Experimental Investigations of Inclusive Fitness Theory in a Multiple-Queen Ant

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Abstract

Experimental investigations of kin-selected conflict in the eusocial Hymenoptera have proved essential in investigating inclusive fitness theory, the leading theory for social evolution. The aim of this thesis was to test both novel predictions, and existing predictions requiring further investigation, in this area. To this end, I performed four experiments using the facultatively multiple-queen ant *Leptothorax acervorum* as a model system. In the first experiment I tested the prediction that workers in multiple-queen colonies favour their most related queen during social interactions. The results showed that workers did not discriminate between nestmate queens based on relatedness. In the second experiment I tested the novel prediction that the extent to which workers prepare themselves for future reproduction is a function of colony social structure (i.e. queen number), given that single-queen colonies are more likely to provide a future opportunity for worker reproduction than multiple-queen colonies. The results were as predicted, with workers in previously single-queen colonies expressing higher levels of reproduction following queen removal than workers in previously multiple-queen colonies. In the third experiment I tested whether the workers that went on to reproduce after the removal of their queen(s) prepared for future reproduction by altering their behaviour. The results showed that the behaviour of future reproductive workers differed from that of other workers in the presence of the queen. In the fourth experiment I tested for an effect of maternal caste, colony social structure and egg age on worker policing. The results showed that workers policed non-nestmate worker-laid eggs at a higher level than non-nestmate queen-laid eggs, and that colony social structure and egg age had no effect on worker policing. The results of the second and third experiments are as predicted by inclusive fitness theory: workers are more highly related to their own sons than to nestmates' sons, and hence should attempt to maximise their chances of producing their own male offspring in the future. The results of the first and fourth experiments are not as predicted by inclusive fitness theory based on relatedness alone, but fit within the theory when costs and constraints are considered.

Contents

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Chapter 1

Chapter 1: General introduction

In 1964 W. D. Hamilton proposed his theory of inclusive fitness (also known as kin selection), the theory that evolutionary fitness consists of two components: direct fitness (fitness achieved through direct reproduction) and indirect fitness (fitness achieved through the reproduction of relatives) (Hamilton 1964a, b). Direct fitness was already a key concept within natural selection theory in the 1960s, but the idea of indirect fitness was new, and the assumptions upon which it was based proved to be invaluable to the field of evolutionary biology. The first key assumption of Hamilton's inclusive fitness theory was that the unit of selection is the gene, not the individual as previously thought. This assumption led to the second: that a gene must be selected on the basis of the effect it has upon the fitness of not only its bearer, but also the effect it has upon any other individual sharing a copy of itself. The most likely circumstance to cause two individuals to share the same gene is kinship, and hence a gene for social behaviour should be subject to selection on the basis of its effect upon kin.

Hamilton's inclusive fitness theory has proved an essential part of evolutionary biology because of its key role in identifying the true biological unit upon which natural selection acts and the importance of relatedness in natural selection. Inclusive fitness theory has also been essential to the understanding of social evolution, especially the evolution of altruism. Altruism can be defined as a social interaction where one individual (the actor) performs a behaviour that increases the direct fitness of another individual (the recipient), but which decreases the direct fitness of the actor. The most extreme form of altruism found in nature is the forfeiting of reproduction in order to rear the offspring of others, especially when the forfeiting of reproduction is made permanent by the evolution of specialised castes. Such reproductive altruism can be found in eusocial organisms such as the eusocial Hymenoptera (ants, bees and wasps), where reproduction is divided between group members in such a way that some members of the group (often only one) reproduce and are specialised in doing so, whilst others do not (or rarely) reproduce, and instead specialise in performing group tasks, such as rearing the brood of their reproductive nestmates. Before inclusive fitness theory was proposed, altruism represented a problem for natural selection theory, because, according to Darwin's original outline of natural selection (Darwin 1859), organisms were expected to evolve only traits that allowed them to increase their own lifetime reproductive success. Hence altruistic traits such as forfeiting reproduction in order to help others presented a potentially fatal flaw in natural selection theory (Darwin 1859). However, inclusive fitness theory explained the evolution of altruism by identifying the indirect component of an individual's fitness and highlighting the importance of shared genes between related individuals. Hamilton proposed that, on the basis of inclusive fitness theory, altruism can evolve provided that the fitness costs suffered by the performer of an altruistic behaviour (the actor) are outweighed by the fitness benefits gained by the recipient once weighted by the value of relatedness between recipient and actor.

The conditions for the evolution of altruism can be put into a simple inequality known as Hamilton's rule, where altruism can evolve if $rb > c$, where c is the cost to the actor's direct fitness (e.g. the number of offspring lost through the altruistic interaction), b is the benefit to the recipient's direct fitness (e.g. the number of offspring gained through the altruistic interaction), and r is the relatedness between actor and recipient. Hamilton's rule clearly shows that for altruism to evolve, relatedness must be greater than zero. Hamilton's rule of inclusive fitness can also be adapted to predict the circumstances under which the other three main categories of social interaction (cooperation, selfishness and spite) can evolve. In terms of relatedness, cooperation (where both actor and recipient benefit from the interaction in terms of direct fitness) and selfishness (where the actor gains direct fitness from the interaction but the recipient loses direct fitness) are predicted to evolve under any value of relatedness between actor and recipient, whereas spite (where both actor and recipient lose direct fitness through the interaction) is predicted to evolve only under negative values of relatedness (Hamilton 1964a, b; Lehmann & Keller 2006; West *et al.* 2007b; Bourke 2011b).

In inclusive fitness theory, relatedness can be informally defined as the probability of two individuals sharing a gene relative to the average probability of any two individuals in the population sharing the gene (Bourke 2011b). Hence when the probability of two individuals sharing a gene is more than the population average, then relatedness takes on a positive value, whereas when the probability of two individuals sharing a gene is less than the population average, relatedness takes on a negative value. The formal, technical definition of relatedness is as a regression coefficient (Hamilton 1972), where relatedness is the gradient of the line when the frequency of a gene in potential recipients is regressed against the frequency of a gene in potential actors (Pamilo & Crozier 1982; Bourke & Franks 1995; Bourke 2011b). In the context of social evolution in the eusocial Hymenoptera, relatedness is usually presented in a "life-for-life" format (as it is throughout this thesis), where regression relatedness is adjusted by the 'sex-specific reproductive value' (Hamilton 1972; Grafen 1986; Bourke & Franks 1995). The sex-specific reproductive value is relevant to haplodiploid species, such as the Hymenoptera, because females are diploid and males are haploid, so a female offers twice the opportunity for a gene to be passed to the next generation that a male does, and hence females have double the reproductive value of males (Hamilton 1972; Grafen 1986; Bourke & Franks 1995).

Criticism of inclusive fitness theory

Despite inclusive fitness theory being widely acknowledged as playing a key role in understanding the evolution of altruism and all other forms of social behaviour, there is still some debate over the relevance, applicability and importance of the theory in the study of social evolution. For example, inclusive fitness theory has been challenged with the claim that the theory overestimates the importance of relatedness and kinship in social evolution, and that other factors besides relatedness and kinship were more important during the evolution of eusociality, such as pre-adaptive behaviour (e.g. progressive brood provisioning), resources of high value requiring defence (e.g. nest or food resources), and a single-point mutation causing altruistic behaviour (e.g. a change in the gene for dispersal causing offspring to stay with their parents) (Wilson 2005, 2008; Wilson & Hölldobler 2005; Nowak *et al.* 2010). Critics of inclusive fitness theory have also suggested that where kinship is found to be in close association with eusociality, it is as a consequence rather than a cause of eusocial behaviour (Wilson 2005; Wilson & Hölldobler 2005; Nowak *et al.* 2010). Furthermore, critics have accused supporters of inclusive fitness theory of placing too much emphasis on the fitness of the altruistic offspring (i.e. the 'workers' in eusocial societies) in explaining the evolution of eusociality (Nowak *et al.* 2010). Instead critics have suggested that the altruistic behaviour of offspring can be explained through selection based on their mother's interests (Nowak *et al.* 2010), a concept similar to that proposed in old theories of altruism based on parental manipulation (Alexander 1974). These criticisms challenging the importance of relatedness and kinship in the evolution of eusociality have themselves been challenged. In terms of factors that have been promoted as alternative means to the evolution of eusociality besides kinship (e.g. pre-adaptive behaviours, a valuable shared resource and a simple mutation triggering altruistic behaviour), these are all factors that are already acknowledged as important within inclusive fitness theory, and therefore evidence of these factors playing a role in the evolution of eusociality does not falsify the importance of relatedness and kinship (Bourke 2011a). Regarding the suggestion that kinship is a consequence rather than a cause of eusociality, supporters of inclusive fitness theory dispute this claim on the basis that there is strong evidence showing that eusociality arose strictly from family groups with high relatedness (Helanterä & Bargum 2007; Boomsma 2009; Boomsma *et al.* 2011). Finally, in response to the criticism of taking a "worker-centred" approach (Nowak *et al.* 2010) to the evolution of altruism, supporters of inclusive fitness have emphasised the fact that the fitness requirements of workers must be considered in explaining altruism, because attempts at parental manipulation by the mother (i.e. the 'queen' in eusocial societies) should be met with counter-adaptations by the workers, and there is no reason to assume the workers should lose such a conflict (Bourke & Franks 1995). Furthermore, there are many known examples of workers actively pursuing their own inclusive fitness interests in conflict with the queen (e.g. workers sometimes destroy the queens' brood in order to manipulate offspring sex-ratio (Sundström *et al.* 1996) or even kill the queen herself in order to pursue their own reproduction (Bourke 1994a)), in contrast to what we would expect if workers were passive to parental manipulation from the queen (Bourke 2011a).

Another major theme among criticisms of inclusive fitness theory is the importance of group selection (i.e. selection upon biological groups, including colonies and species, based upon variation in group success) in social evolution. Group selection has been described as an alternative method of selection relative to that based upon inclusive fitness (Wilson & Hölldobler 2005; Wilson 2008). However, many supporters of inclusive fitness theory view the debate over group selection versus inclusive fitness theory as largely irrelevant, because both forms of selection (when considering group selection in its intrademic form) when modelled mathematically produce the same conditions for the evolution of altruism (Bourke & Franks 1995; Foster *et al.* 2006; West *et al.* 2007c). Critics of inclusive fitness have also suggested that the theory offers little insight into social evolution and is too abstract to apply to practical investigations

(Nowak *et al.* 2010). However, these claims have been strongly disputed on the basis that inclusive fitness theory has, via simple predictions based on Hamilton's rule, led to the understanding of a vast range of social behaviours as diverse as intragenomic conflict and eusocial behaviour (Abbot *et al.* 2011; Bourke 2011a, b; Strassmann *et al.* 2011).

Here I have described just a few of the criticisms made against inclusive fitness theory, and it should be known that others exist (summarised by Bourke 2011b). However, so far, as described in a recent summary of the criticisms made against inclusive fitness theory and the counter-arguments made in its support (Bourke 2011b), inclusive fitness theory has yet to face a criticism by which it is truly challenged, and the wealth of evidence in support of the theory has allowed inclusive fitness to keep its position as the lead theory for social evolution

The eusocial Hymenoptera

Inclusive fitness theory and social evolution have been investigated across a number of different social taxa, particularly in animals that express a reproductive division of labour. For example, inclusive fitness theory has been investigated in cooperatively breeding mammals (such as the meerkat, *Suricata suricatta* (e.g. Clutton-Brock *et al.* 2000; Clutton-Brock *et al.* 2001)), eusocial mammals (such as the naked mole-rat, *Heterocephalus glaber* (e.g. Reeve 1992; Jacobs & Jarvis 1996)), cooperatively breeding birds (such as the Seychelles warbler, *Acrocephalus sechellensis* (e.g. Richardson *et al.* 2002, 2003, 2007)), cooperatively breeding fish (such as the cichlid *Neolamprologus pulcher* (e.g. Stiver *et al.* 2005; Le Vin *et al.* 2010)), eusocial shrimp (of the genus *Synalpheus* (e.g. Duffy & Macdonald 2010)), and eusocial insects (such as the termites, epifamily Termitoidae (e.g. Korb 2006; Atkinson *et al.* 2008)). Social microbes are also an increasingly popular choice of study system for investigating social evolution (e.g. West *et al.* 2007a). Many studies of inclusive fitness have focused upon the eusocial Hymenoptera, which provide excellent study systems for investigating the theory for a number of reasons. To begin with, the eusocial Hymenoptera exhibit a wide range of complex social behaviours, including, as previously mentioned, the most extreme form of altruism known − that of a reproductive division of labour based on morphological castes. A reproductive division of labour is only expressed between females in the eusocial Hymenoptera, with females known as queens exhibiting high levels of reproduction, and females known as workers exhibiting either low levels of reproduction or no reproduction at all. Whilst the queens specialise in reproduction, the workers specialise in tasks such as foraging, nest maintenance, defence and brood care.

In addition to their wide range and high levels of social behaviour, the eusocial Hymenoptera also make excellent study systems for investigating inclusive fitness theory because of their considerable variation in colony kin structure both within and between species. Colony kin structure is principally affected by colony social structure (i.e. the number of reproducing queens in a colony and the number of males with which they have mated), although it is also affected by other factors, such as the extent of worker reproduction and the level of inbreeding (Bourke & Franks 1995). The eusocial Hymenoptera show variation in their social structure both within and between species, with single-queen (monogynous) or multiple-queen (polygynous) colonies, and with singly-mated (monandrous) or multiply-mated (polyandrous) queens. A number of predictions of inclusive fitness theory are concerned with the effect of kin structure on social behaviour (further discussed below under 'Kin conflict'), and the eusocial Hymenoptera provide the opportunity to test these predictions both within and between species.

Another aspect of the eusocial Hymenoptera that makes the group particularly suitable for the study of inclusive fitness theory is the fact that they have a haplodiploid sex determination system (i.e. females are derived from fertilised, diploid eggs, and males from unfertilised, haploid eggs). Haplodiploidy creates unusual patterns of relatedness between kin (e.g. workers are three times more related to their full-sisters than brothers), and these patterns can be used to formulate many predictions of inclusive fitness theory, particularly regarding kin conflicts (further discussed below). Furthermore, haplodiploidy has the effect that workers have retained their ability to produce male offspring in many Hymenopteran species. Although in the majority of eusocial Hymenoptera species workers have lost their ability to mate and so to produce fertilised (diploid/female) eggs, workers of many species have retained their ovaries and therefore their ability to produce unfertilised (haploid/male) eggs (Bourke 1988b). The ability of workers to produce viable male offspring is another factor that can be used to create predictions with which to test inclusive fitness theory (further discussed below).

Finally, on a practical note, many species of eusocial Hymenoptera can be easily maintained and manipulated in a laboratory environment, allowing detailed and controlled tests of inclusive fitness theory to be performed on complete colonies.

Kin conflict

One of the most successful and informative areas of investigation into inclusive fitness theory has been kin conflict. Kin conflict is conflict predicted to occur between members of non-clonal societies over group reproduction. The reason why inclusive fitness theory predicts kin conflict to occur is because, in non-clonal societies, group members differ in their relatedness to different classes of reproductive offspring (e.g. male versus female offspring, or offspring produced by one female versus offspring produced by another female), which in turn means that group members also differ in which outcome of group reproduction can offer them the greatest inclusive fitness (Hamilton 1964a, b; Bourke & Franks 1995). Hamilton alluded to the possibility of kin conflict in his early papers on inclusive fitness theory (Hamilton 1964a, b, 1972), but Trivers was the first person to formally propose the concept of conflict within family groups over variation in fitness optima (Trivers 1974). Originally Trivers predicted kin conflict between parents and offspring (Trivers 1974), but since then many predictions have been developed regarding kin conflict, particularly in the eusocial Hymenoptera (Trivers & Hare 1976; Ratnieks & Reeve 1992; Bourke & Franks 1995; Ratnieks *et al.* 2006). For example, predictions include conflict between queens and workers over sex-allocation (i.e. conflict over whether to produce male or female reproductive offspring) (Trivers & Hare 1976), conflict between queens and workers and among workers over whether to rear queen- or worker-produced males (Hamilton 1964b, 1972; Trivers & Hare 1976; Ratnieks 1988), conflict between members of different genetic lineages over queen rearing (i.e. conflict over whether to rear queens belonging to one matriline or patriline over another) (Hamilton 1964a, b; Visscher 1986), conflict between queens and workers over whether to invest in colony growth or reproduction (Pamilo 1991a), and conflict between female brood items and the rest of the colony over caste determination (i.e. conflict over whether a brood item develops into a queen or a worker) (Ratnieks & Reeve 1992). Such predictions have provided a means with which to test inclusive fitness theory by observing the behaviour of modern-day eusocial insects. In doing so they have been, and continue to be, essential to the study of social evolution.

Although inclusive fitness theory predicts a number of potential kin conflicts to occur on the basis of kin structure, not all potential conflicts are expected to result in actual conflict (Ratnieks & Reeve 1992). For example, potential conflict may not result in actual conflict if there are high colony-level costs associated with overt conflict, or if there is a lack of essential information required for individuals to alter the outcome of colony reproduction towards their own optima (Ratnieks & Reeve 1992). A good example of a potential conflict that never seems to result in actual conflict is that predicted between members of different genetic lineages over queen rearing. Inclusive fitness theory predicts that, other things equal, individuals belonging to a particular matriline (in a polygynous colony) or patriline (in a polyandrous colony) should greatly benefit in terms of fitness by preferentially rearing members of their own genetic lineage as the colony's new queens (Hamilton 1964a, b; Visscher 1986). However, there is little evidence to suggest that nepotistic queen rearing occurs (Breed *et al.* 1994; Keller 1997; Tarpy *et al.* 2004; Ratnieks *et al.* 2006), perhaps as a result of costs associated with the behaviour (Ratnieks & Reeve 1991, 1992) or a lack of variation in chemical recognition cues between genetic lineages belonging to the same colony (Ratnieks 1991; Boomsma *et al.* 2003). Therefore, based upon current evidence, conflict between members of different genetic lineages over queen rearing can be seen as a potential rather than actual conflict. A good example of a potential conflict that does result in actual conflict is that of conflict over male production. According to inclusive fitness theory, other things equal, queens and workers should favour their own production of the colony's males, because a female Hymenoptera is always more related to her own sons than any other type of male relative (Trivers & Hare 1976). Hence conflict is predicted between queens and workers over which party should contribute to the colony's male offspring (Trivers & Hare 1976). Conflict between reproductive and non-reproductive workers over male production is predicted in colonies with low average worker-worker relatedness, because in such colonies, non-reproductive workers are more related to queen-produced males and should therefore be selected to oppose worker reproduction (Ratnieks 1988). Evidence of actual conflict comes from a number of directions (Bourke & Franks 1995; Ratnieks *et al.* 2006), such as the fact that both queens and workers have been observed performing 'policing' behaviour, where they prevent successful worker reproduction by attacking reproductive workers and destroying their eggs (Ratnieks & Visscher 1989; Kikuta & Tsuji 1999; Wenseleers *et* *al.* 2005a, b), and the fact that there is a clear pattern across species for a higher proportion of males to be worker-produced in species where workers are more related to workers' sons than queens' sons (Wenseleers & Ratnieks 2006).

Many experimental and empirical investigations have been performed to test the predictions of kin conflict in the Hymenoptera (Bourke & Franks 1995; Ratnieks *et al.* 2006). These studies have used a number of different approaches, such as testing for a relationship between kin structure and kin conflict by looking at natural variation across species (e.g. Hammond & Keller 2004; Wenseleers & Ratnieks 2006), testing for a relationship between kin structure and kin conflict within species (those with a facultative kin structure) either by looking at natural variation (e.g. Foster & Ratnieks 2000) or through experimental manipulation (e.g. D'Ettorre *et al.* 2004), and testing for signs of kin conflict within colonies of a single kin structure using experimental manipulation (e.g. Ratnieks & Visscher 1989; Kikuta & Tsuji 1999). Through such investigations, many forms of actual kin conflict have been identified, thus providing strong support for inclusive fitness theory (Bourke & Franks 1995; Ratnieks *et al.* 2006). Where these conflicts exist, considerable effort has gone in to trying to understand how these conflicts are resolved in order to allow the existence of stable societies based on cooperation (Ratnieks *et al.* 2006). In the case of investigations that have revealed some conflicts to be only potential rather than actual, these have stimulated considerable investigation into why some potential conflicts do not overtly occur in Hymenopteran societies, such as potential conflict between genetic lineages in polyandrous and polygynous colonies (Keller 1997; Boomsma *et al.* 2003). Hence investigations into kin conflict have led to a greater understanding of many factors that affect social evolution, and so have been proved essential to the study of inclusive fitness theory.

Thesis aims

New techniques for investigating social evolution are being developed, particularly in the field of genomics, an area of research that looks set to provide exciting new insights into the genetics of social behaviour (Bonasio *et al.* 2010; Smith *et al.* 2011a; Smith *et al.* 2011b; Wurm *et al.* 2011). However, behavioural investigations of social evolution and inclusive fitness theory still have much to offer, and there are still important aspects of kin conflict that require further investigation in the eusocial Hymenoptera. For example, there are important predictions of kin conflict that remain to be tested using reliable methods that can provide clear, unambiguous results. One such prediction is the conflict expected to occur between genetic lineages over reproduction in polyandrous and polygynous colonies. There are also important predictions of kin conflict that remain to be tested in a sufficiently wide context. For example, the effect of polyandry on conflict among workers over whether to rear queen- or worker-produced males has been well tested, but the effect of polygyny remains to be sufficiently explored. Furthermore, we still have much to learn about the information cues used during acts of kin conflict. For example, it is still unclear as to whether the cues used by individuals to distinguish queen-laid eggs from worker-laid eggs can be transferred across colonies, or whether these cues breakdown as eggs age. In addition to the fact that there are still important predictions of kin conflict that remain to be understood, the field of kin conflict also requires further investigation due to the fact that there are still new predictions to come from the theory that can, upon testing, improve our understanding of inclusive fitness theory and social behaviour. For example (and as described in more detail below), in this thesis I develop and test the novel prediction that workers belonging to species with a facultative social structure should have evolved to assess the current risk of their colony becoming queenless (i.e. losing all of its queens) because of the opportunity queenlessness can provide for workers to gain direct fitness. The overall aim of this thesis, therefore, is to provide insight into inclusive fitness theory, social evolution and social behaviour by investigating areas of kin conflict that have yet to be tested sufficiently, by investigating specific aspects of kin conflict that remain to be understood, and by investigating novel predictions based on kin conflict and inclusive fitness theory. I use the facultatively polygynous ant *Leptothorax acervorum* as my study species.

***Leptothorax acervorum* as a study system**

L. acervorum (Fabricius) is a small myrmicine ant found in Asia, Europe and North America. Some populations of the ant are facultatively polygynous (Buschinger 1968; Douwes *et al.* 1987; Heinze *et al.* 1995b), whereas others are functionally monogynous (i.e. colonies contain multiple queens but only one of these queens reproduces) (Ito 1990; Felke & Buschinger 1999; Gill *et al.* 2009). *L. acervorum* is an excellent species with which to empirically investigate inclusive fitness theory for a number of reasons. First, the facultatively polygynous populations of *L. acervorum* provide a special opportunity to test the many predictions of inclusive fitness theory concerning the effect of kin structure on social behaviour. Although interspecific studies are essential to observe the effect of kin structure across the Hymenoptera, species with a facultative social structure (and consequently facultative kin structure) permit tests of inclusive fitness theory that control for the effect of species. Second, a considerable amount of background knowledge is available on the behaviour, social structure and kin structure of *L. acervorum*. For example, it is already known that *L. acervorum* queens in polygynous colonies are related (Douwes *et al.* 1987; Stille *et al.* 1991; Heinze *et al.* 1995a, b; Bourke *et al.* 1997; Chan *et al.* 1999; Hammond *et al.* 2001, 2006); that polygyny occurs through the re-adoption of daughter queens that mate near the nest (Hammond *et al.* 2001), while other queens participate in mating swarms (Franks *et al.* 1991); that *L. acervorum* queens tend to mate only with a single male each (Heinze *et al.* 1995b; Hammond *et al.* 2001); that mating occurs at random in the species (i.e. inbreeding is rare) (Stille *et al.* 1991; Heinze *et al.* 1995a, b; Bourke *et al.* 1997; Heinze *et al.* 2001; Hammond *et al.* 2001, 2003); that queen turnover is high (Bourke *et al.* 1997; Hammond *et al.* 2001, 2006); and that workers can produce male offspring but tend to do so at low frequency (Bourke 1991; Heinze *et al.* 1997b; Hammond *et al.* 2003). Concerning a population in Norfolk, UK, it is also known that sex-ratios are split between social structures, with monogynous colonies producing mainly female sexual offspring, and polygynous colonies producing mainly male sexual offspring (Chan & Bourke 1994; Chan *et al.* 1999), a pattern that is achieved through workers rearing a higher proportion of female brood as queens (rather than workers) in monogynous colonies to produce a female-biased sex-ratio, rather than by destroying male brood (Hammond *et al.* 2002).

There are also a number of practical reasons why *L. acervorum* makes a good study species for experimental investigations. *L. acervorum* colonies have a monodomous nest structure (i.e. a single colony occupies just a single nest) and they form their nests within material such as dead twigs and tree bark, which together means that complete colonies can be collected from the field. Furthermore, colony sizes are small so entire colonies can easily be kept in a laboratory environment. *L. acervorum* ants are also highly tolerant to light conditions, allowing colonies to be observed under natural light without disruption to their behaviour.

Thesis outline

As stated above, the aim of this thesis is to provide insight into social evolution and inclusive fitness theory by investigating areas of kin conflict that have yet to be tested sufficiently, by investigating specific aspects of kin conflict that remain to be understood, and by investigating novel predictions based on kin conflict and inclusive fitness theory. I aim to achieve these goals through the use of four experimental investigations, as described below:

In Chapter 2, I perform a test of within-colony kin discrimination to assess whether workers in polygynous *L. acervorum* colonies favour (i.e. spend more time interacting with) whichever of their nestmate queens is most related to them. Within-colony kin discrimination, the act of individuals favouring their closest relatives within a colony (and usually described in the context of distinguishing between members of the same sex and maternal caste (but see Wenseleers 2007)) has been investigated in a number of different social contexts, such as queen rearing (as described above under 'Kin conflict'). The act of discriminating between group members on the basis of relatedness is an important prediction of inclusive fitness theory, and yet the ability of the eusocial Hymenoptera to do so remains surprisingly unclear, partly because of the limitations of the methods used to study the phenomenon. In Chapter 2, I assess the occurrence of within-colony kin discrimination in *L. acervorum* by recording the behaviour of individual workers in detail, with the aim of providing clear, unambiguous results to form a reliable conclusion about the occurrence of kin discrimination.

In Chapter 3, I study an aspect of worker reproduction that has not been investigated before. Inclusive fitness theory predicts that, other things equal, workers should favour their own production of males over the queen's production of males, and yet in many species of Hymenoptera, worker reproduction only occurs at a high level in the absence of the queen(s) (i.e. after queen death). In Chapter 3, I test whether *L. acervorum* workers maximise their chances of achieving future direct fitness as a function of the likelihood of their colony becoming queenless (i.e. losing all of its queens). I predict workers to assess the social structure of their colony (single-queen or multiple-queen), and if finding themselves in a single-queen colony (i.e. a colony with a relatively high chance of suddenly becoming queenless), to prepare themselves for reproduction.

In Chapter 4, having presented evidence for preparation for future reproduction by *L. acervorum* workers in Chapter 3, I investigate whether such preparation takes the form of a change in worker behaviour whilst the colony still contains a queen. Few studies have investigated how workers that refrain from reproduction in the presence of the queen might prepare themselves for future reproduction. I also speculate as to whether such a change in worker behaviour might inflict costs upon the colony, and also whether these costs might be less severe than those potentially associated with workers participating in full reproduction in the presence of the queen.

In Chapter 5, I explore three aspects of worker policing (policing performed by workers) that require further investigation in order to fully understand the behaviour. First I test whether queen-laid eggs appear to have a generic queen-signal that can be interpreted across *L. acervorum* colonies, enabling non-nestmate workers to distinguish between queen-laid eggs and worker-laid eggs, and to police worker-laid eggs in favour of queen-laid eggs. Second I test whether the level of worker policing performed by *L. acervorum* is affected by social structure (monogyny versus polygyny), a hypothesis that has so far received relatively little attention in the context of polygyny. Finally I test whether the age of worker-laid eggs affects their likelihood of being policed by *L. acervorum* workers. The effect of egg age on the acceptance of worker-laid eggs has not been investigated before.

Chapter 2

Chapter 2: Absence of within-colony kin discrimination in a multiple-queen ant, *Leptothorax acervorum*

Abstract

Inclusive fitness theory predicts that, other things equal, individuals within social groups should direct altruistic behaviour towards their most highly related group-mates in order to maximise indirect fitness benefits. In the social insects, most previous studies have shown that within-colony kin discrimination (nepotism) is absent or weak. However, the number of studies that have investigated within-colony kin discrimination at the level of individual behaviour remains relatively low. I tested for within-colony kin discrimination in the facultatively multiple-queen (polygynous) ant, *Leptothorax acervorum*. Specifically, I tested whether workers within polygynous colonies treated queens differently as a function of their relatedness to them. Colonies containing two egg-laying queens were filmed to measure the rate at which individually-marked workers fed, groomed and antennated each queen. Relatedness between individual queens and workers was calculated from their genotypes at four microsatellite loci. The results showed that there was no difference in the mean relatedness of workers to the queen they spent more time feeding or grooming and the queen they spent less time feeding or grooming. Likewise, there was no difference in the mean relatedness of workers to the queen they spent more time antennating and the queen they spent less time antennating. Workers interacted preferentially with their potential mother queen with respect to grooming/feeding but not with respect to antennation. However, because of high queen turnover, the number of workers with their potential mother queen still present within the colony was low. Overall, therefore, for workers as a whole, I found no evidence for within-colony kin discrimination in the context of workers' individual treatment of queens in polygynous *L. acervorum* colonies.

Introduction

Hamilton's (Hamilton 1964a, b) inclusive fitness theory predicts that, other things equal, members of animal societies should discriminate socially in favour of more closely-related nestmates or groupmates, a behaviour known as within-colony kin discrimination, or nepotism. The social insects, especially the eusocial Hymenoptera (ants, bees and wasps), have been widely used to test for within-colony kin discrimination because of the large range of social and genetic structures exhibited by their colonies (Breed *et al.* 1994; Keller 1997; Visscher 1998; Tarpy *et al.* 2004; Ratnieks *et al.* 2006). Within-colony kin discrimination is predicted to occur in social insect colonies with either multiple patrilines (polyandrous colonies) or matrilines (polygynous colonies). Such within-group kin discrimination has been found in societies of vertebrates, e.g. carnivores (Van Horn *et al.* 2004; Wahaj *et al.* 2004) and primates (Silk 2009), but firm evidence of the behaviour has not been found in the social insects.

