Regulation of worker reproduction in ants: The role of kinship

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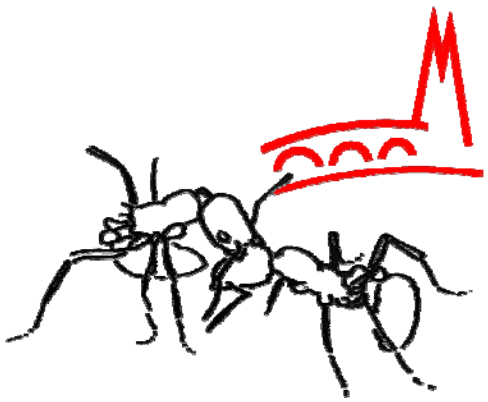

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Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Dissertation selbständig und ausschließlich unter der Verwendung der angegeben Quellen und Hilfsmittel angefertigt zu haben.

Diese Arbeit wurde bisher weder einer Prüfungsbehörde vorgelegt noch veröffentlicht.

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I. General Introduction

Social insects like ants, bees and wasps are characterised by sophisticated communication, cooperation and division of labour in which individuals are specialized of different tasks, including foraging for food, brood care, defence and reproduction (Hölldobler and Wilson 1990). Most fundamentally, queens and males specialize in reproduction, while workers are generally sterile, completely forgo their own reproduction and instead help rearing the offspring of their mother. The evolution of reproductive division of labour constitutes the essence of altruism and self-sacrifice, however, seems contrary to Darwin's theory of natural selection, where genes conferring greater survival and reproduction should spread in a given population.

A few decades ago, the British biologist William D. Hamilton provided the key theory to this apparent paradox in evolutionary theory. According to Hamilton's kin selection theory (Hamilton 1964a,b), also known as inclusive fitness theory, individuals can transmit more copies of their own genes, indirectly, by helping relatives to rear their offspring, than directly, through their own reproduction. How many genes are transmitted depends on three factors defined by Hamilton's rule, $C < rB$, where C is the fitness cost to the altruistic individual, B is the fitness benefit to the recipient of the altruistic behaviour and r is the relatedness between the two actors. The cost is measured in the average number of offspring the altruistic individual could have produced instead of helping, and the benefit is measured in the number of the recipient's offspring due to the help by the altruistic individual. The degree of relatedness is a measure of the genetic similarity between the two individuals. Hence, a general description of Hamilton's rule is that altruistic acts are more likely to be selected for when individuals are closely related and when the decrease in the actor's personal fitness is relatively small compared to the increase in the recipient's fitness.

Due to the haplodiploid sex determination, workers in Hymenoptera, like ants, bees and wasps are highly related to each other. In these societies males derive from unfertilized eggs (arrhenotokous parthenogenesis) and are haploid while fertilized eggs develop, depending on environmental or social conditions, into either diploid female sexuals or workers. As a consequence, in societies with simple mating structures, e.g. colonies headed by a single once-mated queen (monogyny and monandry), sisters share half the genes derived from their mother and all the genes derived from their father resulting in an overall relatedness of 0.75. As females transmit half of their genes to their own offspring, they are

closer related to their sister than to their sons and daughters (r = 0.5). Females therefore gain extraordinarily high indirect fitness gains from helping their mothers to rear their sisters rather than producing their own offspring. Hamilton's kin theory, also known as the inclusive fitness theory, therefore elegantly explains the multiple evolution of sterile workers castes in ants, social bees and wasps (e.g. Bourke and Franks 1995; Queller and Strassmann 1998; West-Eberhard 1975).

Ever since Hamilton's rule was published, the role of kinship in the evolution and organization of insect societies is extensively discussed among researches of eusocial insects (e.g. Foster et al. 2006; Hughes et al. 2008; Korb and Heinze 2004; Wilson and Hölldobler 2005). Recently, kin selection theory has even been put in to question by proponents of "new" group selection theory leading to an ongoing dispute (West et al. 2007; West et al. 2008; Wilson and Wilson 2007; Wilson DS 2008; Wilson EO 2008). Indeed, the importance of high relatedness among females for the evolution and maintenance of eusociality has been overemphasized in many studies (e.g Wenseleers and Ratnieks 2006a). The advance in the genetic analyses of social insects has shown that in many species the social structures deviate from the simple pattern of monogyny and monandry. For example, in several species colonies may contain more than one inseminated queen (polygyny) or queens may be multiple mated (polyandry), leading to a nestmate relatedness below the prominent value of 0.75 (Figure 1). While kinship is undisputable a major force in the evolution of social insect societies, the degree of relatedness may be less important for their maintenance (Korb and Heinze 2004). The other two factors in Hamilton's equation, the costs and benefits of helping, may simultaneously be of great importance in maintaining sociality. However, these factors are widely neglected in studies of social insects, which may be partly due to the difficulty in quantifying them (Bourke and Franks 1995).

