allows the colony to cope with a broader range of environmental conditions (Fewell & Page, 1993; Page *et al.*, 1995; Oldroyd & Fewell, 2007); it reduces queen-worker conflict over sex allocation (Moritz, 1985; Boomsma & Grafen, 1991; Ratnieks & Boomsma, 1995; Sundström & Boomsma, 2001); and it leads to efficient division of labour (Crozier & Page, 1985; Hughes *et al.*, 2003; Mattila & Seeley, 2007; Oldroyd & Fewell, 2007), although there is conflicting evidence as to whether genetically diverse colonies perform tasks more efficiently than those with lower diversity (Fjerdingstad *et al.*, 2003; Schwander *et al.*, 2005; Fournier *et al.*, 2008). Importantly, the occurrence of polyandry and polygyny (more than one queen per colony), which is also a costly behaviour, are negatively correlated in the eusocial Hymenoptera (Hughes *et al.*, 2008b; Qian *et al.*, 2011; Haapaniemi & Pamilo, 2012). This relationship confirms that high intracolonial genetic diversity can be achieved through two different, and costly, strategies. The resulting fitness benefits are likely to have played a role in the evolution of both behaviours (Hughes *et al.*, 2008b).

1.4 Altruism under conditions of low relatedness

Low within-colony relatedness reduces the inclusive fitness of workers and consequently the benefits of altruistic behaviour. Why then, in species with high levels of polyandry or polygyny, do workers continue to act altruistically? In many species, morphological differences between queens and workers result in irreversible altruism – workers lack functional ovaries or are incapable of sexual reproduction, hence they cannot produce daughters or, indeed, reproduce at all (Bourke, 1988). Polyandry is a derived trait in all social insect lineages and, importantly, it seems that obligate or high levels of polyandry tend to evolve when workers have lost their reproductive totipotency (Hughes *et al.*, 2008a).

Another factor that prevents workers defecting from a cooperative strategy is coercion. Coercive behaviour acts to decrease the benefits of defection from a

cooperative strategy, or increase the costs, therefore reducing the benefits of direct reproduction in comparison to rearing siblings. Such actions by cooperative individuals can decrease the number defecting from a cooperative strategy, or prevent defection altogether (Ratnieks, 1988; Clutton-Brock & Parker, 1995; Frank, 1995; Lehmann & Keller, 2006; Wenseleers & Ratnieks, 2006a; Ratnieks & Wenseleers, 2008; Ratnieks & Helanterä, 2009).

A well studied example of coercive behaviour in the social Hymenoptera is worker policing in species where workers have retained the ability to produce sons parthenogenetically (Ratnieks, 1988). In colonies headed by a singly-mated queen, workers are expected to favour their own sons over nephews, and both of these over their brothers, the offspring of the queen. In colonies with a polyandrous queen, where female offspring fall into genetically distinct patrilines, average worker relatedness to nephews decreases and so workers should favour production of brothers over nephews, but still their own offspring over any other (Bourke, 1988; Ratnieks, 1988). Here, there is a clear conflict of interest between both workers and queens, and amongst workers. Workers favour production of their own sons, even at a cost to the colony (West-Eberhard, 1975; Cole, 1986), the cost being a result of workers that are less willing or able to undertake tasks such as foraging or defence if they are attempting to reproduce (Ratnieks, 1988; Ratnieks & Wenseleers, 2005). In colonies with multiple patrilines, an allele causing worker 'policing' behaviour, whereby workers prevent the production of sons by their sisters, either by destroying worker-produced larvae or punishing individuals who reproduce, should spread (Ratnieks, 1988). This lessens the fitness gained through worker reproduction because fewer worker sons are reared, reducing the frequency of such behaviour within the colony and even removing the motivation for individuals to act selfishly (Wenseleers *et al.*, 2004a; Wenseleers *et al.*, 2004c; Wenseleers & Ratnieks, 2006a). Empirical evidence for policing of worker-laid eggs comes from a variety of species, and recent evidence shows that cheating individuals can be reliably identified through their cuticular hydrocarbon profile (Ratnieks & Visscher, 1989; Foster & Ratnieks, 2000; D'Ettorre *et al.*, 2004; Endler *et al.*, 2004; Wenseleers & Ratnieks, 2006b; Helanterä & Sundström, 2007; Smith *et al.*, 2009). This behaviour is a classic example of how punishment imparted on individuals defecting from a cooperative strategy can lower levels of actual intracolonial conflict. Interestingly, in the

Ponerine ant species *Pachycondyla inversa*, policing is carried out by only a subset of workers and so is subject to division of labour (van Zweden *et al.*, 2007). This suggests that policing evolves because it benefits the colony as a whole, by improving its efficiency (Bourke, 2007). Policing of worker reproduction can also occur in societies lacking genetic conflicts, as recent evidence from the parthenogenetic ant *Cerapachys biroi* has shown (Tsuji & Yamauchi, 1995; Kronauer *et al.*, 2013). In this queenless species, larvae inhibit oogenesis of workers, synchronising the colony reproductive cycle (Ravary & Jaisson, 2002). Individuals that reproduce outside of the colony cycle can be recognised by their distinct hydrocarbon profile and are accordingly executed by their nestmates (Teseo *et al.*, 2013). Thus, worker policing can also act to enforce colony phenotypes and maintain group-level cooperation, even in the absence of genetic conflict.

1.5 Caste determination mechanisms

1.5.1 Environmental caste determination

The mechanism underlying Hymenopteran caste determination varies markedly between taxa. It was originally thought to be reliant solely on environmental cues, specifically the nutritional environment. In species with morphological castes, larvae are totipotent until a point at which queen or worker development is stimulated and becomes irreversible. Caste differentiation is controlled by the endocrine system, which itself is primarily regulated by external cues, including nutrition, temperature, maternal effects and inhibitory cues released by functional reproductives (Elmes, 1987; Keller & Nonacs, 1993; Hartfelder, 2000; Hoover *et al.*, 2003; Davidowitz & Nijhout, 2004; de Menten *et al.*, 2005; Schwander *et al.*, 2008; Cahan *et al.*, 2011).

Although it is not yet fully understood how trophic information is processed, the amount of food a larva receives during early development affects juvenile hormone (JH) titres, which subsequently determine which developmental pathway is triggered. Thus, an amount of food above a certain threshold will trigger queen development, and below this a larva will develop as a worker (Wheeler, 1986). Endogenous hormone titres reveal that queen larvae have a higher haemolymph JH

titre than do worker larvae (Rembold, 1987; Rachinsky *et al.*, 1990; Rembold *et al.*, 1992; Bloch *et al.*, 2000). Differential expression of ecdysteroids, or moulting hormones, is also implicated in caste determination (Suzzoni *et al.*, 1983; Hartfelder *et al.*, 2000) and these differences are the result of caste-specific activity of endocrine glands (Ulrich & Rembold, 1983; Rachinsky & Hartfelder, 1990; Hartfelder & Engels, 1998). Caste-regulatory hormones act during critical periods of larval development (Nijhout & Wheeler, 1982; Wheeler, 1991); JH sensitive periods occur during each larval stage and are usually concurrent with ecdysteroid peaks (Riddiford, 1994). Thus, there is strong evidence that JH and ecdysteroids act in concert to regulate the processes of caste determination and metamorphosis (Hartfelder & Engels, 1998; Hartfelder *et al.*, 2000).

1.5.2 Genetic caste determination

Despite compelling evidence that environmental factors play an important role in caste determination (Wheeler, 1986; Hölldobler & Wilson, 1990; Wheeler, 1991; Crozier & Pamilo, 1996), this does not equate to evidence that systems are inevitably insensitive to genetic variation in the predisposition of individuals to a particular caste (Schwander *et al.*, 2010). Indeed, evidence for a genetic component to this process is becoming increasingly prevalent in the literature. The most extreme example of genetic caste determination is in the ant species *Pogonomyrmex barbatus* and *P. rugosus*, whereby workers are the result of inter-lineage hybrid matings, whereas queens are the offspring of within-lineage matings (Cahan *et al.*, 2002; Julian *et al.*, 2002; Cahan & Keller, 2003; Anderson *et al.*, 2006a). A similar mechanism acts in the fire ant *Solenopsis xyloni*, colonies of which contain multiple monandrous queens. In areas where *S. xyloni* occurs in sympatry with *S. geminata*, queens are able to mate with males of either species. Conspecific matings result in new queen offspring, and hybrid matings produce workers (Hung & Vinson, 1977; Cahan & Vinson, 2003). Interestingly, this mode of caste determination seems restricted to *S. xyloni* (Cahan & Vinson, 2003).

Other examples of species with extreme genetic caste determination include *Cataglyphis cursor*, *Vollenhovia emeryi* and the little fire ant, *Wasmannia auropunctata*. In all three species, workers are produced from fertilised eggs, but new queens are clonal, produced by thelytokous parthenogenesis (Pearcy *et al.*,

2004; Fournier *et al.*, 2005; Ohkawara *et al.*, 2006; Foucaud *et al.*, 2007; Kobayashi *et al.*, 2008). This method of caste determination could potentially result in males, who usually gain fitness through their reproductive daughters, having a fitness of zero. However, this is circumvented by males producing clonal sons by eliminating the female genome from fertilised eggs (Fournier *et al.*, 2005; Kobayashi *et al.*, 2008), or by the fertilisation of anucleated oocytes (Foucaud *et al.*, 2007), leading to the complete separation of the male and female gene pools.

Not all examples of a genetic influence on caste determination involve such exceptional phenomena as social hybridogenesis and parthenogenesis. In a number of polyandrous species, certain paternal lineages are over-represented in the queen caste, whilst others are more likely to develop into workers (Keller *et al.*, 1997; Tilley & Oldroyd, 1997; Châline *et al.*, 2003; Moritz *et al.*, 2005; Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Nanork *et al.*, 2011). The mechanism behind this genetic effect on caste determination is as yet unknown, but it may involve genotypic differences in response thresholds to environmental cues, intrinsic differences in the ability of larvae to manipulate nurse workers, such as increased begging or attractiveness of chemical signals, or compatibility between maternal and paternal genomes (Hughes & Boomsma, 2008; Schwander & Keller, 2008).

A genetic effect on caste determination has also been proposed in the meliponine stingless bees. In some *Melipona* species, colonies produce an excess of gynes that far outstrips the number needed for the colony to maximise its reproductive fitness and consequently, many are executed (Kerr, 1950b; Imperatriz-Fonseca & Zucchi, 1995; Wenseleers *et al.*, 2004b). The genetic mechanism underlying this system is thought to involve two or three genetic loci, depending on species. Essentially, the model states that only double or triple heterozygotes, respectively, at these loci are able to develop into queens, providing they receive enough food during development (Kerr, 1950a; Michener, 1974; Velthuis & Sommeijer, 1991; Hartfelder *et al.*, 2006). Irrespective of the genetic mechanism, certain genetic markers are associated with the queen phenotype in *Melipona* (Hartfelder *et al.*, 2006).

Intermorphic queens, or queens exhibiting some worker characteristics, have been reported in the ants *Leptothorax* sp. A and *Harpagoxenus sublaevis*, and have

revealed another genetic effect on caste determination. In both species, 'normal' queens mating with the son of a normal queen produce a significantly higher queen: worker offspring ratio than do intermorphic queens mating with sons of queens of the same type. It has been proposed that in these species, caste determination is influenced by a locus with a dominant allele that impairs the development of queen-like characteristics and increases the likelihood of worker development, compared to larvae with the homozygote recessive genotype. However, a difference in the penetrance of such an allele in the two species suggests other factors are also affecting caste development (Winter & Buschinger, 1986; Heinze & Buschinger, 1989).

1.5.3 Gene by environment interactions

The influence of genetic and environmental factors on caste determination varies widely between taxa. Species exhibiting only environmental caste determination or only genetic caste determination are, in fact, rare, and indeed most species that have been studied fall somewhere on a continuum between the two extremes (Schwander *et al.*, 2010). Thus, in many species, the interaction between genetic and environmental effects, whereby genotypes respond differently to environmental cues, means that caste determination is a plastic process and the effect of an individual genotype may depend strongly on the environmental conditions it encounters (Chapman *et al.*, 2007; Hughes & Boomsma, 2007).

Environmental influences include those provided by the social environment, and so interactions between different genotypes within the colony can also affect the expression of caste in developing larvae. In many species, workers regulate larval nutrition, thereby constraining the nutritional environment larvae will experience. Thus, the expression of queen and worker phenotypes can be influenced by the interaction between nurse and larval genotypes (Osborne & Oldroyd, 1999; Beekman *et al.*, 2000; Pankiw *et al.*, 2002; Allsopp *et al.*, 2003; Linksvayer & Wade, 2005; Linksvayer *et al.*, 2009a; Linksvayer *et al.*, 2009b; Jarau *et al.*, 2010). For example, in the ant *Temnothorax curvispinosus*, gyne and worker mass, and caste ratio, are affected by a combination of direct (self) and indirect (maternal and sibsocial) genetic effects (Linksvayer, 2006). This interaction means that the effects of individual genotype are context dependent, and the contribution of non-self

genotypes to individual phenotypes can be subject to selection (Linksvayer, 2006; Linksvayer, 2007).

1.5.4 Worker caste

In addition to reproductive division of labour, approximately 15% of ant species have polymorphic worker castes, effectively dividing colonial tasks such as brood care, foraging and defence within the colony (Oster & Wilson, 1978; Hölldobler & Wilson, 1990). For example, one of the most extreme cases of worker polymorphism is found in the army ant species *Eciton burchellii*, in which there are four morphologically distinct worker castes: minors, which are primarily employed in brood care within the nest; medias, which play a general role in colony maintenance and foraging; submajors, which are well adapted for efficient transport of prey; and majors, which defend the colony (Topoff, 1971; Franks, 1985). Division of labour amongst the worker caste increases colony efficiency and ultimately reproductive output (Hölldobler & Wilson, 1990; Schmid-Hempel, 1992; Bourke & Franks, 1995; Anderson & Ratnieks, 1999; Ratnieks & Anderson, 1999; Wetterer, 1999; Yang *et al.*, 2004). Social insect workers can even be described as 'super-donors' (West-Eberhard, 1975); the advantage of helping increases with its efficiency. So, the extreme morphological and behavioural specialisations of workers in some social Hymenoptera result from selection to maximise efficiency as a helper, and hence indirect fitness (Wilson, 1971).

The determination of worker caste during development is, again, influenced by both environmental and genetic factors (Stuart & Page, 1991; Wheeler, 1991; Passera *et al.*, 1996; Fraser *et al.*, 2000; Hughes *et al.*, 2003; Julian & Fewell, 2004; Schwander *et al.*, 2005; Jaffé *et al.*, 2007; Smith *et al.*, 2008a) and the expression of worker phenotypes are plastic, allowing a colony's collective phenotype to respond to changing conditions (Hughes & Boomsma, 2007). The underlying mechanism for worker division of labour is likely to involve variable response thresholds for stimuli eliciting certain behaviours (Beshers *et al.*, 1999; Beshers & Fewell, 2001).

The evolution of worker caste diversity is influenced by both queen-worker divergence and intracolonial genetic diversity (Fjerdingstad & Crozier, 2006). The early divergence of queen-worker development may facilitate the evolution of

worker polymorphism by allowing more time for the differentiation of worker phenotypes (Wheeler, 1986). For example, in the fire ant, *Solenopsis invicta*, a species in which workers can vary up to 30-fold in weight (Tschinkel, 2006), queen-worker caste determination occurs as early as the first or second instar (Robeau & Vinson, 1976). Worker diversity is associated with polyandry and polygyny, and shows a negative relationship with relatedness in polygynous colonies (Fjerdingstad & Crozier, 2006). This could result from intracolonial genetic diversity itself favouring the expression of a diverse worker phenotype (Crozier & Page, 1985; Fuchs & Moritz, 1999; Crozier & Fjerdingstad, 2001). Genetic variation also favours the repression of worker reproduction and this may in turn mean that workers are more likely to lose the ability to reproduce, thus allowing worker diversity to evolve more easily (Fjerdingstad & Crozier, 2006).

In contrast, most social Hymenoptera do not have morphologically variable worker castes. Here, worker polyethism is commonly temporal, with young workers being predisposed to carry out tasks within the colony, such as brood care and maintenance, whilst older workers perform activities that carry more risk, such as foraging and defence (Michener, 1974; Robinson, 1992). Temporal polyethism is perhaps best studied in the honey bee, *Apis mellifera*, in which young workers take on the role of nurses, tending to larvae within the colony, whilst older workers leave the colony to forage (Seeley, 1982). Colonies also contain a smaller proportion of highly specialised individuals who undertake tasks such as removing corpses (undertaking behaviour), or guarding the nest entrance (Visscher, 1983; Moore *et al.*, 1987). Division of labour in the honey bee is flexible; the age at which certain behaviours are stimulated may vary depending on variable internal and external conditions (Robinson, 1992; Gordon, 1996; Pankiw *et al.*, 1998; Schulz *et al.*, 1998; Le Conte *et al.*, 2001).

In *Apis mellifera* workers, juvenile hormone titre increases with age, and thus is associated with age polyethism (Robinson, 1987; Robinson *et al.*, 1987; Huang *et al.*, 1994; Jassim *et al.*, 2000). JH is not directly responsible for regulating behaviour, or for altering behaviour in response to variable conditions, but it does regulate the rate at which behaviour develops (Robinson & Vargo, 1997; Sullivan *et al.*, 2000; Robinson, 2002). The plasticity of development is regulated, in part, by

environmental effects on JH levels (Bloch *et al.*, 2002). This includes the social environment; older workers inhibit the maturation of younger workers, perhaps via chemical signals, a process requiring direct social contact (Huang & Robinson, 1992; Huang *et al.*, 1998; Beshers *et al.*, 2001). An increase in octopamine levels in the brain is also associated with age-related polyethism in *A. mellifera* (Wagener-Hulme *et al.*, 1999). Treatment with high levels of octopamine result in bees being more likely to engage in foraging behaviour (Schulz & Robinson, 2001).

In addition to a range of physiological and environmental influences, there is also genetic variation for both the age at which individuals switch tasks, and task specialisation itself (Calderone & Page, 1988; Calderone *et al.*, 1989; Breed *et al.*, 1990; Page & Fondrk, 1995; Giray *et al.*, 2000). Again, we see that division of labour is controlled by a complex interaction between genotype and the environment (Pankiw *et al.*, 2002). Interestingly, a genetic polyethism has been shown to occur within the worker caste of species with morphologically polymorphic workers (Waddington *et al.*, 2010; Eyer *et al.*, 2013), and genotypes also differ in their ability to perform tasks (Constant *et al.*, 2012). That a genetic polyethism has now been observed suggests that this phenomenon could be widespread throughout the social insects, thus strengthening the argument that behavioural plasticity of the workforce is important if colonies are to respond to variable environmental conditions.

1.5.5 Social species without a queen caste

Within the social Hymenoptera, there are four main subfamilies that lack a morphologically distinct queen caste: Ponerinae in the ants, Halictinae in the bees, and Stenogastrinae and Polistinae in the wasps. Although this social structure is thought to be ancestral in the bees and wasps, it is secondarily derived in the ponerine ants, which evolved from a highly eusocial ancestor (Peeters, 1991; Danforth, 2002; Hines *et al.*, 2007). Primitively eusocial species are characterised by small colony size and a simple social structure. Here, all females within the colony are physiologically capable of sexual reproduction, and the reproductive female, or gamergate, maintains her position by means of a dominance hierarchy (Peeters, 1991; Field & Cant, 2009). Rank is at least partially determined by age, and young subordinate workers queue to take over reproductive dominance (Hughes & Strassmann, 1988; Higashi *et al.*, 1994; Shreeves & Field, 2002). High ranking

workers tend to carry out brood care within the nest. In contrast, older individuals are more likely to carry out tasks that have a higher mortality rate, such as foraging, waste removal and nest defence (Ito & Higashi, 1991; O'Donnell, 1998; Cant & Field, 2001; Cronin & Field, 2007; Asher *et al.*, 2013).

Individuals in a social queue face a trade-off between increasing their indirect fitness by putting a lot of effort into working, or increasing their chance of inheriting reproductive dominance by undertaking fewer risky behaviours, thereby increasing their survival probability and fecundity. Thus, high ranked individuals undertake less risky behaviour because they stand to lose more, in terms of direct fitness, through working than do low ranked individuals. Equally, individuals of the same rank tend to work less hard in larger colonies, as they potentially have more to lose by working than they do in small colonies (Field *et al.*, 2006).

1.5.6 The molecular basis of caste determination

Recent advances in sequencing and molecular technology mean that we are now able to look beyond basic sequence information, at how differential gene expression and epigenetic phenomena affect caste determination. The specific genes underlying genetic effects on division of labour are yet to be identified, but current evidence shows that several pathways associated with fundamental life-history traits contribute to division of labour: nutrition, maternal care, diapause and reproduction (Smith *et al.*, 2008b). Already, a vast number of genes have been shown to be differentially expressed in queens and workers, although it can be difficult to determine whether differences in gene expression are either the cause or the consequence of caste differences (Pereboom *et al.*, 2005; Gräff *et al.*, 2007; Grozinger *et al.*, 2007; Patel *et al.*, 2007; Toth *et al.*, 2007; Smith *et al.*, 2008b).

Differential gene expression in the honey bee has received much attention, aided by the sequencing of the *A. mellifera* genome (The Honeybee Genome Sequencing Consortium, 2006). One of the main outcomes of this research has shown that caste determination involves the activation of specific genes in both queens and workers; worker is not the ‘default’ phenotype (Evans & Wheeler, 1999). Queen-destined larvae have been shown to upregulate metabolic enzymes, such as ATP-synthase and cytochrome oxidase I, both directly associated with increased metabolic rate (Evans

& Wheeler, 2000). This may reflect the high growth rate of queen larvae during late development (Bishop, 1961). The insulin signalling pathway has also been implicated as an intermediary between nutritional cues and caste-specific development; queens show an upregulation of a number of insulin-like growth factor genes (Wheeler *et al.*, 2006). This pathway is highly conserved, regulating growth and size (Nijhout, 2003b).

Further to the study of gene expression, another rapidly expanding field is that of epigenetic control of caste determination. Sequencing of the *A. mellifera* genome has shown the honey bee to have a fully functional DNA methylation system (The Honeybee Genome Sequencing Consortium, 2006; Wang *et al.*, 2006), and subsequently, it has been shown that epigenetic regulation is involved in caste determination. DNA methylation leads to gene silencing, and, in general, the worker phenotype is associated with high methylation levels; RNAi knockdown of DNA methylation enzymes leads to larvae developing as queens in the absence of royal jelly (Kucharski *et al.*, 2008). Genes that are differentially expressed between castes tend to be more highly methylated than those related to basic biological processes (Elango *et al.*, 2009). Interestingly, DNA methylation in the honey bee is associated with gene splice sites, and it may be that alternative splicing is one mechanism through which differential gene expression can act (Lyko *et al.*, 2010). DNA methylation is widespread across the eusocial insects, although the extent of methylated sites varies widely between species (Kronforst *et al.*, 2008), thus insights from the honey bee epigenome are likely to be widely applicable across the group.

1.6 Conflict over caste determination

In some ways, the eusocial Hymenoptera epitomise cooperative behaviour, but, as in any social system, conflicts of interest invariably arise. Individuals are not clones, and so behaviour that is advantageous to one individual may not be the optimal strategy for others, or the colony as a whole. Conflict over caste determination is no exception. In most species, larvae are initially totipotent, able to develop into either a queen or a worker. Each individual will theoretically maximise their inclusive fitness by developing as a queen (Bourke & Ratnieks, 1999; Reuter & Keller, 2001;

Wenseleers *et al.*, 2003), even though this may result in lower colony productivity (Ratnieks, 2001; Reuter & Keller, 2001; Wenseleers *et al.*, 2003; Anderson *et al.*, 2006b). The conflict arises because the queen and workers will maximise their inclusive fitness by raising only as many queens as will maximise colony productivity, whereas individual larvae will favour development as a queen irrespective of this (Nonacs & Tobin, 1992; Bourke & Ratnieks, 1999; Reuter & Keller, 2001). This conflict is most pronounced in polyandrous species, a result of increased genetic variation and lower average relatedness between developing female larvae and their female siblings. This reduces the indirect fitness gained from raising sisters and thus the benefits associated with being a worker.

It seems logical, then, that cheating genotypes, able to bias their development towards that of a potential queen, may arise in social insect colonies and that they will do so despite costs to colony productivity, thus fulfilling the definition of a cheat as defined by Ghoul *et al.*(2013). However, the extent to which theoretical conflict is translated into actual conflict is unclear (Ratnieks & Reeve, 1992; Korb & Heinze, 2004; Ratnieks *et al.*, 2006). In many instances, costs at the colony level, lack of information, or limitation of power can allay potential conflicts, or prevent their expression altogether (Ratnieks & Reeve, 1992; Ratnieks *et al.*, 2006). In the case of caste determination, in many species it is the workers that provision larvae with food and so have control over their developmental pathway (Hölldobler & Wilson, 1990). This results in a lack of self-determination in developing females (Bourke & Ratnieks, 1999; Beekman & Ratnieks, 2003); workers hold the power in terms of caste determination, not developing larvae themselves.

It is likely, however, that individuals able to bypass worker control, thus having at least some degree of self-determination, will evolve. One prediction is the occurrence of small, or ‘dwarf’, queens, able to develop queen-like characteristics, despite being fed a worker diet (Bourke & Ratnieks, 1999). Dwarf queens are known in a number of ant and stingless bee species (Imperatriz-Fonseca & Zucchi, 1995; Heinze, 1998; Rüppell *et al.*, 2001). *Schwarziana quadripunctata* dwarf queens, that develop in worker cells and whose weight is the same as that of workers, are able to reproduce and head colonies, but they have reduced fecundity and are known to be killed by workers (Wenseleers *et al.*, 2005). This reduces the fitness benefits of this

developmental strategy, thus explaining why it is rare, with less than 1% of individuals reared in worker cells developing as dwarf queens. Other mechanisms by which larvae can have at least some control over their food intake, and thus potential caste fate, include brood cannibalism, chemical manipulation and increased begging behaviour (Beekman & Ratnieks, 2003; Creemers *et al.*, 2003; Kaptein *et al.*, 2005; Le Conte & Hefetz, 2008; Rüger *et al.*, 2008). Alternatively, certain genotypes might influence the allocation of resources prior to caste determination; the genes implicated in caste determination are involved in nutrition and metabolism, and it may be that these pathways could be manipulated, thus altering an individual's likelihood of developing as a certain caste (Smith *et al.*, 2008b).

Consequently, the overrepresentation of certain genotypes in the queen caste may also be the result of conflict over caste determination. These 'royal' genotypes are essentially gaining the direct fitness benefits of reproduction without paying the cost, the cost being that a proportion of individuals with the genotype will forgo reproduction and develop as workers. Genetic caste-bias is most easily identified in colonies headed by a single polyandrous queen, as they consist of a number of full-sister patrilines that differ only in their paternal genotype. Accordingly, this phenomenon has been identified in number of species: *Apis mellifera* (Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005), *A. florea* (Nanork *et al.*, 2011), *Acromyrmex echinatior* (Hughes & Boomsma, 2008), *Pogonomyrmex badius* (Smith *et al.*, 2008a), *P. rugosus* (Schwander & Keller, 2008) and *Formica sanguinea* (Qian *et al.*, 2011). It has been proposed that in *P. rugosus*, this effect is the result of compatibility between maternal and paternal genomes; different crosses between males and females produce markedly different proportions of queens and workers (Schwander & Keller, 2008). Such compatibility effects would mean that selfish paternal genotypes would only be so in combination with particular maternal genotypes, and not across the population as a whole, thus offering one explanation for the rarity of 'royal' genotypes at the population level. However, such genotypes are also rare within colonies, suggesting that they are constrained by frequency-dependent selection, resulting from costs to colony efficiency or direct suppression by cooperative genotypes (Ratnieks *et al.*, 2006; Hughes & Boomsma, 2008).