Although some early investigations reported within-colony kin discrimination during brood rearing in the polyandrous honey bee, *Apis mellifera* (Getz & Smith 1983; Visscher 1986; Page *et al.* 1989), these studies have since been criticised (Breed *et al.* 1994; Visscher 1998). One of the main criticisms was that the methods used (a) facilitated within-colony kin discrimination, for example by employing visible genetic markers (Carlin & Frumhoff 1990) or colonies with abnormally low numbers of patrilines or matrilines (Hoogendoorn & Velthuis 1988; Carlin & Frumhoff 1990), or (b) caused investigators to falsely detect within-colony kin discrimination, for example via inappropriate statistical tests (Oldroyd *et al.* 1990) or lack of blind observations (Alexander 1991).

The improvement of molecular techniques since the early 1990s has allowed studies of within-colony kin discrimination that avoid many of the difficulties associated with earlier experiments. However, the majority of recent studies have found within-colony kin discrimination to be absent in the social insects, despite investigating a number of different social contexts, including adult-adult interactions, such as worker interactions with nestmate queens in polygynous colonies (DeHeer & Ross 1997; Strassmann *et al.* 1997; Blatrix *et al.* 2000; Kirchner & Arnold 2001; Gilley 2003; Châline *et al.* 2005;

Atkinson *et al.* 2008; Zinck *et al.* 2009), adult-brood interactions (Strassmann *et al.* 2000; Blatrix & Jaisson 2002; Holzer *et al.* 2006), colony fission (Heinze *et al.* 1997a; Kryger & Moritz 1997; Rangel *et al.* 2009) and inbreeding avoidance (Keller & Fournier 2002). A few recent studies have detected within-colony kin discrimination in social insects, in the context of, for example, adult-adult interactions (Tarpy & Fletcher 1998; Adams & Balas 1999), adult-brood interactions (Osborne & Oldroyd 1999; Hannonen & Sundström 2003) and colony fission (Seppä *et al.* 2008). Some of these studies found only weak effects (Adams and Balas 1999), or ones that were not necessarily attributable to kin-selected nepotism (Tarpy & Fletcher 1998; Seppä *et al.* 2008). Two studies suggested that their results showed evidence for strong within-colony kin discrimination, one in the honey bee (Osborne and Oldroyd 1999) and the other in the ant *Formica fusca* (Hannonen & Sundström 2003), but the latter study has been criticised on the grounds that variations in queen fecundity may have produced the observed results (Holzer *et al.* 2006). A study by Korb (2006) found evidence of nepotism in colonies of the termite *Cryptotermes secundus*. However, *C. secundus* is unusual in that multiple matrilines and patrilines are brought together by colony fusion, so in this case nepotism could stem from between-colony kin discrimination. Overall, therefore, the extent and strength of within-colony kin discrimination in social insects remain unclear.

A possible reason for this mixed picture is that many previous studies have searched for within-colony kin discrimination by analysing the outcomes of behaviours, rather than the actual behaviours themselves (Visscher 1998). This introduces the possibility of within-colony kin discrimination remaining undetected when it is present, since nepotism by members of different patrilines or matrilines could often cancel out, rendering nepotism undetectable in terms of the kin composition of the brood reared (Visscher 1986, 1998). Hence there remains a need for further studies testing for within-colony kin discrimination at the level of individual behaviour. In the present study I observed the behaviour of individual workers within polygynous colonies of the ant *Leptothorax acervorum* to investigate whether workers express within-colony kin discrimination while interacting with nestmate queens. I used *L. acervorum* because whole colonies are easy to maintain and observe in the laboratory, polygynous colonies contain multiple matrilines (Bourke *et al.* 1997; Hammond *et al.* 2006) and workers potentially gain a large indirect fitness benefit from within-colony kin discrimination. In the study population, nearly all queens mate singly, nestmate queens are related on average and queen turnover is high (Heinze *et al.* 1995a; Bourke *et al.* 1997; Hammond *et al.* 2001, 2003, 2006). Although polygyny is thought to arise through the adoption of daughter-queens in the study population (Hammond *et al.* 2001), high queen turnover suggests that coexistence of mother-daughter pairs among queens is transient (Bourke *et al.* 1997). Under these conditions, other things equal, workers would maximise their indirect fitness benefits by preferentially caring for their most closely related queen.

Previous studies have tested for within-colony kin discrimination in *L. acervorum* during egg cannibalism by queens (Bourke 1994b) and colony fission (Heinze *et al.* 1997a), but kin discrimination was not detected in either case. However, potentially nepotistic interactions with queens have not previously been investigated in this species. I tested for within-colony kin discrimination in *L. acervorum* by observing the behaviour of individually-marked workers towards individually-marked queens and assessing their relatedness using microsatellite markers.

Methods

Field collection and colony sampling

Colonies of *L. acervorum* were collected from a population within Thetford Forest, near Santon Downham, Norfolk, UK, over three days between April and June 2008. This population is facultatively polygynous, with 20-50% of colonies containing multiple, related queens (means of 2–5 queens per colony), 95% of which are singly mated (Heinze *et al.* 1995a; Bourke *et al.* 1997; Chan *et al.* 1999; Hammond *et al.* 2001, 2002, 2003, 2006). *L. acervorum* colonies nest within cavities in dead twigs and have a monodomous structure, allowing complete colonies to be collected by gathering occupied twigs. Fifty-six colonies were taken at random from the population by collecting all nests found during the three days of collection. Once transported to the laboratory, colonies were transferred from their twigs to artificial nests within five days. The artificial nests were of standard design consisting of two glass microscope slides separated by a card wall (1.5–2mm thick) to leave a cavity (area 39×64mm or 26×63mm). Each nest was kept inside a square Petri dish (10×10×2cm), the inner walls of which were coated with polytetrafluoroethylene (Sigma Aldrich) to prevent the ants from escaping. The colonies were kept at a constant 18°C with a 12h light: 12h dark cycle. Food was provided three times a week in the form of dilute honey and adult *Drosophila*.

Out of 34 colonies collected in May and June 2008, eight were identified as polygynous from the presence of multiple, egg-laying dealate queens. Egg-laying queens were identified by isolating each dealate queen for 24h in a Petri dish (5cm diameter) at least 2.5 weeks before the start of behavioural observations. Six of these polygynous colonies were randomly selected to be used in the experiment (SD0827, SD0828, SD0831, SD0833, SD0849 and SD0850). Colonies collected earlier in the year (April 2008) were used only to provide data on the frequencies of microsatellite marker alleles for use in the calculation of relatedness.

Three out of the six experimental colonies (SD0828, SD0831 and SD0833) had been used in an earlier pilot study during which all workers, dealate queens and 26 larvae had been separated from the remaining brood for 20–21 days. During the separation period the remaining brood was looked after by non-nestmate workers until the nestmate adults were returned, at which point the non-nestmate workers (plus any new workers that had eclosed under their care) were removed from the original nests. As part of the study the adults had been marked with paint (a technique also employed in the current experiment). These three colonies were given 18 days to re-adjust to their original nest and full colony size before the start of filmed observations for the current study.

Paint-marking of individuals

In the six experimental colonies (Table 1), all dealate queens and up to 64 workers were marked with two paint dots (Testors Racing Finish, Pactra ®), one on the thorax and one on the gaster, to give each ant a unique colour code for individual identification. Paint-marking was performed at least a week before the start of the filmed observations (mode = 11 days, range 7–11 days), although lost paint marks had to be renewed on 50% of workers in colony SD0849 and all queens in colony SD0850 one or two days (respectively) before the start of filmed observations. In the case of the three colonies that had previously been paint-marked, new marks were only applied to those individuals who had lost their original paint.

Of the five dealate queens initially in colony SD0850, one was killed by workers between the original paint-marking and the start of filmed observations, and two died (at least one, probably both, were killed by workers) between paint-marking and the completion of the first 6h of filmed observations. Hence these three queens were not included in the behavioural analyses. Queen deaths possibly occurred because of damage to the queens during the paint-marking. One of the remaining two queens in SD0850 lost her paint mark during filming, as did some workers in all colonies (Table 1).

Behavioural observations

The behaviour of individual workers towards queens was recorded by filming the colonies under a stereomicroscope (magnification ×10) using a digital camera (JVC, TK-C1480E). The colonies were filmed within nests with an internal area of 68×18mm. The field of view was 14×18mm and was centred on whichever section of the nest contained the largest number of eggs. Filming was performed in 2-hour bouts between 0900 and 2000, with the order of filming randomly varied among colonies. Before the start of each bout, colonies were given a brief period (>5 min) to adjust to conditions under the microscope. A single colony was never filmed more than once in the same 0900–2000 period. Each colony was filmed for a total of 40h (20×2h bouts) between14 July and 16 August 2008. In each colony at least one queen was filmed laying an egg over the observation period, and five of the six colonies produced at least one adult alate or sexual pupa over the observation period, suggesting that colonies were in their reproductive phase over the course of the study. Once filming was complete, all colonies were killed within five days by freezing at -20°C.

The frequencies and durations of the following interactions performed by workers towards queens were recorded from viewing the films: (1) antennation (including interactions that involved ≥5s antennation alone or ≥5s antennation interrupted by a brief grasp of the queen's body), and (2) grooming/feeding (including grooming or trophallactic feeding interactions preceded by ≥ 5s of antennation or interrupted by any duration of antennation). Only interactions that occurred with a focal queen fully on screen were recorded. Interactions between the same two individuals interrupted by a period of inactivity less than 10s long were merged to form one interaction with the duration of both combined. Grooming was only included in grooming/feeding if a part of the body surface other than a paint mark was groomed (17% of all grooming interactions occurred only on paint marks and hence were omitted from the analysis). Grooming and feeding interactions were combined because feeding interactions proved to be rare (only 37 feeding interactions occurred compared to 644 grooming interactions). Feeding was combined with grooming rather than antennation under the assumption that both behaviours are beneficial to queens, whereas the effect on queens of antennation is unknown.

Ovarian dissections

Dealate queens were classified as egg-layers or not according to whether they were observed egg-laying either in the filmed bouts or during the period of isolation of queens (see above). To confirm queens' mating status, ovarian dissections were performed on all dealate queens in the colonies at the experiment's end (n=15) and on the dealate queens in SD0850 that had died (n=3). The ovaries of each queen were extracted under distilled water using fine forceps and viewed under a compound microscope (Bourke 1991). A queen was classified as mated if she had a sperm receptacle containing sperm and as unmated if not.

Molecular analysis and estimation of relatedness

All dealate queens and all marked workers still in the six focal colonies at the end of the experiment, along with five workers each from 12 other colonies randomly selected from those gathered across all three collection days, were genotyped at four microsatellite loci each. DNA was extracted using the HotSHOT method (Truett *et al.* 2000). For each extraction, the head of the focal ant was submerged in 75μl of lysis reagent (25mM NaOH, 0.2mM EDTA), heated for 1h at 85–90°C, then neutralised using 75μl of neutralising reagent (40mM Tris-HCl). All samples were typed at the four microsatellite loci *LXA GA1* (Bourke *et al.* 1997), *L18* (Foitzik *et al.* 1997), *LX GT 218* (Hamaguchi *et al.* 1993) and *MYRT 3* (Evans 1993), as used in previous investigations of the study population (Bourke *et al.* 1997; Hammond *et al.* 2001, 2002, 2003, 2006). Polymerase chain reactions (PCR) were performed using a QIAGEN® Multiplex PCR Kit. Samples were run in a 10μl mixture (5μl QIAGEN Multiplex PCR Master Mix, 1μl primer mix, 3μl distilled water and 1μl DNA solution) in a Biometra TProfessional Standard Thermocycler or an MJ Research DNA Engine Tetrad 2 Peltier Thermal Cycler on the following PCR program: 95°C initial denaturation step (15m), 33 cycles of 94°C denaturation step (30s), 59°C annealing step (90s), 72°C extension step (60s), and a final 60°C extension step (30m). Samples were genotyped using an ABIPrism 3730 capillary sequencer. A proportion (15%) of all samples were re-genotyped (i.e. via a repeated PCR) to estimate the level of genotyping error. Genotype results were analysed using GeneMapper® Software Version 4.0 (Applied Biosystems). GENEPOP 4.0 (Raymond & Rousset 1995) was used to test whether the four loci deviated from Hardy-Weinberg equilibrium. Expected heterozygosity for each locus was calculated using the program GenAlEx 6.1 (Peakall & Smouse 2006).

For each colony, pairwise relatednesses between all individuals were calculated from the multilocus genotypes using the program RELATEDNESS 5.0.8 (Goodnight Software: http://www.gsoftnet.us/GSoft.html) which calculates regression relatedness using the formula developed by Queller and Goodnight (1989).

Statistical analyses

The number of egg-laying, dealate queens used in the final analyses was two in each of the six colonies. This was because, of queens present on collection, some queens proved to be non-laying virgin queens and some queens died (Table 1). As a result each worker had two queens to choose between: their ‘more related queen’ and their ‘less related queen’. The workers included in the statistical analyses were those that had retained both their paint marks throughout the experiment and interacted with at least one of their nestmate queens during filming (Table 1).

To confirm that workers within the same colony were capable of expressing differential preferences for queens, I first used Wilcoxon signed-rank tests (one for each focal colony) to confirm that, on average, each worker expressed a significant preference for (antennated or groomed/fed at a higher rate) one queen over the other, and then used chi-square tests to test whether workers within a colony all preferred the same queen. Rate was calculated (for these tests and all other tests described below) as the number of seconds a worker spent antennating or grooming/feeding a queen per hour the queen was on screen. In the chi-square tests I compared the overall numbers of workers in each colony favouring each queen, the null hypothesis being that each queen was favoured by 50% of the workers.

I tested the hypothesis that workers preferentially antennated or groomed/fed (i.e. performed the behaviour at a higher rate towards) the more related of a pair of nestmate queens using two generalised linear mixed models (GLMMs) (one for antennation and one for grooming/feeding). The time each worker spent antennating or grooming/feeding one nestmate queen was assumed to be independent of the time they spent antennating or grooming/feeding their other nestmate queen. This independence was assumed on the basis that workers interacted with their nestmate queens at extremely low rates (Table 2) and spent the majority of time performing no work at all, as observed in past studies of *L. acervorum* (Franks *et al.* 1990 estimated workers to spend 72% of their time resting). Hence the amount of time a worker spent with one queen should not have interfered with the amount of time they had available to spend with the other queen. In each GLMM, number of seconds spent antennating or grooming/feeding a queen (depending on the behaviour being tested) was the response variable, worker-queen relatedness was the single, fixed explanatory variable, and the log of the number of hours spent by the queen on screen was used as an offset. For the purpose of these analyses, worker-queen relatedness was converted from a continuous scale to a binary scale (using a one or a zero to describe whether a queen was the more related or less related queen to a focal worker). A binary scale was used because it was predicted to provide a more reliable test of kin discrimination than a continuous scale, given that workers are unlikely to alter their overall time spent antennating or grooming/feeding nestmate queens depending on where their own relatedness values sit within the range exhibited by the population. The question of interest therefore is simply whether a worker always cares for their more related nestmate queen at a higher rate than their less related nestmate queen, regardless of exact relatedness values. Colony, queen and worker identity were fitted as random effects in the GLMMs (with queen ID and worker ID nested separately within colony ID) to control for repeated use of colonies (n=6), queens (n=12) and workers (each worker was included twice in the model – once for each nestmate queen). A Poisson error distribution with a log link function was assumed in each model, and an observation-level random effect was fitted to account for overdispersion in the data.

A pair of preliminary GLMMs (one for antennation, one for grooming/feeding) was used to demonstrate the validity of our binary classification of worker-queen relatedness, with continuous relatedness as the response variable, binary relatedness as the fixed explanatory variable, and worker ID and queen ID (both nested separately within colony ID) as random effects. Gaussian error distributions were fitted.

Further analyses were performed in light of the results of the GLMMs used to test for a preference expressed by workers towards their more related nestmate queen (as described under 'Results', relatedness was found to have no effect on the rate of antennation or grooming/feeding towards queens). The aim of the analyses was to investigate to what extent the workers would need to increase their rate of antennation and grooming/feeding towards the more related queen in order for nepotism to be detected in the current experiment, and to assess if this increase resulted in a rate that could be reasonably expected from *L. acervorum* workers. The analyses were performed by first adding one second to the amount of time each worker antennated or groomed/fed each of their nestmate queens (to account for zeros in the dataset), and then by increasing the number of seconds each worker interacted with their more related queen by a fixed percentage. I then used GLMMs with the same factors as those used in the initial models (including the log of the time spent on screen by the queens as an offset) to analyse the simulated data until a significant increase in rate of antennation and grooming/feeding towards the more related queen was detected.

I also tested the hypothesis that the magnitude of difference in relatedness to nestmate queens may affect the degree of discrimination (i.e. the difference in the rate spent interacting with each queen) exhibited by a worker. I tested this hypothesis by using two GLMMs (one for antennation and one for grooming/feeding), with the difference in interaction rate between nestmate queens as the response variable and the difference in relatedness to each nestmate queen as the fixed explanatory variable. Differences were calculated by subtracting values associated with a worker's less related queen from those associated with their more related queen. I fitted colony ID as a random effect, along with the ID of the more related queen and the ID of the less related queen (nesting more related and less related queen separately within colony) to control for the repeated use of colonies and queens. A Gaussian error distribution was fitted.

As an alternative to kin discrimination based purely on relatedness values, I tested the hypothesis that workers preferentially direct care towards whichever nestmate queen is their mother. To test this hypothesis I conducted exclusionary parentage analyses within colonies using the genotypes of workers and queens (a reproductive queen that shared at least one allele at every locus with a worker was included as a potential mother of that worker). I use the word ‘potential’ because a matching genotype only suggests, rather than confirms, maternity (thus introducing some inevitable noise into the analysis). In the case of colony SD0849, one queen was discovered to be a virgin and hence could not have been the mother of nestmate workers. Therefore this queen was included in the analysis as a known non-mother (i.e. if a worker matched the genotype of only this queen, the queen was not considered as a potential mother, and if a worker matched the genotypes of both queens, only the mated queen was considered a potential mother). I pooled workers with one potential mother (as opposed to zero or two) across colonies in order to obtain a sufficient sample size with which to test (using a chi-squared test) whether workers preferred (antennated or groomed/fed at a higher rate) their potential mother over their non-mother queen.

Statistical analyses were carried out using the statistical software *R* version 2.12.0 (R Development Core Team 2010). GLMMs were performed using the lmer function (lme4 library). The results of the GLMMs (χ^2, df and p) are those obtained from likelihood ratio tests comparing models with and without the variable of interest. All means are reported ± one standard error unless otherwise stated. Statistical significance is reported on the basis of $\alpha = 0.05$.

Results

Reproductive status of queens

The six focal colonies contained 18 dealate queens on collection, with 2–5 queens per colony (Table 1). The number of queens included in the final behavioural analyses was two egg-laying, mated queens per colony, except in the case of SD0849, which contained one egg-laying, mated queen and one egg-laying, virgin queen. Overall queen numbers in the analyses differed from those on collection either because of queens proving to be non-laying virgins or because of queen deaths (Table 1).

Relatedness

The number of alleles for the four loci were 25 (*LXA GA1,* expected heterozygosity, $H_E = 0.89$), 10 (*L18,* $H_E = 0.78$), 2 (*LX GT 218*, $H_E = 0.48$) and 5 (*MYRT 3*, $H_E = 0.66$). The estimated levels of genotyping error (% alleles erroneously scored) were 0.8% (*LXA GA1*), 0% (*L18*), 0% (*LX GT 218*) and 0.8% (*MYRT 3*). The four loci did not significantly deviate from Hardy-Weinberg equilibrium.

Consistent with previous studies of the same population (Heinze *et al.* 1995a; Bourke *et al.* 1997; Hammond *et al.* 2001, 2003, 2006), the mean relatedness between nestmate egg-laying queens across all six focal colonies was 0.49 ± 0.08 (n=15 egg-laying queens, including all five original egg-laying queens in SD0850). The mean relatedness among workers across the six colonies was 0.56 ± 0.08 (n=16–48 workers per colony). The mean absolute difference in relatedness of workers to each of the two surviving, egg-laying queens within their colony (across all six focal colonies) was 0.23 (range of means, 0.16–0.32). This showed that workers were indeed differentially related to queens within the study colonies and hence that the potential for within-colony kin discrimination existed.

Test for variation in behaviour within colonies

On average, individual workers within colonies exhibited significant preferences for a particular queen, although not all nestmate workers preferred the same queen (see below). Hence in each colony, on average, each worker antennated or groomed/fed one queen (their 'preferred queen') at a significantly higher rate than the other (their 'non-preferred queen') (Table 2). With respect to antennation, one of each pair of queens was the preferred queen of a significant majority of workers in three out of the six focal colonies (SD0831: χ^2=9.85, df=1, p=0.002; SD0833: χ^2=7.35, df=1, p=0.007; SD0850: χ^2=11.6, df=1, p=0.001), whereas no single queen was consistently the preferred queen of a majority of workers in the remaining three colonies (SD0827: χ^2=0.31, df=1, p=0.577; SD0828: χ^2=2.58, df=1, p=0.108; SD0849: χ^2=0.09, df=1, p=0.763). With respect to grooming/feeding, one of each pair of queens was the preferred queen of a significant majority of workers in three out of the six focal colonies (SD0827: χ^2=10.7, df=1, p=0.001; SD0833: χ^2=6.37, df=1, p=0.012; SD0850: χ^2=11.8, df=1, p=0.001), although only two of these three colonies were the same as those in which one queen was preferentially antennated by a majority of workers. No single queen was consistently the preferred queen of a majority of workers with respect to grooming/feeding in the remaining three colonies (SD0828: χ^2=0.25, df=1, *p*=0.617; SD0831: χ^2=0.04, df=1, *p*=0.835; SD0849: χ^2=3.60, df=1, *p*=0.058). Therefore, overall, workers in half of the colonies did not show a consistent preference for either queen during both interaction types, showing that workers within a colony are indeed capable of differing in their choice of queen.

Test for within-colony kin discrimination

A total of 532 antennation interactions and 681 grooming/feeding interactions were used to test for within-colony kin discrimination, with a mean of 89 ± 19 antennation interactions and 113 ± 23 grooming/feeding interactions per focal colony. The mean number of workers in the six focal colonies included in the analyses was 22 ± 3 (antennation analyses) and 19 ± 2 (grooming/feeding analyses) (Table 1).

The binary classification of relatedness was found to give a valid measure of worker − queen relatedness: each worker was on average significantly more related to the queen classified as their ‘more related’ queen than to the queen classified as their ‘less related’ queen. This was the case for both the antennation dataset (GLMM: χ^2=102.9, df=1, *p*<0.001) (Table 3, Fig. 1a) and the grooming/feeding dataset (GLMM: χ^2=93.6, df=1, *p*<0.001) (Table 3, Fig. 1b).

Worker-queen relatedness (measured on the binary scale) had no significant effect on the rate at which a worker antennated a queen (GLMM: χ^2=0.20, df=1, *p*=0.651) (Table 3, Fig. 2a). Similarly, the rate at which a worker groomed/fed a queen was not affected by whether a queen was the more or less related of a pair (GLMM: χ^2=0.29, df=1, *p*=0.590) (Table 3, Fig. 2b). The analysis of simulated datasets showed that workers would have needed to increase their recorded rates of antennation towards their more related queen by at least 50% in order for a significant preference for the more related queen to have been detected in the current experiment (GLMM at 50% increase: χ^2=3.88, df=1, *p*=0.049). Similarly workers would have needed to increase their recorded rates of grooming/feeding towards their more related queen by at least 58% in order for a significant preference for the more related queen to have been detected (GLMM at 58% increase: χ^2=3.88, df=1, *p*=0.049).

I also found that the magnitude of difference in relatedness between a worker's more and less related nestmate queen did not significantly affect the magnitude of discrimination (difference in the rate of interaction with queens) expressed by a worker during antennation interactions (GLMM: χ^2=0.04, df=1, *p*=0.838) (Table 3, Fig. 3a) or grooming/feeding interactions (GLMM: χ^2=0.58, df=1, *p*=0.445) (Table 3, Fig. 3b).

Test for a preference for the mother queen

Consistent with high queen turnover in the study population (Bourke *et al.* 1997), few adult workers could be assigned a potential mother (only 25% of all workers still marked at the end of filming). In the case of antennation, a mean of five workers per colony could be assigned one potential mother. In the case of grooming/feeding, a mean of four workers per colony could be assigned one potential mother. Therefore, as described in 'Methods', I pooled workers across colonies to obtain sufficient sample sizes (antennation: n=34, grooming/feeding: n=30). Workers showed no significant preference for any queen during antennation (χ^2=1.88, df=1, *p*=0.170) but showed a significant preference for the potential mother queen during grooming/feeding (χ^2=4.8, df=1, *p*=0.028).

Discussion

I tested for within-colony kin discrimination in the multiple-queen ant *L. acervorum* by observing whether individual workers preferentially antennated, groomed or fed nestmate queens according to their pairwise relatedness. My results show that within-colony kin discrimination in this context is absent. I found no difference in the rate of antennation and grooming/feeding performed by workers towards their more related and less related queen (Table 3, Fig. 2), and furthermore I found that workers did not vary their degree of discrimination between queens with increasing values of difference in relatedness to them (Table 3, Fig. 3). Workers did not favour or disfavour their potential mother queen during antennation, but workers did groom or feed their potential mother queen at a significantly higher rate. However, because of high queen turnover in the study population, the frequency of workers with a potential mother queen still living in their colonies was low.

I am confident that the lack of nepotism detected in the study is a true representation of worker behaviour in *L. acervorum*. The study's dataset covered the full range of relatedness values (and differences in relatedness values) that might be expected in a *L. acervorum* colony, yet I consistently found no effect of relatedness on worker behaviour, as is clear from Figures 2 and 3. The statistical analyses performed to estimate the magnitude of a preference that would be required for nepotism to be detected indicate that a strong preference for the more related queen was unlikely to have been missed in this experiment. The analyses estimated that workers would have needed to increase their rates of antennation and grooming/feeding towards their more related queen by 50% and 58% respectively (compared to the rates they actually expressed) in order for nepotism to be detected in this experiment. Although these figures represent fairly large percentages, they translate into low rates. Returning to the raw data and taking means from all workers pooled across colonies, an increase of 50% in the rate of antennation towards the more related nestmate queen gives a mean rate of 1s/h, and an increase of 58% in the mean rate of grooming/feeding towards the more related queen gives a mean rate of 7s/h. The fact that these rates are so low even after a 50% and 58% increase suggests that workers could have easily expressed antennation and grooming/feeding towards their more related queen at a rate high enough to be detected as nepotism. However, despite the apparent opportunity to favour their more related nestmate queens at a detectable rate, the workers did not do so, further adding to the conclusion that *L. acervorum* workers do not express within-colony kin discrimination whilst antennating and grooming/feeding their nestmate queens.

Overall, the findings of the study are in agreement with the majority of recent studies of within-colony kin discrimination in the social insects, most of which have found such discrimination to be either absent or weak (see 'Introduction'). In light of these findings, a number of explanations have been proposed to explain why social insects might not have evolved within-colony kin discrimination. These explanations can be broadly divided into two, overlapping categories: those that suggest that it would be difficult for within-colony kin discrimination to evolve, and those that suggest that it would not be beneficial for within-colony kin discrimination to evolve.

With respect to the former category of explanation, the main hypothesis as to why social insects might not be able to evolve kin discrimination is that there may not be enough variation in chemical cues within colonies to provide information on kinship (Ratnieks 1991; Arnold *et al.* 2000; Boomsma *et al.* 2003; Dani *et al.* 2004). Genetically derived chemical cues are a means by which individuals can recognise their kin, as shown in a study where workers, having been reared apart from their colony, were able to recognise their mother queen upon being reunited (El-Showk *et al.* 2010). However, there may not be enough variation in genetically derived chemical cues within colonies for an individual to tell two nestmates apart based on their relatedness. One cause of low cue variation within colonies could be the accidental scrambling of chemicals between nestmates during social interactions such as trophallaxis and allogrooming (Arnold *et al.* 2000; Dani *et al.* 2004). Another could be the deliberate scrambling of chemical cues by individuals that conceal their own or their offspring's genetic identity from nestmates who might discriminate against them (Keller 1997). Alternatively, selection for effective nestmate recognition might favour low variation in recognition cues between nestmates within colonies (Keller 1997; Dani *et al.* 2004). Another factor that could limit within-colony variation in relatedness cues is kin discrimination itself (Ratnieks 1991). If individuals were to favour their close relatives, then genes for rare phenotypes would gradually become purged from the population, reducing the high phenotypic variation required for within-colony kin discrimination, and thus preventing the behaviour from becoming established (Crozier 1986; Ratnieks 1991). However, if other selective pressures were to favour high variation at genetic loci underpinning recognition, then enough diversity might be retained to allow kin discrimination to persist (Crozier 1986; Gardner & West 2007; Rousset & Roze 2007).

With respect to the second category of explanation, within-colony kin discrimination might not have evolved in social insects because of the potential costs associated with the behaviour. For example, attempts to recognise kin within colonies could lead to recognition errors being made, and a frequent occurrence of recognition errors could cancel out any benefit of discrimination (Reeve 1989; Ratnieks 1991; Ratnieks & Reeve 1992). Similarly, harmful behaviour directed towards less related kin could also outweigh any benefit of nepotism (Ratnieks & Reeve 1992). If the costs of individuals discriminating against their distant kin were to outweigh the benefits of individuals favouring their close kin, then the evolution of within-colony kin discrimination would be prevented. Finally, time and energy expended on within-colony kin discrimination might be too costly in terms of reductions in overall colony productivity, again preventing its evolution (Ratnieks & Reeve 1991, 1992).

Although it appears *L. acervorum* workers do not preferentially interact with their more related nestmate queen, I did find some evidence to suggest that workers preferentially direct grooming and feeding interactions towards their mother queen. This result is consistent with a study carried out by Gill and Hammond (2011) using a functionally monogynous population of *L. acervorum* where they discovered workers exhibit a preference towards their mother when deciding which queen should become the colony's reproductive leader (despite the fact that more related full-sister queens were present in the nest). This behaviour makes sense in terms of inclusive fitness theory because a worker will always be more related to the future offspring of their mother than any other queen in the colony (once averaged across the sexes) (Field *et al.* 2010; Gill & Hammond 2011). However, as already mentioned, in the population of *L.acervorum* used in this study, workers often do not have the opportunity to favour their mother queen because the rate of queen turnover is high and workers' developmental time is relatively long, with the result that a worker's mother is usually not present in the colony once a worker becomes adult (Bourke *et al* 1997; present study). Nonetheless, my findings suggest that workers have evolved to favour their mother whenever she is present.

In terms of future study, insect societies appear to differ from some kinds of vertebrate societies in which within-group kin discrimination has been found (Van Horn *et al.* 2004; Wahaj *et al.* 2004; Silk 2009), and it would be useful to investigate why insect and vertebrate societies appear to differ in this respect. Although a start has been made (e.g. Boomsma *et al.* 2003; Komdeur *et al.* 2008), the field has still not reached a full understanding of the circumstances under which within-group kin discrimination does and does not evolve.

Tables

Table 1 The number of paint-marked workers and dealate queens in each *Leptothorax acervorum* colony at the start and end of the filmed observations, and the number of each included in statistical analyses.