Organisational traits maintaining social life within societies, such as sex ratio allocation, the partitioning of reproduction and conflict resolutions are similarly explained by relatedness patterns within the societies (Boomsma and Grafen 1990; Boomsma and Grafen 1991; Johnstone 2000; Reeve and Ratnieks 1993; Wenseleers and Ratnieks 2006a). The investment allocation towards female and male sexuals produced within a population, are expected to vary with differential kin structures resulting in different sex-ratio optima for workers and queens (Bourke and Franks 1995; Trivers and Hare 1976). Similarly, conflicts over reproductive rights in the colony and how these conflicts are resolved, are hypothesized to be influenced by varying kin structures resulting from alterations in queen mating frequencies or the number of reproductive queens per colony (Bourke and Franks 1995;

Monnin and Ratnieks 2001; Ratnieks 1988; Ratnieks et al. 2006; Ratnieks and Reeve 1992; Ratnieks and Wenseleers 2005).

Conflict over male parentage is of particular importance in eusocial insects. Though, reproduction is often monopolized by the queen, workers in most species have retained ovaries and are able to lay unfertilized eggs which develop into males, however, in most cases, only use this option once the queen dies or is experimentally removed from the colony (Bourke 1988; Choe 1988). In monogynous and monandrous societies workers are more related to their own sons (r = 0.5) or the sons of other workers (r = 0.375) than to males produced by the queen (r = 0.25; Figure 1). Workers in these societies should therefore be selected to selfishly produce their own sons and favour sons produced by other workers over queen-produced males. In contrast in polygynous or polyandrous colonies workers are still more closely related to their own sons, but at an effective queen mating frequency above two, their average relatedness to other worker's sons (r = 0.125; Figure 2) is lower than to the queen's sons (r = 0.25; Figure 1). In this case, workers can increase their average inclusive fitness by preventing each other from reproducing through aggression or eating eggs laid by workers, which is termed "worker policing" (Crozier and Pamilo 1996; Monnin and Ratnieks 2001; Ratnieks 1988; Starr 1984; Woyciechowski and Łomnicki 1987). Queens are similarly expected to police workers attempting to reproduce as they are more related to their own sons (r = 0.5) than to their grandsons (r = 0.25). Therefore, conflicts arise between queens and workers and among workers about reproductive rights in the colony and the occurrence and resolution of these conflicts are hypothesised to depend on kin structures within the society.

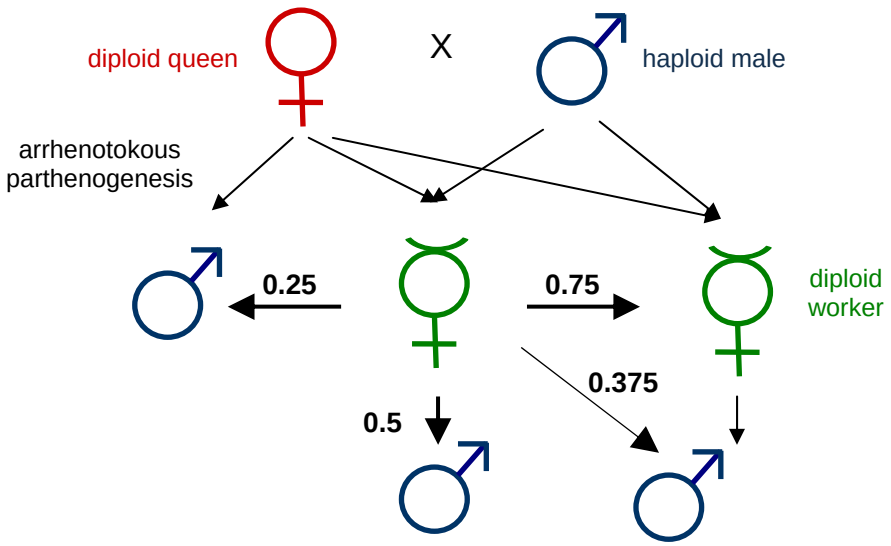

Figure 1. Genetic relationship in a hymenopteran society with a single, singly mated queen (monogyny and monandry). The numbers illustrate the "life-for-life relatedness" of a worker to her brother, her own son, and to the son of another worker.