Recently, it has been shown theoretically that ‘queen-gene’ (a gene imparting a higher propensity to develop as a queen) imprinting may account for observed patterns of genetic caste-bias (Dobata & Tsuji, 2012). In polyandrous societies, a queen-gene would be severely detrimental to colony productivity when present as a matrigene (inherited from the mother) as, in a monogynous colony, approximately half the developing larvae would harbour the gene. However, when present as a patrigene this would not be the case, providing the conditions exist for the evolution of patrigene-dependent expression, or matrigene silencing (Dobata & Tsuji, 2012). Interestingly, this model can also explain the paternal effect on caste-determination shown in the monandrous Argentine ant, *Linepithema humile* (Libbrecht *et al.*, 2011), colonies of which contain multiple unrelated queens. If new males are more susceptible to the cost of caste-biasing than new queens, then it is matrigenes that bear this relative cost; matrigenes therefore have less incentive to contribute to queen development and may be silenced, even under monandry (Dobata & Tsuji, 2012).

1.7 Caste determination in the honey bee, *Apis mellifera*

Perhaps the best studied example of genetic caste-biasing come from the honey bee, *Apis mellifera*. In this species, the nutritional environment plays a significant role in larval development. All larvae are fed a proteinaceous glandular secretion, or larval jelly, produced by nurse workers (Haydak, 1970), but the composition of worker jelly and royal jelly (and also drone jelly) differ in their sugar, vitamin and protein content (Thrasyvoulou, 1983; Brouwers, 1984; Brouwers *et al.*, 1987). Royal jelly is directly responsible for larval development as a queen, at least in part through the action of the protein royalactin, which increases body size and ovary development, and shortens development time (Kamakura, 2011).

Virgin queens are reared by the colony under two circumstances. First, when the colony reaches a critical size, workers will rear a number of new queens. Subsequently, the colony splits (colony fission), with one derivative half being headed by the old queen, and the other by a new queen (Winston, 1987; Seeley, 1995). Under these circumstances, the existing queen will usually lay eggs into pre-designated queen cups. Second, when the existing queen dies or leaves the colony

suddenly, workers will rear ‘emergency queens’ from existing queen-laid larvae. The majority of emergency queens are reared from larvae of less than two days old, but workers will also raise some queens from larvae that are three and even four days old (Fell & Morse, 1984).

Although the nutritional environment has a known role in honey bee caste determination, the genotype of developing larvae also has an effect. *A. mellifera* queens are highly polyandrous (Estoup *et al.*, 1994), and a number of studies have shown that emergency queen rearing is not random; individuals from some patrilines have a higher chance of developing as a queen than do those from others (Estoup *et al.*, 1994; Moritz *et al.*, 1996; Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005). Here, individual genotype affects an individual’s likelihood of developing as a queen, but during emergency queen rearing it is workers who select which larvae are to be reared. The selection process undertaken by workers is, at present, poorly understood. Workers are able to petition for particular larvae during emergency queen rearing, through exposure to pheromones produced by the Nasonov gland (Al-Kahtani & Bienefeld, 2011). However, the larval characteristics that initiate this response from workers, and that lead to an eventual group decision as to which larvae are reared as queens, are currently unknown (Al-Kahtani & Bienefeld, 2011). Genetic variation in larval attractiveness has been shown in the honey bee (Tilley & Oldroyd, 1997; Beekman *et al.*, 2000; Calis *et al.*, 2002) and it seems likely that certain heritable traits make certain larvae either more suitable, or just more attractive, for rearing as queens.

1.8 ‘Royal cheats’ in leaf-cutting ants

A similar phenomenon to the genetic caste-bias of *A. mellifera* has also been found in the leaf-cutting ant *Acromyrmex echinatior*, but under normal conditions rather than those of emergency queen rearing. *Ac. echinatior* is a polyandrous species from the tribe Attini (Bekkevold *et al*. 1999; Sumner *et al*. 2004). The *Ac. echinatior* worker caste is polymorphic; small workers are generally employed within the nest, caring for the brood, tending the fungus garden and protecting the colony from

pathogens, whereas large workers tend to forage outside the nest (Weber, 1972; Wetterer, 1999; Hughes *et al.*, 2002; Poulsen *et al.*, 2002; Hughes *et al.*, 2003).

Similar to *A. mellifera*, in *Ac. echinatior* certain rare patrilines are overrepresented in the queen caste, suggesting that there is a significant genetic influence on queen-worker caste determination. Gynes from these biased patrilines have been termed "royal cheats" (Hughes & Boomsma, 2008), because they are effectively cheating their sisters out of a fair chance of reproduction. Approximately one-fifth of *Ac. echinatior* patrilines are able to bias their caste destiny in favour of becoming queens (Hughes & Boomsma, 2008). This is thought to involve two distinct mechanisms (Figure 1.2), one in which patrilines are overrepresented in both queen and large-worker castes (royal–LW patrilines) and one in which patrilines are overrepresented in the queen and small worker castes (royal–SW patrilines). Both may result from changes in response thresholds for environmental cues, such as nutrition, that control larval development. However, the suggested mechanism underlying royal–LW patrilines involves both worker caste propensity and queen-worker propensity; the threshold levels of food required for both development into a large worker and development into a gyne are slightly lower than in normal patrilines. This selfish caste-biasing may only be a pleiotropic side-effect of a mechanism that has evolved because of its beneficial effect on worker caste determination (Hughes *et al.*, 2003; Waibel *et al.*, 2006; Hughes & Boomsma, 2007).

Conversely, because royal–SW patrilines only affect queen-worker determination, they are likely to have arisen for the selfish benefits gained by becoming a queen (Hughes & Boomsma, 2008). It is possible that here, although the cue-threshold for becoming a large worker is the same as in normal patrilines, the cue-threshold for becoming a queen may be reduced by a much greater extent than in the royal–LW patrilines, resulting in the production of a high proportion of queens and a very low proportion of large workers. Compared to normal patrilines, royal–LW genotypes would gain very little direct fitness, whereas royal–SW genotypes increase their direct fitness by approximately 500%. Furthermore, within colonies, royal–SW patrilines are significantly less common than normal and royal–LW patrilines, and at the population level only 20% of patrilines show significant cheating. This is in line with evolutionary theory, which predicts a rarity of cheating genotypes (Frank,

1998). Such high fitness benefits, coupled with the rarity of royal-SW patrilines, suggest that individuals with such genotypes are genuine cheats (Hughes & Boomsma, 2008).

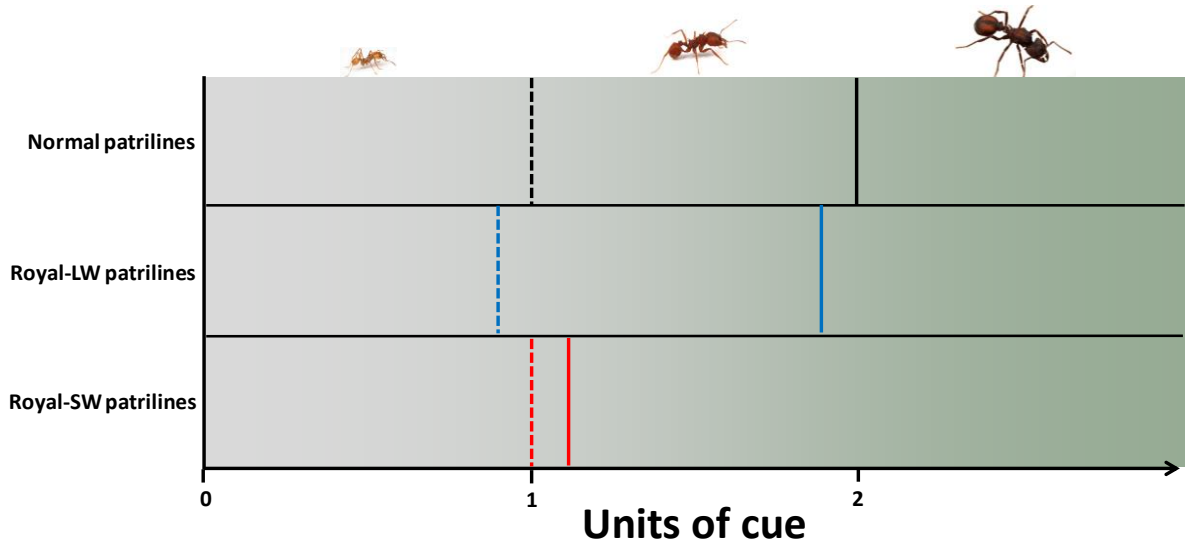

Figure 1.2 A mechanism for patriline caste-biasing in *Acromyrmex echinatior* as proposed by Hughes and Boomsma (2008). For each patriline, the dashed line represents the level of caste-determining environmental cue (e.g. food) required to switch between the small worker (SW) and large worker (LW) developmental path. The solid line represents the level of cue required to switch between the worker and queen developmental path. Both thresholds are lower for royal–LW patrilines than for normal patrilines, whereas royal–SW patrilines have a lower threshold for the queen-worker switch only. Figure adapted from Hughes and Boomsma (2008).

1.9 Nepotism in the eusocial Hymenoptera

In the eusocial Hymenoptera, the concept of nepotism, the preferential treatment of kin (Hamilton, 1987), in the context of queen rearing is controversial. It was originally postulated that patriline caste-bias in *A. mellifera* emergency queen rearing could be the result of nepotistic interactions between workers and their full-sister larvae (Page & Erickson, 1984; Visscher, 1986; Page *et al.*, 1989), but these findings have been heavily criticised on both statistical and biological grounds (Oldroyd *et*

al., 1990; Frumhoff, 1991; Breed *et al.*, 1994). Such logic could also be applied to the case of *Ac. echinatior* 'royal cheats', and yet a conclusive demonstration of nepotism in any social insect group remains conspicuously absent (Queller *et al.*, 1990; DeHeer & Ross, 1997; Tarpy *et al.*, 2004a; Châline *et al.*, 2005; Holzer *et al.*, 2006; Goodisman *et al.*, 2007; Zinck *et al.*, 2009). Most recently, a study of *Formica fusca* showed that in colonies with multiple queens, workers were able to favour their closest kin when rearing eggs and larvae (Hannonen & Sundström, 2003). However, this work has suffered criticism because the findings can also be explained by variation in the egg viability of different queens, a phenomenon shown in polygynous colonies of *Formica exsecta* (Holzer *et al.*, 2006).

It is not particularly surprising that queen-rearing nepotism is rare, if it occurs at all; if such behaviour reduced colony productivity, or workers frequently made mistakes in discriminating between full- and half-sisters, then nepotism would be selected against (Ratnieks & Reeve, 1992). In terms of recognition, information available to workers may indeed be limiting. Nestmate recognition in social Hymenoptera relies on cuticular hydrocarbons, the profile of which differs between colonies and genetic lineages (Ratnieks, 1991; Vander Meer & Morel, 1998; D'Ettorre & Lenoir, 2010). Accurate discrimination of matrilines and patrilines within a colony would require substantial variation at loci associated with chemical recognition (Wenseleers, 2007) and this may be limited in a number of species (Dani *et al.*, 2004; van Zweden *et al.*, 2010). Additionally, varied individual odours within a colony combine to form a colony gestalt odour, which is used as a template against which to recognise foreign individuals (Crozier & Dix, 1979). It is thought that the resulting gestalt odour may be too strong for intracolonial genetic discrimination to occur, thus implying that discrimination between full- and half-sisters based on chemical cues is unlikely (van Zweden *et al.*, 2010; Martin *et al.*, 2012). However, it is important to note that recent work on *Ac. octospinosus* has revealed that in this species, at least, genetic variation in cuticular hydrocarbons is such that full- and half-sisters can be distinguished with high accuracy, contradicting previous assertions that such recognition is unlikely to occur and demonstrating the potential for within-colony patriline discrimination in the social insects (Nehring *et al.*, 2011).

1.10 Causes and consequences of genetic caste-bias in the eusocial Hymenoptera

Despite its apparent ubiquity across the eusocial Hymenoptera, many of the genetic effects on caste determination have been reported relatively recently. Thus, we have a limited knowledge of the specific genes involved and, more broadly, the mechanisms underlying genetic caste determination (Smith *et al.*, 2008b). Undoubtedly, the key to understanding caste determination lies in understanding the interaction between environmental and genotypic effects, but also between different genotypes within the colony. Rapid advances in sequencing technology mean that we are gaining an ever increasing insight into the differences between individual genomes and expression profiles (The Honeybee Genome Sequencing Consortium, 2006; Sumner, 2006; Wang *et al.*, 2006; Smith *et al.*, 2008b). However, elucidating the ultimate causes of the genetic caste-bias observed in a number of species will require a combination of genomic, genetic and behavioural analyses; it is not an individual's genome alone that determines caste, but the environmental and social context in which it is expressed.

Equally, the consequences, in terms of direct fitness and colony productivity, of genetic caste-bias are also important if we are to understand the selective forces that govern such behaviour. In the case of *Schwarziana quadripunctata*, dwarf queens have reduced fecundity in comparison to their 'normal' counterparts and consequently, this strategy is rare relative to developing as a worker (Wenseleers *et al.*, 2005). The costs to self-determination of caste are obvious in this species, but in others they may be more subtle, and the occurrence of such strategies are likely to be, in part, governed by the trade-off between direct fitness gained through selfish behaviour and indirect fitness lost through costs to colony productivity.

In this thesis, I investigate the effect of physiological factors on caste determination and caste-biasing in the highly eusocial Hymenoptera, as well as some of the possible fitness costs accrued by genotypes able to bias their development towards that of a queen. First, I consider whether genetic variation in the intrinsic growth rate of *Apis mellifera* larvae is linked to the likelihood of developing as an emergency queen. I then go on to question whether these differences could result from genotypic

differences in the physiological response to developmental hormones. I then investigate, for the first time, whether parasites, themselves important drivers of evolution, have an effect on the physiology of developing *A. mellifera* larvae and hence their likelihood of being reared as an emergency queen.

To investigate the role played by workers in brood recognition, I next look at the recognition and survival of concolonial and allocolonial brood in *Ac. octospinosus*. Heritable recognition cues have been identified in *Ac. octospinosus* at a resolution sufficient to distinguish patrilines within colonies (Nehring *et al.*, 2011), but it is unknown whether these cues could be used by nepotistic workers during queen-rearing. Here, I investigate the treatment and survival of both nestmate and non-nestmate brood by *Ac. octospinosus*, and consider whether any observed differences in treatment might be extended to patrilines within the colony. Finally, having addressed some potential physiological determinants of queen development, I then examine the consequences of being a 'royal cheat' in the leaf-cutting ant *Acromyrmex echinatior*, in terms of body size, a trait important in determining individual reproductive fitness, and fluctuating asymmetry, a measure used to assess developmental stability and which is often associated with other fitness-related traits.

2. Raising royalty: genotypic variation in larval growth rate affects royal destiny in honey bees

Abstract

Phenotypic plasticity is a widespread and key adaptation, allowing many organisms to respond to a changing environment. It is exemplified by the reproductive castes of social insects such as honey bees, where larvae develop either into reproductive queens or sterile workers. Although long thought to be determined solely by environmental cues such as nutrition, genotypic differences in the propensity of larvae to develop into different castes have now been shown in many social insect species. These findings have profound implications for the evolution of cooperation and conflict, but the mechanisms responsible for genotypic variation in caste propensity are as yet unknown. Here, I determined growth rates of honey bee larvae of different genotypes (patrilines) under controlled conditions to investigate whether genetic influences on intrinsic growth rate affect the propensity of larvae to develop into queens rather than workers. I found significant genotypic variation in the growth rate of larvae in all four of the colonies examined. Importantly, royal patrilines, in which larvae were disproportionately likely to develop into queens, had significantly higher growth rates than patrilines in which larvae showed no caste-bias or tended to become workers. The results of this study show that in honey bees, genetic variation in growth rate is partly responsible for genotypic differences in the propensity of larvae to be reared as royalty. This provides a genetically based mechanism of caste-biasing upon which selection can act, but also a route to cheating the socially controlled altruistic behaviour in the honey bee.

2.1 Introduction

Phenotypic plasticity is essential to both the survival and success of many species, and is epitomised by the reproductive castes of the social insects. Reproductive division of labour is arguably the key trait underlying eusociality, and it is this division of labour between reproductive queens and effectively sterile female workers that has led, at least in part, to the extreme ecological and evolutionary success of the social insects (Wilson, 1987; Crespi & Yanega, 1995). In many species, female castes are polymorphic and extreme phenotypic variation can arise from the same genotypes; queens are specialised for dispersal and reproduction, whereas workers are comparatively small and may be morphologically suited to specific colonial tasks (Wilson, 1953; Oster & Wilson, 1978; Wheeler, 1986; Hölldobler & Wilson, 1990). Understanding the proximate factors that influence such extreme plasticity is essential to our understanding of the origins and maintenance of polymorphism and reproductive division of labour. Ultimately, however, it also provides answers to questions of how conflict and cooperation are mediated in social systems.

Caste determination, the suite of processes that determine the developmental pathway of a totipotent female larva, was traditionally thought to be dependent on environmental influences alone, likely mediated by the amount of food received, which subsequently affects juvenile hormone titres. This is based around the idea of a 'nutritional switch' that controls development as either a queen or a worker (Wheeler, 1986; Rachinsky *et al.*, 1990; Crozier & Pamilo, 1996; Hartfelder & Engels, 1998). However, subsequent work has shown that in many species, genetic factors also play a role in determining caste (Heinze & Buschinger, 1989; Tilley & Oldroyd, 1997; Cahan *et al.*, 2002; Julian *et al.*, 2002; Volny & Gordon, 2002; Châline *et al.*, 2003; Hughes *et al.*, 2003; Moritz *et al.*, 2005; Hartfelder *et al.*, 2006; Jaffé *et al.*, 2007; Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Frohschammer & Heinze, 2009); species exhibiting only environmental caste determination (ECD) or only genetic caste determination (GCD) are, in fact, rare, and indeed most species that have been studied fall somewhere on a continuum between extreme ECD and GCD (Schwander *et al.*, 2010).

The process of caste determination can lead to within-colony conflict between the interests of workers and that of the developing individual. Sexual offspring produced by the colony are generally the only means by which workers can contribute genes to the next generation (Hamilton, 1964b) and so the inclusive fitness of workers is strongly linked to colony productivity; workers will maximise their fitness by rearing only as many queens as will maximise the colony's lifetime reproductive output (Bourke & Ratnieks, 1999; Reuter & Keller, 2001). In contrast, individual larvae will favour development as a queen, even if it is not advantageous in terms of overall colony productivity (Bourke & Ratnieks, 1999; Reuter & Keller, 2001). This conflict is most pronounced in polyandrous species because such colonies are made up of a number of full sister lineages (patrilines), who share their haploid paternal genotype. Workers in different patrilines are only half-sisters and consequently average relatedness between workers and their female siblings is lower than in full-sib societies, reducing the indirect fitness gained from raising sisters and thus increasing the relative benefits gained from development as a queen (Estoup *et al.*, 1994).

Thus, cheating genotypes, able to bias their development towards that of a potential queen, may arise in social insect colonies (Ratnieks, 2001; Reuter & Keller, 2001; Wenseleers *et al.*, 2003). Indeed, in a number of species a genetic caste-bias exists, whereby certain rare patrilines are overrepresented in the reproductive caste (Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Haapaniemi & Pamilo, 2012). This phenomenon is best known in the highly polyandrous honey bee, *Apis mellifera*, during the emergency rearing of larvae as queens; when the resident queen is lost from the colony suddenly, larvae from some rare patrilines show a higher propensity to be reared as emergency queens than would be expected by chance (Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005). Although it is possible that this phenomenon can be caused either by 'royalty alleles', the compatibility of maternal and paternal genotypes, or a more complex interaction between brood and worker genotypes (Osborne & Oldroyd, 1999; Schwander & Keller, 2008; Schwander *et al.*, 2010; Libbrecht *et al.*, 2011), the precise mechanism behind patriline caste-biasing is still unknown, and the fascinating question of how some genotypes are able to significantly increase their direct fitness by manipulating their chances of developing as a reproductive individual remains. Here, I determined growth rates of honey bee larvae of different

genotypes (patrilines), reared under controlled conditions on a standardised diet, to investigate whether genetic influences on intrinsic growth rate affect the propensity of larvae to develop into queens rather than workers. A a genetic component to body size has been shown in a number of insect species (Hunt et al., 1998; Bargum et al., 2004; Fjerdingstad, 2005; Smith et al., 2008a; Mitchell et al., 2012). Thus, I hypothesise that patrilines will differ in their growth rate, despite being reared on a standardised diet under controlled conditions. Additionally, insulin signalling pathways involved in caste differences have also been shown to integrate nutrition and metabolism with the regulation of growth and size in *A. mellifera* (Nijhout, 2003b; Wheeler *et al.,* 2006), and so I hypothesise that the growth rate of queen-biased patrilines will differ significantly from that of worker-biased patrilines.

2.2 Methods

2.2.1 Growth rate quantification

Larvae were collected from each of four colonies of the European honey bee *Apis mellifera*, each headed by an unrelated, naturally mated queen. Larvae were reared individually in 48-well tissue culture plates on a diet of 50% royal jelly, 6% D-glucose, 6% D-fructose and sterile deionised water (Aupinel *et al.*, 2005; Jensen *et al.*, 2009). One to two-day-old larvae were transferred from honeycomb directly onto a 20 µl droplet of larval diet within a cell culture plate. The plates were then placed in sealed boxes containing a pool of 0.04% K_2SO_4 in order to establish high relative humidity and maintained at 34°C. Larvae were fed 40 µl, 20 µl, 30 µl, 40 µl and 50 µl of diet on days 1, 3, 4, 5 and 6 after transfer, respectively. Each larva was photographed on days 1, 3, 5 and 7 after transfer using a Moticam 2300 mounted on a Leica MZ8 microscope. Measurements were made using ImageJ 1.42q and were calibrated using a 0.1 mm graticule. Relative growth rate (RGR) was calculated for each individual as:

$$L_2 - L_1 / (L_1 (t_2 - t_1))$$

where L_1 is length at time 1 (t_1) and L_2 is length at time 2 (t_2).

RGR was calculated for a total of 133 larvae from Colony 1, 202 larvae from Colony 2, 162 larvae from Colony 3 and 220 larvae from Colony 4. To quantify measurement error a randomised subset of sixteen individuals were measured three times at each time interval. Measurement error was estimated as the average coefficient of variation (CV) for each individual, using Haldane's correction for small sample size (Haldane, 1955; Lynch & Hayden, 1995). The average measurement error at each of the four time points was less than 1%.

2.2.2 Royal propensity

The four colonies used for the measurement of larval growth rate were split into two queenless halves, each with an equal amount of brood, food and workers, in order to stimulate the production of new queens. The frame containing the colony's queen was placed into a nuc box with food and a subset of workers. After one week, all queen cells were collected from the queenless subcolonies and a new frame containing eggs laid by the respective queen in her nuc was then placed into each queenless subcolony. This process was repeated four times over a period of four weeks. A total of 22 emergency queens were collected from Colony 1, 65 from Colony 2, 24 from Colony 3 and 49 from Colony 4.

2.2.3 Genotyping

Both the larvae reared under controlled conditions and the queens reared by each colony were genotyped. DNA was extracted from whole larvae and pupae using 5% Chelex 100 (BioRad) suspended in 10 μM Tris buffer, boiled for 15 minutes. All samples were genotyped at seven polymorphic microsatellite loci: A7, A14, A35, A79, A107, B124 and AP243 (Estoup *et al.*, 1994). Markers A7, AP243 and B124 were amplified in a multiplex PCR with an initial denaturing step of 94°C for 3 min, followed by a touchdown sequence of five cycles, with the conditions 94°C for 30 s, 60 to 56°C for 45 s, decreasing by 1°C per cycle, and 72°C for 45 s. This was followed by 30 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 45 s, with a final elongation step of 72°C for 7 min. A14, A35, A79 and A107 were amplified in a multiplex reaction with an initial denaturing step of 94°C for 3 min, followed by a touchdown sequence of three cycles, with the conditions 94°C for 30 sec, 62 to 58°C for 45 sec, decreasing by 2°C per cycle, and 72°C for 45 sec. This was followed by 30 cycles of 94°C for 30 sec, 54°C for 45 sec and 72°C for 45 sec, with a final

elongation step of 72°C for 7 min. Reactions were performed in 15 µl volumes containing 2 µl DNA template, 1x reaction buffer, 0.25 mM dNTPs, 0.2 µM of each primer and 0.8 U GoTaq® DNA polymerase. PCR products were run in an ABI 3130*x1* capillary sequencer. Allele sizes were scored by comparison with internal size markers using Genemapper® software and multi-locus offspring genotypes were used to determine the genotypes of the colony queens and their multiple mates. This allowed individuals to be assigned to a particular patriline within each colony. Individuals for which the paternities could not be reliably determined were excluded from the analysis (~3.5% of individuals). I genotyped and assigned successfully 138 larvae and 22 emergency queens from Colony 1, 215 larvae and 48 queens from Colony 2, 230 larvae and 19 queens from Colony 3, and 226 larvae and 46 queens from Colony 4. Patrilines for which there were ≤ 2 individuals in the larvae sample were excluded.

Queen-worker skew, the amount by which a patriline is skewed towards one or other caste compared to the colony average, was calculated on a scale from -1 to 1, by subtracting the expected proportion of queens assuming no skew from the observed proportion of queens, and multiplying by two. Patrilines with a queen-worker skew greater than 0.4 were classified as queen-skewed, and less than -0.4 were classified as worker-skewed. Those with a queen-worker skew between -0.4 and 0.4 were therefore non-skewed. The sample lacked the very strongly queen-skewed patrilines that are rare in colonies (Moritz *et al.*, 2005), and therefore a threshold of ≥0.4 was used so that all three categories contained at least four patrilines and our power of detecting a large effect was 0.47. The categorisation of queen- and worker-skewed patrilines at cut-off values ranging from 0.3 to 0.5 did not affect either the direction or size of the effect (Figure A2.1a). Additionally, in models excluding non-skewed patrilines, the effect size was greater when a skew cut-off of 0.4 or above was used (Figure A2.1b). The analysis was repeated, including two additional patrilines from Colony 1 with a queen-worker skew of 0.39 in the queen-skewed group, making a total of 6 queen-skewed patrilines. The nature of the relationship between queen-worker skew and RGR did not change (Figure A2.1c). However, reclassifying these patrilines increased our power of detecting a large effect to 0.54 but decreased the size of the observed effect, indicating a trade-off between potential power and effect size.

2.2.4 Statistical analysis

Analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA). All analyses were carried out by fitting generalised linear models (GLM) with gamma error structure unless otherwise stated. Non-significant interactions were removed stepwise from the full models to obtain the minimum adequate models. The effect of colony and patriline on RGR were determined using RGR as the response variable and colony and patriline nested within colony as fixed factors. A repeated measures analysis of variance was used to assess whether the growth response of colony and patriline differed over time. The RGRs for each individual calculated at three time points – days 1 – 3, days 3 – 5 and days 5 – 7 – were used as the repeated measure, with colony and patriline nested within colony as fixed factors. The Greenhouse-Geisser correction was used to correct for the data violating the assumption of sphericity. To assess the relationship between patriline queen-worker skew and larval growth rate, the residual RGR was calculated by subtracting the mean colony RGR from the RGR of each individual in the respective colony. RGR differed significantly between colonies and so this gave a measure of the growth rate of each individual in comparison with the colony norm. To determine whether queen-skewed, non-skewed and worker-skewed patrilines differed in their growth rate, residual RGR was used as the response variable, with skew type and colony as fixed factors. Effect size (ω) was calculated as detailed in Field (2005). ω is an unbiased estimate of *r* (Field, 2005), thus 95% confidence intervals were calculated as for *r* (Baguley, 2012).

To determine whether patrilines with low, medium and high residual RGR differed in their queen-worker skew, skew was used as a response variable with growth rate and colony as fixed factors. Low, medium and high residual RGRs were classified by dividing the range of residual RGRs into three equal groups, -0.157 – -0.072, -0.073 – 0.012 and 0.013 – 0.097 respectively. The group with low residual RGR contained 10 patrilines, medium residual RGR 18 patrilines and high residual RGR 10 patrilines.

2.3 Results

2.3.1 Genetic variation in growth rate

There was a significant difference between the four colonies ($\chi^2 = 90.5$, d.f. = 3, $P < 0.0001$) and between patrilines nested within colony ($\chi^2 = 122.1$, d.f. = 34, $P < 0.0001$) in the relative growth rate (RGR) of larvae reared on a standardised diet under controlled laboratory conditions (Figure 2.1). Repeated measures ANOVA showed RGR to differ significantly between time points ($F_{2, 1106} = 1683.5$, $P < 0.0001$). RGR was highest during the earliest stage of development, and decreased between each time period. There was a significant interaction between time and colony ($F_{5, 1106} = 48.9$, $P < 0.0001$) and time and patriline nested within colony ($F_{55, 1106} = 1.75$, $P < 0.001$), suggesting that both colonies and patrilines differed in their growth rate over time.