Colony	Number of workers					Number of dealate queens		
	Initially present	Marked at the start	Still marked at the end	Included in antennation analyses	Included in groom/ feed analyses	Present at collection and marked	Included in analyses[a]	Not included in analyses
SD0827	55	55	42	29	24	2	2	0
SD0828	33	33	27	19	16	3	2	1[b]
SD0831	52	52	39	26	23	3	2	1[b]
SD0833	32	32	29	23	19	3	2	1[b]
SD0849	27	27	13	11	10	2	2	0
SD0850	82	64	35	22	19	5	2	3[c]
Totals	281	263	185	130	111	18	12	6

[a] All egg-laying, mated queens except for one queen in SD0849, which was an egg-laying, virgin queen.

[b] Not included in analyses because a non-laying, virgin queen.

[c] Not included in analyses because died before, or shortly after, the start of filmed observations.

Table 2. The number of marked workers in each *Leptothorax acervorum* colony that antennated or groomed/fed at least one nestmate queen, and the rate (seconds per hour the queen was on screen) spent interacting with their preferred and non-preferred queens. The significance of an effect is indicated by NA (p>0.05), * (p<0.05), ** (p<0.01) or *** (p<0.001) (Wilcoxon signed-rank tests). n gives the number of workers showing the behaviour.

Colony	Antennation				Grooming/ feeding			
	n	Mean rate (s/h) towards preferred queen	Mean rate (s/h) towards non-preferred queen	Sig.	n	Mean rate (s/h) towards preferred queen	Mean rate (s/h) towards non-preferred queen	Sig.
SD0827	29	1.65 ± 0.55	0.20 ± 0.08	***	24	10.46 ± 2.38	1.01 ± 0.37	***
SD0828	19	0.73 ± 0.14	0.18 ± 0.06	***	16	6.26 ± 1.61	2.52 ± 1.11	***
SD0831	26	2.06 ± 0.35	0.68 ± 0.17	***	23	10.15 ± 2.84	3.93 ± 1.55	***
SD0833	23	0.47 ± 0.05	0.11 ± 0.03	***	19	13.20 ± 2.91	1.86 ± 0.84	***
SD0849	11	1.01 ± 0.24	0.44 ± 0.17	***	10	1.65 ± 0.78	0.27 ± 0.12	**
SD0850	22	1.93 ± 0.50	0.67 ± 0.32	***	19	17.77 ± 5.54	2.15 ± 1.49	***

Table 3. The effect of (1) binary relatedness on continuous *Leptothorax acervorum* worker-queen relatedness, (2) binary relatedness on the rate at which workers interacted with nestmate queens, and (3) the difference in worker-queen relatedness on the difference in rate of interaction with nestmate queens. Effects are shown for (a) antennation and (b) grooming/feeding. Intercepts, effect sizes and standard errors of effect sizes (SE) are as presented in the GLMM outputs from *R*. The reported effect sizes represent the difference between means (in the case of the first two models where binary relatedness is the fixed effect) or the gradient of the line of regression (in the case of the third model where difference in relatedness is the fixed effect). The significance of an effect is indicated by NA ($p>0.05$), * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

Response variable	Fixed effect	Intercept	Effect size	SE	Sig.
(1) Continuous relatedness:					
(a) Antennation	Binary relatedness	0.432	0.202	0.016	***
(b) Grooming/ Feeding	Binary relatedness	0.411	0.208	0.017	***
(2) Rate (s/h):					
(a) Antennation	Binary relatedness	-1.256 [a]	-0.116 [a]	0.255[a]	NA
(b) Grooming/ Feeding	Binary relatedness	-0.825 [a]	0.251 [a]	0.452[a]	NA
(3) Difference in rate:					
(a) Antennation	Diff. in relatedness	-0.140	0.226	1.009	NA
(b) Grooming/ Feeding	Diff. in relatedness	-1.467	-6.364	8.439	NA

[a] Numbers are log transformed, as presented in the model outputs for GLMMs with Poisson error distributions.

Figures

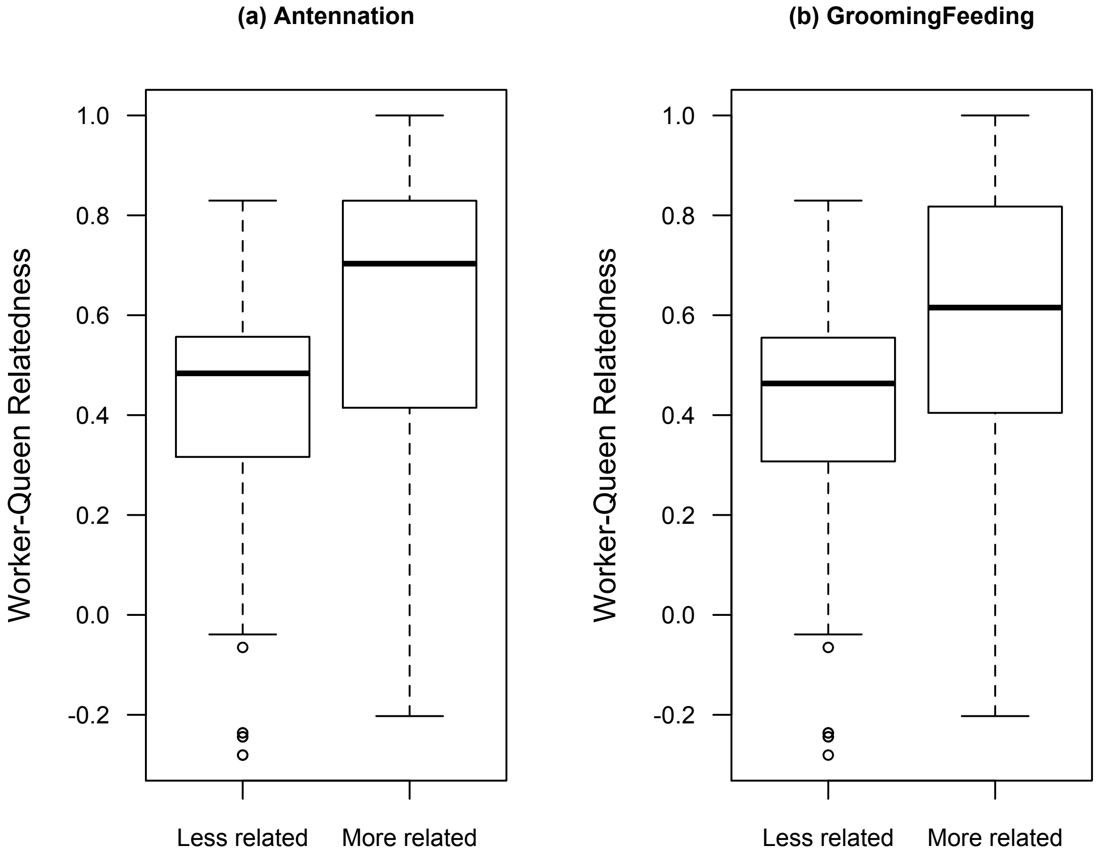

Figure. 1. The relatedness of *Leptothorax acervorum* workers to their less related and more related queen for (a) workers involved in antennation interactions (n=130 workers) and (b) workers involved in grooming/feeding interactions (n=111 workers).

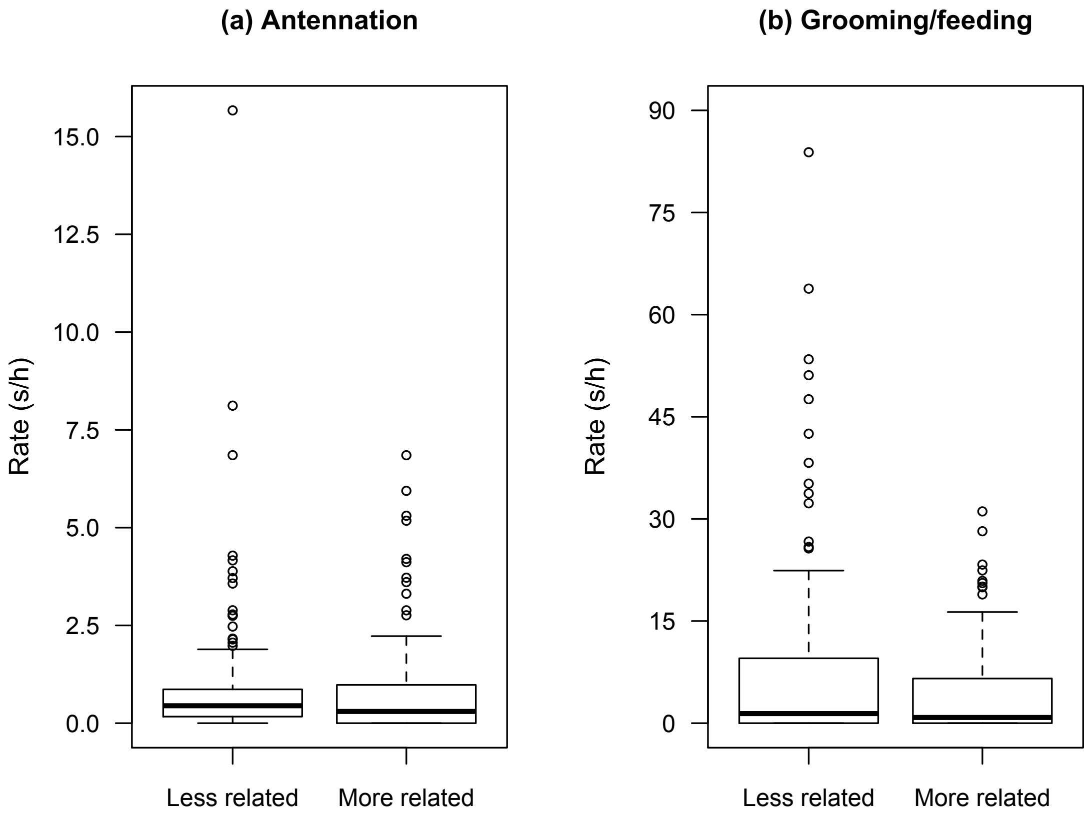

Figure. 2. The rate (seconds per hour the queen was on screen) at which *Leptothorax acervorum* workers (a) antennated (n=130 workers) and (b) groomed/fed (n=111 workers) their less related and more related nestmate queens across the six focal colonies.

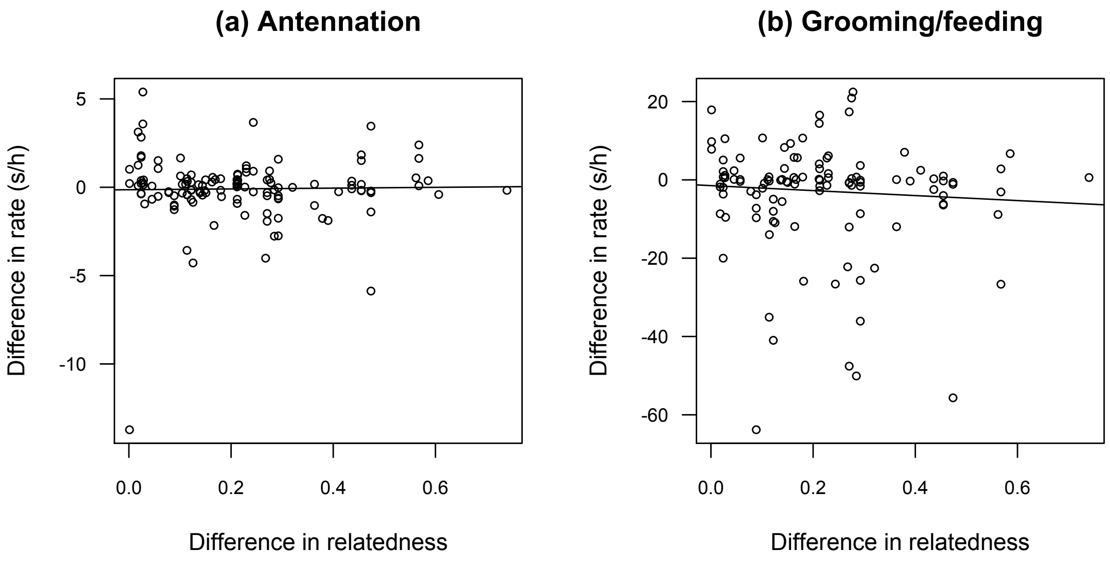

Figure. 3. The effect of the difference in relatedness between a *Leptothorax acervorum* worker's more and less related nestmate queen on the difference in the rate (seconds per hour the queen was on screen) of (a) antennation (n=130 workers) and (b) grooming/feeding (n=111 workers) towards those same queens. Lines show the predicted coefficients (using GLMMs) of the relationship between difference in relatedness and difference in rate.

Chapter 3

Chapter 3: The effect of previous social structure on worker reproduction in queenless colonies of a facultatively polygynous ant

Abstract

According to inclusive fitness theory, eusocial Hymenopteran workers should favour their own direct reproduction over the production of queen-derived males. For the workers of many species, queenless conditions represent a unique opportunity to reproduce directly. Furthermore, worker reproduction is often the only method by which queenless colonies can continue to achieve inclusive fitness. Given the important role of queenless conditions in worker reproduction and vice-versa, workers may have evolved to assess their current risk of queenlessness and prepare themselves for future reproduction when the risk is high. Here I test this hypothesis with the prediction that workers in monogynous (single-queen) colonies (those at a high risk of suddenly losing all queens) should be more prepared for direct reproduction than those in polygynous (multiple-queen) colonies (those at a low risk of suddenly losing all queens). Preparation for reproduction was assessed by removing queens from monogynous and polygynous colonies and observing the occurrence, latency and extent of worker egg-laying over the following 30 days. Workers within previously monogynous colonies were found to (a) be significantly more likely to produce eggs, (b) start to produce eggs significantly more quickly and (c) produce significantly more eggs per capita than workers in previously polygynous colonies. These results are in agreement with the hypothesis that workers have evolved to assess their current risk of queenlessness and to prepare for direct reproduction when necessary. An additional factor found to affect worker reproduction was colony size. The likelihood of a colony producing worker-laid eggs was found to increase with increasing numbers of workers, whereas among those colonies that did produce eggs, the number of eggs laid per capita was found to decrease with increasing colony size. These results could be caused by the relative costs of preparing for reproduction in large and small colonies, and the size and composition of the pre-existing, queen-produced brood pile.

Introduction

Reproductive division of labour is a fundamental property of eusocial societies. In the eusocial Hymenoptera (ants, bees and wasps) the females divide reproduction between them, frequently resulting in two morphologically distinct castes: the queens (females that reproduce) and the workers (females that do not reproduce or that reproduce less). Under such a system the queens predominantly achieve fitness directly and the workers indirectly (Hamilton 1964a, b). However, in many species of Hymenoptera the workers have retained their ovaries and hence can produce haploid eggs that develop into viable males (due to the haplodiploid sex determination system of the Hymenoptera) (Bourke 1988b). Workers are more related to their own sons (r=0.5) than any other male relative (e.g. brothers: r=0.25, 'full' nephews: r=0.375). Therefore, according to inclusive fitness theory, workers should value their own direct reproduction most highly when it comes to producing the colony's males (Hamilton 1964a, b; Ratnieks 1988). If this prediction is correct then workers should actively attempt to increase their own chances of reproduction whenever possible, perhaps by anticipating when they may be given the opportunity to reproduce and preparing themselves in advance.

Despite the potential fitness benefits, workers tend to produce only a very small number of males in queenright colonies (i.e. colonies with at least one queen present) (Bourke 1988b; Bourke & Franks 1995; Hammond & Keller 2004), and the majority of workers in many species do not even attempt to activate their ovaries in the presence of the queen (Ratnieks 1993; Foster *et al.* 2000; Foster & Ratnieks 2001a). There are a number of reasons why the queen may have a detrimental effect on worker reproduction (Bourke & Franks 1995). First, the queen should actively oppose worker reproduction in favour of producing her own sons on the basis that she is more related to her own sons than any other male relatives in the colony (Trivers & Hare 1976; Ratnieks 1988; Bourke & Franks 1995; Ratnieks *et al.* 2006). Therefore the queen may prevent (or 'police') worker reproduction by physically aggressing workers with active ovaries (e.g. Kikuta & Tsuji 1999; Wenseleers *et al.* 2005b) or eating their eggs (e.g. Bourke 1991; Kikuta & Tsuji 1999; Wenseleers *et al.* 2005a, b). Furthermore, the workers in some queenright colonies adopt the same policing behaviour towards reproductive workers as expressed by the queen (e.g. physical aggression: Gobin *et al.* 1999; Kikuta & Tsuji 1999; Iwanishi *et al.* 2003; Wenseleers *et al.* 2005b, destruction of eggs: Ratnieks &

Visscher 1989; Foster & Ratnieks 2000, 2001a; Pirk *et al.* 2003; D'Ettorre *et al.* 2004; Bonckaert *et al.* 2008; Dijkstra *et al.* 2010; Meunier *et al.* 2010). Reasons why workers may actively oppose worker reproduction include relatedness inequalities (when workers are more related to the queens' sons than other workers' sons) (Ratnieks 1988) and colony-level costs associated with worker reproduction (e.g. reduced colony productivity) (Cole 1986; Ratnieks 1988). Second, workers may develop self-restraint in the presence of the queen if the risk of policing is too great (under an efficient policing regime, egg-laying would be more likely to result in wasted colony resources than successful reproduction) (Ratnieks 1988; Wenseleers *et al.* 2004a, b) or if the indirect fitness costs of reproduction are too high (e.g. in colonies with high relatedness) (Ratnieks 1988; Wenseleers *et al.* 2004b). Third, workers may be unable to activate their ovaries in a queenright colony due to the presence of a control pheromone produced by the queen. Hymenopteran queens are known to produce pheromones that inhibit worker reproduction, although there is controversy over whether these pheromones actively suppress workers' ovary activation or send a signal of the queen's presence that workers willingly respond to (Keller & Nonacs 1993; Heinze & D'Ettorre 2009).

For these reasons, the majority of workers in many species do not attempt reproduction in the presence of the queen, with only a small proportion of workers activating their ovaries (Ratnieks 1993; Foster *et al.* 2000; Foster & Ratnieks 2001a). However, the situation in queenless colonies (those without a queen) is very different. To begin with, under queenless conditions the queen is no longer present to police worker reproduction or to produce control pheromones. Also, workers tend to relax their own policing efforts in the absence of the queen, most likely because worker reproduction represents a last opportunity to gain indirect fitness, at least in species in which colonies cannot re-queen themselves (Miller & Ratnieks 2001; D'Ettorre *et al.* 2004). In general, therefore, queenless conditions provide workers with the opportunity to activate their ovaries and lay eggs with a good chance of survival. Furthermore, the incentive for workers to reproduce under queenless conditions should be even greater than under queenright conditions, given that their own reproduction may represent a last opportunity for the colony to reproduce (Bourke 1988b; Ratnieks 1988). Unsurprisingly, therefore, workers of many species of Hymenoptera have been observed to lay eggs in the absence of the queen (Bourke 1988b; Bourke & Franks 1995).

Given that queenless conditions (a) represent an excellent opportunity for workers to achieve direct fitness, and (b) render worker reproduction the only method of continuing colony reproduction (at least temporarily), we may expect workers to have evolved to ‘anticipate’ and prepare for queenless conditions. One circumstance under which workers may anticipate queenlessness is monogyny. Monogyny (when a single queen heads the colony) is one situation that should carry a high risk of queenlessness, because a single queen death will result in an orphaned colony (Bourke 1988b). Polygyny (when multiple queens head the colony) on the other hand should reduce the risk, because multiple queen deaths must occur before a polygynous colony finds itself queenless. Therefore, at any one time, we expect workers within monogynous colonies to be more prepared for queenless conditions than workers within polygynous colonies.

Here I test the hypothesis that workers have evolved to assess their chances of queenlessness and adjust their preparation for direct reproduction accordingly. I do so by using a facultatively polygynous population of the ant, *Leptothorax acervorum*, to test the prediction that workers within monogynous colonies are more prepared for direct reproduction than workers in polygynous colonies. In the study population, worker reproduction occurs only at a low level under queenright conditions (Hammond *et al.* 2003), and workers have been observed to lay eggs under queenless conditions (LF personal observation). I measure preparation for reproduction by recording the likelihood, latency and extent of worker reproduction after queen removal. I predict that, following queen-removal, workers in previously monogynous colonies will (a) be more likely to lay eggs, (b) lay eggs sooner and (c) lay more eggs per capita than workers in previously polygynous colonies.

Method

Colony collections and maintenance

L. acervorum colonies were collected at random from the Santon Downham area of Thetford Forest, Norfolk, in June and October 2009 (37 and 43 colonies respectively) following methods described in Chapter 2. Within this population, 20-50% of colonies are polygynous (with means of 2-5 related queens per polygynous colony across years) and 95% of all queens (monogynous and polygynous) are singly mated (Heinze *et al.* 1995a; Bourke *et al.* 1997; Chan *et al.* 1999; Hammond *et al.* 2001, 2002, 2003, 2006). Polygyny is thought to arise in the population through the readoption of newly-mated queens into their natal colonies (Hammond *et al.* 2001). *L. acervorum* only lay eggs during the spring and summer each year, hence the colonies collected in June were in the egg-laying season whereas the colonies collected in October were not. Once in the laboratory, colonies were transferred to artificial nests (two microscope slides separated by a card wall, internal cavity 64.0×39.0×1.5mm) within five days of collection. Nests were kept within foraging arenas (10×10×2cm Petri dishes with polytetrafluoroethylene (Sigma Aldrich) coated walls) inside an incubator. In the case of the June colonies, the incubator was initially set at 18^{o}C/10^{o}C (14h day/10h night) and then changed to 23^{o}C/13^{o}C 16 days before the start of the experimental queenless stage. Colonies collected in October were taken through a period of hibernation over 13.5 weeks (reaching minimum temperatures of 10^{o}C/0^{o}C (10h day/14h night)) to induce a new egg-laying season when returned to summer temperatures. Following hibernation the October colonies were brought up to the same temperature regime as the June colonies over 3 weeks, reaching a 23^{o}C/13^{o}C (14h day/10h night) cycle 51 days before the experimental queenless stage.

Experimental procedure

Two replicates of the experiment were performed: the first took place in summer (July-August) 2009 using the June 2009-collected colonies, and the second took place in spring (March-May) 2010 using the October 2009-collected colonies. All those colonies that were queenright, had maintained a constant social structure (monogyny or polygyny) since collection, and had more than approximately 15 workers were selected for the experiment. In total there were 36 monogynous colonies (13 collected in June, 23 collected in October) and 23 polygynous colonies (10 collected in June, 13 collected in October) (Table 1). Colonies were moved to either small (40.0×40.0×1.5mm) or large (64.0×39.0×1.5mm) nests (depending on their size) at least 4 days before the start of queen removals, and were kept inside an incubator set on a 23^{o}C/13^{o}C (14h day/10h night) cycle for the duration of the experiment. All dealate queens and eggs were removed from the experimental colonies to create queenless conditions and allow the detection of newly-laid, worker-derived eggs. The dealate queens were frozen the day after removal. Alates (males and gynes) were also removed to simulate the natural process of dispersion. The queen-removal process was staggered across a number of days (seven days in summer 2009 and eleven days in spring 2010). Roughly equal numbers of monogynous and polygynous colonies were made queenless each day, but a greater number of monogynous than polygynous colonies meant that on the last day or two of queen-removals only monogynous colonies remained. The number of eggs in each colony was counted once a day between 0815h and 1530h for 30 days after queen-removal. Counts were taken by observing each nest under a stereomicroscope and counting the eggs until at least two consistent, consecutive counts were obtained. On the day of the final egg-count (30 days after the last colonies to be orphaned lost their queens), all colonies were frozen and an accurate census of the number of workers in each colony was taken. Of the 59 colonies used in the experiment, 10 monogynous and 10 polygynous colonies were also involved in another, simultaneous study for which their workers and queens were marked with paint and filmed before and after queen removal (Chapter 4).

Ovarian dissections

In order to confirm the social structure of the sample colonies, all dealate queens removed from the colonies to create queenless conditions, along with any that had eclosed during the experiment, were dissected in distilled water to remove their ovaries (Bourke 1991). Once removed the ovarioles of each queen's ovaries were measured under a compound microscope to assess the level of ovary activation. Ovarioles were measured (using the software package Auto-montage (Synoptics Ltd)) by taking the average length of the central ovariole of each of a queen's two ovaries. If the central ovariole was damaged through dissection then a different ovariole was chosen at random. If only one ovary remained intact after dissection then only a single measurement was taken. A queen was considered to have active ovaries if her average ovariole measurement was at least 2.00mm (the shortest length found in a mated queen in this experiment, with 2.15mm being the shortest length found in a mated queen with corpora lutea present in the ovaries). The spermatheca of each queen was also examined to assess mating status (full if mated, empty if not). Colonies that contained only one queen with active ovaries before queen removal were classified as monogynous. Colonies that contained at least two queens with active ovaries before queen removal were classified as polygynous.

Statistical analyses

Three analyses were performed to test for a difference in the egg-laying behaviour of workers within previously monogynous and previously polygynous queenless colonies:

1) Likelihood of egg-laying: a generalised linear model (GLM) with a binomial error distribution and logit link function was used to test whether workers in previously monogynous colonies were more likely to lay eggs than workers in previously polygynous colonies in 30 days following queen removal. Each colony was classified as either having produced worker-laid eggs (if ≥ 3 eggs were present in the nest on at least one of the 30 queenless days) or not (if the nest only ever contained <3 eggs). A minimum of three eggs was chosen to classify a colony as producing worker-laid eggs to reduce the risk of mistaking queen-laid eggs as worker-laid eggs (in the event that a couple of eggs were missed during the removal of queen-laid eggs) and to control for the low level of worker egg-laying that is known to occur in *L. acervorum* under queenright conditions (Hammond *et al.* 2003) (and hence does not represent reproduction initiated by queen-loss). Whether or not a colony's workers laid eggs was used as the binary response variable in the GLM, and previous social structure (monogyny or polygyny), colony size (the number of adult workers at the time of freezing) and experimental replicate (summer 2009 or spring 2010) were used as explanatory variables (the latter two to control for the effects of size and replicate whilst considering previous social structure, the variable of interest). The model was initially fitted with all three explanatory variables and their interactions. In order to obtain the minimal adequate model, explanatory variables were removed one by one (starting with the interactions) until only those that could not be removed without causing a significant change in deviance remained (Crawley 2005).

2) Latency to egg-laying: a survival analysis was performed on all 59 colonies to test whether there was a difference in latency to laying eggs between workers within previously monogynous and previously polygynous queenless colonies. Latency to egg-laying (the first day on which at least three eggs were present in the nest) was used as the response variable, and previous social structure, colony size and experimental replicate were used as explanatory variables. A censoring indicator was also added to the model to indicate whether a colony had or had not produced worker-laid eggs at the end of the 30 queenless days (to account for the inclusion of colonies that had not laid

eggs during the experiment). A Weibull distribution was fitted to the model. As described above, all variables and their interactions were initially fitted but then reduced one by one to obtain the minimal adequate model.

3) Extent of egg-laying: a GLM was used to test for a difference between the mean number of eggs produced per worker in previously monogynous and previously polygynous colonies over 30 days following queen removal. The number of eggs in a colony on day 30 after queen removal was used as the response variable, the log of the number of workers per colony was used as an offset, and previous social structure, colony size and experimental replicate were used as explanatory variables. A quasi-Poisson error distribution with a log link function was fitted to account for the use of overdispersed count data. As described above, all variables were initially fitted but then reduced one by one to obtain the minimal adequate model. The GLM was performed twice, once including all colonies, and once excluding those colonies with non-laying workers (i.e. those colonies whose nests never contained at least 3 eggs on any one day in the queenless phase).

As a final analysis I also tested whether the precise number of queens in a colony had an effect on the future reproductive success of workers (i.e. the number of eggs produced per capita) under queenless conditions. This analysis was performed in light of the results of the experiment (see ‘Results’ below) in order to investigate the possibility that workers in previously polygynous colonies expressed lower levels of reproduction under queenless conditions because of higher previous exposure to suppressive queen pheromones than workers in previously monogynous colonies. If this were the case then we would expect previous queen number to be negatively correlated with reproductive success in previously polygynous colonies. I tested this hypothesis using a GLM with the number of eggs in a colony on day 30 after queen removal as the response variable, the log of the number of workers per colony as an offset, and previous number of dealate queens before queen removal as the explanatory variable. A quasi-Poisson error distribution with a log link function was fitted to account for the use of overdispersed count data.

All tests were performed with the statistical software *R* version 2.12.0 (R Development Core Team 2010). The survreg R function was used to perform the survival analysis.

The results shown for each variable are those obtained upon their removal from the models. Statistical significance is reported on the basis of $\alpha = 0.05$. Effect sizes and confidence intervals stated in the results are as provided by the output of the minimal adequate models.

Results

Ovarian dissections

The ovarian dissections confirmed that 36 sample colonies were monogynous and that 23 were polygynous. All ovary-active queens were mated with the exception of seven queens in three polygynous colonies (five in SD0919, one in SD0927 and one in SD0929), and possibly one queen in a single monogynous colony (SD0946) whose mating status was unknown because her spermatheca was damaged during dissection before it could be fully examined (Table 1).

Likelihood of egg-laying

Workers within previously monogynous colonies were significantly more likely to lay eggs than workers in previously polygynous colonies within 30 days following queen removal (GLM: χ^2=8.59, df=1, *p*=0.003) (Table 2, Fig.1), with polygyny decreasing the odds of worker reproduction by a factor of 0.17 (95% CI = 0.05,0.59). The size of a colony was also found to affect the likelihood of egg-laying (GLM: χ^2=4.59, df=1, *p*=0.032) (Table 2), with each additional worker increasing the odds of a colony producing worker-laid eggs by a factor of 1.01 (95% CI = 1.00,1.02). Experimental replicate did not significantly affect the likelihood of egg-laying (GLM: χ^2=1.18, df=1, *p*=0.277), and no significant interactions were found between previous social structure, colony size and experimental replicate (GLM: all interactions *p*>0.05) (Table 2). Raw values for the likelihood of egg-laying are provided in Table 1.

Latency to egg-laying

Workers were found to lay eggs significantly sooner following queen removal in previously monogynous colonies than in previously polygynous colonies (survival analysis: χ^2=7.59, df=1, *p*=0.006) (Table 2, Fig.2), with workers taking on average 1.61 (95% CI = 1.12, 2.33) times longer to lay eggs in previously polygynous colonies (predicted mean = 36.0 days) than in previously monogynous colonies (predicted mean = 22.3 days). Colony size and experimental replicate were found not to have a significant effect on latency to egg-laying (survival analysis: colony size χ^2=3.78, df=1, *p*=0.052; experimental replicate χ^2=1.19, df=1, *p*=0.276) (Table 2). None of the interactions between previous social structure, colony size and experimental replicate were found to be significant (survival analysis: all interactions *p*>0.05) (Table 2). Raw values for the latency to egg-laying are provided in Table 1.

Extent of egg-laying

When all colonies were included in the dataset, workers within previously monogynous colonies were found to lay a significantly higher number of eggs per capita in the 30 days after queen-removal than workers in previously polygynous colonies (GLM: $F_{1,57}$ =14.62, *p*<0.001) (Table 2, Fig. 3a), with workers laying on average 0.19 (95% CI = 0.07, 0.53) times fewer eggs per capita in polygynous colonies (predicted mean = 0.03 eggs per worker) than in monogynous colonies (predicted mean = 0.18 eggs per worker). Colony size did not have a significant effect on the number of eggs laid per capita (GLM: $F_{1,56}$=1.75, *p*=0.191), and neither did experimental replicate ($F_{1,55}$=0.15, *p*=0.703) (Table 2). Furthermore there were no significant interactions between previous social structure, colony size and experimental replicate (GLM, all interactions *p*>0.05) (Table 2).