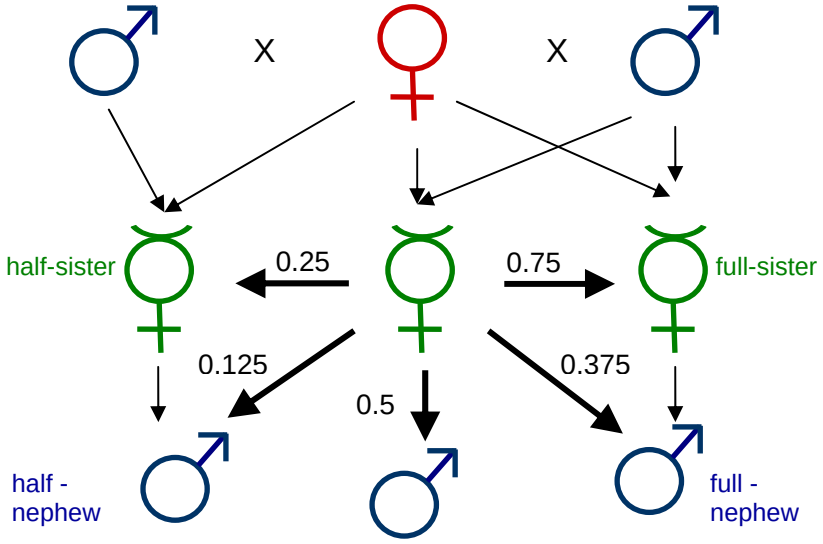

Figure 2. Genetic relationship in a hymenopteran society with a single, double-mated queen (monogyny and polyndry). The numbers illustrate the "life-for-life relatedness" of a worker to her half-sister, her full-sister, her half-nephew, her own son, and her full-nephew.

Particularly in bees and wasps the occurrence of conflicts and their resolutions seem to be in agreement with assumptions made by relatedness structures (Foster and Ratnieks 2001a; Foster et al. 2001; Peters et al. 1999; Ratnieks and Visscher 1989; Tóth et al. 2002a), while only few evidence is found in ants (e.g. Kronauer et al. 2006; Sundström 1994). In the honey bee, where worker policing was predicted on theoretical grounds (Ratnieks 1988), queens are typically mated to several males. As expected from kin theory, eggs laid by workers in the presence of the queen are almost completely removed by other nestmate workers (Ratnieks 1993; Ratnieks and Visscher 1989; Visscher 1996). Furthermore, in agreement with kin theory, policing by eating of worker-laid eggs was found in the polyandrous *Vespula* wasps (Foster and Ratnieks 2001a), whereas in monandrous stingless bees (Meliponinae) and *Dolichovespula* wasps no policing was found, and workers highly contribute to males produced in then colony (Foster and Ratnieks 2001a; Peters et al. 1999; Tóth et al. 2002a). Moreover, facultative worker policing has been found in the wasp *Dolichovespula saxonica,* in which the single queen is either singly or multiply mated and worker-laid eggs were found to be policed only in colonies headed by a multiply mated queen (Foster and Ratnieks 2000). In ants, agreements with kin theory are found in *Formica truncorum*, were colonies bias the sex ratio in response to relatedness asymmetries caused by inter-colony variations in queen mating frequency (Sundström 1994). The absence of worker reproduction in polyandrous army ants similarly seems consistent with predictions made by kin theory (Kronauer et al. 2006).

Despite these observations in agreement with kin theory, a constantly growing number of studies in eusocial insects about worker reproduction and conflict resolution, do not meet the predictions based on kin structure alone. Males are almost exclusively produced by the queen and how conflicts are resolved within the colony are usually independent of the queen's mating frequency (Hammond and Keller 2004; Heinze 2004). Though expected on relatedness grounds, workers do not contribute to male-production in queenright colonies of many monogynous and monandrous species (e.g.; ants: Heinze 1997; Arévalo et al. 1998; Walin et al. 1998; Helanterä and Sundström 2005; stingless bees: Palmer et al. 2002; Tóth et al. 2003; wasps: Foster et al. 2000). While workers lay eggs in colonies of facultatively polygynous *Myrmica tahoensis* (Evans 1998), they neither contribute to the egg pile in monogynous nor polygynous colonies of the ant *Leptothorax acervorum* (Hammond et al. 2003). Similarly, conflict resolutions seem independent of colony kin structures as worker policing occurs in monogynous, monandrous species such as the ponerine ant *Diacamma* sp. (Kikuta and Tsuji 1999) and the wasps *Vespa crabro* (Foster et al. 2002). Even in the parthenogenetic ant *Platythyrea punctata*, in which colony-members are essential clones and thus equally related to all offspring in the colony, worker policing has been observed (Hartmann et al. 2003). To explain this discrepancy between predictions from kin theory and empirical data several hypothesis have been suggested in which different constraints are made responsible why individuals refrain or are prevented from pursuing their own egoistic interests (Figure 3).