2.3 2 Growth rate and royal propensity

Each of the four colonies had one patriline with substantial queen-bias (queen-worker skew greater than 0.4). There was a total of nine worker-skewed patrilines, with a queen-worker skew less than -0.4 (Figure 2.2). Although a patriline skew cut-off of 0.4 was used, using values ranging from 0.3 to 0.5 to define queen-skewed and worker-skewed patrilines did not alter either the size or direction of the effect (Figure A2.1a). There was a significant difference in residual RGR between queen-skewed, non-skewed and worker-skewed patrilines ($\chi^2 = 9.20$, d.f. = 2, $P = 0.01$; Figure 2.3a). Queen-skewed patrilines had a significantly higher residual RGR than worker-skewed patrilines ($P = 0.01$), while the differences in residual RGR between non-skewed patrilines and queen-skewed or worker-skewed patrilines were marginally non-significant ($P = 0.08$ and $P = 0.06$ respectively). Queen-skewed patrilines had a residual RGR greater than the colony norm whereas worker-skewed patrilines had a residual RGR lower than the colony norm.

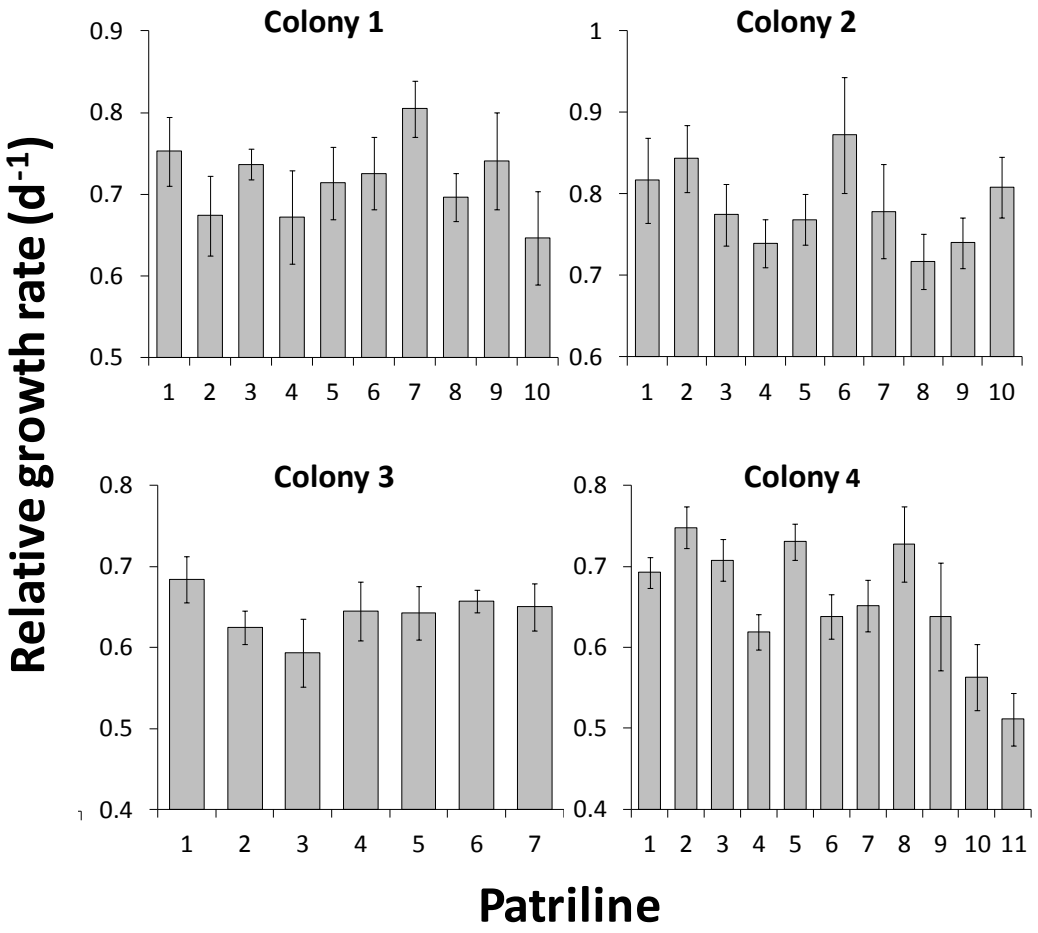

Figure 2.1 Mean ± SEM relative growth rate of honey bee larvae from different genotypes (patrilines) within four colonies. The larvae were kept under controlled conditions in the laboratory and fed identical quantities of a standardised diet.

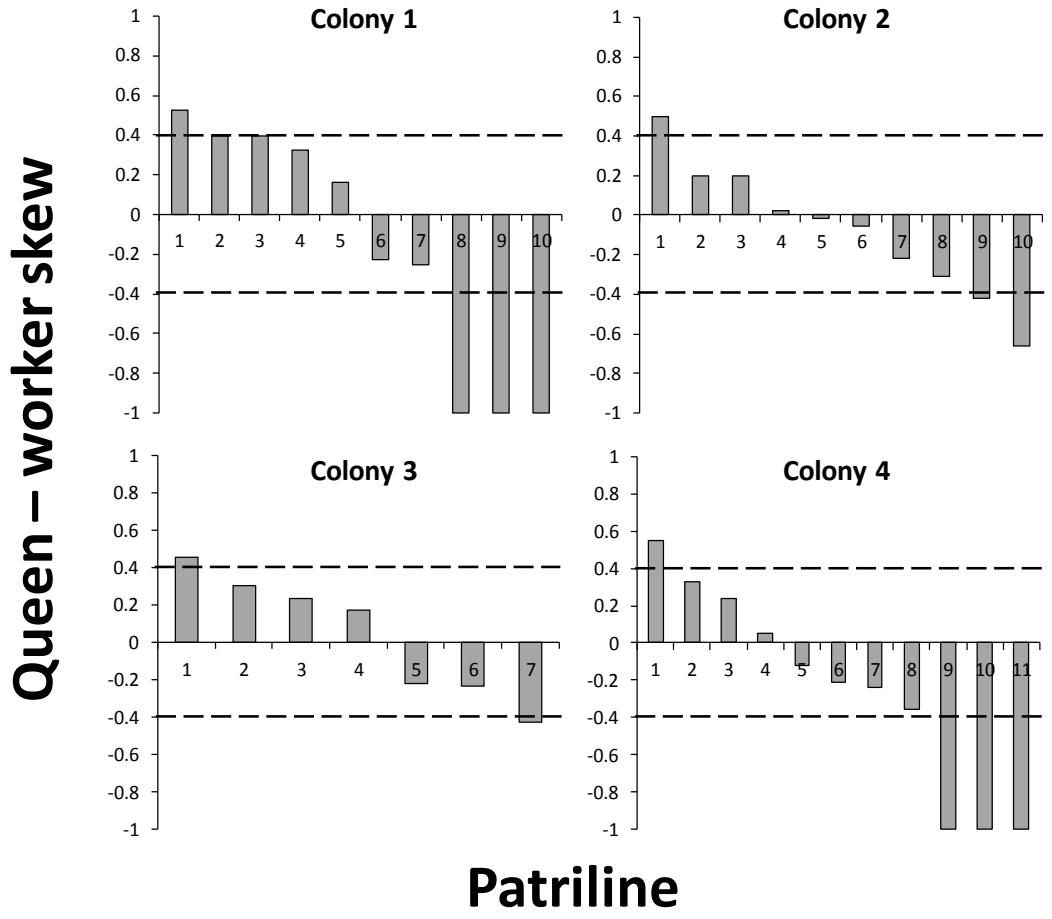

Figure 2.2 Queen-worker skew for genotypes (patrilines) in each of four honey bee colonies, as determined by the queen-worker destiny of larvae in their respective colonies. Dashed lines represent the skew at which patrilines are classified as queen-skewed (0.4) and worker-skewed (-0.4).

There was a significant difference in mean queen-worker skew between patrilines with a low, medium and high residual RGR ($\chi^2 = 170.1$, d.f. = 2, $P < 0.0001$; Figure 2.3b). All three groups differed significantly from each other (low – medium RGR $P < 0.0001$, low – high RGR $P < 0.0001$, medium – high RGR $P = 0.031$). Patrilines with a high growth rate had the highest queen-worker skew, followed by those with an intermediate growth rate, and those with a low growth rate had the lowest skew. Patrilines with an intermediate and low growth rate had, on average, a worker-bias whereas those with a high growth rate tended to be queen-biased.

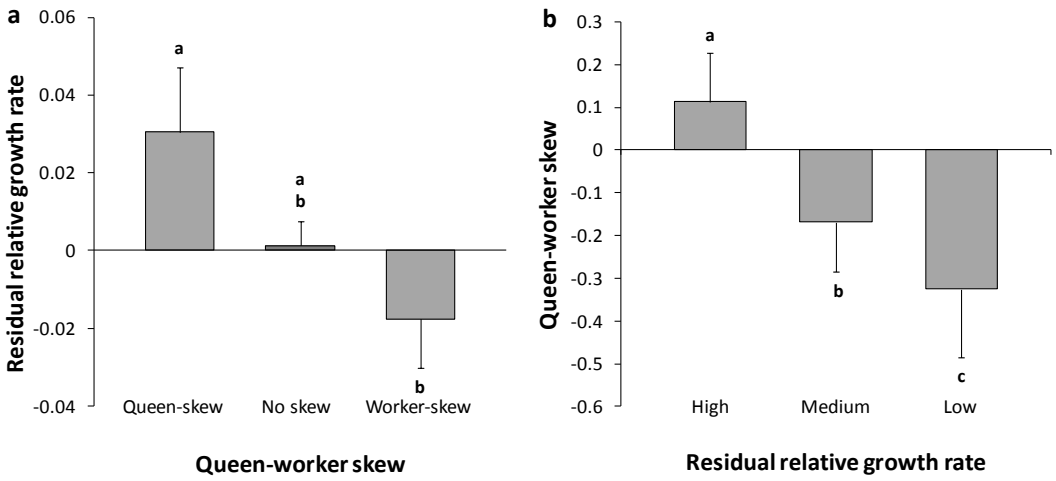

Figure 2.3 a) Mean ± SEM residual relative growth rate of honey bee larvae from genotypes (patrilines) that were either queen-skewed, worker-skewed, or showed no queen-worker skew. Queen-skewed patrilines were defined as having a queen-worker skew of 0.4 or above and worker-skewed a skew of less than -0.4, although varying this threshold between 0.3 and 0.5 did not affect the size or direction of the effect (Figure A2.1). Sample sizes were: queen-skewed N = 48; no-skew N= 553; worker skew N = 116. **b)** Mean ± SEM queen-worker skew of larvae from genotypes (patrilines) that had a high, medium and low residual relative growth rate. Sample sizes were: high RGR N= 155; medium RGR N= 387; low RGR N = 175. Letters indicate significant differences between groups at $P < 0.05$. Relative growth rate was quantified for larvae reared under controlled laboratory conditions on a standardised diet. Queen-worker skew was determined based on the queen-worker destiny of larvae in the respective colonies.

2.4 Discussion

Here, I show a genetic effect on larval growth rate in the honey bee, *Apis mellifera*, both over the entire duration of larval development and in changes in growth rate over time. Honey bee queens have an extremely high mating frequency, leading to high intracolonial genetic diversity (Estoup *et al.*, 1994; Hughes *et al.*, 2008b). One of the advantages of high genetic diversity is task specialisation; within a colony, certain genotypes are more likely to perform particular tasks, thereby increasing colony efficiency through division of labour and task partitioning (Calderone *et al.*,

1989; Robinson, 1992; Beshers & Fewell, 2001; Hughes *et al.*, 2003; Chapman *et al.*, 2007; Oldroyd & Fewell, 2007). Consequently, genetic variation in developmental responses, for example growth rate, to environmental stimuli may increase colony fitness.

Body size is intrinsically linked to both growth rate and development time (Davidowitz & Nijhout, 2004), and a genetic effect on body size has been shown in a number of different social insect species (Hunt *et al.*, 1998; Bargum *et al.*, 2004; Fjerdingstad, 2005; Smith *et al.*, 2008a; Mitchell *et al.*, 2012). Therefore, on an individual as well as a colony level, differences in growth rate could have implications for fitness, as large individuals tend to have a greater fecundity and mating success than those that are small (Honěk, 1993; Carvalho *et al.*, 1998; Bonduriansky, 2001; Jennions *et al.*, 2001; Kovacs *et al.*, 2008; Tarpy *et al.*, 2011). In social insects, maximising reproductive success is imperative both to the fitness of reproductive queens and to that of sterile workers, whose inclusive fitness is dependent on the reproduction of their sisters (Hamilton, 1964a; Hamilton, 1964b; Trivers & Hare, 1976). In addition to increased reproductive success, in the honey bee a faster growth rate could increase direct fitness by increasing survival probability; colonies raise a number of emergency queens and in general, the queen that emerges first has the greatest chance of eventually becoming the colony queen, through the destruction of the cells of slower to emerge rivals (Schneider & Degrandi-Hoffman, 2003). These results suggest that differences in caste propensity of *Apis mellifera* lineages result, in part, from differential developmental responses to the nutritional environment (Linksvayer *et al.*, 2011); they show that both genotypes and colonies differ significantly in their intrinsic growth rate when reared under constant environmental conditions on a standardised diet.

Although the nutritional environment is undoubtedly key to caste determination in the honey bee, the ultimate caste fate of an individual larva is evidently dependent on more than just differential feeding triggering differences in gene expression, as shown by the variable propensity of patrilines to be reared as queens demonstrated here and in a number of other studies of honey bees (Moritz *et al.*, 1996; Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005). The exact cause of this genetic caste-biasing is as yet unknown but the results

presented here show that it may be the result of genotypic differences in growth rate; queen-skewed patrilines have a significantly higher growth rate than worker-skewed patrilines, with non-skewed patrilines having an intermediate growth rate. In addition, genotypes with a high growth rate were more likely to develop into reproductive queens than genotypes with either a medium or low growth rate. In *A. mellifera* queens, genes in the insulin signalling pathway are upregulated (Wheeler *et al.*, 2006). This pathway is also thought to integrate nutrition and metabolism with the regulation of growth and size (Nijhout, 2003b) and thus provides a viable mechanism through which nutrition, growth and caste determination may be linked in the social insects.

Accessing a particular nutritional environment, and utilising it once it is available, depends heavily on both genetic and social factors and it is the workers who select larvae to be reared as emergency queens. Although it seems that workers are able to petition for particular larvae via chemical signals, we have yet to understand the criteria against which larvae are selected (Al-Kahtani & Bienefeld, 2011). It may be that larvae that are comparatively large for their age are more attractive to nurse workers because of the reproductive fitness benefits associated with large body size, or because individuals with a high growth rate are more likely to develop successfully as queens. Indeed, genetic variation in rearing attractiveness has been shown a number of times in honey bees (Tilley & Oldroyd, 1997; Beekman *et al.*, 2000; Calis *et al.*, 2002). Alternatively, it may be that larvae better able to utilise nutritional resources are also better able to signal their 'queen-potential' to nurse workers, either chemically or through begging behaviour (Kaptein *et al.*, 2005; Le Conte & Hefetz, 2008) .

A genetic component to caste determination may take the form of specific 'royal alleles'. Such variants could show strong phenotypic effects, but equally the effect may instead be more subtle, consisting of a combination of genetically-influenced characteristics that increase an individual's likelihood of following a particular developmental trajectory (Cahan *et al.*, 2002; Volny & Gordon, 2002; Ohkawara *et al.*, 2006; Anderson *et al.*, 2008). Alternatively, the compatibility between paternal and maternal genotypes, or the interaction between worker and brood genotypes, may affect caste propensity (Tilley & Oldroyd, 1997; Schwander & Keller, 2008;

Schwander *et al.*, 2010; Libbrecht *et al.*, 2011). Regardless of the genetic mechanism driving differences in growth rate, the results here show a proximate physiological mechanism behind genotypic variation in caste propensity in this system, providing the first example of a genetically-influenced physiological trait directly influencing caste fate.

3. Genotypic variation in the effect of juvenile hormone on the growth of honey bee larvae

Abstract

The social insects are defined by reproductive division of labour, a trait that has contributed to the extreme success of this group. Usually, female caste is dependent on the amount of food received by developing larvae, but in a number of species, including the honey bee, *Apis mellifera*, there is evidence for genotypic variation in the propensity of individuals to become queens. The mechanisms underlying this phenomenon are as yet unknown, but it is possible that genotypes may vary in their physiological response to caste-determining environmental cues. Here, I investigate whether treatment with the juvenile hormone analogue methoprene affects the growth rate of honey bee larvae fed a standardised diet, and whether any effect is genotypically variable. Juvenile hormone has a pleiotropic function in the social insects, and is involved in pathways determining growth and body size, and caste determination. There was a significant interaction between the effects of genotype and hormone treatment on larval growth rate. Larvae treated with the hormone had, on average, a higher growth rate than controls during early development, but a lower growth rate during the latter stages of development, and the magnitude of this response differed significantly between both genotypes and colonies. These results show that genotypic variation in the physiological response to developmental hormones is present in *A. mellifera*, and such variation could potentially give rise to genotypes that vary in their response to the nutritionally controlled caste determination system.

3.1 Introduction

Caste determination is a complex process that underlies the extreme evolutionary and ecological success of the social insects (Wilson, 1987; Wilson, 1990). The development of totipotent female larvae into either reproductive queens or sterile workers is, in most species, governed by an interaction between genetic and environmental factors (Anderson *et al.*, 2008; Schwander *et al.*, 2010). Typically, the amount of nutrition a larva receives during development determines whether it will follow a particular developmental trajectory; larvae will switch to a worker developmental pathway if they receive insufficient nutrition to develop as a queen (Wheeler, 1986). Although the paradigm that nutritional cues are involved in caste determination is well established, there are a number of species in which a genetic component to caste has been identified (Cahan & Keller, 2003; Cahan & Vinson, 2003; Hughes *et al.*, 2003; Pearcy *et al.*, 2004; Hartfelder *et al.*, 2006; Ohkawara *et al.*, 2006; Foucaud *et al.*, 2007; Schwander *et al.*, 2010). In species where queens are polyandrous, such a genetic influence often takes the form of a genetic caste-bias, whereby certain genotypes are over-represented in the queen caste (Châline *et al.*, 2003; Moritz *et al.*, 2005; Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Nanork *et al.*, 2011; Qian *et al.*, 2011). Although the mechanism underlying genetic caste-bias is as yet unknown, it is likely to involve genotypic variation in the physiological response to caste-determining environmental cues (Hughes & Boomsma, 2007).

Larval nutrition can affect caste determination through its interaction with developmental hormones, primarily juvenile hormone (JH) and ecdysteroids, which are involved in development and moulting in all insects (Hartfelder & Engels, 1998; Hartfelder *et al.*, 2000). These hormones act during critical periods of larval development (Nijhout & Wheeler, 1982; Wheeler, 1991) and JH sensitive periods occur during each larval stage, usually concurrent with ecdysteroid peaks (Riddiford, 1994). In general, ecdysteroids stimulate larval-larval moults in the presence of JH, but in the final larval instar JH levels decrease and ecdysteroids stimulate pupation (Nijhout, 1994; Davidowitz & Nijhout, 2004).

JH has a varied role in the insects. As well as mediating moulting cycles, in many insects it acts to stimulate and regulate the production of vitellogenin, a haemolymph

protein involved in oocyte development (Hagedorn & Kunkel, 1979; Raikhel & Dhadialla, 1992; Valle, 1993; Bownes, 1994). However, in the highly eusocial Hymenoptera, JH has lost its gonadotrophic function (Robinson & Vargo, 1997; Hartfelder *et al.*, 2002) although it is involved in the generation of different morphs during caste determination. In the honey bee, *Apis mellifera*, dramatic differences in JH titres in queen- and worker-destined larvae have been recorded between the third and fifth instars, a period concurrent with the point at which worker and queen larvae are fed differing diets by workers (Rembold, 1987; Rachinsky *et al.*, 1990; Rembold *et al.*, 1992). In this species, JH has been shown to affect cell proliferation in the ovaries, and to stimulate an earlier increase in ecdysteroid production in queen larvae, which in turn affects caste-specific protein synthesis in the ovaries (Schmidt Capella & Hartfelder, 1998; Hartfelder, 2000). In addition to its role in caste determination, in *A. mellifera* JH is also involved in worker polyethism and high JH levels are associated with flight activity in queens, drones and workers (Robinson *et al.*, 1991; Giray & Robinson, 1996; Tozetto *et al.*, 1997). Thus, the function of JH has evolved, modulating its role in determining reproductive physiology and acting to integrate social behaviour and colony function (Hartfelder, 2000).

Here, I investigate the growth response of honey bee larvae to the *in vitro* application of the juvenile hormone analogue methoprene, and consider whether any difference in the response observed is dependent on genotype. Caste determination in the honey bee is known to be regulated by the diet fed to developing larvae, with queens being fed a diet unavailable to developing workers (Haydak, 1970; Winston, 1987). However, when the colony queen dies or leaves the colony suddenly, workers rear a number of emergency queens from existing larvae. During this process certain rare genotypes (patrilines) are over-represented in the queen caste, signifying a genetic component to caste determination (Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005). Growth rate, which is also influenced by both genotype and nutrition, has been shown to play a role in this phenomenon (Nijhout, 2003b; Davidowitz *et al.*, 2004; Chapter 2). Consequently, it is possible that genotypic variation in either hormone titres in the haemolymph, or variation in the secretion of hormones in response to a particular amount of nutritional cue, may be responsible for marrying genotypic variation in queen-worker propensity and the physiological response of larvae to developmental

hormones. Thus, I hypothesise that genotypes within a honey bee colony will differ in their growth response to juvenile hormone treatment as a result of differing genotypically determined response thresholds to internal hormone titres.

3.2 Methods

3.2.1 Juvenile hormone treatment

Larvae were collected from three colonies of the European honey bee *Apis mellifera*, each headed by an unrelated, naturally mated queen. Larvae were reared individually in 48-well tissue culture plates on a diet of 50% royal jelly, 6% D-glucose, 6% D-fructose and sterile deionised water (Aupinel *et al.*, 2005; Jensen *et al.*, 2009). One to two-day-old larvae were transferred from brood frames directly onto a 60 µl droplet of larval diet within a cell culture plate. A methoprene stock of 10 mg/ml was made using 100% acetone, which was subsequently diluted for experimental use with sterilised deionised water. Larvae in the treatment group were fed 2 µl of methoprene (Sigma Aldrich) at a concentration of 0.2 mg/ml on day 2 after transfer, and 5 µl of methoprene at 1 mg/ml on day 5 after transfer. Control larvae were fed either sterilised deionised water or acetone diluted to the same extent as the methoprene (day 2: 1:50; day 5: 1:10) in equal volumes and at the same time as the methoprene treated larvae. Experimental methoprene dosages were based on those used by Wheeler and Nijhout (1983) and a preliminary dose-response experiment. The plates were kept in sealed boxes containing a pool of 0.04% K_2SO_4 in order to establish high relative humidity and maintained at 34°C. Survival was monitored for 7 days and larvae were fed 20 µl, 30 µl, 40 µl and 50 µl of diet on days 3, 4, 5 and 6 after transfer, respectively. Only larvae that survived the full seven day period were used in subsequent analyses.

3.2.2 Growth rate quantification

Each larva was photographed on days 1, 4 and 7 after transfer using a Moticam 2300 mounted on a Leica MZ8 microscope. Measurements were made using ImageJ 1.42q and were calibrated using a 0.1 mm graticule. Relative growth rate (RGR) was calculated for each individual as:

$L_2 – L_1 / (L_1 (t_2 – t_1))$

where L_1 is length at time 1 (t_1) and L_2 is length at time 2 (t_2).

RGR was calculated for a total of 460 larvae treated with JH, 378 treated with acetone control and 309 treated with water controls. To quantify measurement error a randomized subset of sixteen individuals were measured three times at each time interval. Measurement error was estimated as the average coefficient of variation (CV) for each individual, using Haldane's correction for small sample size (Haldane, 1955; Lynch & Hayden, 1995). The average measurement error at each of the four time points was less than 1%.

3.2.3 Genotyping

All surviving larvae after 7 days were genotyped. DNA was extracted using 5% Chelex 100 (BioRad) suspended in 10 µM Tris buffer, and boiled for 15 minutes. All samples were genotyped at seven polymorphic microsatellite loci: A7, A14, A35, A79, A107, B124 and AP243 (Estoup *et al.*, 1994). Markers A7, AP243 and B124 were amplified in a multiplex PCR with an initial denaturing step of 94°C for 3 min, followed by a touchdown sequence of five cycles, with the conditions 94°C for 30 s, 60 to 56°C for 45 s, decreasing by 1°C per cycle, and 72°C for 45 s. This was followed by 30 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 45 s, with a final elongation step of 72°C for 7 min. A14, A35, A79 and A107 were amplified in a multiplex reaction with an initial denaturing step of 94°C for 3 min, followed by a touchdown sequence of three cycles, with the conditions 94°C for 30 sec, 62 to 58°C for 45 sec, decreasing by 2°C per cycle, and 72°C for 45 sec. This was followed by 30 cycles of 94°C for 30 sec, 54°C for 45 sec and 72°C for 45 sec, with a final elongation step of 72°C for 7 min. Reactions were performed in 15 µl volumes containing 2 µl DNA template, 1 x reaction buffer, 0.25 mM dNTPs, 0.2 µM of each primer and 0.8 U GoTaq® DNA polymerase. PCR products were run in an ABI 3130*x1* capillary sequencer. Allele sizes were scored by comparison with internal size markers using Genemapper® software and multi-locus offspring genotypes were used to determine the genotypes of the colony queens and their multiple mates. This allowed individuals to be assigned to a particular patriline within each colony Individuals for which the paternities could not be reliably determined were excluded

from the analysis (3.6% of individuals). A total of 414 larvae from 10 patrilines were genotyped in Colony 1, 356 larvae from 17 patrilines in Colony 2 and 291 larvae from 10 patrilines in Colony 3 were successfully genotyped.

3.2.4 Statistical analysis

All analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA). All analyses were carried out by fitting generalised linear models (GLM) with gamma error structure. Non-significant interactions were removed stepwise from the full models to obtain the minimum adequate models. Residual RGR (the difference between an individual's RGR and the colony norm) was calculated by subtracting the mean RGR of control larvae from the RGR of all methoprene treated larvae in the respective colony. Thus, a positive residual RGR indicates a positive effect of methoprene on growth compared to the colony norm and a negative residual RGR represents a negative effect of methoprene on growth rate. The effect of colony and patriline on residual RGR was analysed using residual RGR as the response variable and colony and patriline nested within colony as fixed factors. Data from the time periods days 1 – 4, days 4 – 7 and days 1 – 7 were analysed using separate models. The effect of colony, patriline and treatment on RGR was determined using a model with RGR as the response variable and colony, patriline nested within colony and treatment as fixed factors.