The effect of previous social structure on the extent of egg-laying remained significant when colonies that did not produce worker-laid eggs were excluded from the dataset, with workers laying on average 0.34 (95% CI = 0.13, 0.87) times fewer eggs per capita in previously polygynous colonies than in previously monogynous colonies (GLM: $F_{1,37}$=6.59, *p*=0.014) (Table 2, Fig. 2b). Furthermore, on exclusion of colonies that did not produce worker-laid eggs, colony size was found to have a significant, negative effect on the number of eggs laid per capita (GLM: $F_{1,37}$=5.09, *p*=0.030) (Table 2), with each additional worker reducing the number of eggs-laid per worker by a factor of 0.996 (95% CI = 0.992, 1.000). As before, experimental replicate had no significant effect on the number of eggs laid per worker (GLM: $F_{1,36}$=0.0002, *p*=0.989), and there were no significant interactions between previous social structure, colony size and experimental replicate (GLM, all interactions *p*>0.05) (Table 2).

Finally, the previous number of dealate queens in previously polygynous colonies was found to have no effect on the number of eggs laid per worker under queenless conditions (GLM: $F_{1,21}$=1.88, p=0.185), suggesting that queenless workers do not increasingly suffer from long lasting effects of suppressive queen pheromones with increasing numbers of previous nestmate queens.

Discussion

By removing all queens from monogynous and polygynous colonies of the ant *L.acervorum*, I tested the hypothesis that workers in the eusocial Hymenoptera adjust their reproductive response to queenlessness according to the likelihood of its occurrence. The results confirmed the hypothesis in that, on three measures of reproductive response (likelihood of, latency to, and extent of egg-laying), workers in previously monogynous colonies (those with a higher risk of queenlessness occurring) were more ready to reproduce after queen removal than workers in previously polygynous colonies (those with a lower risk of queenlessness occurring). Hence workers appeared more prepared for future reproduction when they had been living under conditions more likely to lead to queenlessness. By assessing their social structure and preparing for queenless conditions in advance, workers should be able to take full advantage of the opportunity to gain direct fitness and continue the reproduction of the colony. The findings of this study provide multiple lines of support for inclusive fitness theory. First they show that workers are capable of pursuing their own inclusive fitness interests, and second, that the reproductive behaviour of workers is strongly influenced by their social environment. Third they indicate that workers strive to optimise the trade-off between the direct and indirect fitness components of their inclusive fitness, in this case by refraining from full direct reproduction until the direct fitness benefits of doing so outweigh the indirect fitness costs. The results of this study also suggest that workers can assess the social structure of their colonies, as is consistent with the findings of Chan and Bourke (1994) who found that *L. acervorum* workers produce unequal sex ratios depending on colony social structure.

There are two alternative interpretations of the findings in this study (i.e. alternatives to workers maximising their inclusive fitness by being primed to respond to queenlessness differentially according to colony social structure). The first is that the observed difference in reproduction between workers in previously monogynous and previously polygynous queenless colonies might be caused by a difference in prior exposure to queen pheromones. If *L. acervorum* queens do release a control pheromone then we would expect workers in polygynous colonies to receive a higher quantity of the pheromone than workers in monogynous colonies (simply due to the difference in queen numbers). Hence the effects of the control pheromone could be more severe in polygynous colonies (Keller & Nonacs 1993) and take longer to wear off following queen removal, thus delaying worker reproduction and resulting in the findings of this study. However, there are reasons to doubt that a queen control pheromone is responsible for the results observed. To begin with, in a study performed on queenright *L. acervorum* colonies from the Thetford population, workers were found to produce the same percentage of male-destined eggs in monogynous and polygynous colonies (Hammond *et al.* 2003). This finding strongly suggests that workers are not more affected by queen pheromones in polygynous than monogynous colonies. Also, if the difference in reproduction between workers within previously monogynous and previously polygynous colonies in the current study was due to a difference in exposure to queen pheromones, then we would expect worker reproduction in previously polygynous colonies to decrease with increasing previous queen number. However, I found that previous queen number had no effect on the number of eggs laid per worker in previously polygynous colonies. Furthermore, although Hymenopteran queens are known to produce pheromones that are unique to their caste and affect worker reproduction, there is still no firm evidence to suggest that these pheromones do anything other than signal the presence and fertility of the queen (Keller & Nonacs 1993; Heinze & D'Ettorre 2009). If the main purpose of the queen pheromone is to send an honest signal to workers then there is no reason to expect the pheromone to have an additive effect on worker reproduction in polygynous colonies (Keller & Nonacs 1993). Finally, there is good evidence to suggest that *L. acervorum* queens do not produce a pheromone that affects the fecundity of their rival nestmate queens (Bourke 1993, 1995), even in a functionally monogynous population where just a single queen per multiple-queen colony monopolises reproduction (Coston *et al.* 2011), and it seems unlikely that the queens would have evolved such a tool that works exclusively on workers. Instead queens in the Thetford Forest population of *L. acervorum* appear to control worker reproduction by eating worker-produced eggs, often whilst they are still being laid (Bourke 1991; LF personal observation).

The second alternative way in which the findings of this study might be interpreted is that instead of workers in previously monogynous queenless colonies expressing higher levels of reproduction than those in previously polygynous queenless colonies, worker reproduction may be more heavily policed in previously polygynous colonies than previously monogynous colonies. However, as discussed in the introduction, worker policing is generally thought to be relaxed under queenless conditions (Miller & Ratnieks 2001; D'Ettorre *et al.* 2004), rendering this hypothesis unlikely. Furthermore, in a recent study in which *L. acervorum* workers from the Thetford population were offered non-nestmate worker-laid eggs, monogynous and polygynous colonies did not differ significantly in their levels of policing (Chapter 5).

The act of preparing for reproduction under queenright conditions must benefit the individual workers who later achieve direct reproduction, but it is difficult to predict the effect of this behaviour on the fitness of the rest of the colony. On the one hand it should benefit all members of the colony in terms of indirect fitness if their workers are prepared for reproduction and the colony suddenly becomes queenless, but on the other hand, if workers somehow alter their behaviour in order to prepare themselves for reproduction (e.g. perhaps by reducing their work effort, increasing their intake in resources or participating in dominance interactions) then this could reduce overall colony productivity. Although there is evidence to suggest that workers in queenless colonies participate in costly behaviours associated with reproduction (Cole 1986; Gobin *et al.* 2003), less is know about the behaviour of future reproductive workers (i.e. those that reproduce under queenless conditions) whilst still in the queenright stage. One study has shown that future reproductive workers of the ant *Temnothorax unifasciatus* do behave differently to non-future reproductives prior to queen removal, but not necessarily by participating in behaviours that are likely to prove costly for the colony (Brunner & Heinze 2009). In contrast, other studies have shown that workers of the ants *Harpagoxenus sublaevis* and *Leptothorax gredleri* seem to prepare themselves for future reproduction by forming dominance hierarchies in the presence of the queen through aggressive dominance interactions (Bourke 1988a; Heinze & Oberstadt 1999) that have the potential to reduce colony productivity (Cole 1986).

In addition to reproducing themselves, another way in which workers in queenless colonies could continue colony reproduction is to adopt a new queen (Bourke & Franks 1995). Polygyny is thought to arise in *L. acervorum* through the readoption of newly mated queens into their natal nests (Hammond *et al.* 2001), so the same behaviour could be used under queenless conditions for the purposes of requeening the colony. Given their previous experience with queen adoption, we might expect workers in previously polygynous queenless colonies to requeen themselves more readily than those in previously monogynous queenless colonies. Such behaviour could reduce the need for workers in polygynous colonies to prepare for direct reproduction and so help to explain the results of the experiment.

In addition to kin structure, colony size was also found to have a significant effect on worker reproduction under queenless conditions. To begin with, an increase in colony size was found to significantly increase a colony's chances of producing worker-laid eggs. One possible explanation for this observed pattern could involve the potential costs associated with preparation for reproduction. If, for example, workers do reduce their work effort in order to prepare for reproduction, then perhaps only larger colonies can afford for some of their workers to participate in such costly behaviour (because each individual worker represents a smaller proportion of the workforce than in small colonies). A similar prediction has been made regarding the effect of colony size on worker reproduction under queenright conditions (Walter *et al.* 2011). In contrast to the effect of colony size on the likelihood of egg-laying, colony size was actually found to be associated with a decrease in worker reproduction when considering the number of eggs laid per worker (i.e. of the colonies that did reproduce, smaller colonies produced more eggs per capita than larger colonies). This observed pattern could perhaps be linked to the proportion of pre-existing, sexual brood in the colony (i.e. male brood or queen-destined brood). Given that large colonies of *L. acervorum* in the study population have been shown to contain a greater proportion of sexual brood than small colonies (Bourke & Chan 1999), perhaps the best inclusive fitness strategy for workers in large queenless colonies is to balance their direct reproduction with rearing pre-existing brood, whereas the best strategy for workers in small queenless colonies may be to invest heavily in their own direct reproduction because the pre-existing brood has little to offer in terms of indirect fitness (at least compared to large colonies).

Tables

Table 1. The numbers of dealate queens and adult workers in the experimental *Leptothorax acervorum* colonies, and the response of the workers in each colony to queenless conditions, i.e. following queen removal, whether at least three (worker-laid) eggs were present on any single day, latency to worker egg-laying, the number of worker-laid eggs present on the final day, and the number of eggs laid per worker. Colonies SD091-39 were collected in June 2009, and colonies SD0941-83 were collected in October 2009.

Colony	No. queens with active ovaries (& no. of these mated)	No. adult workers (end of expt.)	≥ 3 eggs present on a single day?	Latency to 3 eggs	No. eggs day 30	No. eggs per worker in 30 days
Polygynous:						
SD097	2 (2)	162	Yes	11	15	0.09
SD098	2 (2)	96	No	n/a	0	0.00
SD0910	5 (5)	135	Yes	8	2	0.01
SD0919	24 (19)	366	No	n/a	0	0.00
SD0924	6 (6)	224	No	n/a	0	0.00
SD0927	2 (1)	184	Yes	28	5	0.03
SD0928	2 (2)	33	No	n/a	0	0.00
SD0929	4 (3)	170	Yes	22	3	0.02
SD0934	4 (4)	131	No	n/a	0	0.00
SD0938	4 (4)	74	No	n/a	0	0.00
SD0942	2 (2)	80	No	n/a	1	0.01
SD0943	14 (14)	114	Yes	16	9	0.08
SD0948	5 (5)	155	Yes	11	22	0.14
SD0954	4 (4)	232	Yes	14	16	0.07
SD0956	15 (15)	500	Yes	17	13	0.03
SD0958	3 (3)	87	No	n/a	2	0.02
SD0962	2 (2)	148	Yes	13	22	0.15
SD0965	5 (5)	245	No	n/a	1	0.00
SD0966	3 (3)	105	No	n/a	0	0.00
SD0970	2 (2)	183	No	n/a	1	0.01
SD0971	2 (2)	148	No	n/a	0	0.00
SD0977	2 (2)	195	Yes	10	19	0.10
SD0980	2 (2)	82	Yes	26	3	0.04
Mean (all polygynous)	**5**	**167**	**n/a**	**n/a**	**6**	**0.03**

Table 1 continued.

Colony	No. queens with active ovaries (& no. of these mated)	No. adult workers at the end of the expt.	≥ 3 eggs present on a single day?	Latency to ≥3 eggs	No. eggs present on day 30	No. eggs per worker in 30 days
Monogynous:						
SD091	1 (1)	55	Yes	14	19	0.35
SD092	1 (1)	104	Yes	13	59	0.57
SD093	1 (1)	306	Yes	23	8	0.03
SD099	1 (1)	66	Yes	3	0	0.00
SD0913	1 (1)	165	Yes	18	12	0.07
SD0915	1 (1)	86	No	n/a	0	0.00
SD0916	1 (1)	80	Yes	3	115	1.44
SD0920	1 (1)	102	No	n/a	0	0.00
SD0925	1 (1)	98	Yes	19	8	0.08
SD0931	1 (1)	127	Yes	21	11	0.09
SD0936	1 (1)	204	Yes	12	65	0.32
SD0937	1 (1)	96	No	n/a	0	0.00
SD0939	1 (1)	33	No	n/a	0	0.00
SD0941	1 (1)	211	Yes	18	53	0.25
SD0944	1 (1)	49	Yes	17	10	0.20
SD0945	1 (1)	132	Yes	5	17	0.13
SD0946	1 (unknown)	162	Yes	21	9	0.06
SD0947	1 (1)	134	Yes	13	22	0.16
SD0950	1 (1)	71	Yes	16	26	0.37
SD0952	1 (1)	324	Yes	23	11	0.03
SD0955	1 (1)	156	Yes	12	39	0.25
SD0957	1 (1)	209	Yes	14	72	0.34
SD0959	1 (1)	61	No	n/a	0	0.00
SD0963	1 (1)	224	Yes	21	9	0.04
SD0964	1 (1)	67	Yes	17	11	0.16
SD0967	1 (1)	48	Yes	29	4	0.08
SD0969	1 (1)	97	No	n/a	0	0.00
SD0972	1 (1)	163	Yes	15	11	0.07
SD0974	1 (1)	188	Yes	16	13	0.07
SD0975	1 (1)	140	Yes	24	5	0.04
SD0976	1 (1)	123	Yes	14	113	0.92
SD0978	1 (1)	280	Yes	18	33	0.12
SD0979	1 (1)	87	Yes	13	27	0.31
SD0981	1 (1)	266	Yes	12	67	0.25
SD0982	1 (1)	299	Yes	12	87	0.29
SD0983	1 (1)	154	No	n/a	0	0.00
Mean (mono.)	**1**	**144**	**n/a**	**n/a**	**26**	**0.20**

Table 2. The effect of previous social structure, colony size, replicate and their interactions on (1) the likelihood of queenless *Leptothorax acervorum* colonies producing worker-laid eggs, (2) the latency to workers laying eggs, and (3) the extent of worker egg-laying (i.e. number of eggs per capita) in queenless colonies, (a) including and (b) excluding colonies that did not lay eggs. The significance of an effect is indicated by NS (p>0.05), * (p<0.05), ** (p<0.01) or *** (p<0.001). The explanatory variables remaining in the minimal model are highlighted in bold.

GLM/survival model	χ^2	F	df	p	Sig.
(1) Likelihood of egg-laying					
Previous social structure	**8.59**	**n/a**	**1**	**0.003**	**
Colony size	**4.59**	**n/a**	**1**	**0.032**	*
Replicate	1.18	n/a	1	0.277	NS
Previous social structure: Colony size	1.39	n/a	1	0.239	NS
Previous social structure: Replicate	0.07	n/a	1	0.792	NS
Colony size: Replicate	0.06	n/a	1	0.801	NS
Three-way interaction	0.43	n/a	1	0.514	NS
(2) Latency to egg-laying					
Previous social structure	**7.59**	**n/a**	**1**	**0.006**	**
Colony size	3.78	n/a	1	0.052	NS
Replicate	1.19	n/a	1	0.276	NS
Previous social structure: Colony size	0.01	n/a	1	0.936	NS
Previous social structure: Replicate	0.05	n/a	1	0.822	NS
Colony size: Replicate	0.21	n/a	1	0.648	NS
Three-way interaction	0.28	n/a	1	0.595	NS
(3a) Extent of egg-laying, including colonies that did not produce eggs					
Previous social structure	**n/a**	**14.62**	**1,57**	**<0.001**	***
Colony size	n/a	1.75	1,56	0.191	NS
Replicate	n/a	0.15	1,55	0.703	NS
Previous social structure: Colony size	n/a	0.01	1,52	0.913	NS
Previous social structure: Replicate	n/a	0.79	1,53	0.378	NS
Colony size: Replicate	n/a	1.35	1,54	0.250	NS
Three-way interaction	n/a	0.01	1,51	0.913	NS
(3b) Extent of egg-laying, excluding colonies that did not produce eggs					
Previous social structure	**n/a**	**6.588**	**1,37**	**0.014**	*
Colony size	**n/a**	**5.090**	**1,37**	**0.030**	*
Replicate	n/a	0.000	1,36	0.989	NS
Previous social structure: Colony size	n/a	0.044	1,33	0.835	NS
Previous social structure: Replicate	n/a	0.652	1,34	0.425	NS
Colony size: Replicate	n/a	2.243	1,35	0.143	NS
Three-way interaction	n/a	0.042	1,32	0.840	NS

Figures

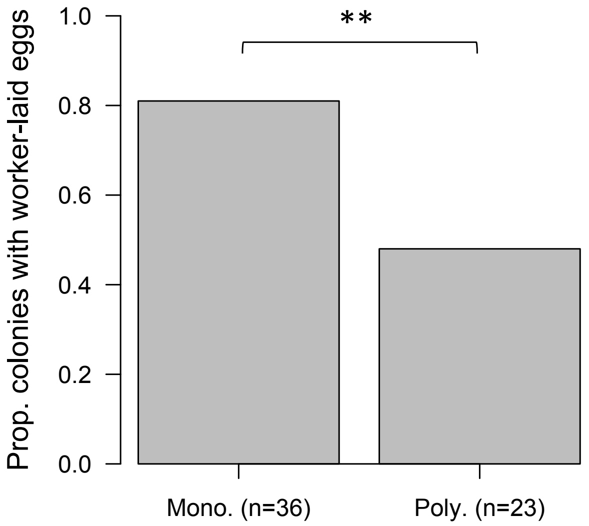

Figure 1. The proportion of previously monogynous and previously polygynous *Leptothorax acervorum* colonies that produced worker-laid eggs within 30 days of queenless conditions. The difference in proportions between previously monogynous and previously polygynous colonies is significant (GLM: $p<0.01$, indicated by **). The sample sizes given in brackets represent the number of colonies used in the experiment.

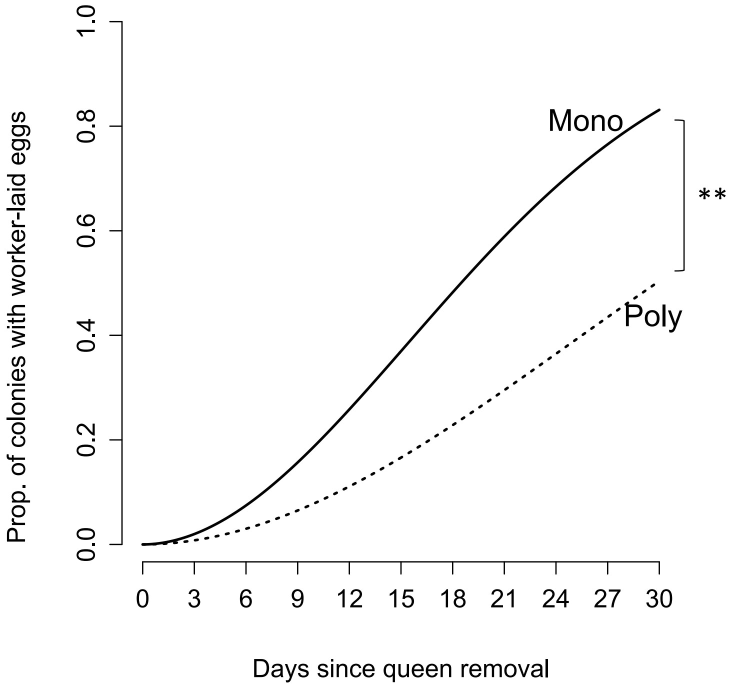

Figure 2. The proportion of previously monogynous (n=36) (solid line) and previously polygynous (n=23) (dashed line) *Leptothorax acervorum* colonies producing worker-laid eggs over 30 days of queenless conditions, as predicted by the minimal model of a survival analysis. The difference in the predicted curves for previously monogynous and previously polygynous colonies is significant (survival analysis: $p<0.01$, indicated by **).

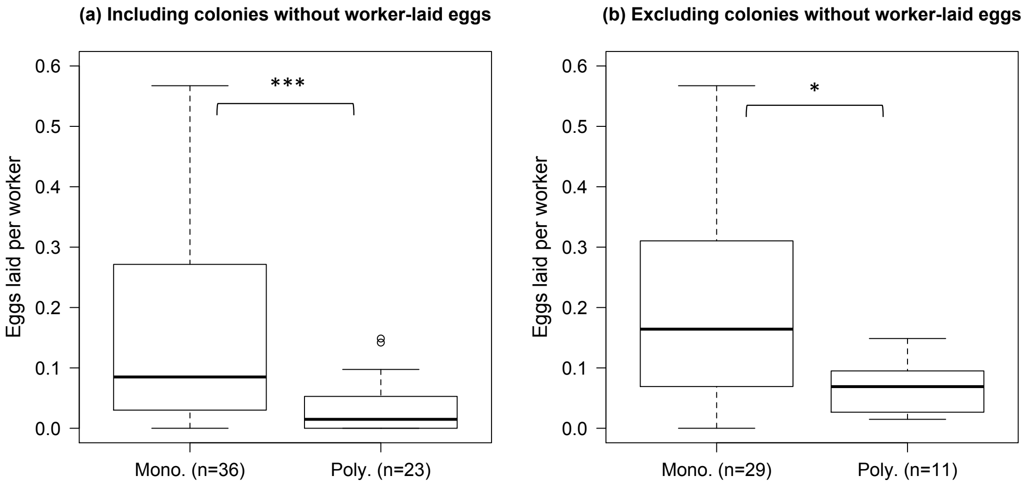

Figure 3. The number of eggs laid per worker in previously monogynous and previously polygynous *Leptothorax acervorum* colonies over 30 days of queenless conditions (a) including colonies that did not produce any worker-laid eggs, and (b) excluding colonies that did not produce any worker-laid eggs. Two data points regarding previously monogynous colonies (eggs laid per worker = 0.92 and 1.44) that were included in the GLM analyses have been removed from both (a) and (b) to facilitate diagram interpretation. The difference in the number of eggs laid per worker in previously monogynous and previously polygynous colonies is significant in both plots (GLM: $p<0.001$ indicated by ***, $p<0.05$ indicated by *). The sample sizes given in brackets represent the number of colonies used in the experiment.

Chapter 4

Chapter 4: The behaviour of future reproductive worker ants in the presence and absence of the queen

Abstract

Queenright Hymenopteran colonies (those containing queens) are a potentially hostile and costly environment for workers to perform direct reproduction within. However, direct reproduction can provide workers with high fitness returns. In contrast to workers in queenright colonies, workers in queenless colonies (those without queens) are often highly successful at producing their own offspring. Therefore, as an alternative to becoming fully reproductive under queenright conditions, workers might instead limit themselves to preparing for future reproduction in anticipation of future queenless conditions. If workers are able to prepare themselves for future reproduction then we might expect them to do so by altering their behaviour. Here I test this hypothesis by filming the behaviour of marked workers of the ant *Leptothorax acervorum* under queenright and then queenless conditions, and testing whether the workers that reproduced under queenless conditions ('future reproductive workers') behaved differently under queenright conditions to non-reproductive control workers. The results showed that, compared to control workers, future reproductive workers showed significantly higher levels of brood care, feeding from larvae, aggression towards (and from) workers, and non-aggressive behaviours towards (and from) dealate queens, and showed significantly lower levels of grooming and feeding towards other workers. These differences in behaviour may allow future reproductive workers to form dominance hierarchies, build up energy resources and monitor the queen's health, but they could also impose a cost upon colony productivity. I also test the hypothesis that the behaviour of future reproductive workers becomes more costly once they have become fully reproductive under queenless conditions. Again I do so by using films of marked *L. acervorum* workers and testing whether future reproductive workers change their behaviour between queenright and queenless conditions. The only significant change observed was a drop in brood care between queenright and queenless conditions, suggesting that workers could perhaps save their colonies from the cost of reduced brood care by refraining from full reproduction in the presence of the queen. These findings add a valuable insight into the methods Hymenopteran workers use to balance the direct and indirect components of their inclusive fitness.

Introduction

In many species of eusocial Hymenoptera the workers have retained their ability to lay male-destined, haploid eggs (Bourke 1988b). However, among these same species, workers are often found to produce only a very small proportion of male offspring in 'queenright' colonies (i.e. those containing at least one queen), despite the high fitness benefits of direct reproduction (Bourke 1988b; Bourke & Franks 1995; Hammond & Keller 2004). There are two main, non-mutually exclusive theories as to why successful worker reproduction tends to rarely occur in the presence of the queen. First, workers may be prevented from reproducing by other workers and the queen(s), who may 'police' reproductive workers with aggression (e.g. Kikuta & Tsuji 1999; Wenseleers *et al.* 2005b), egg-cannibalism (e.g. Ratnieks & Visscher 1989; Kikuta & Tsuji 1999; D'Ettorre *et al.* 2004; Wenseleers *et al.* 2005b), and possibly queen pheromones (Keller & Nonacs 1993). Secondly, workers may express reproductive self-restraint in the presence of the queen due to the potential inclusive fitness costs associated with worker reproduction (Cole 1986; Ratnieks 1988), especially in colonies where efficient policing reduces the likelihood of workers gaining direct fitness from their attempts at reproduction (Ratnieks 1988).

In contrast to their behaviour under queenright conditions, workers under queenless conditions (i.e. in colonies containing no queens) tend to readily reproduce (Bourke 1988b; Bourke & Franks 1995), because under queenless conditions worker policing is relaxed (Miller & Ratnieks 2001; D'Ettorre *et al.* 2004), queen policing cannot occur, and worker reproduction may provide the last opportunity for colony reproduction (Bourke 1988b; Ratnieks 1988). In my study described in Chapter 3 in which I investigated worker reproduction in a facultatively polygynous (multiple-queen) population of the ant *Leptothorax acervorum*, workers were found to adjust their reproductive response to queenless conditions according to their previous social structure. The study showed that queenless workers within previously monogynous (single-queen) colonies (colonies at a high risk of queenlessness) responded to queen-removal with a higher level of reproduction than workers within previously polygynous colonies (colonies at a low risk of queenlessness). *L. acervorum* workers express low levels of egg-laying under queenright conditions and these levels are similar in both monogynous and polygynous colonies (Hammond *et al.* 2003). Taken together, these findings strongly suggest that workers in queenright monogynous colonies are more prepared for future reproduction (but express no more current reproduction) than those in queenright polygynous colonies.

If queenright *L. acervorum* workers are indeed able to prepare themselves for future reproduction, then they may do so by altering their behaviour whilst still in the presence of the queen. For example, in order to prepare for reproduction, workers might increase their food intake to invest more energy into ovary development (Bourke 1988a). They might also decrease their work effort, or exhibit a tendency to participate only in work tasks centred around the brood so as to avoid risky tasks such as foraging and scouting (van Honk *et al.* 1981; Franks & Scovell 1983; Bourke 1988a; Monnin & Peeters 1999). Workers preparing for reproduction might also exhibit aggressive behaviour in order to establish dominance over their nestmates that will give them a reproductive advantage once queenless. Such behaviour has been observed in queenright colonies of other ant species whose workers appear to predominantly refrain from reproduction until queenless (Bourke 1988a; Heinze & Oberstadt 1999). Workers might also prepare for future reproduction by keeping in close contact with the queen and her brood so as to monitor the likelihood of queenlessness in the near future (Brunner & Heinze 2009). Many of these suggested changes in behaviour could carry colony-level costs. For example, increased aggression is thought to reduce work effort (e.g. Cole 1986; Gobin *et al.* 2003; Bocher *et al.* 2007) and waste energy (Gobin *et al.* 2003). Therefore preparation for reproduction, whilst improving a worker's chances of obtaining direct fitness, could in fact damage their levels of indirect fitness.

So far only a few studies have investigated in detail the behaviour of workers that appear to prepare for future reproduction whilst still in the presence of the queen (Bourke 1988a; Heinze & Oberstadt 1999; Brunner & Heinze 2009), and the findings of these studies are not always consistent. For example the use of aggression in the early formation of dominance hierarchies appears to vary between species. Therefore further investigation is required if we are to improve our understanding of how Hymenopteran workers prepare for reproduction whilst balancing the direct and indirect components of their inclusive fitness. Furthermore, it is particularly important to increase our understanding of preparation for reproduction in *L. acervorum*, since this is the first species, to my knowledge, in which workers have been found to exhibit variation in their preparation for reproduction in response to their current social structure.

In the following study I test the hypothesis that *L. acervorum* workers prepare, by altering their behaviour under queenright conditions, for the possibility of queen-loss and direct reproduction. I do so by filming the behaviour of marked workers before and after the removal of their queens, and testing whether the workers that reproduced after queen-removal behaved differently to other workers before queen-removal.
I also investigate the possibility that workers restrict their reproduction to preparation alone under queenright conditions (rather than becoming fully reproductive) because of the cost full reproductive behaviour has upon colony productivity. I do so by testing the hypothesis that the behaviour of workers when they are prepared for reproduction is different to, and potentially less costly than, their behaviour when they are fully reproductive. Again I test this second hypothesis using the filmed behaviour of marked *L. acervorum* workers before and after the removal of their queens, and test whether workers that reproduced under queenless conditions behaved differently before and after queen-removal.

Methods

Colony collections

In June and October 2009, 80 *L. acervorum* colonies were collected from Thetford Forest and established in the laboratory following the methods described in Chapter 2. The Thetford population of *L. acervorum* is facultatively polygynous, with 20-50% of colonies containing multiple, related queens (means of 2-5 related queens per polygynous colony), and 95% of all queens are singly mated (Heinze *et al.* 1995a; Bourke *et al.* 1997; Chan *et al.* 1999; Hammond *et al.* 2001, 2002, 2003, 2006). Two replicates of the experiment were performed: one in the summer of 2009 using the June-collected colonies and one in the spring of 2010 using the October-collected colonies.

Experimental protocol

At the start of each of the two experimental replicates, five monogynous and five polygynous colonies were selected from all colonies that had maintained a constant social structure since collection and had more than 15 workers (resulting in a total of 20 focal colonies across the two experimental replicates). Both monogynous and polygynous colonies were selected for this experiment for the purposes of another simultaneous experiment (Chapter 3, in which the two social structures are compared). The colonies were selected by their size: they had to be small enough to fit into a 40.0×40.0×1.5mm nest for filming (approximately <150 workers), and the monogynous and polygynous colonies had to roughly match in terms of the number of workers (again for the purposes of Chapter 3).

All workers and dealate queens in the 20 selected colonies were given a unique mark using three dots of paint (Testors Racing Finish, Pactra ®) (one on the head, thorax and abdomen) to allow each to be individually identified. At the same time the focal colonies were moved to small nests (internal cavity 40.0×40.0×1.5mm) to maximise the percentage area of nest that could be filmed at close range. The colonies were then given 1-5 days (mode of 4 days) to adjust to their new paint marks and nests before the filming of their queenright stage began. Colonies were filmed in their nests inside foraging arenas (10×10×1cm Petri-dishes) covered by a sheet of glass. A digital camcorder (Sony DCR-SR32E) was then placed directly on top of the sheet of glass to film a 36×26mm area of the nest containing all or part of the brood pile (depending on the size of the colony). Over an 11 day period, each focal colony was filmed on nine different days for 2.25h at varying times of day, giving a total of 20.25h of film for each colony in its queenright stage.