While kin theory gives the optimal sex ratio or reproductive skew, corresponding to the genetic interest of queens and workers, it does not predict the power each party holds to enforce its own optimum (Beekman et al. 2003; Beekman and Ratnieks 2003). Workers in some ant species have not retained their ovaries and are simply incapable of laying eggs (Bourke and Franks 1995; Hölldobler and Wilson 1990; Oster and Wilson 1978). Species in which workers are still able of laying eggs mainly produce trophic eggs in the presence of the queen and only switch to male-production under queenless conditions (e.g.¸Dietemann and Peeters 2000; Gobin et al. 1998; Koedam et al. 1996). Morphological constraints may limit the power of workers to enforce their own interests again the queen. For example, workers posses less ovarioles than queens and may make males produced by workers more costly than males produced by the queen.

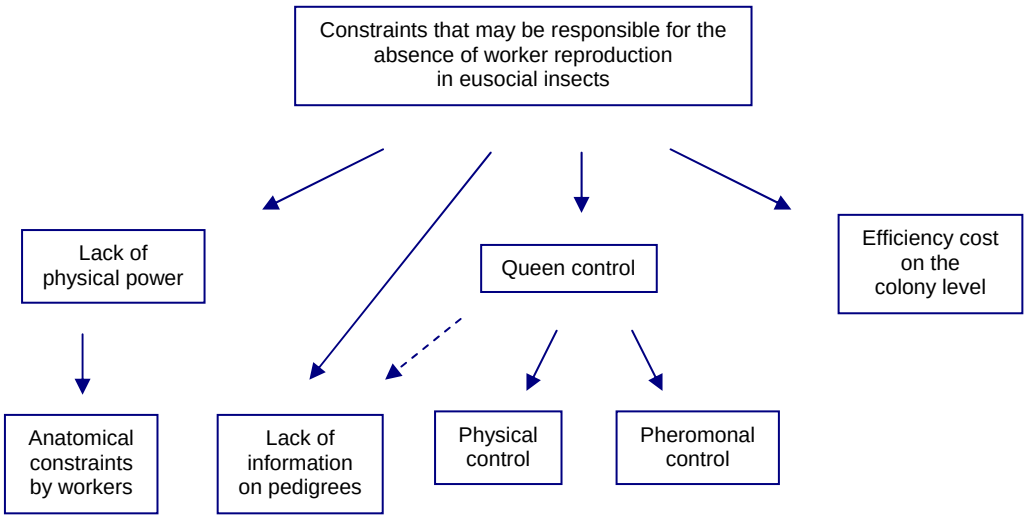

Figure 3. Constraints that may be responsible, other than kin structure, for the absence of worker reproduction in eusocial insects like ants bees and wasps.

Informational constraints on the origin of brood may make workers unable to enforce their own interests. Under given relatedness patterns, the accidental removal of haploid queen- or worker-laid eggs would lower the workers' inclusive fitness, while the accidental removal of diploid queen-laid eggs would decrease the workers' fitness at any time. In the ant *Camponotus floridanus* queens may actively camouflage the sex of their eggs, at least in the early stages of development (Nonacs and Carlin 1990). Such sexual deception by the queen has not been found in the ant *Lasius niger* (Jemielity and Keller 2003), and it is still not fully clear, whether queens actively control the conflict over reproduction through camouflage of their brood.

Nevertheless, reproductive conflicts between queens and workers seem mostly under the control of the queen. Despite in most species workers have retained their ovaries, worker reproduction in the presence of the queen is quite rare (Hammond and Keller 2004; Heinze 2004), while in most other species workers activate their ovaries and produce their own sons only after the experimental removal of the queen, or in nature, after her death (Bourke 1988; Choe 1988; Heinze et al. 1997). The incentive to pursue personal interests may be higher in queens as they suffer a two-fold fitness loss when grandsons are reared rather than her sons, while workers only get a 1.5-fold fitness gain from producing nephews rather than brothers.