3.3 Results

The residual relative growth rate of methoprene treated larvae differed significantly between colonies during days 4 – 7 and days 1 – 7 (days 4 – 7: $\chi^2 = 11.04$, d.f. = 2, $P = 0.004$; days 1 – 7: $\chi^2 = 15.4$, d.f. = 2, $P < 0.001$; Figure 3.1). During these time periods, residual RGR was negative in all colonies, indicating the RGR of methoprene treated larvae was below the colony norm. Larvae from Colony 2 were most affected by the treatment. During early development, days 1 – 4, methoprene treated larvae had a higher RGR than the colony norm (positive residual RGR), with larvae from Colony 2, again, being affected most. However, during this time period, the effect of colony on the net RGR of methoprene treated larvae was marginally

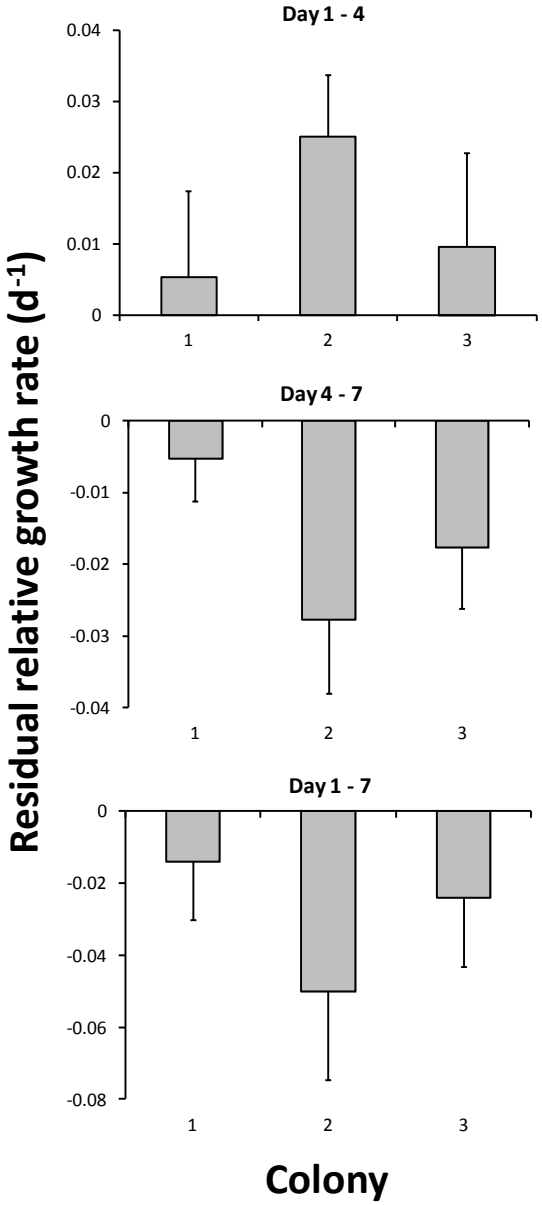

Figure 3.1 Mean ± SEM residual relative growth rate (rRGR) of honey bee larvae treated with methoprene, from each of three colonies. Larvae were treated with methoprene 2 and 5 days after transfer to a standardised diet and controlled laboratory conditions. rRGR was calculated for three time periods: day 1 – 4 after transfer, day 4 – 7 after transfer and days 1 – 7 after transfer and was calculated by subtracting the mean RGR of all control larvae from the respective colony, from the RGR of larvae treated with methoprene. Colony 1, N = 154; Colony 2, N 126; Colony 3, N = 104.

non-significant (χ^2 = 5.58, d.f. = 2, P = 0.061). Additionally, patriline nested within colony had a significant effect on the residual RGR of methoprene-treated larvae during all time periods (days 1 – 4: χ^2 = 175.1, d.f. = 34, P < 0.0001; days 4 – 7: χ^2 = 74.8, d.f. = 34, P < 0.0001; days 1 – 7: χ^2 = 98.0, d.f. = 34, P < 0.001). Over the whole experimental time-period, of seventeen patrilines in Colony 1, eight had a negative residual RGR and nine had a positive residual RGR. In Colony 2, seven patrilines had a negative residual RGR whereas three patrilines had a positive residual RGR. In Colony 3, eight of the ten patrilines had a negative residual RGR and only two patrilines had a positive residual RGR.

Colonies differed significantly the relative growth rate of larvae between days 1 – 4, days 4 – 7 and over the whole study period, days 1 – 7 (days 1 – 4: χ^2 = 88.6, d.f. = 2, P < 0.0001; days 4 – 7: χ^2 = 138.0, d.f. = 2, P < 0.0001; days 1 – 7: χ^2 = 60.97, d.f. = 2, P < 0.0001; Figure 3.2). Larvae from Colony 2 had the lowest growth rate over all three time periods, whereas those from Colony 1 consistently had the highest RGR. There was a significant interaction between treatment and patriline nested within colony (days 1 – 4: χ^2 = 93.5, d.f. = 72, P = 0.045; days 4 – 7: χ^2 = 128.0, d.f. = 72, P < 0.0001; days 1 – 7: χ^2 = 228.3, d.f. = 72, P < 0.001; Figure 3.3); for all time periods, neither treatment nor control larvae consistently had a higher RGR in all patrilines within a colony.

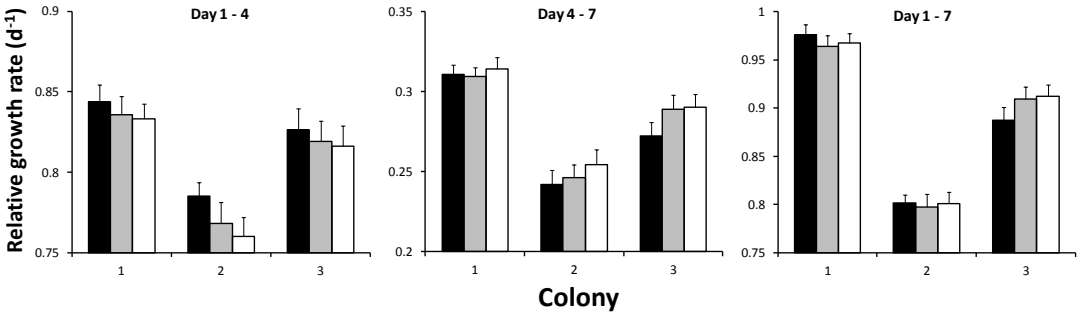

Figure 3.2 Mean + SEM relative growth rate of honey bee larvae, reared on a standardised diet under controlled laboratory conditions, from each of three colonies during three time periods: day 1 – 4 after transfer, day 4 – 7 after transfer and days 1 – 7 after transfer. Larvae were treated with methoprene (black columns), and acetone control (grey columns) or a water control (white columns) on days 2 and 5 after transfer.

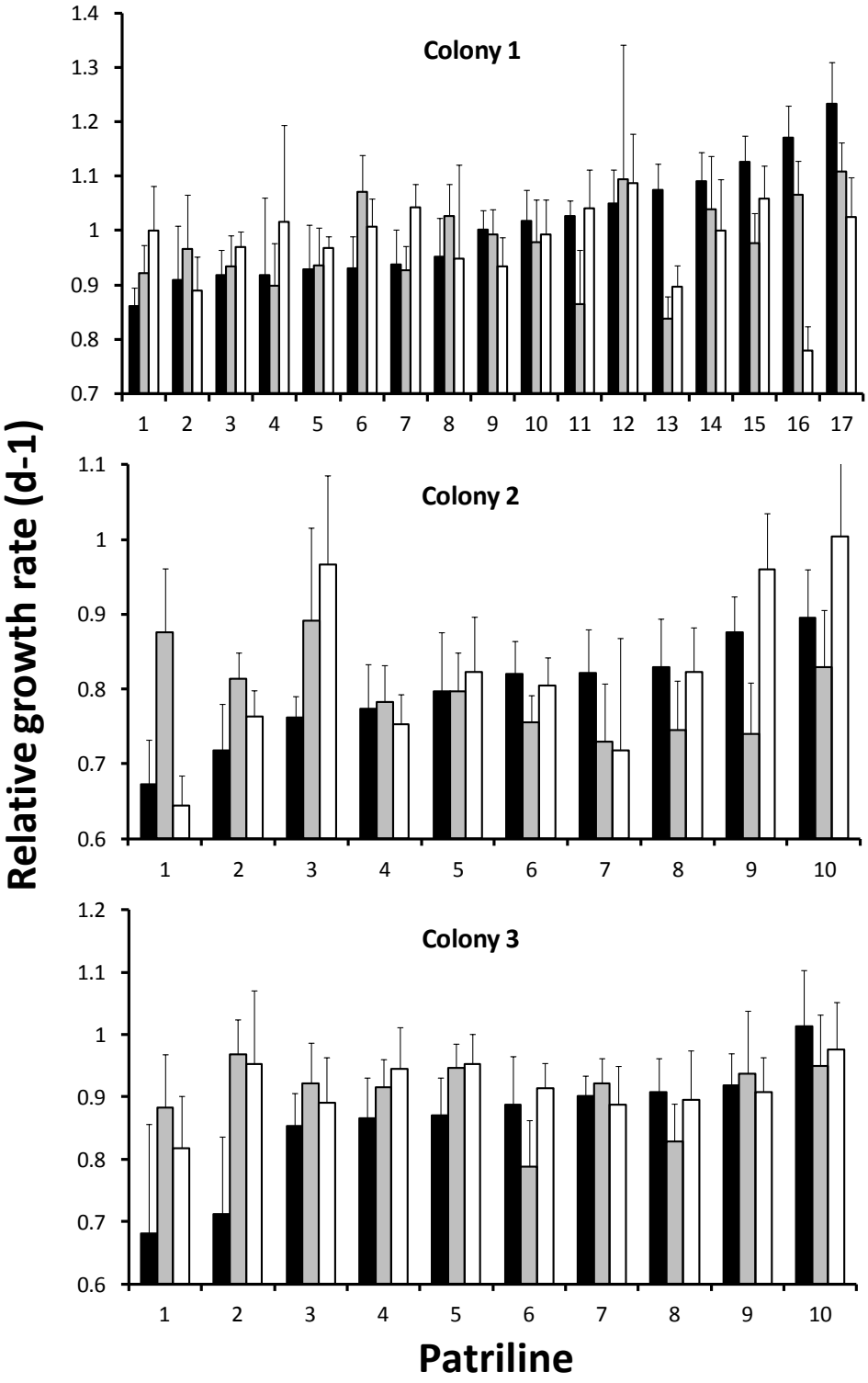

Figure 3.3 Mean + SEM relative growth rate of honey bee larvae, reared on a standardised diet under controlled laboratory conditions, from patrilines in each of three colonies over the whole seven day study period. Larvae were treated with methoprene (black columns), and acetone control (grey columns) or a water control (white columns) on days 2 and 5 after transfer.

3.4 Discussion

Here, I show that larvae from patrilines within honey bee colonies differed in their physiological response, measured as relative growth rate, to treatment with the juvenile hormone (JH) analogue methoprene. The response of patrilines to hormone treatment was not consistent; in some patrilines the RGR was higher when larvae were treated with methoprene than in controls, whereas in other patrilines it was lower. Treatment had no overall effect on larval RGR, but this is likely to be the result of the variation in patrilinial response. In the social insects, JH plays an integral role in caste determination, most likely mediated through the food received by a developing larva (Nijhout & Wheeler, 1982; Wheeler & Nijhout, 1983; Wheeler, 1986; Wheeler, 1991). Here, larvae were fed an identical diet and so it is likely that variation in the larval growth response to JH treatment could be responsible, at least in part, for variation in the propensity of patrilines to be reared as queens in the honey bee and other social insect species (Keller *et al.*, 1997; Tilley & Oldroyd, 1997; Châline *et al.*, 2003; Moritz *et al.*, 2005; Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Nanork *et al.*, 2011). In all insects, JH is involved in a number of developmental and physiological processes (Nijhout & Wheeler, 1982; Nijhout, 1994). Thus, the pleiotropic nature of juvenile hormone makes it a likely candidate to mediate the interaction between growth rate and caste determination, shown previously in *A. mellifera* (Chapter 2).

At the colony level, larvae treated with methoprene had a relative growth rate greater than the colony norm during the early stages of development, but lower than the colony norm during the latter stage of development. In the honey bee, relative growth rate is consistently high during early development, as larvae are small and increase rapidly in size (Chapter 2) and it seems that during this stage, increasing juvenile hormone titres stimulates larval growth. Conversely, during later development when RGR is lower, JH treatment had the opposite effect. The mechanisms underlying this effect are, currently, speculative. During development, JH inhibits the secretion of moulting hormones that initiate metamorphosis, thus prolonging the growth phase (Nijhout, 2003a) and it is possible that increasing JH titre may influence this process. Alternatively, in the honey bee JH is involved in ovary development (Schmidt Capella & Hartfelder, 1998; Hartfelder, 2000) and so it

may be that during the later stages of development, individuals with higher JH titres divert more resources to reproductive development rather than increasing body size.

Whatever the underlying mechanism, colonies differed consistently in their response to JH treatment; Colony 2 was affected most by the hormone treatment during both developmental phases. Interestingly, Colony 2 also consistently had the lowest RGR. This suggests that other factors, such as maternal cues, colony condition or maternal genotype also plays a role in determining the extent to which larvae respond to hormonal cues, as well as in RGR itself.

Ultimately, variation in the physiological response of colonies, and genotypes within them, to treatment with methoprene suggests that genotypic variation, upon which selection can act, in the response to developmental hormones may be present in the honey bee and hence could lead to the evolution of genotypic variation in caste propensity. Consequently, this work provides the first evidence of a physiological mechanism that may, in part, explain genotypic variation in caste destiny not only in the honey bee, but also other species with a genetic caste-bias.

4. Fit for royalty: immune challenge, larval development and caste determination in the honey bee, *Apis mellifera*

Abstract

In all eusocial organisms, caste determination is a process vital for determining both individual fitness, as well as that of all individuals within a colony. In the social insects, caste is determined by a complex interaction between genetic and environmental factors, but whether the interaction with parasites can affect caste determination is as yet unknown. I address this question for the first time by investigating whether the stress of resisting a pathogen affects the larval development and caste fate of a social insect, using the honey bee, *Apis mellifera*, and its fungal pathogen *Ascosphaera apis*. The initial growth rate of larvae reared under controlled conditions was reduced significantly when the larvae had to resist the parasite. However, larvae were subsequently able to compensate for this with a greater growth rate during the latter stages of development, after the infection had been resisted. Importantly, colonies in which larvae suffered most, in terms of growth rate, from parasite-exposure, were also less likely to rear queens from parasite-exposed larvae, whereas colonies in which larvae were better able to resist and compensate for the impact of parasite-exposure, were more likely to rear parasite-exposed larvae as queens. These results show that the indirect effects of parasites on larval development may be an important factor in caste determination that has, so far, received little attention.

4.1 Introduction

Early development is a period vital to determining the fitness of all multicellular organisms. This stage is particularly important in the social insects; it is during this period that a totipotent female larva will develop as either a reproductive queen or sterile worker (Wilson, 1971; Oster & Wilson, 1978). A queen's reproductive potential is important for her own direct fitness, but also for the fitness of her worker sisters whose inclusive fitness relies on the reproductive output of the colony as a whole (Hamilton, 1964a; Hamilton, 1972; Queller & Strassmann, 1998; Boomsma, 2007; Crozier, 2008). Consequently, the production of the highest quality queens is important to all members of a social insect colony.

The caste into which a female larva develops is dependent on both genetic and environmental factors, the relative importance of which differs widely across species (Schwander *et al.*, 2010). Environmental stimuli, primarily nutrition, affect caste determination by altering hormone levels during critical periods of development (Wheeler, 1986; Wheeler, 1991; Hartfelder & Engels, 1998; Evans & Wheeler, 2001; Wheeler *et al.*, 2006; Kucharski *et al.*, 2008), but factors including temperature and interspecific competition have also been shown to affect colony caste ratios (Brian, 1973; Passera *et al.*, 1996). Genotypic effects on caste exist in a variety of species (Cahan *et al.*, 2002; Hughes *et al.*, 2003; Moritz *et al.*, 2005; Hughes & Boomsma, 2008; Smith *et al.*, 2008a; Mitchell *et al.*, 2012), and have been shown to interact with environmental stimuli, leading to plasticity in the genetic influence on caste determination (Robinson, 2002; Hughes & Boomsma, 2007). As yet, however, we have no knowledge of whether parasites can affect caste determination in their social insect hosts, despite extensive research on the relationships between parasites and social insect biology (Schmid-Hempel, 1998; Boomsma *et al.*, 2005; Cremer *et al.*, 2007).

An immune challenge stimulated by contact with a parasite can be costly to an individual, not least during development (Moret & Schmid-Hempel, 2000; Freitak *et al.*, 2003; Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2004; Meylaers *et al.*, 2007). Although host-parasite relationships are important drivers of evolution in many populations, for an individual there is often a trade-off between the fitness costs of resisting a parasite and the costs associated with parasite infection (Frank, 1994;

Boots & Haraguchi, 1999; Webster & Woolhouse, 1999; Zuk & Stoehr, 2002; Cotter *et al.*, 2004). It therefore seems r that the interaction with parasites could affect caste determination in two ways. First, it could affect an individual's ability to develop as a queen, by diverting nutritional resources away from growth, or affecting hormone levels (Khafagi & Hegazi, 2001; Rolff & Siva-Jothy, 2002). Second, a challenge could affect an individual's ability to develop as a high quality queen. Parasite infection during development can affect size, fecundity and reproductive fitness (Fellowes *et al.*, 1999; Rigby & Jokela, 2000; Rantala & Roff, 2005), and in the context of a social insect colony, therefore, the total reproductive output of the colony. Consequently, it could be beneficial for workers to identify developing larvae that had been challenged by a parasite and prevent them being reared as queens, thus focusing resources on more attractive individuals. In many social insect species, workers feed larvae either directly, or by provisioning them with food, and so are directly able to influence caste determination (Hölldobler & Wilson, 1990; Bourke & Ratnieks, 1999).

Here, the effect of an immune challenge by a parasite on larval development and caste fate is investigated using the honey bee, *Apis mellifera*, and a sublethal dose of its fungal pathogen *Ascosphaera apis*. Unlike many other social insect species, the sudden loss of the resident queen from an *A. mellifera* colony results in workers rearing a number of new queens (emergency queens) from existing larvae, one of which will take over reproduction within the colony (Fell & Morse, 1984). Thus, workers have the opportunity to select larvae for rearing as queens and although workers are able to petition for particular individuals via chemical signals, their selection criteria are as yet unidentified (Al-Kahtani & Bienefeld, 2011).

Asc. apis is a common, obligate fungal pathogen which causes the honey bee larval disease chalkbrood. Spores germinate within the digestive tract of larvae, producing hyphae which, if infections are not resisted successfully, spread throughout the body and eventually kill the host insect. Genetic variation in resistance to *Asc. apis* infection has been shown at both the colony level (Tarpy, 2003; Tarpy & Seeley, 2006), and within colonies (Invernizzi *et al.*, 2009; Evison *et al.*, 2013), but the effects of resistance on host physiology and development are unknown. I use a sublethal dose of *Asc. apis* to stimulate an immune response and investigate whether

resisting the parasite has an effect on larval growth under controlled laboratory conditions, indicative of a cost to resistance. Mounting an immune response is costly to all organisms, thus, I hypothesise that the growth rate of larvae challenged by the parasite will be lower than that of control larvae, as a result of resources being diverted from growth to the immune response. I also explore whether resisting a parasite infection affects the selection of larvae to be reared as emergency queens during queen replacement. The inclusive fitness of social insect workers is dependent on fitness of queens produced by the colony and so I also hypothesise that larvae challenged with *Asc. apis* will be less likely to be reared as emergency queens than control larvae. This work is the first to address whether parasite challenge can affect caste determination, providing an initial insight into the interaction between parasites, a significant selective force for all organisms, and caste determination, the key trait defining all eusocial systems.

4.2 Methods

4.2.1 Parasite exposure experiment

Chalkbrood mummies, collected from one colony, were plated onto a media plate and allowed to grow and sporulate. Spores were harvested from the media plate and used to make a spore suspension by grinding ~0.01g of spore material in a glass tissue homogenizer with 50 µl of sterile deionised water. Released spores were made up to a volume of 1 ml with sterile deionised water and left to stand for 20 min to allow asci to settle. A 0.5 ml aliquot of the resulting medium-density spore solution was taken and stored in a separate tube. The concentration of the spore solution was determined using FastRead disposable haemocytometers (Immune Systems) and diluted to 2×10^4 spores per ml. The concentration used was based on serial dilution results over a 10000-fold range, whereby a concentration of 2×10^4 spores per ml of our apiary strain produced less than 10% mortality when fed to larvae reared under controlled laboratory conditions.

Forty-eight larvae from each of ten colonies, and 96 larvae from one additional colony of the European honey bee *Apis mellifera* were collected, each headed by an

unrelated, naturally mated queen. The colonies used were in a different apiary than that from which the chalkbrood mummies were collected and so had not previously been exposed to the *Asc. apis* strain used in the experiment. Larvae were reared individually in 48-well tissue culture plates on a diet of 50% royal jelly, 6% D-glucose, 6% D-fructose and sterile deionised water (Aupinel *et al.*, 2005; Jensen *et al.*, 2009). One to two-day-old larvae were transferred from a brood frame directly onto a 60 µl droplet of larval diet within a cell culture plate. 5 µl of either sterilised water or *Ascosphaera apis* spore suspension (~100 spores) was then applied directly to the mouth of control and parasite-exposed individuals respectively. Each 48 well plate contained 24 control and 24 parasite-exposed larvae. The plates were then placed in sealed boxes containing a pool of 0.04% K_2SO_4 in order to establish high relative humidity and maintained at 34°C. Larvae were fed 20 µl, 30 µl, 40 µl and 50 µl of diet on days 3, 4, 5 and 6 after transfer, respectively.

4.2.2 The effect of Ascosphaera apis treatment on survival and Abaecin expression

Mortality and evidence of infection (hyphal growth) were monitored daily for nine days after exposure using a stereo microscope. In addition, quantitative RT-PCR was used to assess the effect of *Asc. apis* treatment on the expression of the antimicrobial peptide *Abaecin* (Casteels *et al.*, 1990; Casteels-Josson *et al.*, 1994), as this is upregulated in honey bees in response to *Asc. apis* infection and therefore provides confirmation that sublethal infections have challenged larvae (Aronstein & Saldivar, 2005; Aronstein *et al.*, 2010). The housekeeping gene *Ribosomal Protein S5* (*RPS5*) was used as an endogenous control (Evans & Wheeler, 2000; Evans, 2004). 30 larvae (15 control, 15 parasite-exposed) were collected and treated as described above, but four days after treatment were transferred to 50 µl RNAlater and stored at -20°C. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) and used immediately for quantification. Quantitative RT-PCR reactions were carried out using a StepOne Plus real-time PCR thermal cycler (ABI). Reactions were carried out in 10 µl volumes containing 2 µl template, 250 nM TaqMan MGB Probes, 900 nM of each primer and 3.45 µl of TaqMan® Fast Virus 1-Step Master Mix. Primers and probes were as in Evans & Pettis (2005). Standard curve analyses were run for each assay and the calculated efficiencies were 103% for *Abaecin* and 97% for *RPS5* over a 10,000-fold range. Reactions were run in a StepOne Plus instrument (ABI) in

a Fast RT- PCR method of 48°C for 30 min, 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence data were collected each cycle during the extension step at 60°C and the data were analysed using the StepOne Software v2.1 (ABI). *Abaecin* expression was quantified using the comparative C_T method (Schmittgen & Livak, 2008), standardised against *RPS5* quantity. All samples were run in triplicate, with any triplicate technical replicates with high standard deviations (> 0.5 C_T) removed from the analysis. All plates were run with triplicate positive reference samples to allow calibration across plates, and replicate negative controls to check for contamination.

4.2.3 Growth rate quantification

Each larva was photographed on days 1, 4 and 7 after transfer using a Moticam 2300 mounted on a Leica MZ8 microscope. Measurements were made using ImageJ 1.42q and were calibrated using a 0.1 mm graticule. Relative growth rate (RGR) was calculated for each individual as:

$$L_2 - L_1 / (L_1 (t_2 - t_1))$$

where L_1 is length at time 1 (t_1) and L_2 is length at time 2 (t_2). Only larvae that survived for the full experimental period were used in subsequent analyses of RGR. The growth rate was calculated for a total of 204 parasite-exposed larvae and 244 control larvae.

To quantify measurement error a randomised subset of sixteen individuals were measured three times at each time interval. Measurement error was estimated as the average coefficient of variation (CV) for each individual, using Haldane's correction for small sample size (Haldane, 1955; Lynch & Hayden, 1995). The average measurement error at each of the three time points was less than 1%.

4.2.4 Royal propensity

Twelve colonies, ten of which were used for the measurement of larval growth rate, were split into two queenless halves, each with an equal amount of brood, food and workers, in order to stimulate the production of new queens. The frame containing the colony's queen was placed into a nuc box with food and a subset of workers. One

to two-day-old larvae were transferred to 48-well cell culture plates and treated with distilled water or *Asc. apis* spore solution using the same procedure as for measuring growth rate. After 24 h, ten control and ten parasite-exposed larvae were transferred in an alternating pattern onto a frame containing queen cups and one frame was placed into each queenless colony half from their colony of origin. After seven days, frames were removed and the number of capped queen cells were counted. Larvae and pupae inside each queen cell were checked for visual signs of *Asc. apis* infection.

4.2.5 Statistical analysis

All analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA). Survival of *Asc. apis* exposed larvae was compared to that of control larvae using a Cox regression survival model. The level of *Abaecin* expression was compared between control and parasite-exposed larvae using a generalised linear model (GLM) with gamma error structure and a log link function. RQ was the response variable and treatment was a fixed factor.

The relative growth rate of control and parasite-exposed larvae was compared using a generalised linear mixed model (GLMM) with gamma error structure and log link function. Treatment was included as a fixed factor and colony as a random factor. RGR overall (days 1 – 7), RGR between days 1 – 4 and between days 4 – 7 were analysed separately.

The number of emergency queens produced from parasite-exposed and control samples were compared using a GLMM with negative binomial error structure and a log link function, using number of emergency queens as the response variable, treatment as a fixed factor and colony as a random factor. The net growth rate of parasite-exposed larvae, or ‘effect of exposure’, in each colony was calculated as the RGR of parasite-exposed larvae, minus the mean RGR of control larvae. This measure estimates the effect of parasite exposure on larval RGR relative to the colony norm, thereby accounting for intrinsic variation in RGR between colonies (Figure A4.1). A negative effect of exposure then indicates that parasite-exposure reduced larval growth relative the colony norm, while a positive RGR indicates that parasite-exposure increased larval growth. The composition of emergency queens

produced was used to split the colonies into two groups; those that produced 50% or more of their emergency queens from parasite-exposed larvae and those that reared fewer than 50% of emergency queens from parasite-exposed larvae. The effect of exposure was then compared between these two groups of colonies using a GLM with gamma error structure and a log link. Effect of exposure was the response variable and colony-group was included as a fixed factor.

4.3 Results

4.3.1 Effect of treatment with Ascosphaera apis

There was a small, but significant, effect of exposure to the *Asc. apis* parasite on the survival of larvae (Wald = 9.83, d.f. = 1, $P = 0.002$). Of 285 larvae treated with *Asc. apis* spores, 27% died over the seven day period, compared to 16% of the 290 control larvae. However, there was no significant difference between the survival of control larvae and parasite exposed larvae that did not develop *Asc. apis* infection (Wald = 1.77, d.f. = 1, $P = 0.18$), whereas both these groups had significantly greater survival than parasite exposed individuals that did develop *Asc. apis* infection (Wald = 76.73, d.f. = 1, $P < 0.0001$; Wald = 129.6, d.f. = 1, $P < 0.0001$ respectively; 4.1a).

There was no significant difference in survival between colonies (Wald = 13.81, d.f. = 10, $P = 0.18$). Only 18% of the treated larvae died but did not develop *Asc. apis* spores, whereas 8.7% of the larvae died and did develop *Asc. apis* spores, indicating infection with the parasite. None of the control larvae developed *Asc. apis* infection. *Abaecin* was significantly upregulated in larvae four days after treatment with *Asc. apis* spores, in comparison to control larvae ($\chi^2 = 4.99$, d.f. = 1, $P = 0.025$; Figure 4.1b).

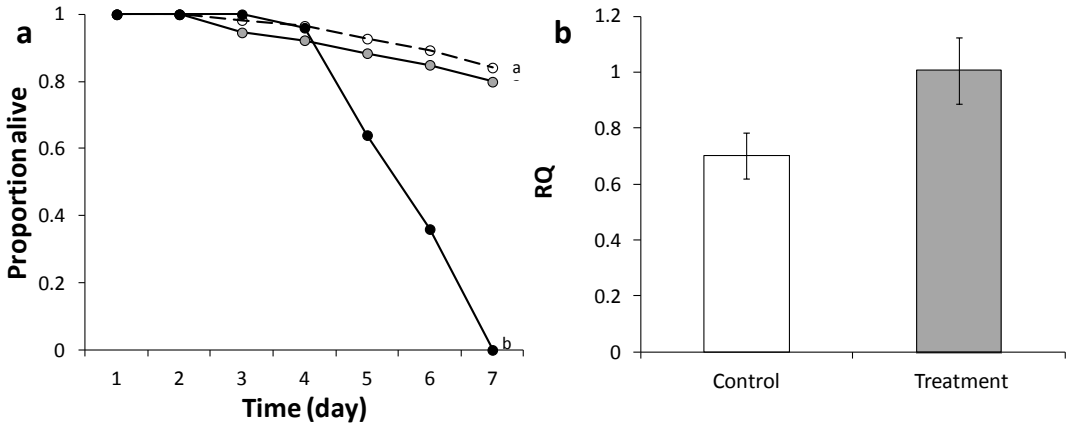

Figure 4.1 a) Proportion of honey bee larvae surviving after exposure to a low dose of the *Ascosphaera apis* fungal parasite and which either did (black circles) or did not (grey circles) become infected, or were treated with control solution (white circles). Different letters represent a significant difference at $P < 0.05$. **b)** Mean ± SEM relative expression (RQ) of the antimicrobial peptide *Abaecin* four days after exposure to a low dose of the *Ascosphaera apis* fungal parasite or a sterilised water control. Honey bee larvae were reared on a standardised diet under controlled laboratory conditions.