Following the filming of the queenright stage, all dealate queens were removed from the colonies to create queenless conditions. These dealate queens were frozen at -20°C the day after removal, and their ovaries were later dissected to confirm the social status of their colonies (as described in Chapter 3). Eggs were also removed from the colonies due to the requirements of another simultaneous study (Chapter 3), as were adult alates (males and gynes), which would otherwise have dispersed under natural conditions. The filming of colonies under queenless conditions began on the day following queen removal. Colonies were filmed using the same cameras and techniques as in the queenright stage, but colonies were sometimes filmed up to 4.5h (2 × 2.25h bouts) in a single day. The queenless colonies were filmed over a period of 29 or 32 days (Replicate 1 and 2 respectively) for a total of 38.25–69.75h each. The amount of filming a colony received was proportional to its level of worker reproduction, with colonies producing the greatest number of eggs receiving the most film time. Colonies were frozen at -20°C up to eight days after the last day of filming.

Identification of reproductive workers and control workers

In over half of the 20 focal colonies (n=11), workers laid no eggs or very few eggs (≤5) during the queenless phase, and hence, given the low chance of identifying any reproductive workers in such colonies, were not used for the remainder of the experiment. Another colony was also excluded from the rest of the experiment because only nine workers retained their unique identification marks until the end of filming, none of which appeared to have developed ovaries when dissected. This left a sample size of eight colonies: six monogynous and two polygynous colonies, which had produced a mean of 29.5 eggs (range 9–56 eggs) per colony by the last day of filming. Details of the composition of these eight colonies are given in Chapter 3 (monogynous colonies: SD092, SD0913, SD0936, SD0945, SD0947, SD0955; polygynous colonies: SD0962, SD0977).

Films of the queenless stage of the eight focal colonies were watched (60.75–69.75h per colony), and the identity of every worker observed laying an egg during these films was recorded. Egg-laying is a fairly conspicuous behaviour in *L. acervorum*, so it is highly likely that all marked workers that did lay eggs during the films were identified. However, there could have been some egg-laying workers that were not identified as such if they only laid eggs off camera. In total 29 different workers were observed laying eggs across the eight focal colonies in the queenless stage, but only 21 of these could still be identified at the end of the experiment (all others had either lost at least one paint mark or had eclosed after paint marks were applied). Three of these 21 workers laid eggs very early on in the queenless stage (less than seven days in) and so were excluded from the rest of the experiment to minimise the possibility of including workers that were already fully reproductive in the queenright stage. These three workers were excluded from the experiment before the detailed recording of behavioural data began (as described below). Hence the decision to exclude the three workers from the experiment was not influenced by any aspect of their behaviour besides the timing of their egg-laying events. The remaining 18 workers that had laid eggs under queenless conditions (from now on referred to as 'future reproductive workers', even when discussed in terms of the queenless stage when they were reproductive) were dissected in distilled water (Bourke 1991) to remove and measure their ovaries (measuring software: Auto-montage by Synoptics Ltd). Measurements were taken as a mean of the length of each worker's two ovarioles. Going by the appearance of their ovaries, 17 of the 18 workers seemed to have become fully reproductive under queenless conditions (each of the 17 workers had a mean ovariole length of ≥1.8mm). These 17 future reproductive workers from the eight focal colonies (1–4 workers per colony) were used for the rest of the experiment.

Non-reproductive control workers were selected for the experiment after the colonies had been frozen. Selection took place by dissecting workers chosen at random from those that had retained their paint marks and had not been recorded as egg-layers during the queenless phase. Of these, workers that had a mean ovariole length of ≤ 1.2mm were used as control workers (the shortest mean ovariole length of the 17 focal future reproductive workers was 1.8mm). Dissections occurred until enough control workers were identified to match the number of reproductive workers in each of the eight colonies (i.e. 17 in total across the eight colonies). Although it was impossible to be sure that these workers had not laid eggs, the relative inactivation of their ovaries strongly suggested they had not, or that they had only done so at very low rates (Fig.1).

Once all reproductive and control workers were selected, the films of the queenright stage for each of the eight focal colonies were watched (20.25h of film per colony). This was to ensure that the control workers were never recorded laying an egg in the queenright stage, and to record any laying events by future reproductive workers prior to queen-removal. For the purposes of the experiment I was primarily interested in workers that became fully reproductive under queenless conditions but refrained from reproduction under queenright conditions. However, I was also interested in workers that became fully reproductive under queenless conditions but which restricted themselves to a low level of egg-laying under queenright conditions, because such workers could also be considered as having demonstrated a level of reproductive self-restraint under queenright conditions. It is impossible to be sure exactly how many of the future workers fell into which of the two categories of self-restraint (full or partial) under queenright conditions (such knowledge could only be gained through continuous observation of the entire nest in each of the focal colonies over a number of weeks), but given the low level of worker egg-laying that occurs in queenright colonies of *L. acervorum*, it is likely that the majority of workers that reproduced under queenless conditions did not lay any eggs under queenright conditions.

Recording behavioural data from the films

Behavioural data were recorded from the films of the queenright stage to assess the behaviour of the 17 future reproductive workers and 17 control workers whilst in the presence of the queen. Five hours of film of the queenright stage were watched per colony, taking 1h from the 1st, 3rd, 5th, 7th and 9th days on which the queenright stage was filmed (i.e. films spanning 10 days). The hour used from each film was that which began 15 minutes into the 2.25h bout of filming. The frequencies with which the 34 focal workers performed (or received) the following behaviours were recorded from the films: aggression (to/from workers), antennation (to/from dealate queens), brood care (grooming and transporting eggs, larvae and pupae), grooming (to/from dealate queens and workers), and trophallactic feeding (to/from dealate queens and workers, and also from larvae). Only clear signs of aggression were recorded in the aggression frequencies (e.g. when a worker rushed towards an individual whilst simultaneously opening her mandibles, threatened an individual by opening her mandibles wide, bit an individual, or dragged an individual). Potential aggression appearing in a more subtle form, such as when a worker took a short rush towards an individual whilst keeping her mandibles closed, was not recorded. Trophallactic interactions between the focal workers and larvae were assumed to always be in the direction of the larvae donating food to the workers, based on the fact that during the interactions, workers kept their mandibles nearly closed (as they do when they are receiving food via trophallaxis from an adult) and usually wagged their gasters up and down (in a focus study of 19 of the recorded trophallactic interactions between workers and larvae, workers were observed to wag their gasters up and down during 84% of the interactions, as they do when they are receiving food via trophallaxis from an adult).

In the case of aggression, antennation, grooming and trophallaxis towards adults, an occurrence of the behaviour was scored each time the behaviour was directed towards/from a single individual. In the case of brood care, a single occurrence of the behaviour was recorded each time the behaviour was directed towards the brood pile as a whole (i.e. not each time the behaviour was directed towards a different individual within the brood pile), unless the type of brood care changed (e.g. if a worker went immediately from grooming the brood pile to moving a brood item to another part of the nest) in which case multiple occurrences of brood care were scored. The time that each focal worker spent on screen was also recorded, although moments where a worker only left/entered the camera's field of view for less than three seconds were not included in calculating the total time on screen. Workers were only classified as on screen when they were in full view of the camera, and behaviours were only scored when the workers were fully on screen.

Behavioural data were recorded from the films of the queenless stage to assess whether the future reproductive workers changed their behaviour once they had become fully reproductive. Two workers that were observed laying an egg under queenright conditions were excluded from this analysis in order to concentrate on the workers that should express the biggest differences in their behaviour between queenright and queenless conditions (due to a complete change from non-reproductive to fully reproductive status across the two stages). Hence only 15 future reproductive workers were used for this analysis. Two hours of film of the queenless stage were watched per future reproductive worker (time limitations prevented the use of five hours of film per worker, as used for the queenright stage), each hour picked at random from the films that were recorded after the first day on which the worker was filmed laying an egg. The hours observed were those starting 15 minutes into the 2.25h bouts of filming. Frequencies of the same behaviours as listed above for the queenright stage were recorded, with the obvious exception of those involving dealate queens, which were absent from the queenless colonies.

Behavioural categories

In the case of each focal worker, the behavioural frequencies recorded from the films of the queenright stage were grouped into eight categories (1–8 below), and from the films of the queenless stage into six categories (1–4 and 7–8 below):

1) Aggression towards workers.
2) Aggression from workers.
3) Brood care (grooming and transporting eggs, larvae and pupae).
4) Trophallactic feeding from larvae.

5) Non-aggressive behaviour (antennation, grooming and trophallactic feeding) towards dealate queens.
6) Non-aggressive behaviour (antennation, grooming and trophallactic feeding) from dealate queens.
7) Grooming and trophallactic feeding towards workers.
8) Grooming and trophallactic feeding from workers.

Statistical analyses

Two sets of statistical analyses were carried out. The aim of the first set was to test the hypothesis that future reproductive *L. acervorum* workers alter their behaviour under queenright conditions compared to other workers. The analyses tested this hypothesis by testing for a difference in the rate with which future reproductive workers (n=17) and control workers (n=17) performed the eight categories of behaviour during the queenright stage. Due to the repeated use of workers from the same colonies, colony rather than worker was used as the unit of replication in the analyses (n=8) (generalised linear mixed models (GLMMs) were not used as an alternative solution to the repeated use of colonies because preliminary GLMMs provided parameter estimates with large confidence intervals, suggesting the behavioural data could not be modelled reliably using such methods, perhaps as a result of the limited sample size). Therefore, for each behavioural category, a mean rate per colony (frequency per hour spent on screen) was calculated for the two types of worker in each of the eight colonies. Mean rates were calculated from 1–4 workers depending on the number of workers sampled per colony (mean rates per worker and per colony are shown in Table A1 of the Appendix). The mean rates of the future reproductive workers were then compared to the mean rates of the control workers using paired *t*-tests (if normally distributed) or Wilcoxon signed rank tests (if non-normally distributed). The mean time spent by workers on screen during the hours of observed film (the 5h from which the behavioural rates were collected) was also compared between future reproductive workers and control workers. This was done using the eight colony means as samples (as for the rate data) and comparing the two worker categories with a paired *t*-test. Time spent on screen was considered a measure of interest because it could be used as a surrogate measure for time spent on or near the brood pile (due to the fact that the camera was always focused on a large portion of the brood pile).

The aim of the second set of statistical analyses was to test the hypothesis that future reproductive workers alter their behaviour in a potentially costly way once they become fully reproductive. The analyses were therefore designed to test for a difference in the rate with which the future reproductive workers (n=15 for this analysis) performed six of the eight categories of behaviour (1–4 and 7–8 listed above) during the queenright stage (when they were prepared for reproduction) and queenless stage (when they were fully reproductive). As before, colony means were used as the unit of replication in the tests (n=8). For each behavioural category, a mean rate (frequency per hour on screen) was calculated per colony for the queenright stage and the queenless stage. Again means were calculated from 1–4 workers depending on the number sampled per colony (mean rates per worker and per colony are shown in Table A2 of the Appendix). In the case of two colonies (SD0955 and SD0962), the mean rates of behaviour calculated for the queenright stage differed to those calculated for the first set of analyses because of the exclusion of a future reproductive worker from each due to the egg-laying behaviour of these two workers under queenright conditions (explained above). The mean rates taken from the queenright stage were then compared to the mean rates taken from the queenless stage using paired *t*-tests (if normally distributed) or Wilcoxon signed rank tests (if non-normally distributed). The proportion of time spent by future reproductive workers on screen during the hours of observed film (5h queenright, 2h queenless) was also compared between the queenright and queenless stage. Again colony means were used as samples (n=8), and the mean proportion of time spent on screen was compared across the queenright stage and the queenless stage using a paired *t*-test.

All statistical analyses were performed using the software programme *R* version 2.12.0 (R Development Core Team 2010). The results are reported as mean rates of behaviour ± the standard error of the mean. Statistical significance is reported on the basis of $\alpha = 0.05$.

Results

Egg-laying rates

Over the course of the films of the queenright stage (20.25h per colony), two (12%) of the 17 future reproductive workers laid one egg each, and the remaining 15 workers did not lay any. Across all 17 focal workers, this represented a mean (±standard error) of 0.14 ± 0.10 eggs laid per worker per day. Each of the two eggs laid in the queenright stage survived in the nest at least until the end of the film in which they were laid. Over the course of the films of the queenless stage (60.75–69.75h per colony) the 17 future reproductive workers laid 1–4 eggs each (29 in total), giving a mean of 0.62 ± 0.09 eggs per worker per day. Out of all the eggs laid by the focal workers in the queenless stage (n=29), 22 survived in the nest until the end of the film in which they were laid, three did not have their fates recorded, and four were eaten by other workers. All four egg-eating events occurred in the same colony (SD0913) and were performed by a single worker which had been excluded from the experiment on the basis that it began egg-laying very early in the queenless stage (see above). During one of these egg-eating events another of the focal future reproductive workers also participated. There were other occasions when egg-eating may have occurred whilst an egg was being laid, but these events were not included in the dataset (or to identify egg-layers), mainly because it was not possible to be certain that oviposition had occurred in these cases, but also because the eggs produced under these conditions may have been non-viable trophic eggs (which would have accounted for their immediate consumption (Bourke 1991)).

The fact that such a small number of future reproductive workers were observed egg-laying in the queenright stage (2 out of 17 future reproductive workers) provides support for the theory that the majority of future reproductive workers probably did not lay eggs until queenless or greatly increased their egg-laying rate once queenless, as does the fact that there was a more than four-fold increase in the egg-laying rate across all future reproductive workers between the queenright and queenless stage. In the case of the two future reproductive workers that were observed laying an egg each in the queenright stage (giving each a mean rate of 1.2 eggs laid per day), their individual egg-laying rates did not appear to increase under queenless conditions (mean rates of 1.1 and 0.4 eggs laid per day). However, these rates are calculated from a very small number of egg-laying events (n=6 in total, two in the queenright stage and four in the queenless stage), making it difficult to be sure of their accuracy.

It should be noted that the mean rate of egg-laying in both the queenright and queenless stage could have been underestimated because the number of eggs laid per day for each worker was calculated using total film time rather time spent on screen (a worker's time spent on screen was only recorded in those films studied in detail to record behavioural rates). It should also be noted that the apparent increase in worker egg-laying rate between the queenright and queenless stage could potentially have been caused by an increase in the frequency with which workers laid eggs in view of the camera (rather than an increase in overall worker egg-laying rate). Workers might be expected to lay eggs away from the brood pile (out of view of the camera) more often in the queenright stage than the queenless stage in order to avoid harassment from the queen when she is present (queens spend the majority of their time on the brood pile (Bourke 1991)). However, *L. acervorum* workers appear to generally lay their eggs on the brood pile in both queenright and queenless colonies (LF personal observation). Furthermore, by immediately placing their own eggs among the queen-laid brood, workers are perhaps able to decrease the likelihood of their eggs being detected by the rest of the colony. Therefore there is no strong basis upon which to expect workers to vary the location of their egg-laying with the presence/absence of the queen.

Do future reproductive workers alter their behaviour under queenright conditions compared to other workers?

In total across the 34 focal workers (17 future reproductives and 17 controls) I recorded 77 acts of aggression between workers (59 performed by the focal workers and 18 aimed towards the focal workers), 1672 acts of brood care, 58 occurrences of feeding from larvae, 83 non-aggressive interactions between focal workers and dealate queens (37 performed by the focal workers, and 46 performed by the queens), and 688 feeding/grooming interactions between workers (341 performed by the focal workers and 347 aimed towards the focal workers). The behavioural rates for individual workers are shown in the appendix at the end of the chapter (Table A1). Future reproductive and control workers were found to differ in their behaviour under queenright conditions in the following ways. First, future reproductive workers were found to express aggression towards other workers significantly more often than control workers (paired *t*-test: t=3.67, df=7, p=0.008; difference in means = 0.77 ± 0.21 occurrences per hour) (Fig. 2a), and to receive aggression significantly more often than control workers (paired *t*-test: t=3.60, df=7, p=0.009; difference in means = 0.33 ± 0.09 occurrences per hour) (Fig. 2b). Future reproductive workers were also found to participate in brood care significantly more often than control workers (paired *t*-test: t=4.62, df=7, p=0.002; difference in means = 9.74 ± 2.11 occurrences per hour) (Fig. 2c), and to feed from larvae significantly more often than control workers (paired *t*-test: $t=6.37$, df=7, $p<0.001$; difference in means = 0.68 ± 0.11 occurrences per hour) (Fig. 2d). In terms of non-aggressive interactions with dealate queens, future reproductive workers were found to antennate, groom and feed dealate queens significantly more often than control workers (paired *t*-test: $t=2.69$, df=7, $p=0.031$; difference in means = 0.50 ± 0.19 occurrences per hour) (Fig. 2e), and to receive the same behaviours from dealate queens significantly more often than control workers (Wilcoxon signed rank test: $w=0$, n=8, $p=0.023$; difference in means = 0.39 ± 0.18 occurrences per hour) (Fig. 2f). The only behavioural category that control workers performed significantly more often than future reproductive workers was cooperative behaviour (grooming and trophallactic feeding) directed towards other workers (paired *t*-test: $t=2.41$, df=7, $p=0.047$; difference in means = 4.95 ± 2.05 occurrences per hour) (Fig. 2g). In contrast, future reproductive workers and control workers did not differ significantly in the amount of cooperative behaviour they received from workers (paired *t*-test: $t=1.34$, df=7, $p=0.223$; difference in means = 2.22 ± 1.66 occurrences per hour) (Fig. 2h). Regarding time spent on screen during the five hours of queenright observation, future reproductive workers were found to spend longer on screen than control workers (paired *t*-test: $t=5.42$, df=7, $p=0.001$; difference in means = 1.75 ± 0.32 hours spent on screen) (Fig. 3). A more conservative test of significance for the nine behaviours investigated (those numbered 1–8 under ‘Behavioural categories’ plus time spent on screen) would have been to apply a sequential Bonferroni correction for multiple tests (Rice 1989). Following such a correction, the only behaviour to lose statistical significance would be cooperative behaviour (grooming and trophallactic feeding) directed towards other workers (paired *t*-test: $t=2.41$, df=7, $p=0.047$), which would narrowly miss the corrected value of statistical significance of $\alpha = 0.044$.

It should be noted that the two future reproductive workers that were observed egg-laying in the queenright stage (worker GGG in colony SD0955 and worker OYY in colony SD0962) expressed similar rates of behaviour to the 15 future reproductive workers that were not observed egg-laying in the queenright stage (Table A1 in the Appendix). Furthermore, the removal of the two egg-laying future reproductive workers from the analyses did not alter any of the results (i.e. the same significant and non-significant differences in behaviour between future reproductive workers and control workers were found). Therefore the observed behavioural differences between future reproductive workers and control workers were not driven by the two future reproductive workers that laid eggs in the queenright stage.

Do future reproductive workers alter their behaviour once fully reproductive?

In total across both the queenright and queenless stage, and across the 15 focal workers (all future reproductives) I recorded 208 acts of aggression between workers (153 performed by the focal workers and 55 aimed towards the focal workers), 1207 acts of brood care, 76 occurrences of feeding from larvae, and 461 feeding/grooming interactions between workers (185 performed by the focal workers and 276 aimed towards the focal workers). The behavioural rates for individual workers are shown in the appendix at the end of the chapter (Table A2). The only behavioural category in which future reproductive workers were found to differ significantly between the queenright and queenless stage of the nest was the brood care category. Here future reproductive workers were found to perform brood care at higher rate in the queenright stage than the queenless stage (after observed laying their first egg) (paired *t*-test: t=3.56, df=7, p=0.008; difference in means = 8.43 ± 2.36 occurrences per hour) (Fig. 4c). In terms of aggressive interactions, there was a strong but non-significant trend for future reproductive workers to increase their rate of aggression towards workers between the queenright and queenless stage (Wilcoxon signed rank test: w=19, n=8, p=0.094; difference in means = 2.72 ± 1.48 occurrences per hour) (Fig. 4a). Similarly there was a strong but non-significant trend for future reproductive workers to receive aggression from workers at a higher rate in the queenless stage compared to the queenright stage (paired *t*-test: t=1.43, df=7, p=0.195; difference in means = 0.95 ± 0.66 occurrences per hour) (Fig. 4b). In terms of the remaining behaviours, there was no significant difference in the rate at which future reproductive workers fed from larvae (Wilcoxon signed rank test: w=17, n=8, p=0.945; difference in means = 0.21 ± 0.36 occurrences per hour) (Fig. 4d), performed grooming and trophallaxis towards other workers (paired *t*-test: t=1.79, df=7, p=0.120; difference in means = 1.76 ± 0.98 occurrences per hour) (Fig. 4e), and received grooming and trophallaxis from other workers (paired *t*-test: t=0.46, df=7, p=0.659; difference in means = 0.38 ± 0.83 occurrences per hour) (Fig. 4f) between the queenright and queenless stage. Regarding the proportion of time spent on screen during detailed hours of observation, there was no difference in the behaviour of future reproductive workers between the queenright and queenless stage (paired *t*-test: t=0.73, df=7, p=0.487; difference in means = 0.04 ±

0.05 proportion of time spent on screen) (Fig. 5). As above, a more conservative test of significance for the seven behaviours investigated (those numbered 1–4 and 7–8 under 'Behavioural categories' plus the proportion of time spent on screen) would have been to apply a sequential Bonferroni correction for multiple tests (Rice 1989). Following such a correction, the difference in the rate at which future reproductive performed brood care between the queenright and the queenless stage (the only behaviour to show a significant difference) would be marginally non-significant (paired *t*-test: t=3.56, df=7, p=0.008), with a new corrected value of statistical significance of $\alpha = 0.007$.

Discussion

By filming and recording the behaviour of *L. acervorum* workers before and after queen-removal, I was able to test the hypothesis that future reproductive workers (those that reproduce under queenless conditions) behave differently to other workers under queenright conditions, when they were either non-reproductive or reproductive at a very low level. The results of the experiment proved this hypothesis to be correct in terms of the majority of behaviours investigated, with future reproductive workers performing some types of behaviour significantly more often than other workers (aggression to and from workers, brood care, feeding from brood, non-aggressive behaviour to and from the queen, time spent on the focal area of the brood pile), and some types of behaviour significantly less often than other workers (grooming/trophallactic feeding towards workers). In terms of reproduction, the majority of future reproductive workers (88%) were not observed laying eggs under queenright conditions, and the average egg-laying rate across future reproductive workers increased more than four-fold between the queenright and the queenless stage. These results suggest that the majority of the future reproductive workers refrained from full reproduction until queenless. I also tested the hypothesis that future reproductive workers change their behaviour between queenright and queenless conditions (following the start of their own reproduction in the queenless stage). This hypothesis proved to be correct in terms of brood care, with the future reproductive workers performing significantly less brood care in the queenless stage than in the queenright stage. There were also strong but non-significant trends for the future reproductive workers to direct more aggression towards other workers and receive more aggression from other workers in the queenless stage than the queenright stage.

By showing that future reproductive workers behave differently to other workers under queenright conditions, this study shows that workers in the eusocial Hymenoptera anticipate future reproductive opportunities by altering their current behaviour, demonstrating that worker reproduction can have social effects even when it is currently absent or only present at a low frequency. Results from the present study are also consistent with the findings of my earlier study (Chapter 3), which showed that workers reproduce more readily in queenless conditions when they belong to colonies that were previously (under queenright conditions) at a high risk of queenlessness. It has been suggested before that queenright workers may keep themselves poised rather than active in terms of reproduction in preparation for queen-death (Bourke 1988a), and evidence of such preparation has been found before, such as in species whose queenright workers establish dominance hierarchies using either aggressive (e.g. Bourke 1988a; Heinze & Oberstadt 1999) or non-aggressive behaviours (Brunner & Heinze 2009) whilst refraining from reproduction until queenless. However, in combination with the earlier study on *L. acervorum* (Chapter 3), this is the first time to my knowledge that a preparation for future reproduction has been shown in a species in which workers adaptively respond to the current risk of queenlessness (Chapter 3). The findings of this study are similar to those that have investigated the behaviour of subordinates in primitively eusocial Hymenopteran societies where reproductive and non-reproductive females are not morphologically distinct, and where subordinate females queue to inherit breeding rights. The opportunity for future direct reproduction is known to influence the behaviour of subordinate females in such societies, with high ranking subordinates (i.e. those nearer the opportunity to breed) expressing more aggression and participating in less work away from the nest than low ranking subordinates (Cant & Field 2001; Cant *et al.* 2006; Field *et al.* 2006).

The behaviour of future reproductive workers under queenright conditions

The way in which future reproductive *L. acervorum* workers were found to vary their behaviour in comparison to control (non-future reproductive) workers is as we might expect from workers aiming to prepare for future reproduction. To begin with, future reproductive workers were found to aggress other workers at a significantly higher rate than control workers, suggesting they were trying to establish reproductive dominance before the onset of queenless conditions. Aggressive dominance behaviour is known to play an important role in the reproductive success of Hymenopteran workers, with the most dominant workers usually achieving the highest fecundity (Cole 1981; Franks & Scovell 1983; Bourke 1988a; Oliveira & Hölldobler 1990), or inheriting the position of gamergate (the main reproductive) in queenless Hymenopteran species (Monnin & Peeters 1999). However, the results of the current study show that aggressive interactions can play a role in determining future reproductive success in species whose workers tend to refrain from laying eggs in the presence of the queen, as has been found in the ants *Harpagoxenus sublaevis* and *Leptothorax gredleri* (Bourke 1988a; Heinze & Oberstadt 1999). In contrast, workers of the ant *Temnothorax unifasciatus* prepare for future reproduction using only non-aggressive behaviours in queenright colonies (Brunner & Heinze 2009), with the exception of interactions involving the policing of experimentally-induced reproductive workers (Stroeymeyt *et al.* 2007).

In the current study, future reproductive workers also received significantly more aggression from other workers than control workers did, probably as a consequence of workers detecting their increased level of reproductive activity and responding either with policing (to prevent full reproduction) or dominance (to compete for future reproduction). Hymenopterans are able to detect the fertility status of their nestmates through their individual chemical profiles (Howard & Blomquist 2005; Monnin 2006) and use the information to respond with aggression when they wish to prevent reproduction (Smith *et al.* 2009).

In terms of cooperative behaviours between *L. acervorum* workers, future reproductive workers were found to feed and groom workers at a significantly lower rate than control workers, yet the two types of workers received an equal amount of feeding and grooming. This behavioural pattern suggests that future reproductive workers reduce their work effort in terms of caring for nestmate workers, but at no direct cost to themselves (given they still receive the same amount of care). By reducing the amount of work effort and food lost on grooming and feeding their nestmates, future reproductive workers may be able to reserve energy for preparing for future reproduction (e.g. by beginning ovary activation). A reduced level of providing liquid food to other workers has been observed in dominant, reproductive workers of the ant *Leptothorax allardycei* (Cole 1981).

In terms of interactions with the brood, future reproductive *L. acervorum* workers were found to care for brood and to feed from larvae significantly more often than control workers. This high level of contact with the brood is also reflected by the fact that future reproductive workers spent significantly more time in the camera's field of view than control workers, which was always focused on a large portion of the brood pile. By regularly caring for brood, future reproductive workers may be able to increase their feeding rate, since regular care may help workers to detect larvae ready to donate food. An increased level of feeding by reproductively dominant workers has been observed in other ant species, but in these species the food came from slave workers (Franks & Scovell 1983; Bourke 1988a) as well as larvae (Bourke 1988a).

Caring for brood might also be a tactic used by future reproductive workers to monitor the fertility of the queen(s) so they know when to expect to take over reproduction (Brunner & Heinze 2009). Monitoring of the queens' fertility is also suggested by the fact that future reproductive workers performed non-aggressive behaviours towards dealate queens significantly more often than control workers did. In return it appears that dealate queens may also monitor the future reproductive workers, since the queens performed non-aggressive behaviours towards future reproductive workers significantly more often than control workers. In the ant *Harpagoxenus americanus*, queens have been suggested to predominantly demand and receive food from dominant rather than subordinate workers so as to limit the amount of resources dominants have to invest in their own reproduction (Franks & Scovell 1983). The high levels of brood care and queen contact exhibited by future reproductive workers in the current experiment is similar to that expressed by future reproductive workers of the ant *T. unifasciatus* (Brunner & Heinze 2009). Future egg-laying workers in colonies of the bee *Bombus terrestris* have also been found to keep close contact with the queen before the onset of egg-laying, although their passive interactions with the queen (such as light antennation and tracking) gradually turn aggressive until the queen is removed from her reproductive position and the workers can start egg-laying (van Honk *et al.* 1981).

A number of the behaviours displayed by future reproductive *L. acervorum* workers in the current study could be associated with costs that reduce colony productivity. For example, the high levels of aggression expressed by future reproductives could cause a reduction in their work effort (Cole 1986; Gobin *et al.* 2003), such as the reduced tendency to groom and feed other workers observed in this study. Furthermore, the increased levels of feeding from larvae by future reproductive workers could restrict the amount of resources available to other colony members, in particular the queens who predominantly feed from larvae in *L. acervorum* (Bourke 1991; LF personal observation). However, the fact that workers appear to refrain from reproduction until their queen is absent suggests that preparation for reproduction may be less costly than active reproduction.

The behaviour of reproductive workers under queenless conditions

The second part of the current study explored the possibility that workers restrict their reproduction to preparation alone under queenright conditions, rather than become fully reproductive, because of the cost their full reproductive behaviour would have upon colony productivity. I predicted that if this were the case then workers should express a change in their behaviour between being prepared for reproduction (in the queenright stage) and being actively reproductive (in the queenless stage), and the changes in behaviour should have potential costs associated with them. The results showed that once workers had begun full reproduction they experienced a potential drop in work effort. Under queenright conditions, when the future reproductive workers were presumably only prepared for reproduction, their main contribution to the functioning of the colony appeared to be brood care (with future reproductive workers performing significantly more brood care than control workers). However, once the future reproductive workers became fully reproductive in the queenless stage, the amount of brood care they performed significantly decreased (the mean rate of brood care dropped by 45%), and this decrease was not counteracted by a significant increase in caring for adult workers in the colony.

Another change in behaviour that could add to the cost of full reproduction is an increase in aggression. Although the change in aggression expressed by the future reproductive workers between the queenless and queenright stage was not significant, future reproductive workers did exhibit a strong tendency to increase their aggression once in the queenless stage (by four fold on average), and the lack of detection of a significant increase could have been largely due to low statistical power in the test. As discussed above, aggression is thought to decrease work effort among reproductive workers and could be responsible for the decreased rate of brood care observed among reproductive workers in the queenless stage (Cole 1986; Gobin *et al.* 2003; Bocher *et al.* 2007).