How queens mediate the control over worker reproduction seems to vary with colony structures. In small colonies queens may be able to physically control workers, while in large colonies, in which workers far outnumber the queen, physical control is no more effective and may be replaced by pheromonal control. Indeed, there is ample evidence that pheromones,

such as cuticular hydrocarbons on the surface of the cuticle, are involved in the regulation of reproduction (reviewed in Monnin 2006). In general, non-polar chemicals on the cuticle act as nestmate recognition cues, protecting the colony from parasites or conspecifics invasion (Hefetz 2007; Lacy and Shermann 1983), but cuticular hydrocarbon patterns also vary with reproductive status (Monnin 2006). Direct evidence that such substances indeed serve as pheromones are rare (Smith et al. 2009) and most studies are only correlative. Initially, pheromones by the queen were thought to actively suppress worker reproduction against the worker's interest (Fletcher and Ross 1985). However, as coercion would trigger an arms race between workers and queens, the queen control hypothesis has been argued evolutionary unstable, and instead queens are thought to honestly communicate their fertility status to which workers could react in their own interest (Keller and Nonacs 1993). Whether queen pheromones are honest signals or manipulative agents is still not fully resolved (Heinze and d´Ettorre 2009).

Conflict resolution among workers may similarly depend on unequal power held by each individual. Especially in small insect societies workers form linear or near-linear dominance hierarchies which are established through aggressive interactions either already in the presence of the socially and reproductively dominant queen or once she has died or been experimentally removed (Franks and Scovell 1983; Heinze 2004). Reproductive status and hierarchy rank are typically closely related and only a few top-ranking workers monopolize egg laying rights within the colony. Dominance thus means that an individual increases its own direct fitness by punishing subordinates. In contrast, worker policing, in which nestmate workers oppose each others egg laying activities, serves the interest of the colony as a whole and increases the indirect fitness of each colony member (Monnin and Ratnieks 2001). In this case aggression is initiated by subordinate towards dominant egg-laying workers. However, policing individuals might also act selfishly in order to gain reproductive rights themselves and in that way increase their own direct fitness (Saigo and Tsuchida 2004; Stroeymeyt et al. 2007; Wenseleers et al. 2005). Aggressive behaviour would then be more related to dominance than policing activities and is independent of kin structures within the colony. However, dominance and policing behaviour are difficult to distinguish and might often be not mutually exclusive.

Worker reproduction may be associated with costs leading to lower colony performance. Reproductive active workers usually perform little or no work useful to the colony (e.g., Cole 1984; Cole 1986; Hartmann and Heinze 2003; Hillesheim et al. 1989) and

energy used, when fighting for a high position in the dominance hierarchy, could be invested into colony maintenance (Molina and O'Donnell 2009). Hence, worker reproduction can cause a ´tragedy of the commons´ (Hardin 1968), with individual exploitation potentially causing costs to the whole group. As colonies compete with other colonies, selection on the colony level might act against worker reproduction (Korb and Heinze 2004). An increase in the individual's direct fitness might thus be cancelled out by an overall decrease in its indirect fitness, leading to the evolution of self-restraint (self-policing, Ratnieks 1988). Even if an individual would still gain higher direct fitness due to own reproduction, the other individual's indirect fitness could still be lowered. Nestmate workers may therefore prevent workers from reproducing through worker policing even when not expected from kin theory (Ratnieks 1988; Ratnieks and Reeve 1992).

Aim of this thesis is to investigate the role of kinship in the occurrence and resolution of reproductive conflicts in eusocial insect societies. By experimental manipulation of colony composition, behavioural observations and genetic and chemical analyses we examined various traits that may influence the regulation of worker reproduction in the two model systems, the ant genus *Temnothorax* and the thelytokous ponerine ant *Platythyrea punctata*.