4.3.2 Relative growth rate

Relative growth rate (RGR) between days 1 and 4 after treatment was significantly higher for control larvae than for larvae exposed to the parasite ($F_{1, 450} = 6.82$, $P = 0.009$; Figure 4.2a). Between days 4 and 7 after treatment, the opposite was true; RGR of treated larvae was higher than for controls. However, this result was marginally non-significant ($F_{1, 450} = 3.49$, $P = 0.062$; Figure 4.2b). Over the full seven day period, there was no significant difference in RGR between control and parasite-exposed larvae ($F_{1, 450} = 0.004$, $P = 0.95$; Figure 4.2c), although there was a significant difference between colonies ($Z = 2.05$; $P = 0.04$; Figure A4.1).

4.3.3 Queen production

The number of emergency queens reared from control and parasite-exposed larvae did not differ significantly ($F_{1,46} = 0.03$, $P = 0.87$; Figure 4.3). However, colonies varied in whether they reared more emergency queens from control or parasite-exposed larvae: of 12 colonies, two produced an equal number of emergency queens

from control and parasite-exposed larvae, four produced more queens from control larvae, and six produced more queens from exposed larvae (Figure 4.3).

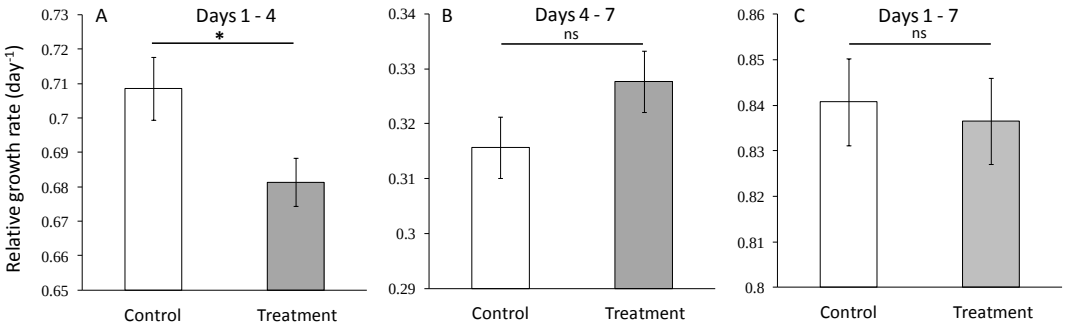

Figure 4.2 Mean ± SEM relative growth rate of honey bee larvae either treated with a low dose of the *Ascosphaera apis* fungal parasite (grey bars) or sterilised water control (white bars). Honey bee larvae were reared under controlled laboratory conditions on a standardised diet. The relative growth rate is show for: **(a)** days 1 – 4, **(b)** days 4 – 7 and **(c)** days 1 – 7. Significant differences are represented by: ns = non significant, * = $P < 0.05$. Parasite-exposed larvae, N = 244; control larvae, N = 204.

During both days 1 – 4 and days 4 – 7, larvae from colonies that reared a low proportion of emergency queens from parasite-exposed larvae showed a more negative effect of parasite-exposure on growth (net RGR) than larvae from colonies that reared a higher proportion of emergency queens from parasite-exposed larvae (Figure 4.4). The effect was strongest between days 4 – 7 with larvae in colonies that reared a high proportion of parasite-exposed larvae as queens showing stronger compensatory growth following exposure ($\chi^2 = 5.17$, d.f. = 1, $P = 0.023$). There was a similar, but non-significant pattern between days 1 – 4, with larvae in colonies that reared a high proportion of parasite-exposed larvae as queens being less negatively affected by the parasite challenge ($\chi^2 = 1.1$, d.f. = 1, $P = 0.29$; Figure 4.4). In colonies that reared a high proportion of their emergency queens from parasite-exposed larvae, these larvae therefore suffered less during early development between days 1 – 4 in terms of reduced growth rate, and were also able to better compensate for this loss during the later stages of development. Accordingly, over

the whole study period (days 1 – 7), there was a highly significant difference in the effect of exposure of the two groups ($\chi^2 = 6.07$, d.f. = 1, $P = 0.008$). Larvae from colonies that reared a high proportion of queens from parasite-exposed larvae in fact showed a positive effect of parasite-exposure; the RGR of parasite-exposed larvae was on average greater than control larvae. Larvae in colonies that reared a low proportion of queens from parasite-exposed larvae showed a negative effect of exposure; parasite-exposed larvae had a lower RGR than control larvae.

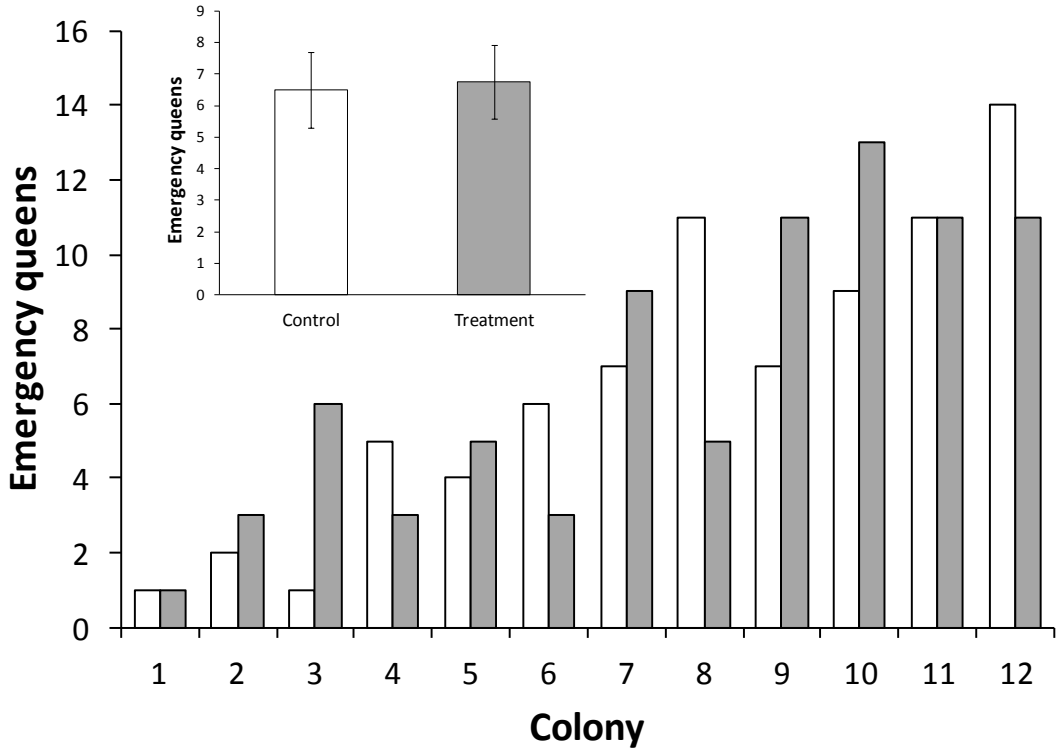

Figure 4.3 Total number of emergency queens reared by each of twelve honey bee colonies from larvae either treated with a low dose of the *Ascosphaera apis* fungal parasite (grey columns) or sterilised water control (white columns). Inset: Mean ± SEM number of emergency queens reared by twelve honey bee colonies from larvae either treated with a low dose of the *Ascosphaera apis* fungal parasite (grey column) or sterilised water control (white column). Queenless subcolonies were provided with a total of 10 control and 10 treated larvae, transferred into queen cups after treatment, from which to select emergency queens.

4.4 Discussion

These results show that an immune challenge, stimulated by treatment with a sublethal dose of the *Ascosphaera apis* fungal parasite, had an effect on the relative growth rate of honey bee larvae. Although the parasite dose with which larvae were treated was relatively low (~100 spores), both the 10% mortality that resulted from parasite treatment and the upregulation of the antimicrobial peptide *Abaecin* show that larvae exposed to *Asc. apis* did face an immune challenge. The effect of parasite-exposure on the likelihood of larvae being selected for rearing as emergency queens was strongly dependent on colony. Colonies in which larvae were more negatively affected by the parasite challenge were less likely to rear parasite-exposed larvae as emergency queens.

The relative growth rate of larvae over the duration of the experiment did not differ between control and parasite-exposed groups. However, between days one and four, RGR was significantly lower in parasite-exposed than control larvae, whereas between days four and seven parasite-exposed larvae had a higher growth rate than the control group, although this was marginally non-significant. This result is in keeping with the survival data; at day four, the survival of larvae that subsequently developed chalkbrood infection dropped sharply and this effect is mirrored in the growth rate data. It appears that for the first few days after exposure to the parasite, an immune response is mounted that diverts resources from growth. The activation of an immune response in honey bee larvae, stimulated by *Asc. apis* treatment, leads to a depletion of nutritional resources resulting from the down-regulation of major storage proteins (Aronstein *et al.*, 2010), and this could have a subsequent effect on larval growth. Only individuals that survived the full duration of the experiment were used in the growth rate analysis and so the lower growth rate of treated larvae early in development is not an artefact created by individuals that would subsequently die from chalkbrood infection.

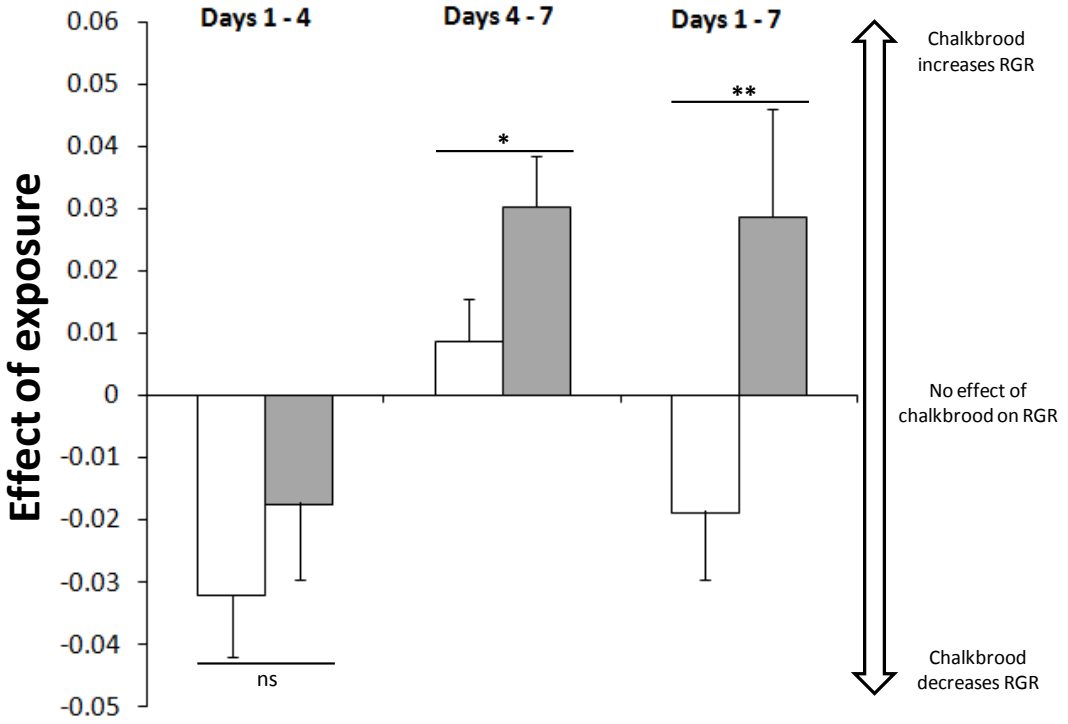

Figure 4.4 Mean ± SEM effect of exposure (RGR of parasite exposed larvae – RGR of control larvae) for honey bee colonies that reared less than 50% of their emergency queens from parasite-exposed larvae (white columns; 6 colonies, N = 113) and colonies that reared 50% or more of their emergency queens from parasite-exposed larvae (grey columns; 4 colonies, N = 75). The effect of exposure is shown for days 1 – 4, days 4 – 7 and days 1 – 7, with negative values indicating that parasite-exposure reduced larval RGR relative to control larvae, and positive values indicating that parasite-exposure increased RGR relative to control larvae. Significant differences are represented by: ns = non significant, * = $P < 0.05$, ** = $P < 0.01$.

The survival data suggest that after approximately four days, larvae had either succumbed to chalkbrood infection, or successfully resisted the parasite, and interestingly, during the later stage of development the growth rate of larvae exposed to *Asc. apis* was greater than that of the control group. This is in accord with growth data from larvae challenged with *Paenibacillus larvae*, the causative agent of American foulbrood, whereby larvae from susceptible lines initially showed a decreased growth rate, but after six days there was no longer a significant difference in size between resistant and susceptible lines (Sutter *et al.*, 1968). Growth rate is

often optimal rather than maximal and compensatory growth, whereby individuals who have experienced a period of growth retardation are able to subsequently increase their growth rate when conditions improve, is well documented in a variety of taxa (Arendt, 1997; Metcalfe & Monaghan, 2001). These results show that having suffered impeded growth, honey bee larvae that have resisted *Asc. apis* infection are able to then compensate for this poor start. However, compensatory growth can leave individuals more vulnerable to nutritional stress and other physiological costs (Clutton-Brock *et al.*, 1985; Gotthard *et al.*, 1994; Samuels & Baracos, 1995; Morgan & Metcalfe, 2001; Yearsley *et al.*, 2004; De Block & Stoks, 2005). Although they may not suffer in terms of net growth, it may be that larvae that have resisted infection, and have already experienced stress through mounting an immune response, lose fitness in other ways associated with nutritional stress, such as learning ability, immune function and fecundity (Jann & Ward, 1999; Siva-Jothy & Thompson, 2002; Riddell & Mallon, 2006).

Another way in which fitness could be affected by exposure to *Asc. apis* is if mounting an immune response affects caste determination. This hypothesis is supported by these results, which show an association between the costs of resisting the parasite, or effect of exposure, and the proportion larvae exposed to the parasite that are reared as queens. It seems that colonies in which parasite-exposed larvae suffered most, in terms of their growth rate, were also the colonies that reared a low proportion of emergency queens from parasite-exposed larvae. These results also reflect other findings that show honey bee genotypes with a high larval growth rate are significantly more likely to be reared as emergency queens than genotypes with a low growth rate (Chapter 2). Although these data are not evidence that the parasite is having a direct effect on caste determination, it appears that the physiological effects of resisting the parasite, shown here in terms of growth rate, can lead to an indirect effect. It may be that larvae with a low growth rate during the early stages of development, or those that are unable to sufficiently compensate for this bad start, are unable to develop successfully as queens, or that workers preferentially select larvae with a higher growth rate at this crucial stage of the colony lifecycle.

The reproductive division of labour between queens and workers is arguably the defining trait of eusocial societies, and several environmental and genetic influences

have previously been shown to be involved in the determination of caste during larval development (Schwander *et al.*, 2010). Our results show, for the first time, that the cost arising from an immune challenge can also affect larval development, and hence caste determination at the colony level. Parasites represent a diverse and widespread selective pressure on all organisms, that can have a multitude of effects on an individual's development and physiology, dependent on factors such as virulence, exposure and resistance mechanisms (Minchella, 1985; May & Anderson, 1990; Frank, 1993; Sheldon & Verhulst, 1996; Thomas *et al.*, 2005). It may therefore be that the indirect effects of parasites on larval development are a more important factor in the determination of royalty in eusocial organisms than has previously been appreciated.

5. Brood recognition capabilities in the leaf-cutting ant *Acromyrmex octospinosus*

Abstract

The expression of altruistic behaviours in all social groups relies on effective kin recognition. In the social insects, nestmate recognition is often important if workers are to maximise their inclusive fitness by directing altruistic behaviour towards related individuals. Discrimination between nestmate and non-nestmate larvae has been documented in a number of social insect species, but brood adoption is also common. However, whether preferential treatment of nestmate brood occurs after adoption, and whether such discrimination can have physiological consequences for developing brood is as yet unknown. Here, workers were shown to discriminate between nestmate and non-nestmate larvae in the leaf-cutting ant *Acromyrmex octospinosus* and discrimination persisted even when larvae had been adopted into the fungus garden of foreign colonies. However, the discrimination response was strongly dependent on the colony from which workers originated, and aggressive interactions were also affected by larval colony-of-origin. Importantly, non-nestmate brood lost significantly more weight prior to pupation than did nestmate brood, suggesting that workers were still able to discriminate brood after it had been incorporated into the fungus garden. These results show that although workers can discriminate larvae based on colony-level cues, strong discriminator-source interactions in this ability may make discrimination at a finer-scale difficult to detect experimentally.

5.1 Introduction

Kin recognition is a trait essential to the evolution of sociality. In social groups, recognition allows the optimal expression of both aggressive and altruistic behaviours, and facilitates social decisions. Whether animals live in groups can be influenced by factors including predator avoidance, resource availability, parental care and disease transmission (Alexander, 1974; Johnson *et al.*, 2002). The trade-off between the costs and benefits of social behaviour is dependent on the relatedness between actor and recipient (Hamilton, 1964a; Hamilton, 1972) and so understanding recognition mechanisms is essential to our understanding of the evolution and maintenance of all social systems.

In many ways, the social insects epitomise cooperative behaviour. In addition to reproductive division of labour, many species have a complex system of task allocation, which increases colony productivity (Hölldobler & Wilson, 1990; Schmid-Hempel, 1992; Anderson & Ratnieks, 1999; Bourke, 1999; Ratnieks & Anderson, 1999). Accordingly, a complex recognition system exists, which is reliant on cuticular hydrocarbons, the composition of which depend upon both individual genotype and colony odour (Jutsum *et al.*, 1979; Heinze *et al.*, 1996; Lahav *et al.*, 1999; Wagner *et al.*, 2000; Akino *et al.*, 2004; Martin *et al.*, 2008; D'Ettorre & Lenoir, 2010; van Zweden *et al.*, 2010; Nehring *et al.*, 2011). Workers eclose with a limited chemical profile and subsequently both synthesise hydrocarbons and acquire them from the environment (Mintzer, 1982; Morel *et al.*, 1988; Stuart, 1992; Dahbi *et al.*, 1998; Liang & Silverman, 2000; Breed *et al.*, 2004). In many species, the strength of aggressive interactions towards foreign individuals have been correlated with differences in cuticular hydrocarbon profiles (Jutsum *et al.*, 1979; Heinze *et al.*, 1996; Roulston *et al.*, 2003; Akino *et al.*, 2004; Richard *et al.*, 2007; Martin *et al.*, 2008; Guerrieri *et al.*, 2009), but recognition cues are also plastic and can vary over time and with colony size or stage (Vandermeer *et al.*, 1989; Stuart, 1991; Provost *et al.*, 1993; Balas & Adams, 1996; D'Ettorre *et al.*, 2004). Thus, the threshold at which non-kin will be accepted is context dependent and will vary depending on the fitness costs of rejecting kin and accepting non-kin (Reeve, 1989; Buczkowski & Silverman, 2005).

In a number of social insect species, workers are able to distinguish brood of differing origins (Klahn & Gamboa, 1983; Lenoir, 1984; Hare & Alloway, 1987; Camargo *et al.*, 2006; Fouks *et al.*, 2011). Brood recognition is crucial in a number of contexts, perhaps most importantly in that of inquiline and slave-making social parasites. Inquiline queens very rarely produce their own work force, but manipulate host workers into rearing parasite brood as reproductive queens, whilst producing no workers themselves (Hölldobler & Wilson, 1990; Bourke & Franks, 1991; Buschinger, 2009). Inquiline social parasites may either accurately mimic the chemical profile of their host colony, or express as few chemical recognition cues as possible, in order to avoid detection by the host colony (Dettner & Liepert, 1994; Lenoir *et al.*, 2001; Lorenzi *et al.*, 2004; Lorenzi, 2006; D'Ettorre & Lenoir, 2010). However, recognition and elimination of parasite brood can greatly increase the inclusive fitness of workers in parasitised colonies (Davies *et al.*, 1989; Lenoir *et al.*, 2001; Lorenzi, 2006). In contrast, slave-making social parasite workers raid nearby colonies of their host species and steal brood, which is subsequently reared as part of the work force (Hölldobler & Wilson, 1990; D'Ettorre & Heinze, 2001; Foitzik & Herbers, 2001). Intraspecific brood raiding also occurs in a number of ant species, often in the context of territoriality disputes (Pollock & Rissing, 1989; Tschinkel, 1992; Gadau *et al.*, 2003; Hölldobler *et al.*, 2011). Here it is the raiding colony that will gain from brood discrimination; workers can increase their inclusive fitness if they are able to distinguish kin and non-kin brood, thus preventing nutritional resources being wasted rearing unrelated female brood as queens, and reducing the chance of cannibalising related brood (Tschinkel, 1993; Bourke, 1994; Clouse, 1995).

Brood recognition may also be adaptive in the absence of selection pressure from social parasites. In polygynous colonies, and colonies with a polyandrous queen, workers could theoretically increase their inclusive fitness by acting nepotistically, preferentially rearing their more closely related kin as queens (Ratnieks & Reeve, 1991; Carlin *et al.*, 1993; Crozier & Pamilo, 1996; Osborne & Oldroyd, 1999; Tarpy *et al.*, 2004a). In the leaf-cutting ant *Acromyrmex octospinosus*, genetic variation in patriline cuticular hydrocarbon profile has been shown to be sufficient for nepotism to occur (Nehring *et al.*, 2011). Despite this, no convincing empirical evidence for intracolonial discrimination has yet been produced, although this does not equate to

evidence for a lack of nepotism outright (Breed *et al.*, 1994; DeHeer & Ross, 1997; Tarpy *et al.*, 2004a; Holzer *et al.*, 2006; Wenseleers, 2007; Zinck *et al.*, 2009). Additionally, the adoption of foreign brood also can be beneficial, strengthening the workforce (Lenoir, 1981; Isingrini *et al.*, 1985; Hare & Alloway, 1987; Fénéron & Jaisson, 1992). Both intra- and interspecific brood adoption has been shown experimentally (Carlin, 1988; Fénéron & Jaisson, 1992; Fouks *et al.*, 2011), although adopted brood may be killed by the host colony upon eclosion (Wilson, 1987). Current research focuses on behavioural interactions, and although it has been shown that survival of heterospecific brood is lower than that of conspecific brood (Carlin *et al.*, 1987; Hare & Alloway, 1987), it is as yet unknown how behavioural discrimination impacts survival of larvae when reared by conspecific but allocolonial workers.

Here, I use a combination of behavioural observations and survival analysis to investigate the relationship between worker behaviour and larval survival in the polyandrous leaf-cutting ant *Acromyrmex octospinosus*. Colonies of this species may be parasitised by the closely related inquiline parasite *A. insinuator* (Schultz *et al.*, 1998; WOHH personal observation), and brood-raiding is also documented within the *Acromyrmex* genus and the closely related *Atta* (Pollock & Rissing, 1989; Fjerdingstad *et al.*, 1998; Adams *et al.*, 2000). In addition, in *Ac. octospinosus* colonies, patrilines differ significantly in their hydrocarbon profile (Nehring *et al.*, 2011). Thus, it is possible that selection for the discrimination of brood on every scale may occur in this species, making it a good model for investigating brood recognition. I hypothesise that *Ac. octospinosus* workers will be able to discriminate between nestmate and non-nestmate larvae, and will modify their behaviour accordingly, directing a higher frequency of aggression but a lower frequency of attentive behaviours, such as grooming, towards non-nestmate workers. Brood of *A. subterraneous subterraneous* have a cuticular hydrocarbon profile that is intermediate between pupae and the fungus with which each colony has an obligate symbiosis (Viana *et al.*, 2001), and so I also investigate whether exposure to foreign fungus prior to interaction with workers contributes to nestmate recognition in *Ac. octospinosus*. I hypothesise that exposure of foreign larvae to worker fungus will disguise allocolonial recognition cues and thus make nestmate discrimination less accurate. However, as individual odour is a combination of genetic and acquired

characteristics, it may not completely eliminate the ability for nestmate discrimination.

5.2 Methods

5.2.1 Experimental setup

Acromyrmex octospinosus colonies, collected from Gamboa, Panama, during May 2010 (Ao1001, Ao1002, Ao1003) and in Trinidad in 2007 (Ao071), were maintained in the laboratory at 26°C and 80% humidity on a diet of privet leaves and rice. Mini-nests were set up from three colonies (Ao1001, Ao1002 and Ao071) and contained five small workers, two medium workers and one large worker, kept in a plastic pot (8 x 5 x 6 cm) with *ad libitum* supply of water and 20% sucrose solution. Small and medium workers were taken from inside the fungus garden. Each mini-nest contained approximately 2.5 cm^3 of fungus in a moist petri dish (5 cm diameter). All workers and larvae visible to the naked eye were removed from the fungus, but eggs and very small larvae remained.

Medium and large larvae were taken from the fungus garden from the same three colonies (Ao1001, Ao1002 and Ao071), plus the fourth (Ao1003), and were kept for 12 hours in a moist petri dish containing either fungus from their colony-of-origin, or fungus from one of the three colonies from which mini-nests were setup. The larvae were then tested with workers of either their own colony or a different colony. This created three larval treatment groups: nestmate larvae exposed to fungus from their own colony-of-origin, non-nestmate larvae exposed previously to the workers' fungus and non-nestmate larvae exposed previously only to their own fungus (Figure 5.1).

5.2.2 Behavioural assay

24 hours after mini-nests had been set up, one larva was placed into each mini-nest outside the fungus chamber and observed for 15 min, during which three worker behaviours were recorded: antennation, grooming, which included licking and manipulation of the larvae with mouthparts but excluded carrying, and aggression,

which included biting and moving the brood to the waste pile (Fouks *et al.*, 2011). The frequency of behaviour was recorded, irrespective of length. An act was counted as a new behavioural event if it was performed by a new individual worker, or a worker that had previously interacted with the brood item but had moved away and subsequently returned. Whether or not brood were placed in the fungus garden during the observation period was also recorded. If a larva was transported to the fungus and subsequently removed within the observation period, this was recorded as not transported. After an initial 15 min observation period, larvae were removed from the mini-nest and rotated for two more observation periods within a group of three mini-nests, constructed from the same colony, in which observations were again made for 15 min. This meant that worker behaviour in each individual mini-nest was observed with a larva from each treatment group to control for differences in behaviour between nests (Figure 5.1). A period of 30 min was left between each 15 min observation period for each mini-nest.

5.2.3 Survival and growth assay

Larvae were tested in the same treatment groups as for the behavioural assay. One larva was placed directly into the fungus garden of each mini-nest. Each nest contained only one focal larva, but also contained a number of very small larvae and eggs, to provide competition for rearing by workers. These larvae were much smaller than the focal larvae and so were easily distinguishable. Survival of the focal larva within each mini-nest was monitored every day for a period of 7 days. Weight was also measured on day 1 and on either the day it pupated, or on day 7 if pupation did not occur. For all larvae that pupated, the weight change up to pupation was calculated as the relative growth rate, thus taking into account the initial size of the larva, and time to pupation, using the formula:

$m_2 - m_1 / (m_1 (t_2 - t_1))$

where m_1 is mass at time 1 (t_1) and m_2 is mass at time 2 (t_2).