The reduction in brood care and possible increase in aggression exhibited by workers once fully reproductive could impose a cost on colony productivity, especially if a large proportion of the colony's workers were to express these changes in behaviour. However, if we assume the data collected in the current experiment to give a fairly accurate estimation of the number of future reproductive workers per colony (1–4 future reproductive workers out of 104–204 total workers in each of the eight focal colonies), then only 0.5–2.4% of a colony's workers appear to be prepared for reproduction under queenright conditions. In the case of such a small percentage of the colony's workforce, it is hard to imagine that the future reproductive workers would actually impose a significant cost on colony productivity by becoming fully reproductive under queenright conditions. Therefore it is difficult to say whether queenright *L. acervorum* workers limit themselves to preparation for reproduction under queenright conditions because of colony-level costs associated with their full reproduction. This study has begun the process of trying to understand this form of self-restraint by highlighting some key changes in the behaviour of future reproductive workers that appear to be associated with the onset of full reproduction. However, a detailed assessment of the costs of worker reproduction in *L. acervorum* would be required in order to estimate the true impact a small number of workers could have on colony productivity by participating in full reproduction. Quantifying the costs of worker reproduction is extremely difficult (Dijkstra & Boomsma 2007) and so far only a few studies have attempted the task (Cole 1986; Gobin *et al.* 2003; Lopez-Vaamonde *et al.* 2003; Dijkstra & Boomsma 2007). Further understanding of why so few workers appear to prepare themselves for future reproduction is also required, especially if preparation for direct reproduction is truly less costly in terms of colony productivity than participation in full reproduction. The future reproductive workers in this study appeared to be some of the larger workers in the experimental colonies (LF personal observation), although body size was never actually measured. Therefore, although there is no formal data to support this hypothesis, perhaps one reason why only a few workers appear to prepare for reproduction is that not all workers are large enough to spare the resources for preparation and to maintain a dominant position among a reproductive hierarchy.

In conclusion, this study has shown that *L. acervorum* workers, which predominantly refrain from reproduction in the presence of the queen, sometimes prepare for reproduction in the future by altering their behaviour in ways that may allow them to establish dominance, reserve energy and monitor the health of the queen. Some of the behavioural changes involved in preparation for reproduction may have the potential to impose a cost on colony productivity, although perhaps less so than some of the behavioural changes involved in participating in full reproduction.

Figures

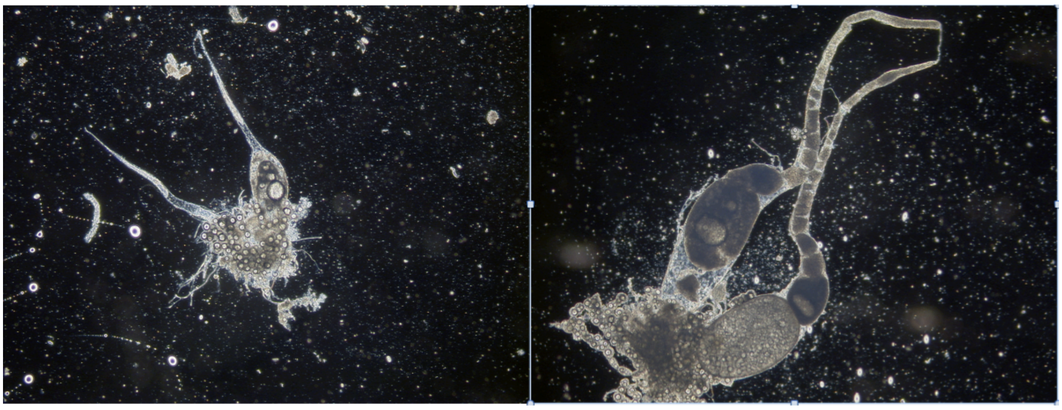

Figure 1. The ovaries of a control *Leptothorax acervorum* worker (left) with a mean ovariole length of 1.2mm, and the ovaries of a future reproductive *L. acervorum* worker (right) with a mean ovariole length of 2.7mm. The two workers are from the same colony and their ovaries are shown at the same magnification.

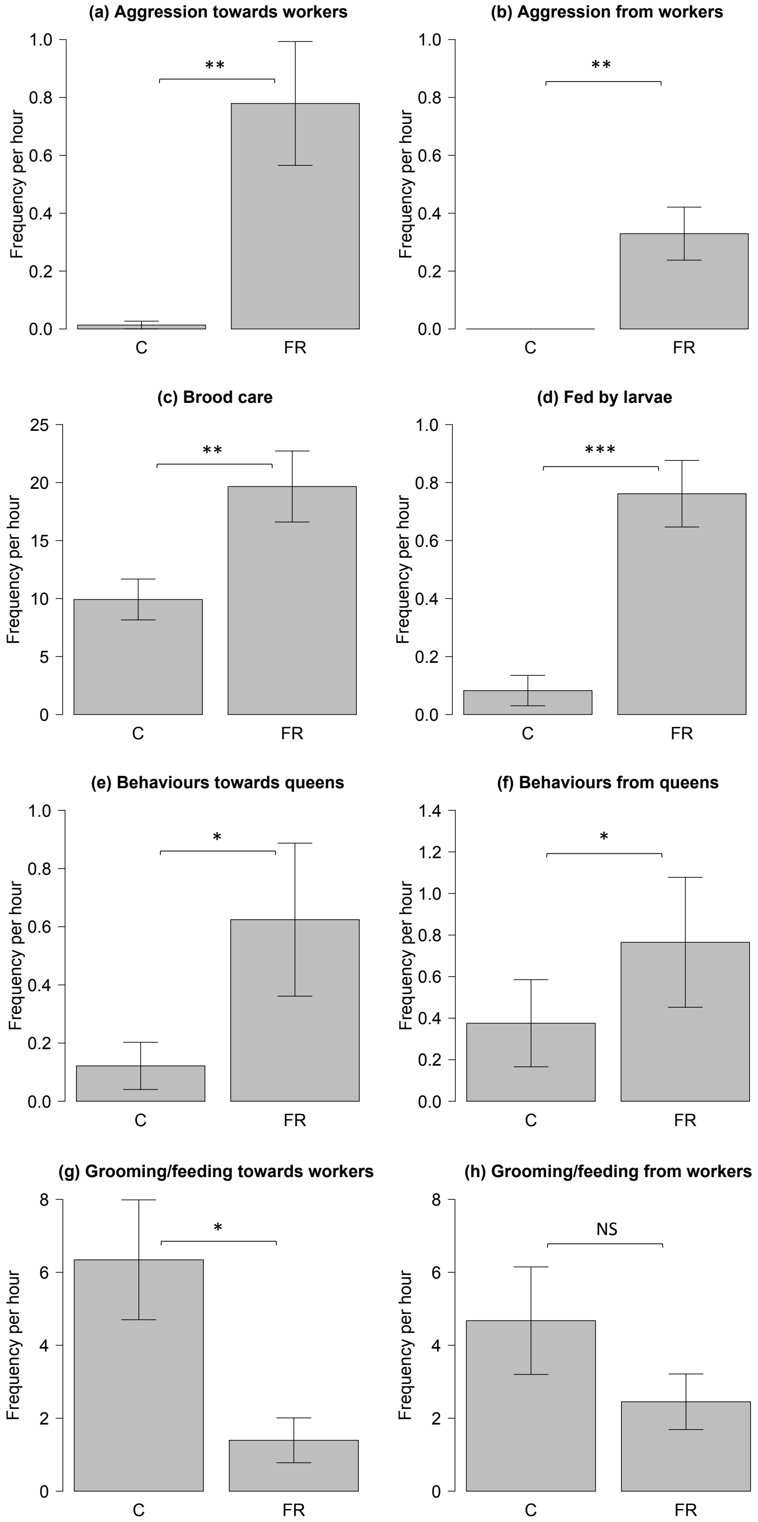

Figure 2. The mean rate (frequency per hour) across colonies (n=8) with which control (**C**) and future reproductive (**FR**) *Leptothorax acervorum* workers participated in various interactions under queenright conditions. Error bars show ± standard error of the mean. Significance values are indicated by NS (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001). Statistical analyses were performed on colony means (n=8), derived from observations of 17 future reproductive and 17 control workers (1–4 of each type of worker per colony) recorded from 5h of film per worker of the queenright stage.

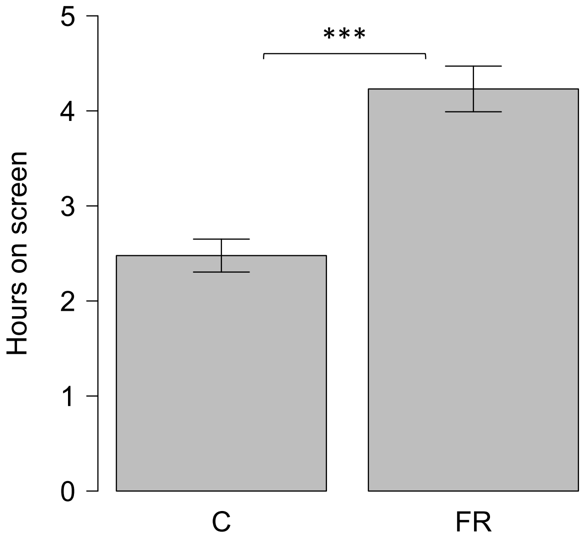

Figure 3. The mean number of hours across colonies (n=8) spent on screen by control (**C**) and future reproductive (**FR**) *Leptothorax acervorum* workers during the five hours of queenright films observed. Error bars show ±standard error of the mean. The difference between means is significant (paired *t*-test: $p<0.001$) indicated by ***. The statistical analysis was performed on colony means (n=8), derived from observations of 17 future reproductive and 17 control workers (1–4 of each type of worker per colony) recorded from 5h of film per worker of the queenright stage.

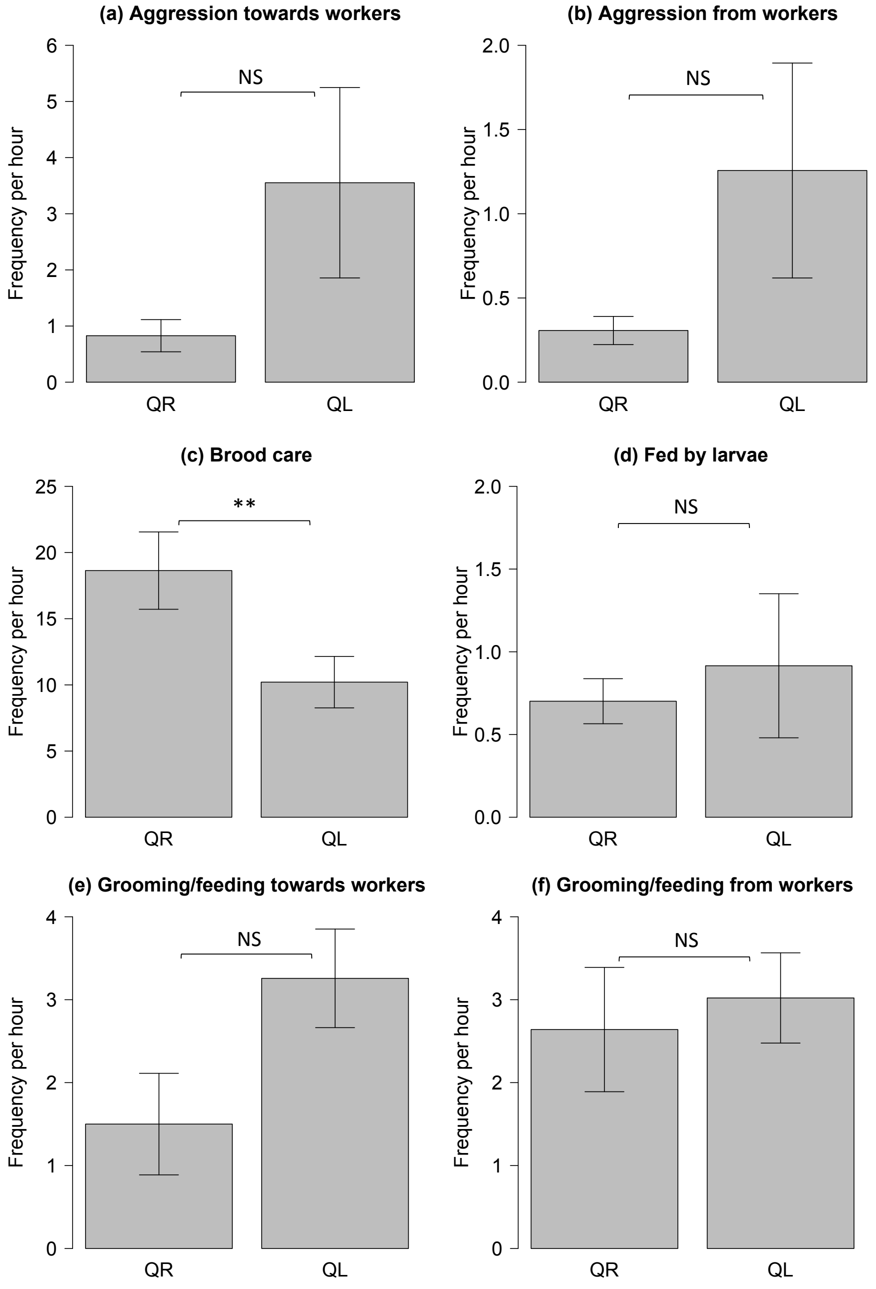

Figure 4. The mean rate (frequency per hour) across colonies (n=8) with which future reproductive *Leptothorax acervorum* workers participated in various interactions under queenright (QR) and queenless (QL) conditions. Error bars show ±standard error of the mean. Significance values are indicated by NS (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001). Statistical analyses were performed on colony means (n=8), derived from observations of 15 future reproductive workers (1–4 per colony) recorded from 5h of film per worker of the queenright stage, and 2h of film per worker of the queenless stage. Mean rates in the queenright stage are not identical to the mean rates for future reproductive workers shown in Fig. 2 because only 15 of the 17 future reproductive workers were used to calculate the colony means shown in the current figure, where as all 17 were used to calculate the colony means shown in Fig. 2

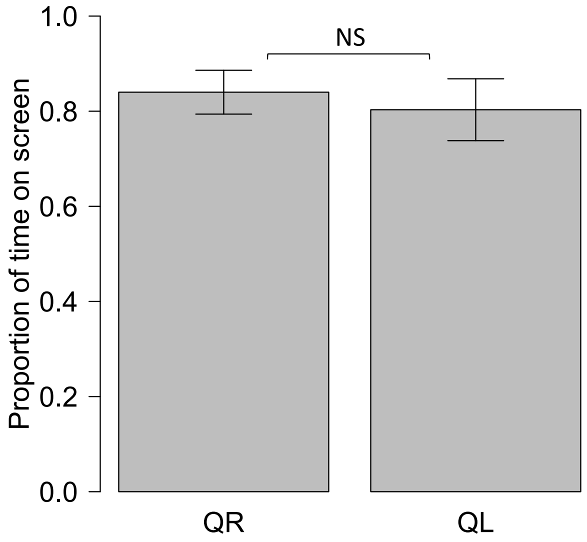

Figure 5. The mean proportion of time across colonies (n=8) spent on screen by future reproductive *Leptothorax acervorum* workers under queenright (QR) and (QL) conditions. Proportions of time are calculated from five hours of film from the queenright stage and two hours of film from the queenless stage. Error bars show ± standard error of the mean. The difference between means is not significant (paired *t*-test: p=0.49), indicated by NS. The statistical analysis was performed on colony means (n=8), derived from observations of 15 future reproductive workers (1–4 per colony) recorded from 5h of film per worker of the queenright stage, and 2h of film per worker of the queenless stage.

Appendix: Table A1. The mean rate (frequency per hour) of behaviours expressed by the 17 control (C) and 17 future reproductive (FR) *Leptothorax acervorum* workers in the queenright stage, and the number of hours (out of a maximum of 5h) spent on screen by each worker. Rates are calculated from observations recorded from 5h of film of the queenright stage per worker. Behaviours shown are aggression (agg.) to and from workers, brood care, feeding from larvae, non-aggressive behaviour (non-agg.) to and from dealate queens, and feeding/grooming behaviour to and from workers. Mean rates of behaviour per colony (n=8) are also shown for each type of worker (control or future reproductive), as are mean times spent on screen.

Colony	Focal worker	Type of focal worker	Agg. to worker (freq/h)	Agg. from worker (freq/h)	Brood care (freq/h)	Fed by larva (freq/h)	Non-agg. to queen (freq/h)	Non-agg. from queen (freq/h)	Feed/ Groom to worker (freq/h)	Feed/ Groom from worker (freq/h)	Time on screen (h)
SD092	WYB	C	0.00	0.00	3.32	0.00	0.00	0.00	3.99	1.33	1.50
SD092	YRO	C	0.22	0.00	11.66	0.00	0.00	0.00	3.45	1.30	4.63
SD092	**Mean**	**C**	**0.11**	**0.00**	**7.49**	**0.00**	**0.00**	**0.00**	**3.72**	**1.31**	**3.07**
SD0913	OOW	C	0.00	0.00	4.17	0.00	0.00	0.00	1.90	2.65	2.64
SD0913	RRG	C	0.00	0.00	0.59	0.00	0.00	0.00	3.54	3.54	1.70
SD0913	RYG	C	0.00	0.00	13.52	0.23	0.00	0.00	3.60	1.35	4.44
SD0913	YYO	C	0.00	0.00	4.55	0.00	0.00	0.00	6.83	8.35	1.32
SD0913	**Mean**	**C**	**0.00**	**0.00**	**5.71**	**0.06**	**0.00**	**0.00**	**3.97**	**3.97**	**2.52**
SD0936	GGY	C	0.00	0.00	10.75	0.00	0.00	0.00	2.26	0.85	3.54
SD0936	GRG	C	0.00	0.00	22.59	0.79	0.00	0.00	1.31	1.84	3.81
SD0936	GYY	C	0.00	0.00	12.80	0.00	0.00	0.00	3.01	1.51	3.98
SD0936	ORG	C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.16	1.39
SD0936	**Mean**	**C**	**0.00**	**0.00**	**11.53**	**0.20**	**0.00**	**0.00**	**1.65**	**1.59**	**3.18**
SD0945	BRY	C	0.00	0.00	11.72	0.00	0.00	1.47	3.42	1.47	2.05
SD0945	**Mean**	**C**	**0.00**	**0.00**	**11.72**	**0.00**	**0.00**	**1.47**	**3.42**	**1.47**	**2.05**
SD0947	BRW	C	0.00	0.00	5.68	0.00	0.00	0.00	16.65	8.12	2.46
SD0947	**Mean**	**C**	**0.00**	**0.00**	**5.68**	**0.00**	**0.00**	**0.00**	**16.65**	**8.12**	**2.46**
SD0955	GYG	C	0.00	0.00	5.27	0.00	0.00	0.00	11.67	1.13	2.66
SD0955	OBY	C	0.00	0.00	4.60	0.00	0.00	0.00	2.30	0.00	2.17
SD0955	**Mean**	**C**	**0.00**	**0.00**	**4.94**	**0.00**	**0.00**	**0.00**	**6.99**	**0.56**	**2.41**
SD0962	BRY	C	0.00	0.00	0.81	0.00	0.00	0.00	11.79	6.50	2.46
SD0962	WWB	C	0.00	0.00	38.45	0.00	1.13	2.26	2.26	14.70	0.88
SD0962	**Mean**	**C**	**0.00**	**0.00**	**19.63**	**0.00**	**0.57**	**1.13**	**7.02**	**10.60**	**1.67**
SD0977	WBG	C	0.00	0.00	12.63	0.41	0.41	0.41	7.34	9.78	2.45
SD0977	**Mean**	**C**	**0.00**	**0.00**	**12.63**	**0.41**	**0.41**	**0.41**	**7.34**	**9.78**	**2.45**

Appendix: Table A1 (continued)

Colony	Focal worker	Type of focal worker	Agg. to worker (freq/h)	Agg. from worker (freq/h)	Brood care (freq/h)	Fed by larva (freq/h)	Non-agg. to queen (freq/h)	Non-agg. from queen (freq/h)	Feed/ Groom to worker (freq/h)	Feed/ Groom from worker (freq/h)	Time on screen (h)
SD092	OWW	FR	2.02	0.20	19.97	1.41	0.20	0.20	0.61	2.42	4.96
SD092	WYW	FR	0.86	0.21	18.45	0.86	0.86	0.43	3.22	0.86	4.66
SD092	**Mean**	**FR**	**1.44**	**0.21**	**19.21**	**1.13**	**0.53**	**0.32**	**1.91**	**1.64**	**4.81**
SD0913	GGW	FR	0.00	0.00	9.57	0.00	0.00	0.00	5.25	12.35	3.24
SD0913	GRY	FR	0.77	0.26	14.31	1.28	0.26	0.26	1.28	4.34	3.91
SD0913	GWB	FR	3.00	0.27	12.81	1.36	0.00	0.00	0.00	7.91	3.67
SD0913	WWB	FR	1.88	0.00	14.39	0.63	0.00	0.00	0.31	5.32	3.20
SD0913	**Mean**	**FR**	**1.41**	**0.13**	**12.77**	**0.82**	**0.06**	**0.06**	**1.71**	**7.48**	**3.50**
SD0936	BGY	FR	0.00	0.00	8.96	0.92	0.00	0.00	2.76	3.45	4.35
SD0936	GGO	FR	0.00	0.00	9.03	0.64	0.43	0.43	4.73	3.65	4.65
SD0936	WGW	FR	0.00	0.00	22.41	0.33	0.00	0.00	1.32	1.65	3.03
SD0936	WYG	FR	0.00	0.00	14.78	0.43	0.00	0.43	12.61	2.61	2.30
SD0936	**Mean**	**FR**	**0.00**	**0.00**	**13.80**	**0.58**	**0.11**	**0.22**	**5.35**	**2.84**	**3.58**
SD0945	BGO	FR	0.78	0.78	17.06	0.26	0.52	1.55	0.26	0.52	3.87
SD0945	**Mean**	**FR**	**0.78**	**0.78**	**17.06**	**0.26**	**0.52**	**1.55**	**0.26**	**0.52**	**3.87**
SD0947	WYG	FR	0.82	0.41	18.86	1.03	0.00	0.00	0.21	1.23	4.88
SD0947	**Mean**	**FR**	**0.82**	**0.41**	**18.86**	**1.03**	**0.00**	**0.00**	**0.21**	**1.23**	**4.88**
SD0955	GGG	FR	0.80	0.20	13.01	0.80	0.00	0.20	0.40	0.40	5.00
SD0955	GYB	FR	0.00	0.20	7.15	0.20	0.41	0.00	1.84	2.86	4.89
SD0955	**Mean**	**FR**	**0.40**	**0.20**	**10.08**	**0.50**	**0.20**	**0.10**	**1.12**	**1.63**	**4.95**
SD0962	OYB	FR	2.18	0.44	26.79	0.44	3.48	3.48	0.44	1.96	4.59
SD0962	OYY	FR	0.60	0.80	37.32	0.80	0.20	0.80	0.20	1.40	4.98
SD0962	**Mean**	**FR**	**1.39**	**0.62**	**32.05**	**0.62**	**1.84**	**2.14**	**0.32**	**1.68**	**4.79**
SD0977	GRY	FR	0.00	0.29	33.44	1.15	1.73	1.73	0.29	2.59	3.47
SD0977	**Mean**	**FR**	**0.00**	**0.29**	**33.44**	**1.15**	**1.73**	**1.73**	**0.29**	**2.59**	**3.47**

Appendix: Table A2. The mean rate (frequency per hour) of behaviours expressed by 15 future reproductive *Leptothorax acervorum* workers in the queenright (QR) and queenless (QL) stage, the number of hours spent on screen by each worker (out of a maximum of 5h of film of the queenright stage and 2h of film of the queenless stage) and the proportion of time spent on screen by each worker. Mean rates are calculated from observations recorded from 5h of film of the queenright stage and 2h of film of the queenless stage per worker. Behaviours shown are aggression (agg.) to and from workers, brood care, feeding from larvae, and feeding/ grooming behaviour to and from workers. Mean rates of behaviour per colony (n=8) are also shown for each colony stage (queenright or queenless), as are mean times spent on screen and mean proportion of time spent on screen.

Colony	Focal worker	Colony stage	Agg. to worker (freq/h)	Agg. from worker (freq/h)	Brood care (freq/h)	Fed by larva (freq/h)	Feed/ Groom to worker (freq/h)	Feed/ Groom from worker (freq/h)	Time on screen (h)	Prop. time on screen
SD092	OWW	QR	2.02	0.20	19.97	1.41	0.61	2.42	4.96	0.99
SD092	WYW	QR	0.86	0.21	18.45	0.86	3.22	0.86	4.66	0.93
SD092	**Mean**	**QR**	**1.44**	**0.21**	**19.21**	**1.13**	**1.91**	**1.64**	**4.81**	**0.96**
SD0913	GGW	QR	0.00	0.00	9.57	0.00	5.25	12.35	3.24	0.65
SD0913	GRY	QR	0.77	0.26	14.31	1.28	1.28	4.34	3.91	0.78
SD0913	GWB	QR	3.00	0.27	12.81	1.36	0.00	7.91	3.67	0.73
SD0913	WWB	QR	1.88	0.00	14.39	0.63	0.31	5.32	3.20	0.64
SD0913	**Mean**	**QR**	**1.41**	**0.13**	**12.77**	**0.82**	**1.71**	**7.48**	**3.50**	**0.70**
SD0936	BGY	QR	0.00	0.00	8.96	0.92	2.76	3.45	4.35	0.87
SD0936	GGO	QR	0.00	0.00	9.03	0.64	4.73	3.65	4.65	0.93
SD0936	WGW	QR	0.00	0.00	22.41	0.33	1.32	1.65	3.03	0.61
SD0936	WYG	QR	0.00	0.00	14.78	0.43	12.61	2.61	2.30	0.46
SD0936	**Mean**	**QR**	**0.00**	**0.00**	**13.80**	**0.58**	**5.35**	**2.84**	**3.58**	**0.72**
SD0945	BGO	QR	0.78	0.78	17.06	0.26	0.26	0.52	3.87	0.77
SD0945	**Mean**	**QR**	**0.78**	**0.78**	**17.06**	**0.26**	**0.26**	**0.52**	**3.87**	**0.77**
SD0947	WYG	QR	0.82	0.41	18.86	1.03	0.21	1.23	4.88	0.98
SD0947	**Mean**	**QR**	**0.82**	**0.41**	**18.86**	**1.03**	**0.21**	**1.23**	**4.88**	**0.98**
SD0955	GYB	QR	0.00	0.20	7.15	0.20	1.84	2.86	4.89	0.98
SD0955	**Mean**	**QR**	**0.00**	**0.20**	**7.15**	**0.20**	**1.84**	**2.86**	**4.89**	**0.98**
SD0962	OYB	QR	2.18	0.44	26.79	0.44	0.44	1.96	4.59	0.92
SD0962	**Mean**	**QR**	**2.18**	**0.44**	**26.79**	**0.44**	**0.44**	**1.96**	**4.59**	**0.92**
SD0977	GRY	QR	0.00	0.29	33.44	1.15	0.29	2.59	3.47	0.69
SD0977	**Mean**	**QR**	**0.00**	**0.29**	**33.44**	**1.15**	**0.29**	**2.59**	**3.47**	**0.69**

Appendix: Table A2 (continued)

Colony	Focal worker	Colony stage	Agg. to worker (freq/h)	Agg. from worker (freq/h)	Brood care (freq/h)	Fed by larva (freq/h)	Feed/ Groom to worker (freq/h)	Feed/ Groom from worker (freq/h)	Time on screen (h)	Prop. time on screen
SD092	OWW	QL	2.48	0.00	25.37	4.33	4.33	1.86	1.62	0.81
SD092	WYW	QL	5.08	0.51	6.60	3.05	3.05	4.57	1.97	0.98
SD092	**Mean**	**QL**	**3.78**	**0.25**	**15.99**	**3.69**	**3.69**	**3.21**	**1.79**	**0.90**
SD0913	GGW	QL	2.50	1.50	6.50	0.00	2.50	0.50	2.00	1.00
SD0913	GRY	QL	10.07	2.24	12.30	3.36	0.00	5.59	1.79	0.89
SD0913	GWB	QL	5.29	1.18	5.29	0.59	5.29	6.47	1.70	0.85
SD0913	WWB	QL	0.57	3.39	5.66	1.70	1.70	4.52	1.77	0.88
SD0913	**Mean**	**QL**	**4.61**	**2.08**	**7.44**	**1.41**	**2.37**	**4.27**	**1.81**	**0.91**
SD0936	BGY	QL	1.77	3.54	11.21	0.00	0.59	0.00	1.70	0.85
SD0936	GGO	QL	9.77	4.11	7.20	2.57	0.51	4.63	1.95	0.97
SD0936	WGW	QL	0.00	0.00	4.10	0.59	0.00	1.17	1.71	0.85
SD0936	WYG	QL	4.67	4.67	9.33	0.00	4.67	0.00	0.64	0.32
SD0936	**Mean**	**QL**	**4.05**	**3.08**	**7.96**	**0.79**	**1.44**	**1.45**	**1.50**	**0.75**
SD0945	BGO	QL	1.01	0.00	6.04	0.00	6.04	4.03	0.99	0.50
SD0945	**Mean**	**QL**	**1.01**	**0.00**	**6.04**	**0.00**	**6.04**	**4.03**	**0.99**	**0.50**
SD0947	WYG	QL	0.53	0.00	11.19	0.53	2.66	1.60	1.88	0.94
SD0947	**Mean**	**QL**	**0.53**	**0.00**	**11.19**	**0.53**	**2.66**	**1.60**	**1.88**	**0.94**
SD0955	GYB	QL	0.00	0.00	7.10	0.00	5.47	1.09	1.83	0.91
SD0955	**Mean**	**QL**	**0.00**	**0.00**	**7.10**	**0.00**	**5.47**	**1.09**	**1.83**	**0.91**
SD0962	OYB	QL	14.45	4.64	5.16	0.00	2.58	3.10	1.94	0.97
SD0962	**Mean**	**QL**	**14.45**	**4.64**	**5.16**	**0.00**	**2.58**	**3.10**	**1.94**	**0.97**
SD0977	GRY	QL	0.00	0.00	20.78	0.90	1.81	5.42	1.11	0.55
SD0977	**Mean**	**QL**	**0.00**	**0.00**	**20.78**	**0.90**	**1.81**	**5.42**	**1.11**	**0.55**

Chapter 5

Chapter 5: Worker policing in the multiple-queen ant *Leptothorax acervorum*: the effects of maternal caste, colony social structure and egg age

Abstract

Studies of worker policing have played a vital role in developing our understanding of inclusive fitness theory and worker sterility in the eusocial Hymenoptera. However, there are still important aspects of worker policing that remain little explored, such as the effect of polygyny (multiple-queens) and egg age on worker policing, and the specificity of the signals used by workers to discriminate between eggs. In this study I aim to improve our understanding of these aspects of worker policing by testing the following three hypotheses using the ant *Leptothorax acervorum*: (1) that workers use a generic egg-marking signal to discriminate against worker-laid eggs in favour of queen-laid eggs, (2) that colony social structure (monogyny (single-queen) vs. polygyny) affects the expression of worker policing and (3) that egg age affects the expression of worker policing. I tested these hypotheses by introducing non-nestmate queen- and worker-laid eggs belonging to two different age classes into monogynous and polygynous colonies and recording the response of workers. The results of the experiment supported only the first hypothesis, with workers being found to favour queen-laid eggs over worker-laid eggs. The fact that workers were able to identify the maternal caste of non-nestmate eggs suggests that *L. acervorum* produce an egg-marking signal that can be detected across colonies. Colony social structure was found not to affect worker policing, with workers from monogynous and polygynous colonies expressing the same level of policing towards worker-laid eggs. Similarly, egg age did not have a significant effect on worker policing, with workers expressing the same level of policing towards young and old worker-laid eggs. These results suggest that *L. acervorum* workers use a generic egg-marking signal to discriminate against worker-laid eggs in favour of queens-laid eggs, but that colony social structure and egg age do not affect the degree to which this discrimination is expressed.