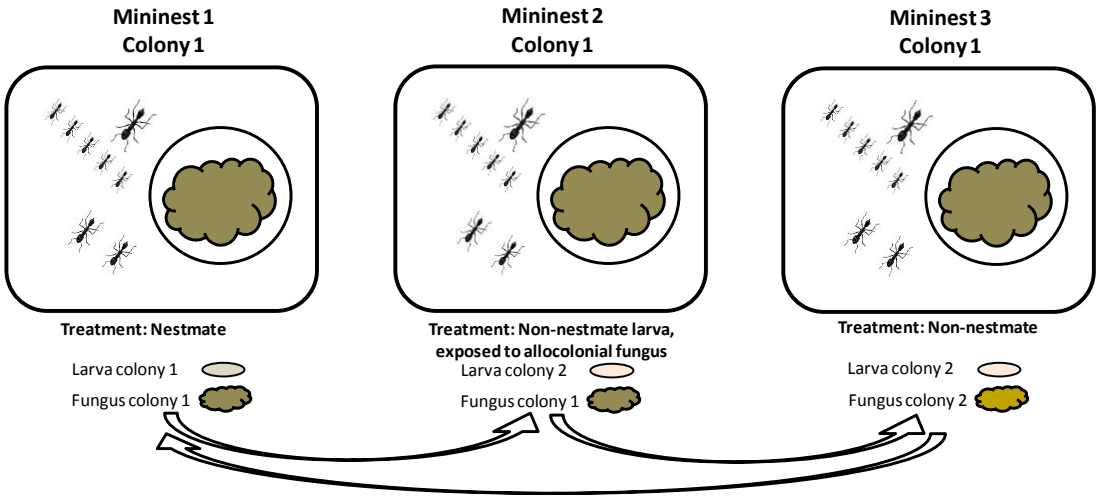

Figure 5.1 Experimental mini-nest set up. Each mini-nest contained a 5 cm diameter Petri dish containing approximately 5 cm^3 of fungus, and five small *Acromyrmex octospinosus* leaf-cutting ant workers, two medium workers and one large worker. Larvae were split into three treatment groups: nestmate larvae exposed to fungus from their own colony-of-origin (Treatment 1), non-nestmate larvae exposed previously to the workers' fungus (Treatment 2) and non-nestmate larvae exposed previously only to their own fungus (Treatment 3). Larvae were placed into the foraging area of the mini-nest and behavioural interactions between workers and larvae were observed for 15 min. Larvae were then rotated, within a group of three mini-nests constructed from the same colony, and observed twice more for 15 min. A total of 19 larvae from Colony Ao1001, 14 from Colony Ao1002, 20 from Colony Ao071 and 16 from Colony Ao1003 were used. Nine mininest were set up from both Colonies Ao1001 and Ao10022, and 18 from Colony Ao1003.

5.2.4 Statistical analysis

All analyses were carried out in PASW Statistics 20 (IBM, Armonk, NY, USA). Behavioural data were analysed with generalised linear mixed models using larval treatment and worker colony as fixed factors and larval colony and mininest identity as random factors. Larval identity within larval colony were included as subjects. Non-significant interactions were removed stepwise from the full models to obtain the minimum adequate models. The frequency of antennation and grooming were analysed using models with a negative binomial error structure and log link functions. Aggression was analysed using an inverse Gaussian error structure and a log link function. Whether larvae were transported to the fungal chamber during

each observation period was analysed using a model with a binomial error structure and probit link.

A Cox regression survival model was used to compare the survival of larvae in each treatment group, and in mini-nests set up from each colony. Weight change to pupation was pooled for individuals from the two non-nestmate treatment groups, as there was no significant difference between the survival of larvae from the two groups. Whether the growth rate of these two groups differed was analysed using a generalised linear model with an inverse Gaussian distribution and a log link function.

5.3 Results

5.3.1 Behavioural assay

There was a significant difference in the frequency of antennation and grooming behaviours performed by workers towards larvae of the three larval treatment groups (nestmate larvae exposed to fungus from their own colony-of-origin, non-nestmate larvae exposed previously to the workers' fungus and non-nestmate larvae exposed previously only to their own fungus; antennation: $F_{2,61} = 42.8$, $P < 0.0001$; grooming: $F_{2,201} = 51.5$, $P < 0.0001$). The frequency of both behaviours was significantly higher for nestmate larvae than for non-nestmate larvae from both groups but there was no significant difference in these two behaviours between the two non-nestmate treatments (Figure 5.2).

The frequency of both antennation and grooming differed significantly between worker colonies (antennation: $F_{2,87} = 14.7$, $P < 0.0001$; grooming: $F_{2,97} = 11.7$, $P < 0.0001$). There was no significant difference between the frequency of these behaviours performed by workers from Colonies Ao1001 and Ao1002 ($P = 0.34$; $P = 0.21$ respectively), but Colony Ao071 showed a significantly higher frequency of both behaviours than the other two colonies ($P < 0.05$ for all comparisons; Figure 5.2).

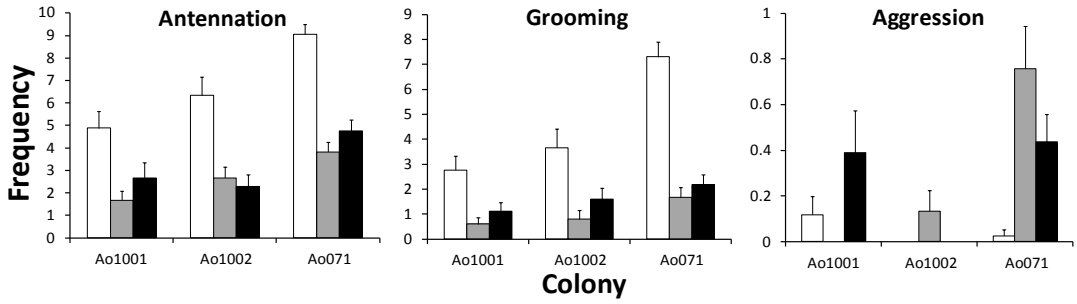

Figure 5.2 Mean + SEM frequency of antennation, grooming and aggressive behaviour by *Acromyrmex octospinosus* leaf-cutting ant workers from each of three colonies directed toward nestmate larvae (white columns), non-nestmate larvae exposed previously to the workers' fungus (grey columns) and non-nestmate larvae exposed previously only to their own fungus (black columns).

There was a significant interaction between the effect larval treatment and worker colony on the frequency of aggression ($F_{4,148} = 4.38$, $P = 0.0002$; Figure 5.2). Additionally, both larval treatment and worker colony had a significant effect on the frequency of aggressive interactions (treatment: $F_{2,197} = 5.83$, $P = 0.003$; worker colony: $F_{2,196} = 12.2$, $P < 0.0001$). The frequency of aggression was significantly higher for non-nestmate larvae than for nestmate larvae (nestmate larvae *vs.* non-nestmate larvae exposed previously to the workers' fungus: $P = 0.01$; nestmate *vs.* non-nestmate larvae exposed previously only to their own fungus: $P = 0.009$). There was no significant difference in the frequency of aggressive behaviour directed towards larvae from the two non-nestmate treatments (0.93). There was no significant difference between the frequency of aggressive behaviours performed by workers from Colonies Ao1001 and Ao1002 ($P = 0.07$), but Colony Ao071 showed a significantly higher frequency of aggressive behaviour than the other two colonies (Ao1001 *vs.* Ao1003: $P = 0.01$; Ao1002 *vs.* Ao1003: $P < 0.001$).

5.3.2 Transportation to fungus garden

Overall, nestmate larvae were more likely to be taken to the fungus garden than non-nestmate larvae ($F_{2,201} = 16.9$, $P < 0.001$; Figure 5.3b). There was no significant difference in the proportion of non-nestmate larvae exposed previously to the workers' fungus and non-nestmate larvae exposed previously only to their own

fungus taken to the fungus garden ($P = 0.06$). Worker colony also had a significant effect on larval transport to the fungus garden ($F_{2,68} = 3.57$, $P = 0.03$; Figure 5.3b). Workers from Colony Ao1002 were least likely to transport larvae to the fungus garden, regardless of their nestmate status, whereas workers from Colony Ao071 were most likely to transport larvae to the fungus garden.

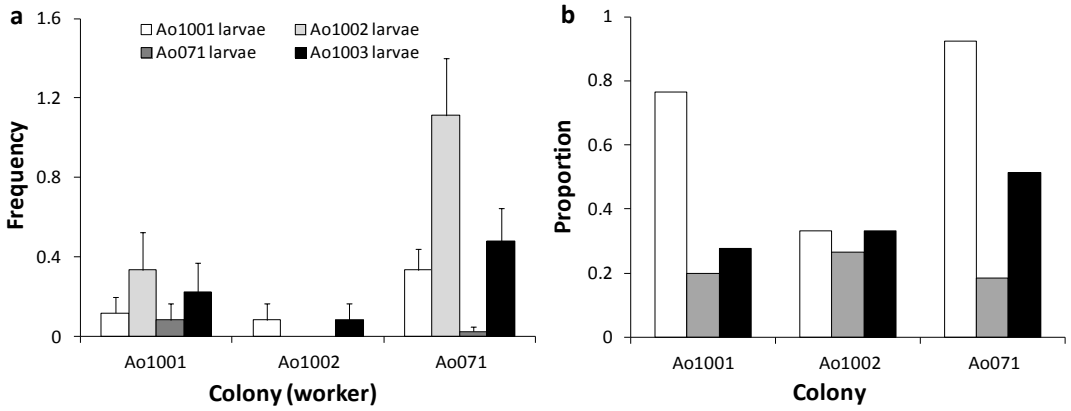

Figure 5.3 a) Mean + SEM frequency of aggressive behaviour by *Acromyrmex octospinosus* leaf-cutting ant workers from each of three colonies (Ao1001, Ao1002 and Ao071) towards larvae from these colonies (white, light grey and dark grey columns respectively) or a fourth colony (Ao1003, black columns). **b)** Proportion of larvae taken into the fungus garden by workers from each of three colonies. Larvae were either nestmate (white columns), non-nestmate larvae exposed previously to the workers' fungus (grey columns) and non-nestmate larvae exposed previously only to their own fungus (black columns).

5.3.3 Survival

Overall, survival of larvae over seven days did not differ significantly between the three larval treatment groups (Wald = 0.91, d.f. = 2, $P = 0.64$). However, worker colony did have a significant effect on larval survival (Wald = 13.7, d.f. = 2, $P = 0.001$; Figure 5.4). Larvae in Colony Ao071 mini-nests were significantly less likely to survive than those in nests from Colonies Ao1001 and Ao1002 (Wald = 7.84, d.f. = 1, $P = 0.005$; Wald = 7.52, d.f. = 1, $P = 0.006$ respectively), but there was no significant difference in survival between larvae in Colony Ao1001 and Colony Ao1002 mini-nests (Wald = 0.029, d.f. = 1, $P = 0.87$).

5.3.4 Weight change to pupation

The growth rate to pupation differed significantly between nestmate and non-nestmate larvae ($\chi^2 = 4.49$, d.f. = 1, $P = 0.03$; Figure 5.4d). On average, nestmate larvae slightly increased in weight up to pupation, whereas the weight of non-nestmate larvae decreased. There was also a significant effect of colony ($\chi^2 = 11.2$, d.f. = 2, $P = 0.004$) and a significant interaction between treatment and colony ($\chi^2 = 6.26$, d.f. = 2, $P = 0.04$). In Colonies Ao1001 and Ao071, nestmate larvae had a higher growth rate than non-nestmate larvae, which, on average, lost weight. In Colony Ao1002, both nestmate and non-nestmate larvae lost weight, and the difference between the two groups was negligible.

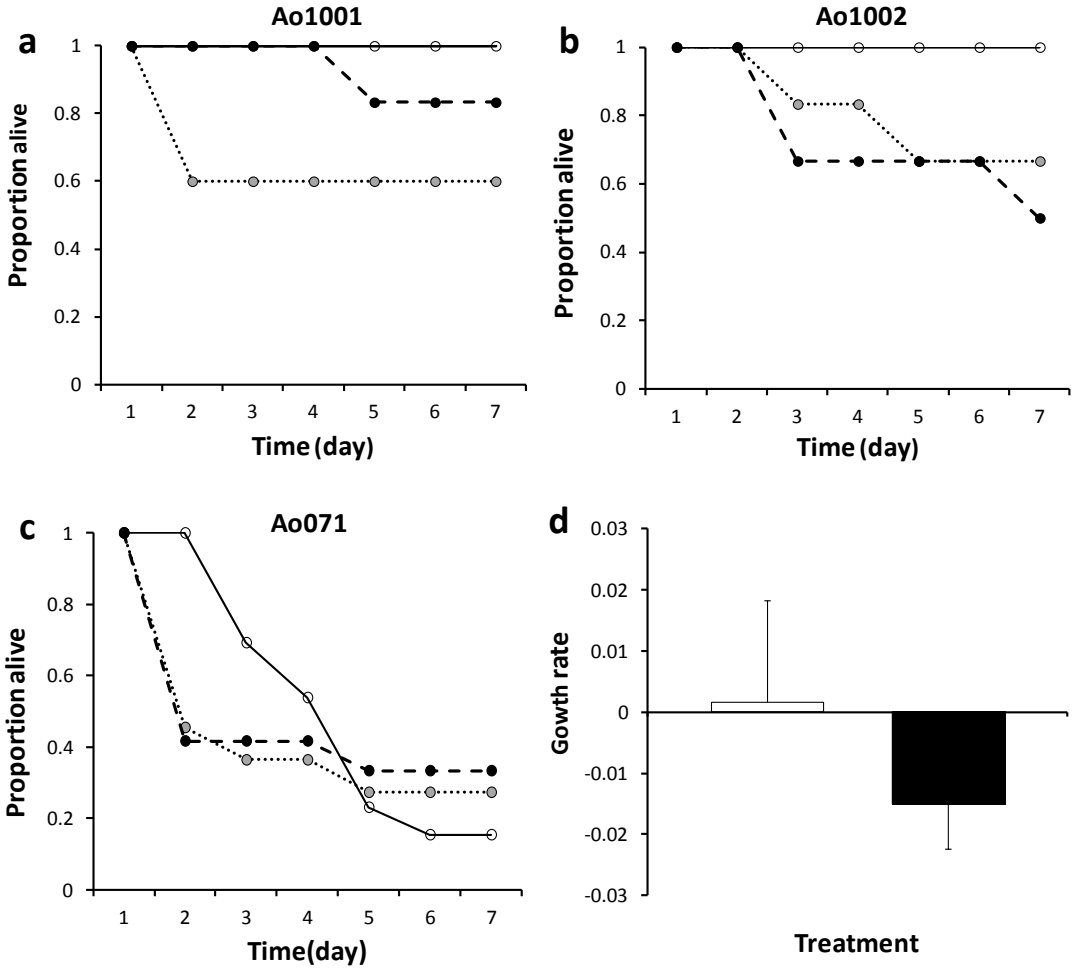

Figure 5.4 Survival of nestmate larvae exposed to fungus from their own colony-of-origin (solid line), non-nestmate larvae exposed previously to the workers' fungus (dashed line) and non-nestmate larvae exposed previously only to their own fungus larvae (dotted line) in mini-nests containing workers and fungus from **(a)** Colony Ao1001 ($N_{t=0}$ = 17), **(b)** Colony Ao1002 ($N_{t=0}$ = 17) and **(c)** Colony Ao071 ($N_{t=0}$ = 36) over a period of seven days. All mini-nests contained a total of thirteen workers; five small workers, two medium workers and one large worker. **d)** Mean ± SEM weight change of nestmate (white column) and non-nestmate (black column) larvae between the time when placed into the mini-nest, and time of pupation. Weight change was calculated as the relative growth rate, thus taking initial weight and time to pupation into account. Nestmate larvae, N = 10, non-nestmate larvae, N = 18.

5.4 Discussion

Here, I show that *Acromyrmex octospinosus* workers were able to discriminate between nestmate and non-nestmate larvae; nestmate larvae were subject to a higher frequency of antennation and grooming behaviours, but a lower frequency of aggressive behaviours than were non-nestmate larvae. Antennation is associated with brood recognition in the social insects. Thus, it is somewhat surprising that a higher frequency of antennation was directed towards nestmate workers and, indeed, this result is in contrast with the findings of Fouks *et al.*(2011), who showed non-nestmate brood to receive a longer duration of antennation than did nestmate larvae. Allogrooming plays an important role in social immunity and also acts to homogenise variation in recognition cues, aiding nestmate discrimination (Boulay *et al.*, 2000; Cremer *et al.*, 2007; Walker & Hughes, 2009). Significantly higher levels of grooming behaviour towards nestmate larvae across all colonies show that *Ac. octospinosus* workers are able both to recognise and act preferentially towards concolonial brood.

Preferential treatment of nestmate brood was also exhibited through aggressive behaviour directed primarily towards non-nestmate larvae. Aggressive interactions between allocolonial and allospecific individuals have revealed the ability to discriminate nestmates in a variety of social insect species (Roulston *et al.*, 2003; Buczkowski & Silverman, 2005; D'Ettorre & Lenoir, 2010; Bos *et al.*, 2011). Aggression towards foreign brood is perhaps an artefact of the aggressive response towards any individual with an unfamiliar hydrocarbon profile. Alternatively, it may be an adaptive response – the genus *Acromyrmex* is affected by a number of closely related inquiline social parasites (Schultz *et al.*, 1998; Sumner *et al.*, 2004a; de Souza *et al.*, 2007) and the ability to identify and reject non-nestmate brood could decrease the fitness costs incurred through parasitism.

Discrimination between nestmate and non-nestmate brood resulted in nestmate larvae being significantly more likely to be transported to the mininest fungus garden than non-nestmate larvae. Studies looking at brood recognition consistently show discrimination by *Acromyrmex* workers, but also a high frequency of foreign brood adoption (Viana *et al.*, 2001; de Souza *et al.*, 2006; Fouks *et al.*, 2011). Seemingly, nestmate discrimination mechanisms are not infallible; in the honey bee, *Apis*

mellifera, overlapping chemical profiles mean that errors in recognition are made (Couvillon *et al.*, 2009). Here, non-nestmate brood was, at least to some extent, tended to and accepted by workers from all colonies. This could be the result of 'mistakes' in recognition, or it may be that similarities in the hydrocarbon profiles of nestmate and non-nestmate brood may make the fitness consequences of mistakenly rejecting nestmate brood too high for such discrimination to be viable, particularly in small colonies.

Interestingly, workers were still able to discriminate between nestmate and non-nestmate brood after non-nestmate brood had been in contact with the workers' own fungus. Additionally, there was still some level of discrimination by workers even after larvae had been incorporated into the fungus garden, with non-nestmate brood losing significantly more weight prior to pupation than nestmate brood. This could indicate a subtle, physiological cost incurred by larvae as a result of discrimination, most likely the result of reduced feeding by workers. *Acromyrmex* larvae have a hydrocarbon profile intermediate between that of the fungus and of pupae (Viana *et al.*, 2001) and the absence of an effect of concolonial *vs.* allocolonial fungus exposure could be a result of insufficient time for the larvae to acquire fungus-derived cues, rather than these cues being unimportant for discrimination. However, that discrimination is still possible even after incorporation in host colony fungus suggests that genetically-derived cues, in addition to those acquired from the environment, influence nestmate discrimination in *Ac. octospinosus*. This could potentially be an important response when defending against the harmful effects of social parasites; recognising and underfeeding parasite brood would mean less of a colony's resources were wasted rearing unrelated brood.

Although overall, *Ac. octospinosus* workers were able to discriminate between nestmate and non-nestmate larvae, this response differed significantly between colonies. Workers from all colonies directed more attentive behaviours towards nestmate larvae, but colonies differed in the frequency of all behaviours performed, most notably aggressive interactions. Colonies also differed in the proportion of larvae taken to the mini-nest fungus garden and the treatment and survival of larvae once adopted into the mini-nest. Consequently, it seems that although workers from all colonies were able to identify nestmate larvae, the subsequent treatment of brood

was not exclusively dependent on their nestmate status, as colonies differed strongly in either their discrimination capabilities, their response to foreign larvae, or, indeed, both these factors.

Clearly, the response to unfamiliar cues is dynamic, dependent on a number of factors such as colony size, variation in diet or other environmental cues (Crosland, 1990; Morel *et al.*, 1990; Stuart, 1991; Balas & Adams, 1996; Liang & Silverman, 2000; Buczkowski & Silverman, 2005; Bos *et al.*, 2011), and, potentially, the interaction between worker and larva genotypes. Such an interaction was demonstrated in Colony Ao071, workers from which showed a much higher frequency of aggression towards larvae from Colony Ao1002 than towards non-nestmate larvae from other colonies. This suggests that workers are not reacting to just a two-dimensional 'friend-or-foe' signal, but rather are making behavioural decisions based on a complex process of template matching (Reeve, 1989).

Ultimately, the results presented here show that workers of *Ac. octospinosus* are able to identify foreign brood when dealing with colony-level cue differences, but the behavioural response towards both nestmate and non-nestmate brood differs between colonies. That workers were still able to discriminate brood once it was incorporated into the fungus garden, and not just when encountering it outside the colony, suggests that they may also be capable of discriminating between brood from a social parasite or an unrelated queen. It could also suggest that workers have the potential to discriminate between brood from different patrilines, particularly in a species such as *Ac. octospinosus* in which sufficient cue diversity is present (Nehring *et al.*, 2011). However, that discrimination is highly dependent on the colony and, to some extent, the interaction between worker colony and brood colony means that any expression of nepotism is likely to depend on the specific genotypes concerned. Such constraints could make intracolonial nepotism difficult to detect experimentally, and could thus explain the current lack of empirical evidence for this phenomenon.

6. Size and asymmetry: are there costs to winning the royalty race?

Abstract

Body size and morphology are key fitness-determining traits that can vary genotypically. They are likely to be important in social insect queens, which mate in swarms and found colonies independently, but genetic influences on queen morphology have been little investigated. Here, the body size and morphology of queens were shown to be influenced by their genotype in the leaf-cutting ant *Acromyrmex echinatior*, a species in which certain lineages (patrilines) bias their development towards reproductive queens rather than sterile workers. There was no relationship between the queen-worker skew of patrilines and the size or morphology of queens, but there was a significant relationship with fluctuating asymmetry, which was greater in more queen-biased patrilines. These results suggest that queen-biased patrilines do not incur a fitness cost in terms of body size, but may face more subtle costs in developmental stability. Such costs may constrain the evolution of royal cheating in social insects.

6.1 Introduction

In plants and animals, body size and morphology are associated with a number of life-history traits, which include dispersal ability, survival probability, fecundity and mating success (Vander Meer *et al.*, 1992; Honěk, 1993; Carvalho *et al.*, 1998; Bonduriansky, 2001; Gonzaga & Vasconcellos-Neto, 2001; Jennions *et al.*, 2001; Araújo *et al.*, 2004; Kingsolver & Huey, 2008; Kovacs *et al.*, 2008; Kajita & Evans, 2010). Size is influenced by both genetic and environmental conditions, but in the insects it is the amount of food available to developing larvae that has the greatest effect on adult body size (Davidowitz *et al.*, 2003; Speight *et al.*, 2008). Many of the highly eusocial insect species are characterized by varied morphology and individuals are often morphologically suited to a particular role. In some ant species, for example, females can develop into a variety of forms, ranging from small workers, generally employed within the nest, to soldiers, morphologically specialised to protect and defend the nest, and reproductive queens which may be hundreds of times larger than their smallest sisters (Hölldobler & Wilson, 1990). The variety of forms into which such genetically similar individuals are able to develop make this an important group in which to study body size and morphology.

Four main factors have been shown to affect brood development and hence body size in the social insects: genotype, maternal and sibsocial (worker) effects and environmental conditions (Wheeler, 1986; Mousseau & Fox, 1998; Hughes *et al.*, 2003; Schwander *et al.*, 2008; Radmacher & Strohm, 2010). Large queens often have a higher colony founding success and greater fecundity than small queens (Vander Meer *et al.*, 1992; Honěk, 1993; Wiernasz & Cole, 2003; Wenseleers *et al.*, 2005). In ants, body size is also associated with divergent dispersal and colony founding strategies. In general, large gynes (virgin queens) have high dispersal ability and high fat and glycogen reserves, a phenotype suited to independent colony foundation (Keller & Passera, 1988; Keller & Passera, 1989). Smaller gynes tend to mate and stay within the natal colony, join a ready established colony, or found a colony close to the natal site with the aid of workers (Ross & Keller, 1995; Sundström, 1995; Rüppell *et al.*, 1998; Rüppell & Heinze, 1999; Peeters & Ito, 2001). It is likely that size dimorphism is a consequence of disruptive selection resulting from a trade-off between survival and reproductive output; independent colony foundation often has

an extremely low success rate and puts individuals at high risk of predation (Schmid-Hempel, 1984; Hölldobler & Wilson, 1990), but queens that successfully found their own colony, on the whole, produce more offspring than those in polygynous nests (Vander Meer *et al.*, 1992).

In ants, a direct effect of genotype on body size has so far been demonstrated in only a few species: *Formica truncorum* (Bargum *et al.*, 2004), *Lasius niger* (Fjerdingstad, 2005), *Solenopsis invicta* (Ross & Keller, 1998) and *Pogonomyrmex badius* (Smith *et al.*, 2008a). The complex social structure of ant societies means that it is often difficult to distinguish genetic effects from maternal and sibsocial effects (Linksvayer, 2006; Meunier & Chapuisat, 2009). However, it is possible to identify genetic effects by looking at size variation in colonies headed by a single, multiply mated queen because individuals can be separated into a number of genetically distinct patrilines that differ only in their paternal genotype.

In an ant colony, larval food intake tends to be controlled by sterile female workers (Oster & Wilson, 1978) and so traits that are determined in the larval stage, such as body size, can be strongly affected by the social environment. In a colony with monomorphic sexuals, the body size of reproductive females can be the cause of intracolonial conflict of interest between the developing gynes and their nursing workers (Herbers, 1990; Backus, 1993) that is not dissimilar to the more traditional parent-offspring conflict (Trivers, 1972). Developing larvae can maximise their size, and hence direct fitness, by taking a disproportionate share of resources. In contrast, workers who, in general, can only facilitate the transmission of their genes through caring for sexual offspring produced by the colony (Bourke & Franks, 1995) will achieve greatest indirect fitness if all gynes develop to a standard optimum size, which may differ from a maximum potential size (Fjerdingstad, 2005). This logic would only be false if workers in polygynous or polyandrous colonies were able to preferentially rear the larvae to which they were most related. Nepotism has long been a controversial area of social insect research and a conclusive demonstration of such behaviour in any social insect group is conspicuously absent (Queller *et al.*, 1990; DeHeer & Ross, 1997; Holzer *et al.*, 2006; Goodisman *et al.*, 2007; Zinck *et al.*, 2009). Consequently, despite evidence for chemical cues specific enough to allow such kin discrimination in the ant *Ac. octospinosus* (Nehring *et al.*, 2011),

nepotism is thought not to occur in colonies with multiple related matrilines or patrilines (Queller *et al.*, 1990; DeHeer & Ross, 1997).

Here, I investigate whether there is a genetic influence on queen size and morphology in the polyandrous leaf-cutting ant species *Acromyrmex echinatior* (Bekkevold *et al.*, 1999; Sumner *et al.*, 2004b). *Ac. echinatior* queens found colonies after their nuptial flight and it is possible that body size may play an important role in all aspects of this process, from acquiring mates, to founding a new colony and defending it from predators. Genetic effects on both queen-worker caste determination and worker caste development have been shown in *Ac. echinatior*, making this species a good model in which to detect genetic effects on within-caste morphology (Hughes *et al.*, 2003; Hughes & Boomsma, 2007; Hughes & Boomsma, 2008). In addition, this species is particularly interesting because certain patrilines show a greater propensity to develop into gynes, thus cheating their nest-mates out of a fair chance of reproduction (Hughes & Boomsma, 2008). Such patriline variation in caste propensity can be due to genetic incompatibilities in some ant species (Schwander & Keller, 2008), but the within-colony rarity of royal patrilines in leaf-cutting ants means it is more likely to result from genotypic variation in the ability to obtain, utilise or respond to key caste-determining environmental cues, and fitness costs selecting for a low frequency of cheats within colonies (Hughes & Boomsma, 2008). Larval nutrition is one such environmental cue and it may be that *Acromyrmex* cheats require less food to trigger gyne development. Thus, I hypothesise that gynes from royal patrilines will be significantly smaller than those from other patrilines in the colony. I also predict that royal patrilines will show signs of developmental instability, resulting from potential nutritional or worker-induced stress during development. This is assessed by examining fluctuating asymmetry, the difference in size between right and left sides in organisms with bilateral symmetry (Van Valen, 1962), which is commonly used as a measure of developmental stability, an individual's ability to buffer development under imperfect environmental conditions (Palmer & Strobeck, 1986).

6.2 Methods

6.2.1 Sample collection

Gynes and workers were sampled from six monogynous *Acromyrmex echinatior* colonies. Samples were collected from two colonies in the field (Ae125 and Ae158) and in the laboratory from four colonies (Ae07P4, Ae48, Ae357, and Ae088) which had been collected in Gamboa, Panama, and maintained in the laboratory at 26°C and 80% RH, on a diet of privet leaves and rice. 96 gynes, 96 large workers and 96 small workers were sampled from each of Ae07P4, Ae357, and Ae088, and 96 gynes and 96 workers from each of Ae48, Ae125 and Ae158. Samples were stored in 100% ethanol at -20°C. Samples from Ae48, Ae125 and Ae158 had previously been used in Hughes and Boomsma (2008).

6.2.2 Genotyping analysis

DNA was extracted from the legs of individual ants using 5% Chelex 100 (BioRad) suspended in 10 µM Tris buffer. 5 µl Proteinase K (5 µl/ml) was added to the samples, which were then incubated at 56°C overnight, then boiled for 15 min. All samples were genotyped at five polymorphic microsatellite loci: Ech1390, Ech3385, Ech4126, Ech4225 and Atco15 (Ortius-Lechner *et al.*, 2000; Helmkampf *et al.*, 2008). All PCR cycles had an initial denaturing step of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, an annealing step of 45 sec, and 72°C for 45 sec, with a final elongation step of 72°C for 15 min completing the amplification process (for PCR conditions, see Appendix Table A6.1).

PCR products were run in an ABI 3130*x*1 capillary sequencer. Allele sizes were scored by comparison with internal size markers, and multi-locus offspring genotypes were used to determine the genotypes of the colony queens and their multiple mates. This allowed individuals to be assigned to a particular patriline within each colony. Individuals for which the paternities could not be reliably determined were excluded from the analysis (~4.5%). I used *G* tests for heterogeneity and Fisher's exact tests to examine whether the patrilines differed from the expected ratio in a uniform direction (Sokal & Rohlf, 1995). Tests for queen-worker-bias and worker-bias were conducted separately. Corrections for

multiple testing were made using QVALUE software (Storey, 2002). Queen-worker skew for each patriline was calculated on a scale from -1 to 1, by subtracting the expected proportion of queens assuming no skew from the observed proportion of queens, and multiplying by two.

6.2.3 Body size

Six body size measurements were made for each gyne sampled: head width, the maximum width across the eyes (HW); forewing length, from the first vein intersection to the base of the wing (WL); forewing width, running parallel to the cross-vein 2r (Brown & Nutting, 1950) (WW); maximum thorax length (TL); maximum thorax width (TW); leg length, the fibula, patella and tibia (LL). Body parts were scanned using an Epson Scan V300 Photo with a resolution of 9600 pixels, with wings being scanned under glass slides. Measurements were made using ImageJ 1.42q and were calibrated using a scanned 0.1 mm graticule. From Colony Ae07P4, 87 gynes were measured, 95 from Colony Ae357, 87 from Colony Ae048, 92 from Colony Ae088, 41 from Colony Ae125 and 18 from Colony Ae158.

To quantify measurement error a randomized subset of ten individuals were measured three times. Measurement error was estimated as the average coefficient of variation (CV) for each character, using Haldane's correction for small sample size (Haldane, 1955; Lynch & Hayden, 1995). The average measurement error of the six characters was less than 1%.

All analyses were carried out in PASW Statistics 18 and measurements were log-transformed prior to analysis. One sample Kolmogorov-Smirnoff tests were used to test for normality within-colony for each character. The length distribution of all characters measured did not differ significantly from a normal distribution. The empirical morphospace – the distribution of realized forms (McGhee, 1991; Stone, 1997) – occupied by each colony was described by a principal components analysis (PCA) of the covariance matrix. Principal component axes with eigenvalues of at least 0.7 were retained (Jolliffe, 1972) and individual principal component scores were subjected to a nested analysis of variance (ANOVA) with patriline nested within colony. Effect sizes (Cohen's f) were calculated using G*Power v3.1.2 (Faul *et al.*, 2007).

6.2.4 Allometry

Allometric relationships between head width, a trait commonly used as a measure of body size in ants (Hölldobler & Wilson, 1990), and all other characters were determined using the equation $y = bx^{\alpha}$, where x and y are the size of the two given traits (Huxley & Teissier, 1936). The log-transformation of this produces the linear equation $\log(y) = \log(b) + \alpha \log(x)$. The allometric coefficient (α), the scaling relationship between two traits, is the slope calculated from log-log plots of the two traits. If both traits are measured in the same dimension, an allometric coefficient of 1 indicates an isometric relationship, whereby the relative size of the traits does not vary with absolute size. Ordinary least squared regression was used to determine the allometric coefficient for each character against head width. A *t*-test was used to determine whether each allometric coefficient differed significantly from 1 and corrections for multiple testing were made using QVALUE software (Storey, 2002).

6.2.5 Fluctuating asymmetry

Both right and left wings of all gynes were measured twice for the fluctuating asymmetry analysis. Repeat measurements were made on separate days and all measurements were made blind to patriline. The mean difference between right and left sides was used as a measure of asymmetry. Both directional asymmetry (DA), where one side of a trait has a propensity to develop more than the other and antisymmetry (AS), whereby one side of a character is larger than the other but there is no bias as to which side is larger (Van Valen, 1962), can inflate estimates of fluctuating asymmetry (Palmer & Strobeck, 1986; Palmer, 1994). They can typically be recognized by skewed and platykurtic asymmetry frequency distributions respectively. Before analyzing FA, the data were tested for the presence of DA and AS, as recommended in Palmer (1994). Measurement error was partitioned from asymmetry using a mixed model ANOVA, with side and replicate as fixed factors and individual as a random factor (Palmer, 1994; Swaddle *et al.*, 1994). Two FA indices were calculated for each patriline: absolute right minus left (FA1 from Palmer 1994) and Palmer's (1994) FA10. FA10 is the only FA index that allows measurement error to be partitioned from the FA estimate (Palmer, 1994). The relationship between patriline FA and queen-worker skew was examined using a mixed model, with colony as a random factor.

6.3 Results

6.3.1 Patriline representation

Four out of the six colonies genotyped contained patrilines that were over-represented in the queen caste, or "royal patrilines" as defined by Hughes and Boomsma (2008; Figure A6.1 and Table A6.2). Colonies Ae357 and Ae48 were each found to have one royal–LW patriline. Ae125 and Ae158 had three and two royal–SW patrilines respectively.

6.3.2 Body size and morphology

The first three principal components (PCs) extracted in the PCA accounted for a total of 86.4% of variation in the data (Table 6.1). In morphological studies, PCA typically results in a first component on which all traits are positively loaded, thus representing size (Blackith & Reyment, 1971; Bookstein, 1989). Here, PC1, which accounted for 56.1% of the total variation, was used as a measure of general body size. Leg length (LL) had a high positive loading on PC2, which explained 16.8% of the variation. Thorax width had a strong positive loading on PC3, whereas all other characters had negative loadings on this component, which accounted for 13.5% of the total variation (Table 6.1). Mean PC scores for each patriline can be found in Table A6.3.

All three principal components analysed showed a significant difference between colonies (PC1: $F_{5, 388} = 123.2$, $P < 0.0001$; PC2: $F_{5, 388} = 214.3$, $P < 0.0001$; PC3: $F_{5, 388} = 29.8$, $P < 0.0001$) and between patrilines nested within colony (PC1: $F_{26, 388} = 4.005$, $P < 0.0001$; PC2: $F_{26, 388} = 2.026$, $P = 0.002$; PC3: $F_{26, 388} = 2.785$, $P < 0.0001$; Figure 6.1).

	Components		
	PC1	**PC2**	**PC3**
Eigenvalue	**3.37**	**1.01**	**0.81**
Variance explained (%)	**56.1**	**16.8**	**13.5**
HW	0.845	0.121	-0.024
TL	0.905	-0.014	-0.033
TW	0.462	-0.108	0.859
WL	0.839	-0.353	-0.149
WW	0.800	-0.367	-0.215
LL	0.525	0.842	-0.047

Table 6.1 Principal component loadings for the six morphological traits used in the analysis: head width (HW); thorax length (TL); thorax width (TW); wing length (WL); wing width (WW); leg length (LL).

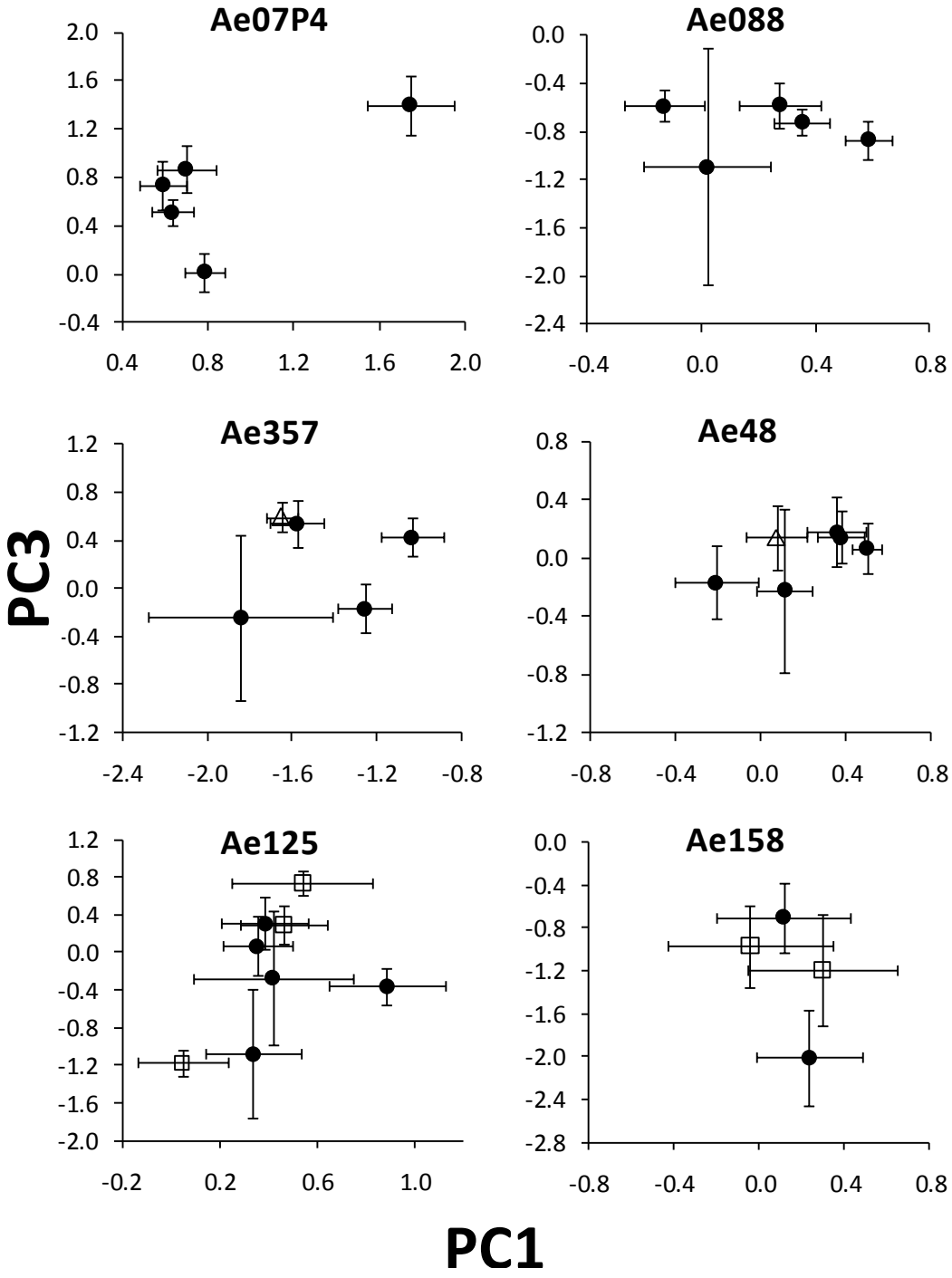

Figure 6.1 Mean ± SEM PC1 and PC3 of patrilines from the six colonies used in the analysis. Black circles represent patrilines with no significant queen-worker-bias. *Royal–LW* patrilines are represented by white circles and *royal–SW* patrilines by white squares.

The effect size of colony was large for all three principal components (PC1: f = 0.831; PC2: f = 0.888; PC3: f = 0.62). For patriline, averaged over all colonies, the effect size was medium for PC1 (f = 0.231) and PC3 (f = 0.350) but small for PC2 (f = 0.139; Cohen, 1977). No significant relationship was found between any of the principal components and queen-worker skew (PC1: $r^2 = 0.014$, $F_{1,31} = 0.456$, $P = 0.51$; PC2: $r^2 = 0.015$, $F_{1,31} = 0.49$, $P = 0.49$; PC3: $r^2 = 0.0$, $F_{1,31} = 0.002$, $P = 0.97$; Figure 6.2).

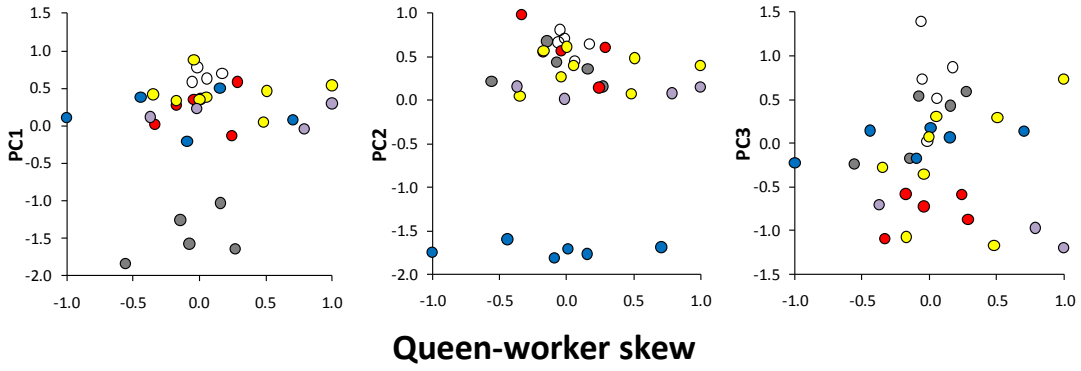

Figure 6.2 Relationship between queen-worker skew and each of the first three principal components (PCs), each PC averaged by patriline. Each point represents one patriline and each colour represents one of six colonies. No significant relationship was found between queen-worker skew and any of the PCs analysed.

TL was the only character measured that scaled isometrically with HW ($\alpha = 1.03$, $t_{418} = 0.56$, $P = 0.57$; Figure 6.3a). TW showed the highest level of negative allometry ($\alpha = 0.38$, $t_{418} = 13.23$, $P < 0.0001$, $Q < 0.0001$; Figure 6.3b). WL and WW showed some negative allometry ($\alpha = 0.76$, $t_{418} = 5.22$, $P < 0.0001$, $Q < 0.0001$; WW: $\alpha = 0.73$, $t_{418} = 5.56$, $P < 0.0001$, $Q < 0.0001$; Figures 3c and d respectively). LL also showed significant negative allometry ($\alpha = 0.77$, $t_{418} = 3.50$, $P < 0.001$, $Q <$ 0.00l; Figure 6.3e), although one colony, Ae48, had a disproportionate effect on this relationship (Ae48 only: $\alpha = 0.31$, $t_{85} = 10.80$, $P < 0.0001$, $Q < 0.0001$; Ae48 excluded: $\alpha = 0.88$, $t_{331} = 2.46$, $P = 0.014$, $Q < 0.01$).

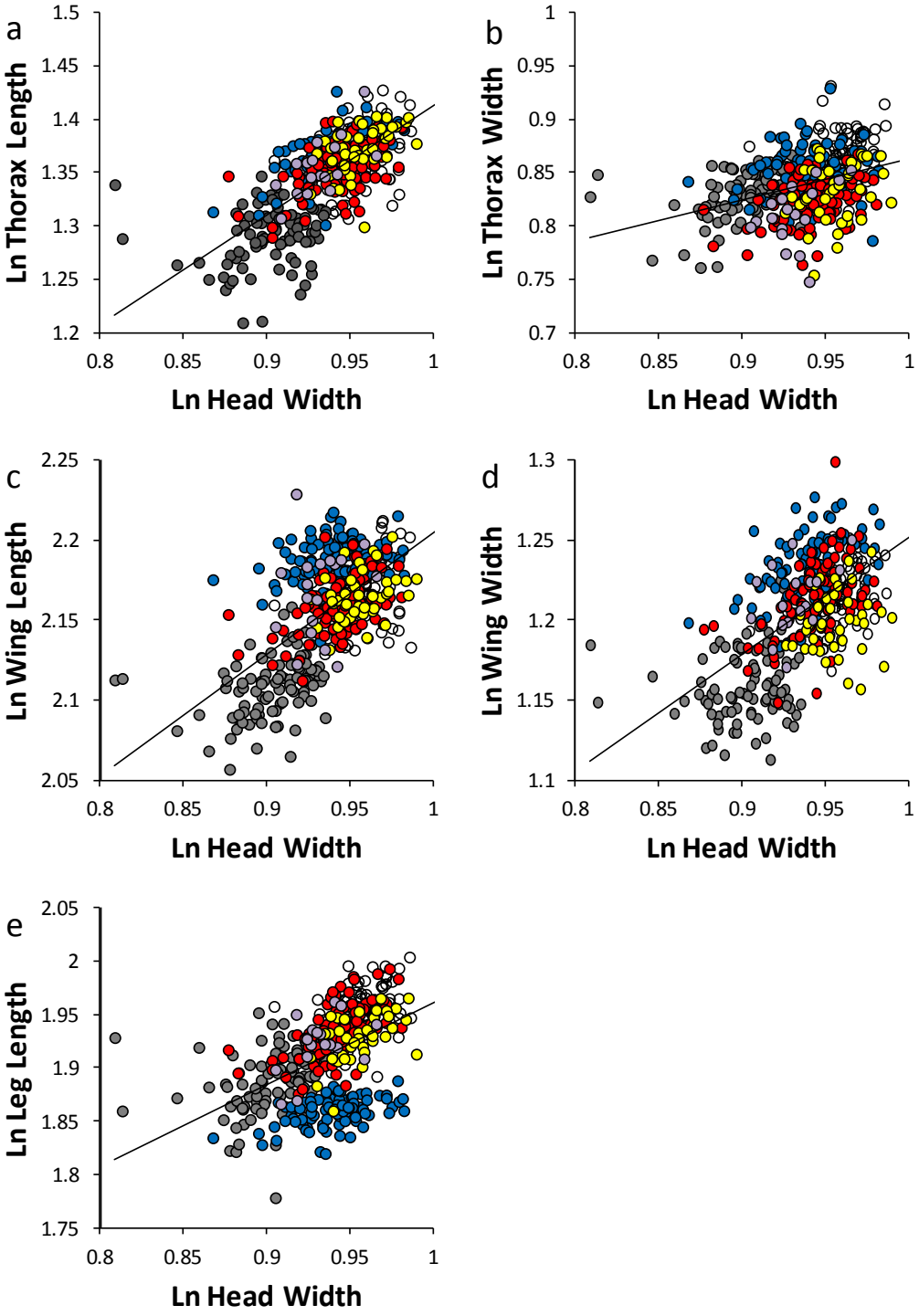

Figure 6.3 Relationship between head width and five morphological traits in *Acromyrmex echinatior* queens; **a)** thorax length (TL), **b)** thorax width (TW), **c)** wing length (WL), **d)** wing width (WW), **e)** leg length (LL). Each circle represents one individual and each colour corresponds to one of six colonies. Lines of best fit are; TL: $y = 1.03x + 0.39$; TW: $y = 0.38x + 0.49$; WL: $y = 0.76x + 1.44$; WW: $y = 0.73x + 0.52$; LL: $y = 0.77x + 1.19$.

6.3.3 Fluctuating Asymmetry

I found no evidence of significant directional asymmetry or antisymmetry. Mean signed asymmetry did not differ significantly from 0 (t_{425} = -1.078, P = 0.28), signed asymmetry showed no evidence of a skewed or bimodal distribution and the distribution of asymmetry did not differ significantly from a normal distribution (Z=0.11, P = 0.21). Thus, deviations from perfect symmetry can be interpreted as fluctuating asymmetry. There was no significant relationship between trait size and asymmetry (ρ=0.02, P = 0.72). Estimated asymmetry was found to be significantly greater than measurement error for the whole sample ($F_{423,846}$ = 10.74, P < 0.0001) and also when each patriline was tested individually (P< 0.001 in all cases). FA1 and FA10 showed a strong positive relationship (ρ=0.77, P < 0.0001), suggesting both offer a robust estimate of FA. There was a significant relationship between queen-worker skew and both FA1 and FA10, with more queen-biased patrilines exhibiting greater FA (FA1: $F_{1,25}$ = 10.0, P = 0.004; Figure 6.4; FA10: $F_{1,25}$ = 4.98, P = 0.035).

6.4 Discussion

Here, I found a genetic effect on the body size of *Ac. echinatior* queens. In many insect taxa, including ants, large female body size is associated with high fecundity and reproductive output (Vander Meer *et al.*, 1992; Honěk, 1993) and in the ants, large queens are more likely to survive the initial stages of colony foundation (Wiernasz & Cole, 2003). It is therefore likely that in *Ac. echinatior*, as well as *P. badius, L. niger* and *F. truncorum* in which similar effects have been shown (Bargum *et al.*, 2004; Fjerdingstad, 2005; Smith *et al.*, 2008a), large queens may have greater fitness than smaller individuals. Thus, those individuals who, as larvae, are better able to signal to workers that they require feeding, or those who are better able to use resources for growth, may be at a competitive advantage over their sisters.

The inclusive fitness of workers is strongly linked to colony productivity because the sexual offspring produced are generally the only means by which workers can contribute genes to the next generation (Hamilton, 1964b). In the insects, adult body

size is partly dependent on the amount of food received during development (Davidowitz *et al.*, 2003; Speight *et al.*, 2008) and in the social insects it is the workers who forage and provide food for developing larvae. If a colony has limited resources with which to raise sexual offspring, workers, too, face a trade-off between number and size of gynes produced. Thus, for workers, variation in the size of sexuals produced by the colony is not advantageous, unless workers possess the ability to recognize and preferentially rear their closest kin, something for which there is currently no conclusive evidence in social insects (Queller *et al.*, 1990; DeHeer & Ross, 1997; Holzer *et al.*, 2006; Goodisman *et al.*, 2007; Zinck *et al.*, 2009).

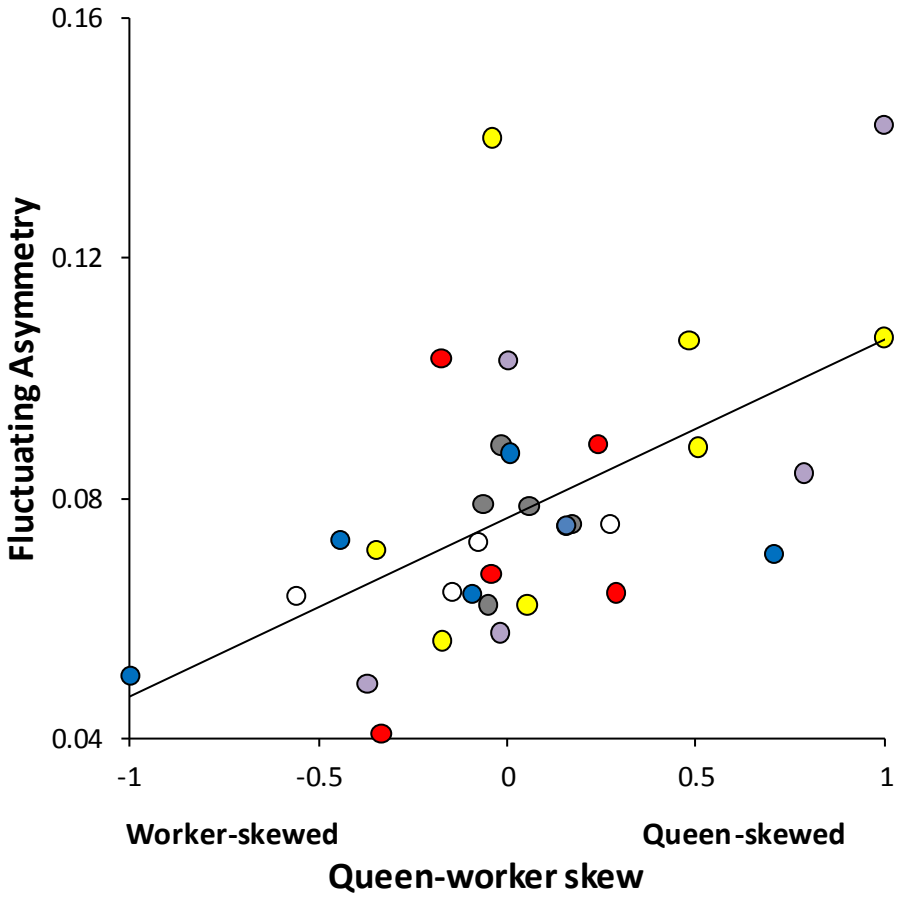

Figure 6.4 Relationship between queen-worker skew and fluctuating asymmetry (FA1). Each point represents one patriline and each colour represents one of six colonies ($y = 0.031x + 0.076$, $r^2 = 0.319$).

It has been proposed that royalty-biasing of patrilines in *Acromyrmex echinatior* could result from changes in response thresholds for environmental cues, such as nutrition, that control larval development (Hughes & Boomsma, 2008). Patrilines exhibiting a queen- and a large worker-bias (*royal–LW* patriline) may have slightly lower nutritional thresholds for the initiation of both gyne and large worker development, whereas in patrilines with a queen- and small worker-bias (*royal–SW* patrilines) it is only the queen-worker nutritional threshold that is reduced, but to a greater extent. Were this hypothesis correct, we might expect that, on average, gynes from these patrilines would have a smaller body size than those from normal patrilines, *royal–SW* patrilines differing more from the colony norm than *royal–LW* patrilines. The average body size of both the *royal–LW* patrilines was towards the low end of the size distribution (Figure 6.1), but the lack of a similar relationship in *royal–SW* patrilines, in addition to a non-significant relationship between body size and queen-worker skew means that the data presented here offer little support for this hypothesis. It may be, therefore, that the mechanism controlling caste determination is decoupled from that which determines queen size. In ant species that exhibit a worker polymorphism, such as *Acromyrmex*, the early divergence of queen and worker development may be crucial, allowing time for the development of worker size and allometric differences (Wheeler, 1986). In the fire ant *Solenopsis invicta*, gyne determination takes place in the first or second instar (Robeau & Vinson, 1976). In contrast, it is unlikely that adult body size is wholly determined at such an early stage of larval development and so the temporal separation of caste determination and morphological development may account for the lack of a relationship between size and queen-worker skew observed here.

In addition to information on body size, the results here offer an interesting insight into morphological differences between *Ac. echinatior* queens. Thorax length was the only character that scaled isometrically with head width. This is perhaps unsurprising, as both measurements are used as indicators of body size in insects (Hölldobler & Wilson, 1990; Berrigan, 1991; Partridge *et al.*, 1994). In contrast, wing length, wing width and leg length showed some negative allometry. In holometabolous insects, adult appendages that develop from imaginal structures do not grow synchronously with the larval body, and so static allometries (allometry among body parts in a single species) in this group often differ from those expected

based on other animal groups (Nijhout & Wheeler, 1996). The effect of colony on PC2, for which leg length had a strong positive loading, was due to a single colony (Ae48) exhibiting a much greater negative allometry than the remainder of the sample. Interestingly, individuals from Ae48 had wings at the high end of the size distribution. The short legs and relatively large wings of queens in this colony could potentially result from appendages competing for nutrients during development (Nijhout & Wheeler, 1996). In winged insects, thorax width is a trait that may influence an individual's flight ability, and consequently dispersal ability and reproductive success. The results show a genetic effect on thorax width (PC3) and, surprisingly, thorax width showed negative allometry far higher than any other body part. This could result from a decoupling of the mechanism controlling the size of flight muscles and those involved in determining other components of body size, or from a restriction in the size of these muscles in terms of optimal flight ability.