Introduction

In social groups of non-clonal organisms, inclusive fitness theory predicts conflict over reproductive behaviours because different outcomes offer different group-members the greatest indirect fitness benefits (Hamilton 1964b; Bourke & Franks 1995; Ratnieks *et al.* 2006). In the eusocial Hymenoptera (ants, bees and wasps), conflict is predicted both within and between female castes (queens and workers) over which caste should produce male offspring (Hamilton 1964b; Trivers & Hare 1976; Bourke & Franks 1995; Ratnieks *et al.* 2006). Inclusive fitness theory predicts such conflict between reproductive and non-reproductive workers when the two types of worker differ in whether they are more related to queen- or worker-derived males, a factor which is affected by colony social structure (i.e. whether a colony is headed by a single-queen or multiple-queens, and whether the queen(s) is singly or multiply mated) (Ratnieks 1988). For example, in colonies with a social structure that creates low average worker–worker relatedness e.g. polyandrous colonies (those containing a multiply-mated queen), non-reproductive workers are more related to the queen's sons than workers' sons (and should therefore prefer to rear queen-produced males), where as reproductive workers are more related to their own sons than the queen's sons (and should therefore prefer to rear worker-produced males). However, in colonies with a social structure that creates high worker–worker relatedness i.e. monogynous/monandrous colonies (those containing a single queen who is singly-mated), both reproductive and non-reproductive workers are more related to the workers' sons than the queen's sons, and so there should be no conflict between workers over which males to rear. Therefore, according to inclusive fitness theory, the social structure of a colony should influence the level of conflict between nestmate workers over male production (Ratnieks 1988).

As a consequence of the conflicts described, workers are predicted to try to prevent the successful reproduction of their nestmate workers in colonies with social structures that reduce average worker–worker relatedness (Ratnieks 1988). Such 'policing' of worker reproduction is a well recorded phenomenon, with workers either selectively rejecting (destroying or neglecting) worker-laid eggs or exhibiting physical aggression towards reproductive workers (e.g. egg rejection: Ratnieks & Visscher 1989; Visscher 1996; Foster & Ratnieks 2000, 2001a; Pirk *et al.* 2003; D'Ettorre *et al.* 2004; Wenseleers *et al.* 2005a, b; Bonckaert *et al.* 2008; Dijkstra *et al.* 2010; Meunier *et al.* 2010; Bonckaert *et* *al.* 2011a, physical aggression: Gobin *et al.* 1999; Kikuta & Tsuji 1999; Iwanishi *et al.* 2003; Wenseleers *et al.* 2005b). Worker policing has been found to occur both irrespective of and in correlation with social structure, depending on the study species (e.g. irrespective: Kikuta & Tsuji 1999; Foster & Ratnieks 2001a; Iwanishi *et al.* 2003; Pirk *et al.* 2003; Hammond & Keller 2004; Wenseleers *et al.* 2005b; Wenseleers & Ratnieks 2006; Meunier *et al.* 2010; Bonckaert *et al.* 2011a, correlated: Ratnieks & Visscher 1989; Foster & Ratnieks 2000; Wenseleers *et al.* 2005a; Wenseleers & Ratnieks 2006; Bonckaert *et al.* 2008; Dijkstra *et al.* 2010). However, few studies have tested the link between social structure and policing by directly observing worker policing in species that have a facultative social structure (Foster & Ratnieks 2000; D'Ettorre *et al.* 2004; Meunier *et al.* 2010; Bonckaert *et al.* 2011b). The benefit of such studies is that they control for between-species variation whilst confirming the presence of worker policing (which can otherwise be difficult to distinguish from worker self-restraint (e.g. Hammond *et al.* 2003)). Furthermore, the majority of studies investigating the link between worker policing and social structure have focused upon monogynous colonies. By contrast, the effect of polygyny (multiple-queens in the same colony) on worker policing remains little explored (D'Ettorre *et al.* 2004; Meunier *et al.* 2010). The extent to which policing occurs in polygynous colonies should depend on how related the queens are and how many queens there are (Pamilo 1991b). In general, if worker policing is linked to social structure, policing should occur in colonies with many, highly related queens, and not in colonies with few, marginally related queens (Pamilo 1991b).

In order to police via selective rejection of worker-laid eggs, workers must be able to detect the maternal caste of an egg. The means by which they do so is thought to be a chemical cue produced by the queen and deposited on her eggs (Ratnieks 1988, 1995; Ratnieks & Visscher 1989), although the exact identity of this chemical signal remains unknown (Katzav-Gozansky *et al.* 2001; Martin *et al.* 2002b; Endler *et al.* 2004; van Zweden *et al.* 2009). A question surrounding the queen egg-marking signal is whether the chemical is colony-specific or if it can override nestmate recognition systems to operate across colonies (Helanterä & Sundström 2007). The majority of policing studies involving the use of non-nestmate eggs suggest there is a generic queen-signal which works across conspecific colonies (Ratnieks & Visscher 1989; Foster *et al.* 2002; D'Ettorre *et al.* 2004; Endler *et al.* 2004; Bonckaert *et al.* 2008), and even subspecies

(Martin *et al.* 2002a; Pirk *et al.* 2003). However, some studies have shown that queen-laid eggs are not recognised across conspecific colonies (Helanterä & Sundström 2007), or at least not within monogynous colonies (Meunier *et al.* 2010). In order to improve our understanding of the queen egg-marking signal, more species need to be studied (Helanterä & Sundström 2007).

One aspect of worker policing which has not been empirically tested is the effect of the age of worker-laid eggs on policing. Previous studies have shown that the majority of worker-laid eggs are policed less than a day after appearing in the colony, whilst just a few survive beyond this time (Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996; Wenseleers *et al.* 2005b). Furthermore, although worker-laid eggs are thought to be policed at a fairly constant rate during the early stages of their development (e.g. during the first 16h after being laid in the case of *Apis mellifera* worker eggs), the overall rate of policing of worker-laid eggs is thought to decrease by the end of the development period (e.g. by the end of the approximate 72h development period in the case of *Apis mellifera* worker-laid eggs) (Ratnieks 1993; Visscher 1996). However, studies have not tested whether this pattern is due to an effect of age, i.e. if worker-laid eggs become less susceptible to policing with age and some eggs, by chance, reach the age above which they are immune (Visscher 1996; Wenseleers *et al.* 2005b). If age does affect the probability of an egg being policed then studies investigating the extent of worker reproduction or worker-policing need to account for this effect. A study by Hammond *et al.* (2003) can be used to explain why. The aim of the study was to test for an effect of social structure on the extent of worker reproduction in the ant *Leptothorax acervorum*. The results showed that an equally small percentage of haploid eggs were present in monogynous and polygynous colonies (suggesting social structure has no effect on worker reproduction), and these percentages did not change between the egg and adult stage (suggesting a lack of policing of worker-laid eggs). The authors concluded that, given the lack of policing, *L. acervorum* workers express a high level of reproductive self-restraint (i.e. rarely attempt to lay eggs) across both social structures. However, the sample of eggs tested for maternity in the experiment may have contained mostly old eggs. Therefore, if policing were to be predominantly directed towards young eggs, the true extent of worker reproduction and policing would not have been detected (Hammond *et al.* 2003). Hence worker policing cannot be ruled out in this species, and in fact has been observed at a low level in colonies of *L. acervorum* in which the queen is absent (Chapter 4).

In the following study I investigated three, non-mutually exclusive hypotheses concerning worker policing in the ant *L. acervorum*. I did so by introducing non-nestmate queen- and worker-laid eggs into conspecific colonies, a technique that has been used in a number of past experiments investigating policing behaviour (D'Ettorre *et al.* 2004, 2006; Meunier *et al.* 2010). First I tested the hypothesis that queen-laid eggs have a generic signal that can be detected across conspecific colonies, and that workers use this signal to discriminate against worker-laid eggs in favour of queen-laid eggs (the queen signal hypothesis). If the queen signal hypothesis is correct, workers should favour non-nestmate queen-laid eggs over non-nestmate worker-laid eggs. Second I tested the hypothesis that worker policing is influenced by social structure (the relatedness hypothesis) by comparing levels of policing in monogynous and polygynous colonies. *L. acervorum* is an excellent species with which to test the effect of social structure because monogynous and polygynous colonies can be compared within the same facultatively polygynous population. Furthermore, average worker–worker relatedness is known to vary with social structure in *L. acervorum*, with workers in monogynous colonies being more related on average to their nestmate workers (r=0.71) than to their nestmate queens (r=0.42), and workers in polygynous colonies being equally related on average to their nestmate workers (r=0.28) and their nestmate queens (r=0.27, based on colonies with a range of 2−8 queens) (Hammond *et al.* 2003). The fact that workers are equally or more related to their nestmate workers than to their nestmate queens under both social structure means that worker policing is not predicted to occur in monogynous or polygynous colonies based on relatedness values alone (Hammond *et al.* 2003). However, should policing occur in *L. acervorum* for reasons such as the mitigation of colony-level costs associated with worker reproduction, then relatedness may affect the extent of the worker policing expressed, with workers in polygynous colonies predicted to express higher levels of policing towards worker-laid eggs (compared to queen-laid eggs) than workers within monogynous colonies. Third I tested the hypothesis that worker-laid eggs become less susceptible to policing with age (the egg age hypothesis) by introducing non-nestmate eggs of two different ages classes to *L. acervorum* colonies. If the egg age hypothesis is correct, workers should police worker-laid eggs belonging to the younger age class more heavily than those belonging to the older age class.

Method

Collection of colonies

L. acervorum colonies were collected from Santon Downham in Thetford Forest, Norfolk, UK (following methods in Chapter 2), in May–June 2010. Between 20–50% of colonies in this population contain multiple, related queens (means of 2–5 queens per colony), and almost all queens (95%) are singly mated (Heinze *et al.* 1995a; Bourke *et al.* 1997; Chan *et al.* 1999; Hammond *et al.* 2001, 2002, 2003, 2006). Colonies were taken to the laboratory and transferred to artificial nests (two microscope-slides separated by a wall of card, internal cavity 64.0×39.0×1.5mm) within three days after collection. Nests were kept in foraging arenas (10×10×2cm Petri dishes with walls coated with polytetrafluoroethylene (Sigma Aldrich)) inside an incubator on a 14h day (23°C, light)/10h night (13°C, dark) cycle. Thirty-eight queenright colonies (i.e. with queens) with >20 workers each were selected for the experiment. These 38 colonies (27 monogynous, 11 polygynous) were divided into 'source' colonies (those colonies that were to donate eggs) and 'discriminator' colonies (those colonies that were to receive eggs) (Table 1). All 11 polygynous colonies were designated as discriminator colonies, as well as 11 monogynous colonies selected to match the polygynous colonies approximately in size (the number of adult workers) (mean colony size: monogynous = 168 workers, polygynous = 149 workers). The remaining 16 monogynous colonies were designated as source colonies. The reason why only monogynous colonies were used as source colonies was to control for social structure amongst donated eggs. As later confirmed by dissection, monogyny was defined by the presence of a single ovary-active queen and polygyny by the presence of more than one such queen (Table 1). A census taken early on in the experiment revealed that the majority of colonies (63% of source colonies, 82% of monogynous discriminator colonies and 82% of polygynous discriminator colonies) contained at least one alate adult (gyne or male) or sexual pupa, suggesting most colonies were in their reproductive stage.

Sampling of queen- and worker-laid eggs from source colonies

In order to obtain queen- and worker-laid eggs, each source colony was split into two halves 11–25 days after the last colony collections. The two halves were housed in nests within separate foraging arenas. One half received the colony queen and was designated the queenright half and the other half thereby became queenless and was designated the queenless half. Workers and brood (other than eggs) from each source colony were split approximately evenly between the two halves. All eggs and alates (gynes and males) present at the time of splitting were removed. Alates that eclosed later during the experiment were removed as often as possible (whenever the nest was opened up to remove eggs or whenever an alate was observed outside the nest), as were any newly eclosed gynes that had time to drop their wings before removal. Almost all eggs sampled from the queenright halves after splitting would have been queen-laid eggs, since *L. acervorum* workers lay very few eggs in queenright conditions (Hammond *et al.* 2003).

The queenless halves of four of the 16 colonies originally designated as source colonies produced very few eggs, so these colonies were not used to donate any eggs (either queen- or worker-derived). A given source colony was only used as an egg donor when both its queenright and queenless halves were producing sufficient numbers of eggs.

Sampling eggs of different age classes

Eggs of different ages were obtained by sampling eggs less than one day old (<1d) and eggs less than four days old (<4d). I chose <1d as the younger class because previous studies have shown the majority of worker-laid eggs are policed within a day of appearing in a colony (Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996; Wenseleers *et al.* 2005b). I chose <4d as the older class because it provided as large a gap as possible between age classes whilst allowing sufficient egg sampling bouts to be conducted during the experiment.

To sample eggs from a given source colony, both halves (queenless and queenright) were cleared of all eggs and started on a cycle to produce <1d and <4d eggs alternately (half the colonies began with <1d and half started with <4d). Each source colony half was used to produce eggs of both age classes in order to control for any natural variation in policing susceptibility between colonies. To obtain <1d eggs, all eggs were removed (and discarded) from a colony half after 1600h on a given day and, on the following day (between 1000h and 1600h), eggs appearing in the colony half were themselves removed and used for the trials. To obtain <4d eggs, an identical procedure was followed except that eggs appearing in the colony half were removed and used in trials on the fourth day following the first removal of eggs. Note that this method meant that the <4d class could have included some <1d eggs. However, the two age classes will have differed in mean age. Towards the end of the experiment the source colonies decreased their egg-production, and hence it was not possible to maintain the strict rotation of age classes in each colony. Instead some colonies (involved in 22% of all trials) were consistently used to produce <1d eggs or <4d eggs as and when one age class was in short supply.

Egg introduction trials

Egg-introduction trials were performed over a period of 56 days, starting 31 days after the last colony collections. In total each of the 22 discriminator colonies received one or two of each of the four combinations of egg (queen-laid/<1d, worker-laid/<1d, queen-laid/<4d, worker-laid/<4d) (Fig. 1), receiving between five and seven eggs in total over the course of the experiment. Each source colony was used to donate eggs to multiple discriminator colonies, and each discriminator colony received eggs from multiple source colonies. Source colonies were used as equally as possible, although the most productive colonies were used more often than others in order to maximise the sample size. Each discriminator colony was only used once in the same day and given at least one day without trials between successive trials.

At the start of each egg-introduction trial the discriminator colony was given five minutes to adjust to conditions under the microscope. An egg was then removed from a source colony and placed 5mm in front of the discriminator colony's nest entrance. Eggs were placed in front of the entrance rather than inside the nest to avoid having to cause severe disruption to the discriminator colony by opening up the nest. A timer was started and I recorded if and when the egg was detected (i.e. actively picked up by a worker). If an egg was not detected within 15 minutes of being placed in front of the nest then the egg was removed and the trial was abandoned (this occurred eight times out of 151 trials). If an egg was detected within 15 minutes then the trial continued and I recorded the workers' response to the egg in terms of the following three measures of policing:

(1) Nest entry: whether an egg was taken into the nest or not. Eggs that were not taken into the nest were instead 'discarded' (defined as left in one place outside the nest for a continuous five minutes), 'destroyed' (defined as eaten or punctured by a worker), or 'carried' (defined as detected but not discarded or destroyed within 15 minutes after initial detection). Eggs that were initially taken into the nest but later discarded in the arena were still classified as taken into the nest (n=2).
(2) Latency to nest entry: the time in seconds between an egg being detected and taken into the nest (this measure was only relevant to eggs that were taken into the nest).
(3) Nest survival: whether an egg survived or did not survive after being taken into the nest (again this measure was only relevant to eggs that were taken into the nest). Eggs were classified as having survived in the nest if they were either 'accepted' (defined as left in one place in the nest for a continuous five minutes) or carried (defined above). Eggs were classified as having not survived in the nest if they were either destroyed (defined above) or removed from the nest and discarded (defined above).

Eggs that were discarded or punctured (i.e. destroyed but not eaten) and left in the arena at the end of the trial were removed. A trial ended once an egg was accepted, discarded or destroyed within 15 minutes following initial detection, or if an egg was carried for 15 minutes following initial detection. Six longer trials showed that the fate of an egg at the end of 15 minutes was generally a good indication of the fate of an egg after a longer time period (a total of 45 minutes). If a queen (dealate or alate) rather than a worker took an egg into the nest or determined its final fate (whether it was accepted, destroyed or discarded) then the trial was not included in the data set (n=2). Trials where queens picked-up an egg for a period of time but did not make the final decision to accept, destroy or discard the egg, or take it into the nest, were included in the data set (n=3).

Ovarian dissections

On the day following the final egg-introduction trial all colonies (source and discriminator) were frozen at -20°C. To confirm the social structure of colonies, all dealate queens were dissected. The ovaries were removed and examined under a compound microscope to determine mating status and ovary activation (Bourke 1991). A dealate queen was classified as mated if her sperm receptacle contained sperm. A dealate queen was classified as having active ovaries if her mean ovariole length was ≥2.9mm (the shortest length found in any queen that had both corpora lutea in her ovaries and sperm in her sperm receptacle in this experiment). Mean ovariole length was taken by measuring the central ovariole of each of a queen's two ovaries (using the software package Auto-montage (Synoptics Ltd)) and taking the mean value.

Statistical analyses

Generalised linear mixed-effects models (GLMMs) were used to test the effect of maternal caste, social structure and egg age on three measures of worker policing. The response variable in each of the three GLMMs was as follows:

(1) Nest entry: a binary measure of whether an egg was taken into the nest or not.

(2) Latency to nest entry: the time in seconds between an egg being detected and taken into the nest (eggs that were not taken into the nest were excluded from this dataset). A reciprocal square-root transformation was used on the latency dataset to normalise the distribution of the residuals.

(3) Nest survival: a binary measure of whether an egg survived or did not survive once inside the nest (again, eggs that were not taken into the nest were excluded from this dataset). Five eggs that were taken into the nest could not be included in this test because their final fate was not recorded.

The explanatory variables (fixed effects) were the same for all three GLMMs: (a) maternal caste (whether an egg was queen- or worker-laid), (b) social structure (whether the discriminator colony was polygynous or monogynous) and (c) egg age (whether an egg was <1d or <4d old). The random effects were also the same for all three GLMMs: (a) discriminator colony identity, (b) source colony identity, (c) discriminator colony size (the number of adult workers present at the time of freezing) and (d) day of the trial (the date on which the trial was performed). Discriminator colony identity and source colony identity were included as random effects to control for the repeated use of colonies. Discriminator colony size was included to control for variation in size between colonies (colony size could potentially affect policing behaviour (Bourke 1999)), and the day of the trial was included to control for any variation in response to eggs over time. A binomial distribution with a logit link function was specified for the GLMMs testing nest entry and nest survival, and the default Gaussian distribution was used for the GLMM testing latency to nest entry.

All fixed and random effects were fitted in the first model of each GLMM. Fixed factors were then removed one by one to obtain the minimal adequate model. Fixed factors were removed in the order of least significance (starting with the interaction terms) using likelihood ratios tests to compare models with and without the factor of interest. Factors that could not be removed without causing a significant change in log likelihood ratio were kept in the model. Random effects were not removed. GLMMs were run using the 'lmer' package (part of the 'lme4' library) in the statistical software program *R* version 2.12.0 (R Development Core Team 2010). The *p*-value shown for each variable is that obtained upon the removal of the variable from the GLMM. Significance is reported on the basis of $\alpha = 0.05$.

Results

Queen signal hypothesis

The maternal caste of an egg had a significant effect on workers' treatment of eggs: queen-laid eggs were significantly more likely to be taken into the nest than worker-laid eggs (nest entry GLMM: $p<0.001$, estimated difference in log odds (logit) = 1.65 ± 0.41 standard error) (Table 2, Fig. 2a), and queen-laid eggs were also taken into the nest significantly sooner than worker-laid eggs (latency to nest entry GLMM: $p=0.0476$, estimated difference in reciprocal square-root means = 0.07 ± 0.04 standard error) (Table 2, Fig. 2b). There was no significant effect of maternal caste on the likelihood of an egg surviving in the nest, although there was a trend for the proportion of eggs that survived in the nest to be higher for queen-laid than for worker-laid eggs (nest survival GLMM: $p=0.245$) (Table 2, Fig. 2c). These results suggest the presence of a queen egg-marking signal that is effective across colonies.

Relatedness hypothesis

The social structure of discriminator colonies had no significant effect on the likelihood of an egg being taken into the nest (nest entry GLMM: $p=0.537$) (Table 2, Fig. 3a), the latency to an egg being taken into the nest (latency to nest entry GLMM: *p*=0.418) (Table 2, Fig. 3d), or the likelihood of an egg surviving in the nest (egg survival GLMM: *p*=0.519) (Table 2, Fig. 3g). This was the case for both queen- and worker-laid eggs (GLMM social structure/maternal caste interaction term: nest entry *p*=0.108 (Table 2, Fig. 3b,c), latency to nest entry *p*=0.192 (Table 2, Fig. 3e,f), nest survival *p*=0.142 (Table 2, Fig. 3h,i)), although the fact that so few worker-laid eggs were taken into the nest meant that the GLMMs for nest survival and latency to nest entry lacked statistical power in testing for an interaction between colony social structure and the maternal caste of eggs. Overall these results suggest that workers within polygynous colonies do not express a higher level of policing behavior towards worker-laid eggs compared to queen-laid eggs than those within monogynous colonies.

Egg age hypothesis

Egg age (<1d or <4d old) had no significant effect on the likelihood of an egg being taken into the nest (nest entry GLMM: *p*=0.354) (Table 2, Fig. 4a), the latency to an egg being taken into the nest (latency to nest entry GLMM: *p*=0.263) (Table 2, Fig. 4d), or the likelihood of an egg surviving in the nest (egg survival GLMM: *p*=0.747) (Table 2, Fig. 4g). Furthermore, the effect of egg age on nest entry, latency to nest entry and nest survival was not significantly affected by the maternal caste of an egg (GLMM egg age/maternal caste interaction term: nest entry *p*=0.226 (Table 2, Fig. 4b,c), latency to nest entry *p*=0.179 (Table 2, Fig. 4e,f), nest survival *p*=0.581 (Table 2, Fig. 4h,i)), although the fact that so few worker-laid eggs were taken into the nest meant that the GLMMs for nest survival and latency to nest entry lacked statistical power in testing for an interaction between egg age and maternal caste. Overall the results provide no evidence to suggest that worker-laid eggs <1d old are more susceptible to policing than worker-laid eggs <4d old.

Discussion

This study tested for effects of three, non-mutually exclusive factors on the likelihood of eggs being policed by workers of the ant *L. acervorum*, namely maternal caste of eggs, social structure of discriminator colonies and age of eggs. The results showed that the only significant predictor of whether eggs were policed was maternal caste of eggs, with queen-laid eggs being more likely to be accepted than worker-laid eggs (significant for two of three measures of acceptance). By contrast, polygynous colonies did not police worker-laid eggs significantly more than monogynous colonies, and young worker-laid eggs were not policed significantly more than older worker-laid eggs.

Queen signal hypothesis

Overall, given that workers selectively rejected worker-laid eggs in two out of the three measures of acceptance (and expressed a trend for doing so in the third measure), these results strongly suggest that *L. acervorum* workers have evolved to police worker-laid eggs. *L. acervorum* can therefore be added to the growing list of eusocial Hymenoptera that have been found to express worker policing in the form of egg-rejection (e.g. Ratnieks & Visscher 1989; Visscher 1996; Foster & Ratnieks 2000, 2001a; Pirk *et al.* 2003; D'Ettorre *et al.* 2004; Wenseleers *et al.* 2005a, b; Bonckaert *et al.* 2008; Dijkstra *et al.* 2010; Meunier *et al.* 2010). Given that workers were found to distinguish between queen- and worker-laid eggs, the results suggest the presence of an egg-marking signal on the surface of the eggs indicating their maternal caste. In general, such egg-marking signals are thought to be produced by the queen (Ratnieks 1988, 1995; Ratnieks & Visscher 1989). Furthermore, given the use of non-nestmate eggs in this study, the results also suggest that the hypothetical queen egg-marking signal is informative to workers even across colonies, as has been found in some species of eusocial Hymenoptera (Ratnieks & Visscher 1989; Foster *et al.* 2002; D'Ettorre *et al.* 2004; Endler *et al.* 2004; Bonckaert *et al.* 2008) but not others (Helanterä & Sundström 2007; Meunier *et al.* 2010).

An alternative interpretation of these results is that workers discriminated between eggs based not on their maternal caste per se but on their ploidy. The worker-laid eggs in this study would have been 100% haploid whereas the queen-laid eggs would have been approximately 16% haploid and 84% diploid (Hammond *et al.* 2002). However, there is no reason to expect workers to discriminate against haploid eggs in favour of diploid eggs unless they are trying to achieve a female bias in the sex-ratio of the colony's reproductive brood. Only workers in monogynous colonies of *L. acervorum* favour a female-bias in the reproductive brood, and they are known to achieve such a bias by increasing the ratio of queens to workers reared from diploid eggs, not by destroying haploid eggs (Hammond *et al.* 2002). Given that workers in both monogynous and polygynous colonies in the current experiment expressed an equal level of aggression towards worker-laid eggs, and that the destruction of eggs is not the usual method of sex-ratio control in *L. acervorum*, it seems unlikely that the results of this experiment occurred as a result of workers favouring diploid eggs over haploid eggs.

Relatedness hypothesis

The social structure of a colony was found to have no significant effect on the level of policing received by worker-laid eggs (and queen-laid eggs), although there was a trend for workers in polygynous colonies to accept fewer worker-laid eggs into the nest than monogynous colonies (as was the predicted direction). The absence of an effect of social structure was found consistently across all three measures of policing. The results of this study are therefore in agreement with the findings of Hammond *et al*. (2003), who showed social structure to have no effect on the frequency of worker-produced male eggs or adult males in *L. acervorum*.

The findings of other studies that have performed intraspecific investigations of the effect of colony social structure on worker policing are mixed. In the facultatively polygynous ant species *Pachycondyla inversa*, worker–worker relatedness is higher on average in monogynous colonies than polygynous colonies (Kellner *et al.* 2007), and workers have been found to express significantly lower levels of policing towards worker-laid eggs in monogynous than polygynous colonies, showing colony social structure to effect the level of worker-policing expressed (D'Ettorre *et al.* 2004). In contrast, in the facultatively polygynous ant species *Formica selysi* in which worker–worker relatedness is higher in monogynous colonies than polygynous colonies (Chapuisat *et al.* 2004), colony social structure has been found to have no effect on the level of policing expressed towards worker-laid eggs when compared to queen-laid eggs (Meunier *et al.* 2010). The results of intraspecific studies investigating the link between colony social structure and policing have even been found to vary when focused on the same species. In a British population of the facultatively polyandrous wasp *Dolchiovespula saxonica*, worker–worker relatedness was found to be positively correlated with the percentage of worker-produced males, a pattern caused by higher levels of worker policing in polyandrous than monandrous colonies (Foster & Ratnieks 2000). However, in a Danish population of the same species of wasp, worker–worker relatedness was found to have no effect on worker reproduction, and estimated levels of policing did not vary with queen mating frequency (Bonckaert *et al.* 2011b).

Given that social structure appears to have no significant effect on worker policing in *L. acervorum*, it is possible that policing is driven by colony-level costs associated with worker reproduction, rather than the relatedness differences of workers to queen- and worker-derived males (Cole 1986; Ratnieks 1988). Costs associated with worker reproduction could include (a) the production of more sexual offspring or general brood than the colony can rear (Hartmann *et al.* 2003; Wenseleers *et al.* 2004b), (b) a male biased sex-ratio (Foster & Ratnieks 2001b) (although as discussed above *L. acervorum* do not control sex-ratio via egg cannibalism (Hammond *et al.* 2002)), and (c) a change in behaviour that causes reproductive workers to spend less time participating in essential work tasks (Cole 1986; Ratnieks 1988). Policing via egg-rejection could be an effective mechanism in preventing colony-level costs associated with worker-reproduction, especially if the risk of policing results in workers expressing reproductive self-restraint. Efficient policing is expected to select for self-restraint because it reduces the probability of workers gaining direct fitness from their attempts at reproduction, leaving workers only to suffer the costs of their reproduction along with the rest of the colony (Ratnieks 1988; Wenseleers *et al.* 2004a).

Egg age hypothesis

The results of the current study do not support the egg age hypothesis: worker-laid eggs were policed at a constant level regardless of whether they were less than one day old or less than four days old, suggesting worker-laid eggs do not become less susceptible to policing with age. To my knowledge this is the first study to directly test whether the age of worker-laid eggs has an effect on worker policing. The only other circumstance that I am aware of egg age being investigated under is that of the acceptance of queen-laid eggs into non-nestmate, queenless, honey bee (*Apis mellifera*) colonies (Gabka *et al.* 2011). In a study by Gabka *et al.* (2011), egg age was found to influence the percentage of queen-laid eggs accepted into queenless honey bee colonies (the percentage of eggs accepted into colonies increased with egg age), depending on the length of time the colony had been queenless and the presence or absence of brood in open cells.

One possible limitation of the current study is that it only tested the effect of egg age on policing using worker-laid eggs reared apart from queen-laid eggs. Therefore, although the study informs us that worker-laid eggs do not lose a worker-specific signal with age, it does not tell us whether worker-laid eggs gain a queen-specific signal with age under natural conditions, as we might predict to occur in colonies where queen- and worker-laid eggs share an egg-pile (Monnin & Ratnieks 2001; D'Ettorre *et al.* 2006). However, there is little evidence to suggest that chemical cues can be transferred between queen- and worker-laid ants' eggs, or that contact with queen-laid eggs makes worker-laid eggs less susceptible to policing (D'Ettorre *et al.* 2006). Therefore there is no reason to expect workers to treat eggs that have been taken from a pile containing only worker-laid eggs differently to those taken from a pile containing both queen- and worker-laid eggs. As well as suggesting worker-laid eggs do not become more queen-like with age, the results of this study also suggest that workers are not less selective when assessing older eggs (Visscher 1996), so workers do not, for example, use egg-age as a proxy for maternal caste.

The egg age aspect of this study also plays an important role in interpreting the results of the study by Hammond et al (2003) described above (see 'Introduction') and furthering our understanding of worker reproduction in *L. acervorum*. The results of the study by Hammond *et al*. (2003) showed that an equally small percentage of both male eggs (3.2−4.2%) and male adults (2.3−4.6%) are worker-derived in queenright *L. acervorum* colonies. The authors concluded that *L. acervorum* workers express a high level of reproductive self-restraint, but they could not rule out the possibility of policing because their sample of male eggs may have largely consisted of older eggs, and hence, had policing predominantly occurred when eggs were young, the study would have underestimated the true level of worker reproduction and policing. The current study shows, however, that egg age does not affect worker policing in *L. acervorum*, and hence the level of worker reproduction estimated by Hammond et al. (2003) can be considered fairly reliable. Therefore it appears that *L. acervorum* workers express a high level of self-restraint in natural queenright colonies.

Reproductive self-restraint is predicted to evolve when the indirect fitness costs (i.e. the colony-level costs) of worker reproduction outweigh the direct fitness benefits (Cole 1986). Policing is thought to contribute to the evolution of self-restraint by reducing the direct fitness benefits gained by workers through their attempts at reproduction (Ratnieks 1988). Given the apparent reproductive self-restraint expressed by *L. acervorum* workers in queenright colonies (Hammond *et al.* 2003), there should be little need for worker policing to occur. However, this study shows that *L. acervorum* workers have the ability to discriminate against worker-laid eggs in favour of queen-laid eggs. One reason for this coexistence of self-restraint and policing ability could be that policing evolved at a time in the past when worker reproduction was more prevalent in *L. acervorum*, as has been suggested for a clonal species of ant that expresses both self-restraint and the ability to police (Hartmann *et al.* 2003). Furthermore, policing is likely to be retained even in a population where the evolution of worker self-restraint has reduced the selection pressure on worker policing, because a drop in policing should encourage outbreaks of worker reproduction, which in turn would renew the selective advantage of policing (Ratnieks 1988). Worker policing could also play a role in queenless *L. acervorum* colonies, where there is some evidence to suggest that a small number of reproductive workers use egg-eating behaviour to dominate other reproductive workers, in which case workers would still need to be able to distinguish newly-laid workers' eggs from the leftover queen-laid eggs in the colony (Chapter 4).

Tables

Table 1. The number of dealate queens with and without active ovaries and the number of workers in the discriminator and source *Leptothorax acervorum* colonies. All queens with active ovaries were mated, and all queens without active ovaries were not mated with the exception of those marked with an asterisk (*). Numbers shown are the total numbers present at the end of the egg-introduction trials (with the exception of the dealate queens without active ovaries which were removed from the colonies as and when they were found over the course of the experiment)**.** The four source colonies that did not lay eggs in their queenless halves (and hence were not used in the introduction trials) are not shown.

Discriminator colony	Number dealate queens with active ovaries	Number dealate queens without active ovaries	Number adult workers	Source colony half	Number dealate queens with active ovaries	Number dealate queens without active ovaries	Number adult workers
Monogynous:				Queenright:			
SD106	1	0	66	SD101	1	1	58
SD107	1	3	266	SD104	1	0	58
SD108	1	5*	188	SD105	1	0	62
SD109	1	2	139	SD1011	1	0	52
SD1017	1	0	150	SD1013	1	0	34
SD1020	1	1	251	SD1023	1	1	25
SD1037	1	0	193	SD1040	1	0	29
SD1038	1	1*	243	SD1048	1	0	19
SD1041	1	0	51	SD1049	1	0	32
SD1050	1	0	186	SD1056	1	0	56
SD1055	1	0	120	SD1061	1	0	27
				SD1062	1	0	15
Polygynous:				Queenless:			
SD102	4	0	269	SD101	0	2	71
SD1018	2	2	156	SD104	0	0	42
SD1024	2	2	117	SD105	0	0	64
SD1025	6	0	214	SD1011	0	0	55
SD1030	3	0	88	SD1013	0	0	48
SD1032	2	1	145	SD1023	0	0	24
SD1033	2	0	73	SD1040	0	0	33
SD1039	2	0	98	SD1048	0	0	21
SD1054	4	4	160	SD1049	0	0	37
SD1058	2	0	76	SD1056	0	2	49
SD1060	8	0	244	SD1061	0	0	31
				SD1062	0	0	10

Table 2. The results of the generalised linear mixed models (GLMMs) for the three measures of policing (a-c below) in *Leptothorax acervorum* colonies. The significance values of the results are indicated by: NS (p>0.05), * (p<0.05), ** (p<0.01), *** (p<0.001). The statistics shown are those obtained when each term is removed from the GLMM. The explanatory variables remaining in the minimal model are highlighted in bold.

Explanatory variables	χ^2	df	*P*	Sig.
(a) Nest entry (n=143).				
Egg age:Maternal caste:Social structure	1.02	1	0.312	NS
Egg age:Maternal caste	1.46	1	0.226	NS
Egg age:Social structure	1.68	1	0.195	NS
Maternal caste:Social structure	2.58	1	0.108	NS
Egg age	0.86	1	0.354	NS
Maternal caste	**16.52**	**1**	**<0.001**	***
Social structure	0.38	1	0.537	NS
(b) Latency to nest entry (n=50).				
Egg age:Maternal caste:Social structure	0.06	1	0.800	NS
Egg age:Maternal caste	1.81	1	0.179	NS
Egg age:Social structure	0.01	1	0.912	NS
Maternal caste:Social structure	1.70	1	0.192	NS
Egg age	1.26	1	0.263	NS
Maternal caste	**3.92**	**1**	**0.048**	*
Social structure	0.66	1	0.418	NS
(c) Nest survival (n=45).				
Egg age:Maternal caste:Social structure	0.00	1	1.000	NS
Egg age:Maternal caste	0.30	1	0.581	NS
Egg age:Social structure	0.07	1	0.794	NS
Maternal caste:Social structure	2.15	1	0.142	NS
Egg age	0.10	1	0.747	NS
Maternal caste	1.35	1	0.245	NS
Social structure	0.42	1	0.519	NS

Figures

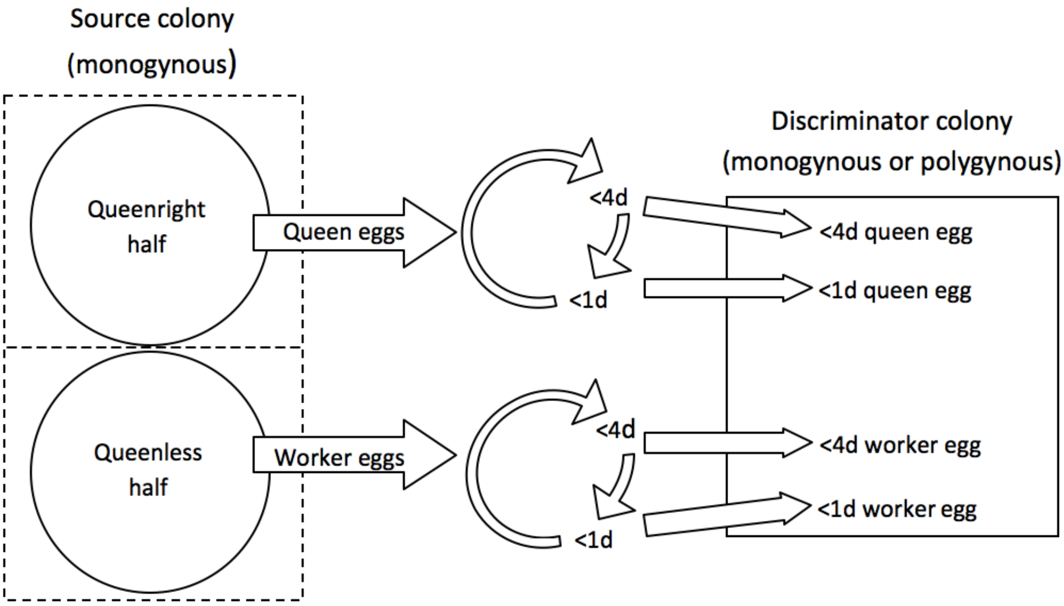

Figure 1. A diagram of the experimental set-up. Each source colony of *Leptothorax acervorum* was split into two halves (one queenright and one queenless) to produce queen- and worker-laid eggs, and eggs were harvested when they were <1d or <4d old in a repeated cycle. Harvested eggs were immediately used in egg-introduction trials during which they were donated to discriminator colonies. Discriminator colonies received between one and two of each of the four types of egg (<4d queen-laid, <1d queen-laid, <4d worker-laid, <1d worker-laid) over the course of the experiment. Each source colony donated eggs to multiple discriminator colonies, and each discriminator colony received eggs from multiple source colonies.

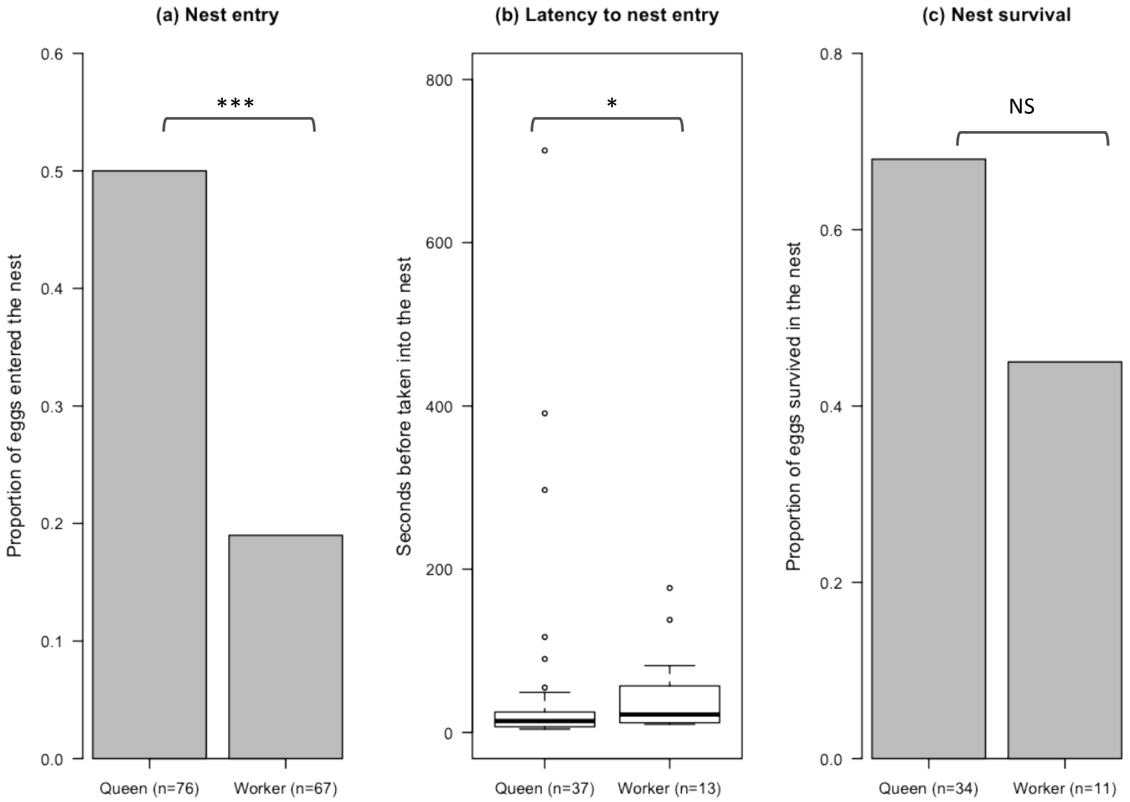

Figure 2. The effect of maternal caste in *Leptothorax acervorum* on (a) the proportion of eggs taken into the nest, (b) the latency to eggs being taken into the nest, and (c) the proportion of eggs to survive in the nest. The highest data point in the latency dataset has not been shown on figure (b) to facilitate diagram interpretation (a queen-laid egg that took 713s to being taken into the nest), but the data point was included in the statistical analyses. The latency to nest entry figure (b) shows untransformed data, but the statistical analysis (GLMM) was performed on transformed (reciprocal square-root) data. Significance values are shown: $p>0.05$ (NS), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). Sample sizes shown in brackets represent numbers of eggs.

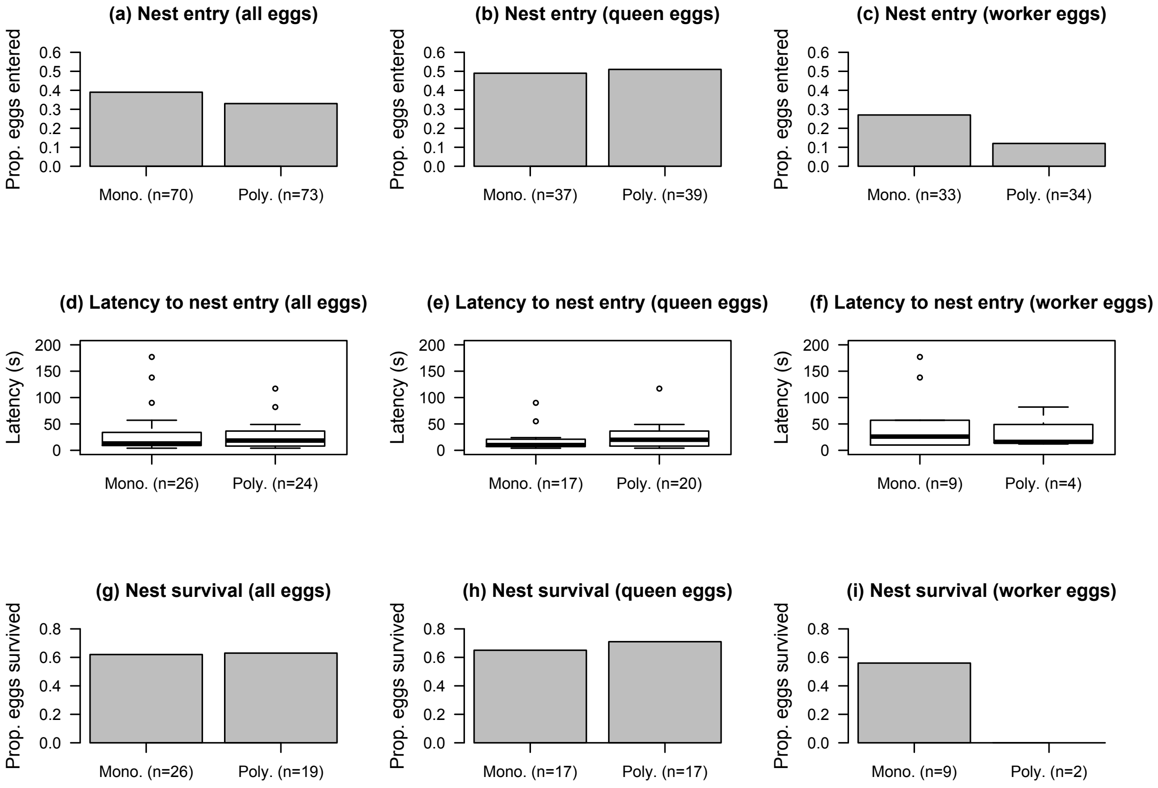

Figure 3. The effect of social structure (whether a colony is monogynous or polygynous) in *Leptothorax acervorum* on (a–c) the proportion of eggs taken into the nest (showing (a) all eggs, (b) queens' eggs and (c) workers' eggs), (d–f) the latency to eggs being taken into the nest (showing (d) all eggs, (e) queens' eggs and (f) workers' eggs), and (g–i) the proportion of eggs to survive in the nest (showing (g) all eggs, (h) queens' eggs and (i) workers' eggs). The highest three data points in the latency dataset have not been shown on figures (g)–(i) to facilitate diagram interpretation (two queen-laid eggs that took polygynous colonies 297s and 713s to take them into the nest, and one queen-laid egg that took a monogynous colony 391s to take it into the nest), but the three data points were included in the statistical analyses. The latency to nest entry figures (d–f) show untransformed data, but the statistical analysis was performed on transformed (reciprocal square-root) data. All differences between monogynous and polygynous colonies in plots (a)–(i) were non-significant. Sample sizes shown in brackets represent numbers of eggs.

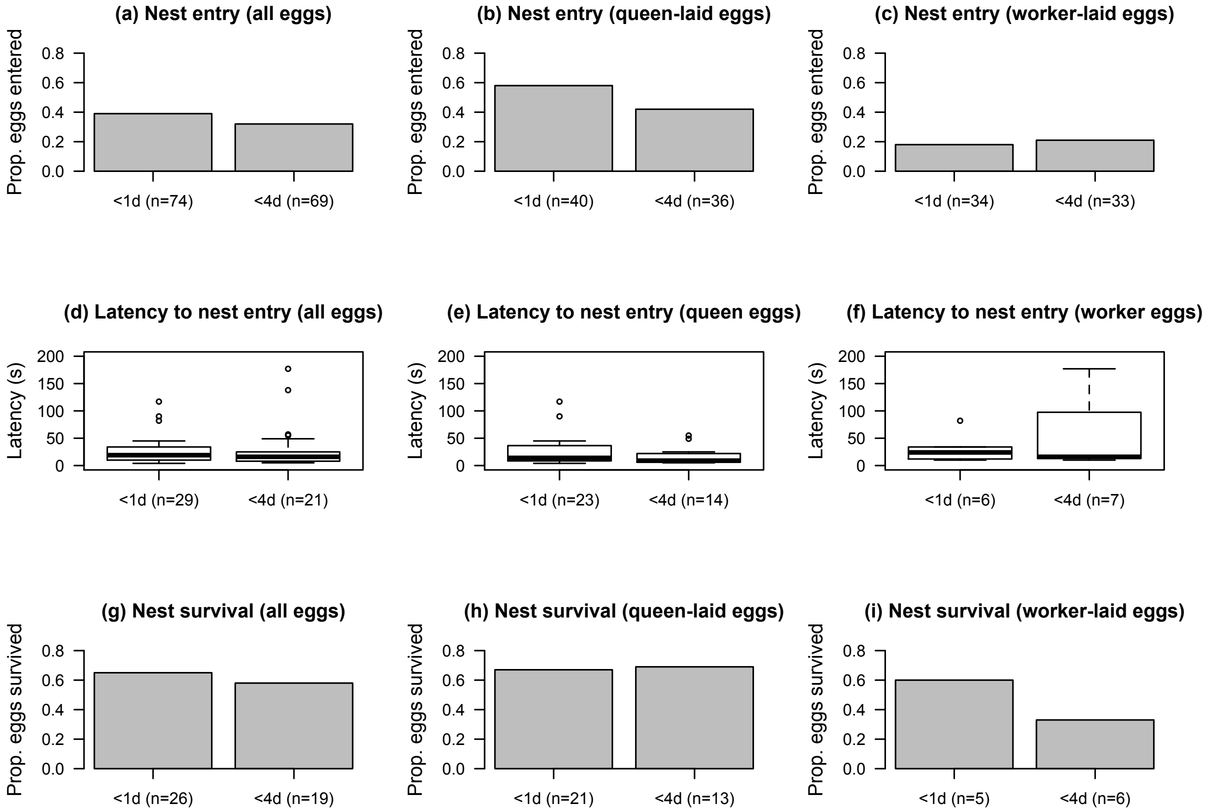

Figure 4. The effect of egg age (whether an egg is <1d or <4d old) in *Leptothorax acervorum* on (a–c) the proportion of eggs taken into the nest (showing (a) all eggs, (b) queens' eggs and (c) workers' eggs), (d–f) the latency to eggs being taken into the nest (showing (d) all eggs, (e) queens' eggs and (f) workers' eggs), and (g–i) the proportion of eggs to survive in the nest (showing (g) all eggs, (h) queens' eggs and (i) workers' eggs). The highest three data points in the latency dataset have not been shown on figures (g)–(i) to facilitate diagram interpretation (three queen-laid eggs <1d old that took 297s, 391s and 713s to be taken into the nest), but the three data points were included in the statistical analyses. The latency to nest entry figures (d–f) show untransformed data, but the statistical analysis was performed on transformed (reciprocal square-root) data. All differences between <1d eggs and <4d eggs in plots (a)–(i) were non-significant. Sample sizes shown in brackets represent numbers of eggs.

Chapter 6

Chapter 6: Conclusion

Experimental and empirical investigations based on predictions of kin conflict in the eusocial Hymenoptera have played a key role in investigating inclusive fitness theory over the past 35 years or so. Such studies have proved essential in both providing evidence for inclusive fitness theory and improving our understanding of social behaviour. However, despite the importance of kin conflict theories and the considerable amount of work that has been invested into testing their predictions, there are still important predictions of kin conflict that have not been tested adequately, and new predictions of kin conflict that have not been tested at all. The overall aim of this thesis has been to test some of the predictions of kin conflict that require further investigation, as well as to test novel predictions of kin conflict arising from inclusive fitness theory. I have done this through the use of four experimental investigations performed using the multiple-queen ant *Leptothorax acervorum*.

An important prediction of kin conflict that I addressed was that of within-colony kin discrimination in the context of individuals discriminating between nestmates based on their genetic lineage (i.e. not their sex or maternal caste) in order to favour their closest relatives during social interactions. Within-colony kin discrimination is in great need of further investigation, because it is a key prediction of inclusive fitness theory, and yet many of the studies used to test the prediction in the past have used unreliable methods, thus leaving the status of the prediction in doubt. Through my own test of within-colony kin discrimination in which I recorded the behaviour of individual *L. acervorum* workers (Chapter 2), I have shown that workers do not perform within-colony kin discrimination whilst antennating, feeding and grooming their nestmate queens. This study provides strong, reliable evidence that within-colony kin discrimination does not occur in this context in the study system, and so adds robust support to the overall conclusion that it does not generally occur in the eusocial Hymenoptera. I hope that in providing such evidence the study will encourage further investigation into the reasons behind why the behaviour is absent or so rare. Inclusive fitness theory predicts that the costs and benefits of a social behaviour are essential to its evolution, as well as the relatedness between actor and recipient (Hamilton 1964a, b). Hence within-colony kin discrimination may be an example of a behaviour where the fitness benefits simply do not outweigh the fitness costs, thus preventing its evolution (Ratnieks & Reeve 1991, 1992). Alternatively the evolution of within-colony kin discrimination may be prevented by information constraints, with members of different genetic lineages expressing too little variation in their chemical recognition cues to be distinguished from one another (Arnold *et al.* 2000; Boomsma *et al.* 2003). Studies have begun to test these hypotheses, but further investigation is required if we are to fully understand the circumstances under which within-group kin discrimination can and cannot evolve. A useful approach could be to compare eusocial insect systems with the few vertebrate systems in which within-group kin discrimination is known to occur (Van Horn *et al.* 2004; Wahaj *et al.* 2004; Silk 2009), and in doing so attempt to identify the key factors that are required for kin discrimination that are present in the vertebrates and not the insects.

In Chapter 3, I describe and test a novel prediction of kin conflict based upon inclusive fitness theory: the prediction that workers should have evolved to prepare for future reproduction when living in colonies that are at a high risk of losing all of their queens. According to inclusive fitness theory, Hymenopteran workers should favour their own direct reproduction over the reproduction of their nestmate queens and workers when it comes to producing the colony's males (Hamilton 1964b; Trivers & Hare 1976; Bourke & Franks 1995; Ratnieks *et al.* 2006). However, in many cases, workers do not achieve successful reproduction in colonies containing a queen (i.e. queenright colonies) and freely reproduce only in colonies without a queen (i.e. queenless colonies) (Bourke 1988b). Based on this information I predicted that workers should have evolved to assess their current risk of queenlessness based on the social structure of their colony (i.e. whether their colony contains a single queen or multiple queens) and to prepare for reproduction in advance when finding themselves in a colony at a high risk of queenlessness (i.e. a single-queen colony). I tested this prediction using a facultatively polygynous (multiple-queen) population of *L. acervorum* and found strong evidence to suggest that workers do prepare for future reproduction as a function of their colony's social structure, exactly as predicted under inclusive fitness theory. To my knowledge this is the first time that a facultative response to social structure in terms of future reproduction under queenless conditions has been proposed. The accuracy of the prediction provides strong evidence in support of inclusive fitness theory, in that it shows that workers express control over pursuing their own inclusive fitness interests, that the reproductive behaviour of workers is strongly influenced by their social environment, and that workers strive to optimise the trade-off between the direct and indirect fitness components of their inclusive fitness. Furthermore, the discovery of a facultative response to social structure in terms of the future reproduction of queenless workers through a study based on inclusive fitness theory demonstrates just how applicable and relevant the theory is to the study of social behaviour. In future, workers within other facultatively polygynous species of eusocial Hymenoptera should be tested for a facultative reproductive response to queen number in order to assess how common the behaviour is.

In addition to driving conflicts based on relatedness asymmetries, inclusive fitness is also predicted to drive intracolonial conflicts based on colony-level costs associated with selfish behaviour. For example, inclusive fitness is thought to drive conflict over worker reproduction not only when colony members differ in their relatedness to queen- and worker-produced males, but also when worker reproduction reduces overall colony productivity (Ratnieks 1988). Having found strong evidence for the occurrence of preparation for future reproduction by *L. acervorum* workers in queenright colonies (Chapter 3), I went on to investigate the possibility that such preparation takes the form of a change in worker behaviour, and that such a change in behaviour could be associated with colony-level costs (Chapter 4). I found strong evidence to suggest that workers do indeed alter their behaviour when preparing for reproduction, and that some of the observed changes in behaviour (such as increased levels of aggression and feeding from larvae) have the potential to result in colony-level costs. I also found some evidence to suggest that the behaviour exhibited by workers when prepared for reproduction may not be as potentially costly as the behaviour exhibited by workers when fully reproductive. Only a small number of studies have provided evidence to suggest that the behaviour of workers in queenright colonies can be driven by preparation for future reproduction (as opposed to participation in current reproduction) (Bourke 1988a; Heinze & Oberstadt 1999; Brunner & Heinze 2009), and this is the first time to my knowledge that evidence of such an occurrence has been found in a species in which workers appear to alter their preparation for future reproduction as a function of social structure (as shown in Chapter 3). As well as improving our understanding of the behaviour of workers under queenright conditions (for example by explaining why some workers appear to be particularly aggressive or to receive high levels of aggression despite their apparent lack of reproductive activity), this specific study into the behaviour of future reproductive workers also highlights an area of social insect behaviour which has received very little attention so far and can be used for studies of kin conflict in the future.

One of the key behaviours in the study of kin conflict in the eusocial Hymenoptera is that of worker policing (i.e. policing performed by workers). Worker policing has provided strong evidence in support of inclusive fitness theory in the past and plays a key role in a number of different kin conflicts, such as conflict between workers over whether to rear queen- or worker-derived males (Ratnieks 1988), conflict over the cost of worker reproduction (Ratnieks 1988), and conflict over sex-allocation when workers wish to create a female bias in the sex-ratio of the colony's sexual offspring (Foster & Ratnieks 2001b). Despite the importance of worker policing in the study of inclusive fitness theory, there are still essential aspects of the behaviour that require further understanding, three of which I have addressed in the study presented in Chapter 5, which investigates worker policing in a facultatively polygynous population of *L. acervorum*. The first aspect of worker policing that I wanted to address was that of the specificity of the egg-marking signals involved in worker policing. Despite the importance of understanding the mechanisms involved in worker policing, there is still much to be learnt about the chemical signals involved in the behaviour. Workers are thought to distinguish queen- from worker-laid eggs with the use of a chemical cue deposited by the queen onto the surface of her eggs (Ratnieks 1988, 1995; Ratnieks & Visscher 1989), but the exact chemical has yet to be identified (Katzav-Gozansky *et al.* 2001; Martin *et al.* 2002b; Endler *et al.* 2004; van Zweden *et al.* 2009). The results of the experiment presented in Chapter 5 showed that workers could discriminate between queen- and worker-laid eggs in favour of queen-laid eggs, even though the eggs had been transferred from a non-nestmate colony. Few species have been used to investigate whether queen egg-marking signals can be transferred across colonies, but the findings of the study in Chapter 5 add to the slowly emerging trend that in most species (but not all e.g. *Formica fusca* (Helanterä & Sundström 2007)) the queen egg-marking signal appears to be generic across conspecific colonies (Ratnieks & Visscher 1989; Foster *et al.* 2002; D'Ettorre *et al.* 2004; Endler *et al.* 2004; Bonckaert *et al.* 2008).

The second aspect of worker policing that I wanted to address with the experiment in Chapter 5 was that of the effect of social structure on worker policing. Only a small number of studies have assessed the effect of social structure on worker policing by directly observing policing behaviour in a species with a facultative social structure (Foster & Ratnieks 2000; D'Ettorre *et al.* 2004; Meunier *et al.* 2010; Bonckaert *et al.* 2011b). Such studies are essential because they test the effect of social structure on policing whilst controlling for between-species variation and whilst confirming that worker policing (rather than worker self-restraint, for example) is responsible for any variation in successful worker reproduction observed. The experiment in Chapter 5 also adds to the small number of studies that have investigated the effect of polygyny (as opposed to polyandry) on worker policing (D'Ettorre *et al.* 2004; Meunier *et al.* 2010). The results of the experiment showed social structure to have no effect on worker policing in a facultatively polygynous population of *L. acervorum*, suggesting that colony kin structure (chiefly influenced by social structure) is not the determining factor behind worker policing in *L. acervorum*, and that other factors such as colony costs associated with worker reproduction could be driving the behaviour instead (Ratnieks 1988).

Finally, the third aspect of worker policing that I wanted to address with the experiment in Chapter 5 was that of the effect of egg age on worker policing. Previous studies of worker policing have shown the majority of worker-laid eggs to be destroyed within 24 hours of being laid (Ratnieks & Visscher 1989; Ratnieks 1993; Visscher 1996; Wenseleers *et al.* 2005b), and it is essential to the interpretation of past studies and the planning of future experiments to know whether this effect is caused by a decrease in susceptibility to policing with egg age. However, the results of the experiment in Chapter 5 suggest that egg age does not effect the chances of worker-laid eggs being policed, suggesting that egg age need not be considered as a confounding factor in studies of worker policing and worker reproduction. Further investigation into the reason why the majority of worker-laid eggs are destroyed soon after laying would greatly improve our understanding of the cues involved in worker policing. A starting point could be to measure variation in the chemical profile of worker-laid eggs and to see if some chemical profiles (perhaps those with a relatively more queen-like profile (Ratnieks 1993; Wenseleers *et al.* 2005b) are less prone to policing than others.

To conclude, through experimental investigations of predictions derived from inclusive fitness theory using the facultatively polygynous ant *L. acervorum*, I have shown that some predictions of kin conflict based on relatedness asymmetries do not result in actual conflict, suggesting that the cost and benefit components of Hamilton's rule are equally as important in the evolution of social behaviour as the relatedness component, and that information constraints may also prevent potential kin conflicts from developing into actual kin conflicts. I have also shown that workers in the eusocial Hymenoptera have evolved mechanisms that allow them to take full advantage of reproductive opportunities when they arise, as predicted by inclusive fitness theory. Finally I have provided another case study of worker policing in a eusocial Hymenopteran. I found *L. acervorum* workers to perform worker policing irrespective of colony social structure, again highlighting the importance of the cost and benefit components of Hamilton's rule. As a concluding point, I hope that I have also managed to demonstrate to some degree just how extremely relevant and applicable inclusive fitness theory is to the study of social evolution, and how essential the theory has been to the field of evolutionary biology as a whole. In this thesis I have used simple predictions of inclusive fitness theory to investigate social behaviour, and in doing so have been able to further our understanding of when relatedness alone determines behaviour, and when the effects of relatedness are outweighed by colony-level costs. I have also been able to demonstrate the importance of inclusive fitness in predicting and discovering previously unobserved behaviours (such as an adaptive response of workers to their current risk of queenlessness, as demonstrated in Chapter 3), and how experiments based on the theory can provide information on the mechanisms behind social behaviour, such as the nature of the cues involved in egg recognition.

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