Levels of fluctuating asymmetry have been investigated in a number of social insects, but previous studies have looked at variation in FA between castes, sex, colonies and species or subspecies (Ross & Robertson, 1990; Keller & Passera, 1993; Crespi & Vanderkist, 1997; Smith *et al.*, 1997; Heinze & Oberstadt, 1999; Fjerdingstad, 2004; Jones *et al.*, 2005). Here, I found a positive relationship between patriline queen-worker skew and FA. Such patriline variation in caste propensity in some ants can result from compatibility between maternal and paternal genomes (Schwander & Keller, 2008), but is more likely in *Acromyrmex* to be due to genotypic variation in the ability to obtain or utilise caste-determining environmental cues such as food (Hughes & Boomsma, 2008). FA is commonly used as a measure of developmental stability, an organism's ability to produce an ideal phenotype (perfect symmetry) under a particular set of environmental conditions (Zakharov, 1992). The relationship shown here could suggest that individuals from queen-biased patrilines have less ability to buffer environmental stress during development. This could result from a trade-off between ability to trigger gyne development and developmental stability. Alternatively, patriline variation in the amount of food required for the initiation of gyne development could lead to gynes from queen-biased patrilines being subject to greater levels of nutritional stress. Equally, gynes from worker-skewed patrilines would be subject to the least nutritional stress, owing to a high nutritional threshold at which gyne development is triggered. Although our

results suggest there is no direct fitness cost from cheating in terms of reduced body size, it appears that there may be a more subtle cost to patriline caste-biasing in *Ac. echinatior*, as indicated by the positive relationship between queen-worker skew and FA. It may be that cheats suffer costs in other fitness related traits, such as offspring survival, immunity or learning ability (Jann & Ward, 1999; Siva-Jothy & Thompson, 2002; Riddell & Mallon, 2006). Such costs may go some way towards balancing out the benefits gained by such individuals and may help to explain, in part, why cheats are rare. These results, however, should be interpreted with caution; although FA has been linked to a number of fitness traits, results are inconsistent and are often dependent on the trait in question (Lens & Van Dongen, 2002).

In conclusion, these results show a genetic effect on the size and morphology of queens in *Ac. echinatior*. In terms of body size, this is most likely to be a direct effect, resulting from between-patriline differences in the ability of larvae to signal to workers that they need food, or in their ability to process and utilize nutrients. However, an indirect genetic effect resulting from differential treatment by workers, although unlikely, cannot be ruled out. A strong colony-effect on both body size and morphology suggests that although some variation in size is genetic, other influences, such as maternal effects, environmental variation and colony condition are also important in determining these characteristics. Analysis of body size provided no direct evidence for the nutritional-threshold hypothesis, proposed by Hughes and Boomsma (2008) to explain the phenomenon of *Ac. echinatior* 'royal cheats', but a positive relationship between queen-worker skew and FA could be suggestive of individuals from queen-skewed patrilines suffering from greater nutritional stress as a result of a lower nutritional threshold for gyne development. It may be that experiments looking directly at preferential rearing by workers and how development is related to nutritional intake are the only way to conclusively test this hypothesis and determine the cause of caste-bias in social insects.

7. General discussion

Caste determination in the social insects is a phenomenon that has fascinated sociobiologists since Darwin questioned how workers could show significant morphological variation without being able to pass on their phenotype through reproduction (Darwin, 1859), and yet it still remains a somewhat elusive process. In all species so far studied, caste determination is non-random and in most it relies on a complex interaction between environmental and genetic factors, and genotypes within the colony (Anderson *et al.*, 2008; Schwander *et al.*, 2010). With few exceptions (Cahan & Keller, 2003; Cahan & Vinson, 2003; Pearcy *et al.*, 2004; Fournier *et al.*, 2005; Ohkawara *et al.*, 2006), however, the mechanisms underlying why any particular individual takes one of these two developmental routes are not clearly understood. In a number of species, a genetic caste-bias, whereby certain genotypes are overrepresented in the queen caste, has been identified (Tilley & Oldroyd, 1997; Moritz *et al.*, 2005; Hughes & Boomsma, 2008; Schwander & Keller, 2008; Smith *et al.*, 2008a; Nanork *et al.*, 2011; Qian *et al.*, 2011). Whatever the mechanism underlying this phenomenon, it is likely that the differing propensity of genotypes to develop as queens is the result of genotypic differences in response thresholds to environmental stimuli, primarily nutrition. Thus, to better understand the ultimate causes of caste determination, we must explore the physiological responses of genotypes to environmental cues.

Here, the honey bee, *Apis mellifera*, and leaf-cutting ants of the closely related species *Acromyrmex echinatior* and *Ac. octospinosus* (Sumner *et al.*, 2004a) were used to investigate some of the causes and consequences of genetic caste-bias. These species share some common life-history traits; all are highly polyandrous, and colonies are exclusively, in the case of *A. mellifera* and *Ac. octospinosus*, or usually in *Ac. echinatior*, headed by a single queen, resulting in colonies that consist of a number of full-sister patrilines that differ only in their paternal genotype (Estoup *et al.*, 1994; Ortius-Lechner *et al.*, 2000). Thus, these species provide a context in which genetic effects can be readily detected. Additionally, genetic variation in queen-worker propensity has been shown in both *A. mellifera* and *Ac. echinatior* (Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005; Hughes & Boomsma, 2008). However, *A. mellifera* produce only a small

number of new queens when the colony is ready to divide by colony fission (Winston, 1987) and genetic caste-bias has been identified only under conditions of emergency queen rearing (Moritz *et al.*, 2005). In contrast, *Acromyrmex* species produce a vast number of gynes that found colonies independently (Hölldobler & Wilson, 1990), and in *Ac. echinatior*, caste-biasing occurs during this period of colony reproduction (Hughes & Boomsma, 2008). Nonetheless, both species offer ideal systems in which to explore the relationship between phenotypic plasticity and genetic caste-bias, both of which are essential to our understanding of caste determination mechanisms.

Both the body size of *Ac. echinatior* queens and the growth rate of *A. mellifera* larvae were shown to have a genetic component. Although not directly equivalent measures, in the Hymenoptera, as in all insects, adults do not grow and so peak larval mass largely determines adult body size (Nijhout, 2003b; Davidowitz *et al.*, 2004). Body size and growth rate are intrinsically linked, as adult body size is determined by both growth rate and time to maturation (Davidowitz & Nijhout, 2004). Thus, both body size and growth rate can affect individual fitness, as large individuals tend to have a greater fecundity, survival probability and ability to attract a mate than do those that are small (Honěk, 1993; Carvalho *et al.*, 1998; Bonduriansky, 2001; Jennions *et al.*, 2001; Kingsolver & Huey, 2008; Kovacs *et al.*, 2008). Furthermore, growth rate may be particularly important in honey bees as it is often the queen first to emerge that eventually takes over reproduction in the colony by killing her slower to emerge rivals (Winston, 1987; Schneider & Degrandi-Hoffman, 2003). Consequently, in a social insect colony, the body size and, indirectly, larval growth rate of queens produced can affect the fitness of all colony members, as the inclusive fitness of workers relies on the reproductive output of the colony as a whole.

Here, I found that *A. mellifera* genotypes with a queen-bias had a higher growth rate than those with no caste-bias or a worker-bias. Accordingly, growth rate is a life-history trait that has the potential to be important in queen rearing across the social Hymenoptera. Reasons as to why a high growth rate might predispose larvae towards queen development are still speculative. A high growth rate may indicate greater efficiency in utilising nutritional resources for growth. Such a response would most

likely be mediated through the action of juvenile hormone (JH) and other hormones, such as the ecdysteroids, that play a role in larval development (Rachinsky *et al.*, 1990; Robinson *et al.*, 1991; Hartfelder, 2000). In insects generally, JH, which works in concert with ecdysteroids to regulate moulting and pupation, is no longer secreted once larvae reach a critical mass and JH titres subsequently fall (Nijhout & Williams, 1974; Baker *et al.*, 1987; Riddiford, 1994). During the period in which JH is removed from the haemolymph, larvae can still eat and grow. Consequently both the duration of this period and larval growth rate have a critical influence on size at pupation (D'Amico *et al.*, 2001; Davidowitz *et al.*, 2003; Davidowitz & Nijhout, 2004).

JH also plays an important role in caste determination (Wheeler, 1986; Wheeler, 1991) and it is likely that the pleiotropic functions of insect developmental hormones tie the processes of growth and caste determination together. In relation to genetic caste-bias, it could be that genotypes differ in their hormonal response to the same nutritional environment, or in their physiological response to haemolymph JH titre. Here, I show that this may indeed by the case in *A. mellifera*, as both patrilines and colonies varied in the growth response of larvae to treatment with the juvenile hormone analogue methoprene. This result suggests that there may be genotypic variation in the physiological response of larvae to endogenous hormone titres and, for the first time, provides evidence for a potential physiological mechanism underlying the variable propensity for genotypes to develop as queens.

Growth rate may also be indicative of other fitness-related traits that influence the queen-rearing process. For example, in *A. mellifera*, activation of an immune response in developing larvae leads to a depletion of nutritional resources (Aronstein *et al.*, 2010), which may consequently result in a decreased growth rate. Indeed, the results presented here show that the growth rate of *A. mellifera* larvae was affected by an immune challenge, stimulated by the fungal pathogen *Ascosphaera apis*. Interestingly, the growth rate of larvae exposed to the parasite was initially reduced compared to control individuals, but this group was then able to compensate for an initial poor start. Parasites are one of the major driving forces of evolution, and resisting parasite infection is vital in determining an organism's fitness (Anderson & May, 1982). Genetic variation in parasite resistance is common across all taxa, and is

one of the major hypotheses proposed to explain multiple mating by females (Schmid-Hempel, 1998; Schmid-Hempel & Crozier, 1999; Hughes & Boomsma, 2004). As such, it would not be surprising to find that parasite resistance played a role in caste determination, and, indeed, here I show that the physiological consequences of parasite resistance, as manifested in growth rate, affected caste determination at the colony level. These findings do not equate to definitive evidence that parasites play a direct role in caste determination, rather that the physiological effect incurred through resisting a parasite may influence this process. Nonetheless, this work is the first to address the impact of an immune challenge on queen rearing in the social insects, thus connecting genetic variation in growth rate (in Chapter 2), caste propensity (Tilley & Oldroyd, 1997; Osborne & Oldroyd, 1999; Châline *et al.*, 2003; Moritz *et al.*, 2005) and resistance (Palmer & Oldroyd, 2003; Tarpy, 2003; Evans, 2004; Evans & Pettis, 2005; Tarpy & Seeley, 2006; Invernizzi *et al.*, 2009; Evison *et al.*, 2013), all of which are present in the honey bee.

As important as the genotype-by-environment interaction is in determining caste, the interaction between genotypes within a colony also plays an important role. In the ant species *Pogonomyrmex rugosus* and *Linepithema humile*, it has been proposed that genetic caste-bias results from the interaction between maternal and paternal genotypes at the individual and colony level (Schwander & Keller, 2008; Libbrecht *et al.*, 2011). Additionally, it is also important to consider how the genotype of colony workers, or the social environment, interacts with that of developing larvae; there is a growing body of evidence that shows the phenotype of developing larvae is dependent both on individual genotype and the genotype of colony workers (Allsopp *et al.*, 2003; Linksvayer, 2006; Linksvayer, 2007; Linksvayer *et al.*, 2009b; Linksvayer *et al.*, 2011). Perhaps the ultimate expression of an interaction between nurse and larva genotypes would be intracolonial nepotism, although there are currently no empirical data to conclusively support the occurrence of this behaviour (Osborne & Oldroyd, 1999; Holzer *et al.*, 2006; Wenseleers, 2007). However, this is far from evidence that nepotism does not occur in any species, rather that if such behaviour does occur, we are so far unable to detect it.

Potentially, nepotistic behaviour could explain the genetic caste-bias observed in *A. mellifera* and *Ac. echinatior*, and this is a particularly interesting prospect in

Acromyrmex, as variation in cuticular hydrocarbons sufficient to reliably identify individual patrilines has been discovered in *Ac. octospinosus* (Nehring *et al.*, 2011). Here, I show *Ac. octospinosus* workers were able to discriminate between nestmate and non-nestmate larvae, both upon initial contact and also once larvae were integrated into the colony. These results that support the findings of other studies using *Acromyrmex* (Viana *et al.*, 2001; Fouks *et al.*, 2011) although results in this genus are somewhat mixed (de Souza *et al.*, 2006). However, this response was heavily dependent on the colony from which workers were taken and, in terms of aggressive interactions, the larval colony-of-origin. Furthermore, the response was not mediated by short contact with fungus from the adoptive colony. Nestmate recognition is dependent on a combination of genetic and environmental cue variation (Lahav *et al.*, 1999; D'Ettorre & Lenoir, 2010), and in *Acromyrmex*, odour results from a combination of genetically variable cuticular hydrocarbons and odour cues from the fungus (Richard *et al.*, 2007; Nehring *et al.*, 2011). Here, *Ac. octospinosus* workers were shown to discriminate between larvae when dealing with colony-level cues, even after larvae were adopted into the fungus garden, and hence may be able to discriminate between larvae from different queens or different patrilines within the nest. However, the variability of discrimination between colonies may make this effect hard to detect experimentally, and could thus account for the current lack of evidence for intracolonial nepotism.

Genetic caste-bias may afford individuals a great fitness advantage. For example, *Ac. echinatior* 'royal cheats' gain up to a 500-fold increase in their direct fitness (Hughes & Boomsma, 2008). It is unlikely, however, that this strategy is without cost. Here, *Ac. echinatior* royal patrilines were not found to suffer fitness costs in terms of body size, but these patrilines had higher fluctuating asymmetry, indicative of other, more subtle, costs incurred by individuals in these patrilines, such as reduced fecundity and immune function. Costs such as these might be caused by developmental stress resulting from the initiation of queen development at a lower nutritional threshold than other patrilines. Such costs may be accrued by the individual, but can also impact on colony fitness. Any process that has a detrimental effect on the reproductive potential of virgin queens leaving a colony will decrease the inclusive fitness of colony workers. The fitness gained by individuals from queen-biased patrilines is likely to be frequency dependent, constrained either by direct

suppression by workers, or costs at the colony level (Hughes & Boomsma, 2008). Such selective constraints, coupled with the fitness costs implied by high levels of fluctuating asymmetry would suggest that queen-biased genotypes should be rare, which is indeed the case in both *A. mellifera* and *Ac. echinatior* (Moritz *et al.*, 2005; Hughes & Boomsma, 2008).

One of the most pressing questions is, perhaps, whether the potential causes and consequences of genetic caste-bias in the social Hymenoptera identified here could apply to all species in which it occurs. Eusociality has evolved independently nine times in the Hymenoptera (Hughes *et al.*, 2008a) and so there is no necessary condition for identical mechanisms to underlie caste determination across the order. Nonetheless, in most species, this process is ultimately mediated by the nutritional environment and so it is not unlikely that queen-biased genotypes would have convergent proximate causes. Equally, it is possible that genetic caste-bias has evolved as an artefact of ancestral genetic variation in the response thresholds that mediate phenotypic plasticity and so its proximate causes would be expected to be similar across taxa. Here, although both *Ac. echinatior* body size and *A. mellifera* growth rate were shown to have a genetic component, it was only growth rate that was shown to interact with patriline caste-bias, despite the two measures being fundamentally related. This comparison does not offer evidence that different mechanisms are acting in these two species; rather, it is a reminder that the relationship between genotype and environment is a complex one, which varies significantly across species (Schwander *et al.*, 2010).

Overall, this thesis has shown that genetically-influenced physiological traits can play a role in the caste determination system of the social Hymenoptera, and may be particularly important in explaining the genetic caste-bias observed in a number of polyandrous species. It also demonstrates that parasites can indirectly affect caste determination through their effects on host physiology. It shows that nestmate recognition in a polyandrous species varies significantly between colonies, suggesting that the social environment is likely to have an effect on nestmate discrimination and, potentially, intracolonial nepotism. Finally, it demonstrates that queen-biased genotypes face physiological costs, which may go some way towards countering the fitness benefits gained through cheating strategies. That queen rearing

in the social insects is non-random has been known for many years, but the importance of this study is that it begins to address both the proximate causes and the fitness consequences of this phenomenon.

All the work presented here invites the prospect of further investigation. The idea that parasites could play a role in caste determination is one that has not previously been investigated and therefore warrants further study. Bearing in mind that parasites are one of the most important drivers of evolution, it is possible that further research could implicate host-parasite dynamics as a factor involved in the evolution of eusociality itself. Equally, investigating the interaction between genotype, developmental hormones and physiology by, for example, exploring genotypic variation in haemolymph hormone titres, in combination with the response of genotypes to externally administered hormones, would help us to better understand the effect genotype can have on phenotypic plasticity.

To date, eleven Hymenopteran genomes have been published (Munoz-Torres *et al.*, 2011) and consequently, identifying genes that are differentially expressed in queens and workers, and during caste determination, is the focus of much current research (Evans & Wheeler, 1999; Evans & Wheeler, 2000; Pereboom *et al.*, 2005; Cristino *et al.*, 2006; Sumner, 2006; Sumner *et al.*, 2006; Wheeler *et al.*, 2006; Feldmeyer *et al.*, 2013). Such research is vital if we are to understand the physiological basis of caste determination, specifically, how genotypes have the potential to produce such varied phenotypes. However, although variation in gene expression can help to explain phenotypic variation, it does not necessarily answer the question of why particular individuals follow the developmental trajectory they do. It is likely that workers hold the key to answering this question. In the honey bee, for example, workers have been shown to petition for certain larvae to be reared as queens, but how the group makes a collective decision as to which larvae are selected is unknown (Al-Kahtani & Bienefeld, 2011). It is crucial that we continue to combine work investigating the behavioural, genetic and environmental factors affecting social insect caste if we are to unravel the complex interactions that lead to the most extreme expression of altruism and, equally, some of the most selfish behaviour observed in the animal kingdom.

8. References

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Appendix

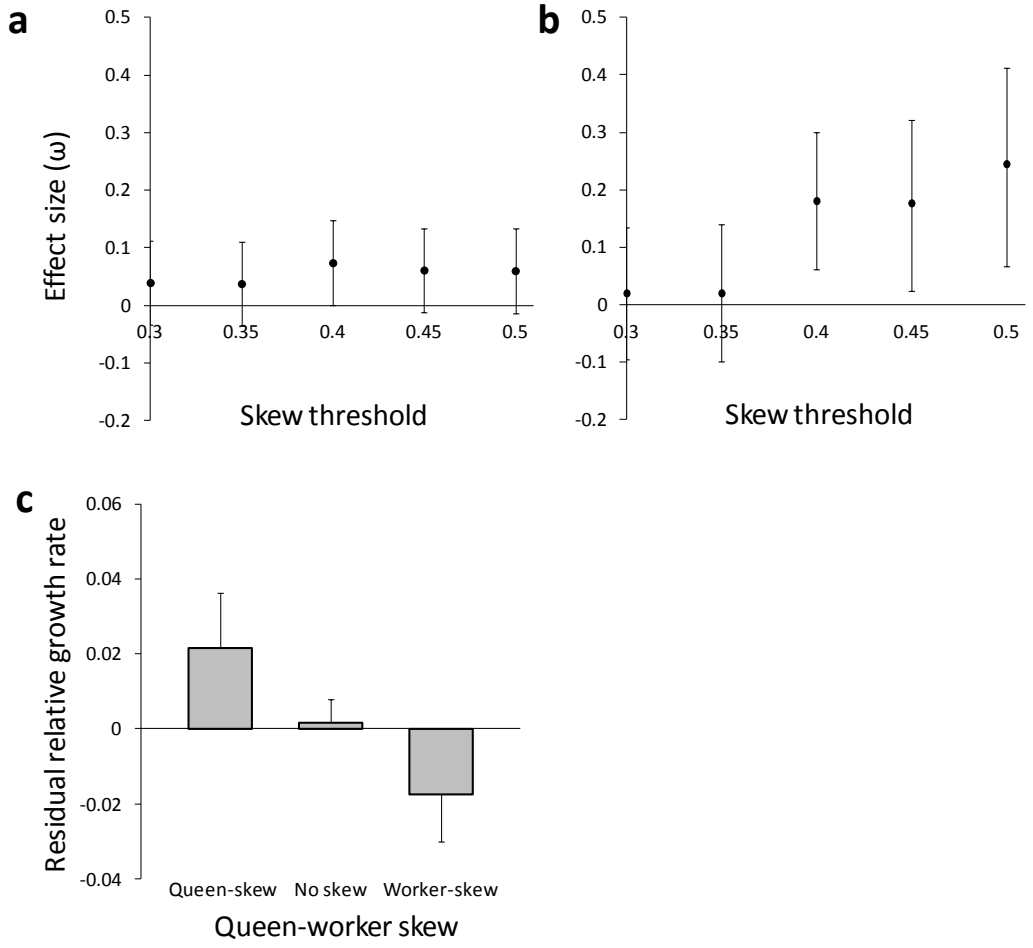

Figure A2.1 Effect size (ω) ± 95% confidence intervals for the effect of patriline queen-worker skew on the residual relative growth rate honey bee larvae. Effect size was calculated for models that used different threshold skew values from ± 0.3 to ± 0.5 for classifying queen-skewed and worker-skewed patrilines. Models included either **(a)** all individuals in the study (queen-skewed, worker-skewed and non-skewed patrilines) or **(b)** individuals from queen-skewed and worker-skewed patrilines only. A skew cut-off of ±0.4 was used in the final analysis of results. There was a significant difference between the residual relative growth rate of queen-skewed, worker-skewed and non-skewed patrilines (GLM: $\chi^2 = 9.20$, d.f. = 2, P = 0.01). The significance of this effect increased when non-skewed patrilines were removed from the analysis (GLM: $\chi^2 = 8.77$, d.f. = 1, P = 0.003). **c)** Mean ± SEM residual relative growth rate of honey bee larvae from genotypes (patrilines) that

were either queen-skewed, worker-skewed, or showed no queen-worker skew, when using a skew threshold of 0.39. In the original analysis, queen-skewed patrilines were classified as having a queen-worker skew > 0.4, resulting in 4 queen-skewed patrilines. Here, we include two extra patrilines from Colony 1 in this group, that each had a queen-worker skew of 0.39, to increase the statistical power. The additional patrilines did not alter the nature of the relationship (GLM: $\chi^2 = 8.42$, d.f. = 2, $P = 0.015$).

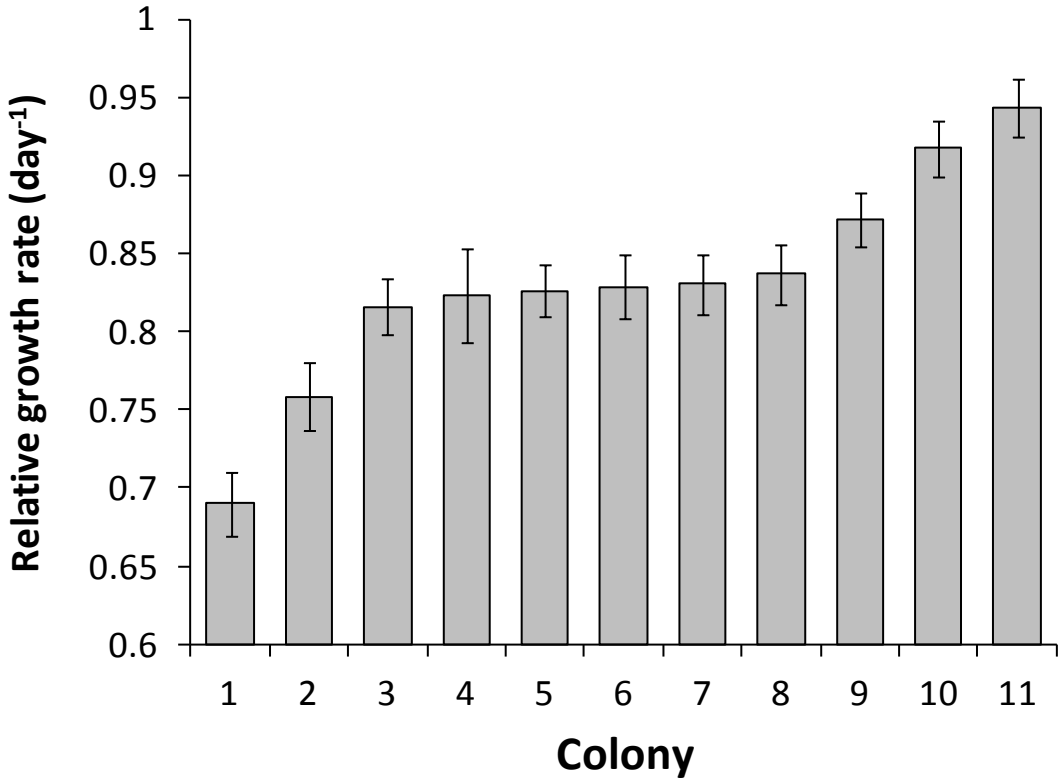

Figure A4.1 Mean ± SEM relative growth rate of honey bee larvae over a seven day period, for each of eleven *Apis mellifera* colonies. Larvae were reared under controlled laboratory conditions on a standardised diet.

Primer	**Ech1390**	**Ech3385**	**Ech4126**	**Ech4225**	**Atco15**
Taq polymerase (Promega) (units/µl)	0.4	0.4	0.4	0.4	0.3
Taq buffer	1x	1x	1x	1x	1x
$MgCl_2$ (mM)	1.62	0.67	0.67	3.38	2.00
Total dNTPS (µM)	270	100	100	340	200
Primer F (µM)	0.4	0.5	0.5	0.5	0.2
Primer R (µM)	0.4	0.5	0.5	0.5	0.2
Template DNA (µl)	1.1	1.1	1.1	1.1	2
Reaction volume (µl)	11.1	11.1	11.1	11.1	12
Annealing temperature (°C)	48	50	57	52	55

Table A6.1 Conditions for the amplification of five polymorphic microsatellites used to identify patrilines in four *Acromyrmex echinatior* colonies.

Colony	Patriline	G test			Fisher's exact test	
		G	*p*	*Q*	*p*	*q*
Ae07P4	1	1.27	0.26	0.852	0.364	1
	2	0.18	0.672	0.852	0.734	1
	3	0.02	0.884	0.884	1	1
	4	0.19	0.666	0.852	0.775	1
	5	0.14	0.71	0.852	0.846	1
	6	0.54	0.464	0.852	0.723	1
	Overall	2.33	0.802	0.178	0.825	0.145
Ae088	1	3.89	0.049	0.074	0.09	0.109
	2	3.47	0.063	0.074	0.123	0.109
	3	0.1	0.755	0.296	1	0.381
	4	0.19	0.663	0.296	0.707	0.314
	5	2.08	0.149	0.088	0.214	0.114
	6	2.54	0.111	0.087	0.195	0.114
	7				0.1	0.109
	Overall	12.08	0.034	0.011	0.013	0.003
Ae357	1	7.75	0.005	0.004	0.02	0.018
	2	0.91	0.34	0.131	0.444	0.202
	3	0.41	0.522	0.161	0.652	0.237
	4	1.13	0.287	0.131	0.396	0.202
	5	17.22	0.00003	0.00005	0.0002	0.00036
	Overall	27.42	0.00002	0.00002	0.00003	0.00003
Ae48	1	4.32	0.038	0.051	0.051	0.053
	2	1.34	0.247	0.132	0.292	0.139
	3	2.64	0.105	0.077	0.187	0.107
	4	0.008	0.929	0.354	1	0.357
	5	0.187	0.665	0.296	0.839	0.342
	6	7.91	0.005	0.013	0.015	0.043
	7	2.47	0.116	0.077	0.056	0.053

	8				0.183	0.107
	Overall	18.8	0.005	0.002	0.001	0.001
Ae125	1	7.82	0.005	0.026	0.002	0.008
	2	6.138	0.013	0.034	0.021	0.039
	3	4.06	0.044	0.077	0.078	0.098
	4	0.13	0.717	0.605	0.744	0.465
	5	0.00005	0.994	0.654	1	0.469
	6	0.06	0.802	0.605	0.87	0.466
	7	2.72	0.099	0.13	0.193	0.145
	8	3.06	0.805	0.605	0.123	0.115
	Overall	24	0.001	0.001	0.002	0.001
Ae158	1				0.021	0.033
	2				0.028	0.033
	3	0.00001	0.997	0.7	1	0.471
	4	0.03	0.857	0.7	0.902	0.471
	5	3.89	0.049	0.103	0.102	0.08
	Overall	2.91	0.23	0.061	0.003	0.0008

Table A6.2 Results of Fisher's exact tests (all patrilines) and G tests (only the more abundant patrilines) comparing actual frequencies of queens and workers with the frequencies expected frequencies in four *Acromyrmex echinatior* colonies.