The study species

The ant genus *Temnothorax* (formerly *Leptothorax* (Myrafant)) belonging to the tribe Formicoxenini are an ideal model system to investigate the role of kinship in worker reproduction. In most *Temnothorax* species colonies contain a single, singly mated queen (monogynous and monandrous), and though workers are capable of laying unfertilized eggs and produce haploid males, they do not reproduce in the presence of a queen (Heinze et al. 1997). When the queen is removed from the colony, workers engage in dominance interactions and form hierarchies in which one or a few top-ranking workers start to develop their ovaries and lay male-destined eggs (Heinze et al. 1997). As workers are more closely related to the sons of other workers than to their brothers, worker reproduction is predicted by kinship theory even in the presence of the queen. Hence, worker sterility cannot be explained by relatedness patterns. In the context of this thesis we try to investigate several other factors which may be important for the absence of worker reproduction in this species. We mainly focus on two species from Central and Southern Europe, *T. unifasciatus* and *T. crassispinus*,

while in one experiment we included four further species: *T. nylanderi*, *T. recedens*, *T. lichtensteini* and *T. affinis*.

Colonies of *Temnothorax* usually contain only a few dozen individuals and are relatively small. They are among the most abundant ants in boreal and temperate habitats, and are very abundant in Central and Southern Europe (Seifert 2007). Particularly in *T. nylanderi* and *T. crassispinus* nest site density can reach up to 200 nests per $25m^2$ (Foitzik et al. 2003; Seifert 2007). Colonies generally live in small acorns, rotten twigs, grass stems, under bark or in small rock cavities, which makes them easy to collect.

The parthenogenetic ponerine ant *P. punctata* represent a prime example why the occurrence of worker policing cannot be solely explained by kin theory. In *P. punctata* all workers are capable of producing diploid offspring from unfertilized eggs by thelytokous parthenogenesis (Heinze and Hölldobler 1995; Schilder et al. 1999a,b). As a consequence, workers may essentially be clone mates and policing is not expected on relatedness grounds, because it is of no relevance to the worker who produces the offspring in the colony (Greeff 1996). Surprisingly, *P. punctata* workers do police new reproductives after reunion of split colonies (Hartmann et al. 2003). Costs to the colony due to worker reproduction might be the driving force of worker policing in this species. Indeed, the addition of brood items in experimental colonies did not lead to a higher reproductive output and colonies may not be able to rear brood from more than a single reproductive worker (Hartmann et al. 2003).

The species of *P. punctata* is distributed in the tropical regions of northern and central America. Colonies are relative small, ranging from a few to over one hundred workers and are found in preformed cavities in rotten wood.

The maintenance of *Temnothorax* and *P. punctata* colonies in artificial laboratory conditions are well established and small colony sizes in both genera are an important prerequisite for behavioural observations in which all colony-members are marked individually.

In **Chapter 1** we demonstrate the association of worker dominance and policing behaviour in the ant *T. unifasciatus*. In a preliminary study to this thesis we could demonstrate selfish policing behavior towards reproductive active workers in *T. unifasciatus* (Stroeymeyt et al. 2007). In undisturbed, queenright colonies of this species, no aggression or overt dominance behaviour can be observed (Heinze et al. 1997), and males are only produced by the queen (Hammond and Keller 2004; Heinze et al. 1997). However, when colonies are split, workers in the queenless colony fragment quickly establish near-linear hierarchies, in which the top-ranking workers soon begin to lay haploid eggs (Heinze et al. 1997). When colonies are reunited, these reproductive workers are attacked by individuals, which later become fertile themselves when the queen is removed from the colony (Stroeymeyt et al. 2007). Therefore, policing and dominance behavior may be associated to some extent. Furthermore, selfish policing in the presence of the queen implies that workers form hierarchies already in queenright colonies even though aggressive interactions are usually restricted only to the period after queen loss. In this study we try to allocate aggressiveness by workers towards policing and/or dominance behavior and investigate whether hierarchies based on subtle, non-aggressive interactions exist in queenright colonies.

In **Chapter 2** we further investigate policing behavior in the parthenogenetic ant *P. punctata*. Similar to *T. unifasciatus*, we found policing individuals increasing their chance of later becoming reproductives themselves. However, as workers are normally clonemates and thus equally related to all offspring in the colony, individuals are not expected to behave selfishly.

Chapter 3 contributes to the understanding of the role of cuticular hydrocarbons in the regulation of reproductive conflicts in *T. unifasciatus*. In many species of social insects, cuticular hydrocarbons correlate with the fertility of an individual and chemical profiles differ between worker- and queen-laid eggs. The worker's abilities to recognize fertile nestmates and to distinguish between worker- and queen-laid eggs are an important pre-requisite of effective policing behaviour. In this study we investigate chemical profiles on the cuticle of queens, fertile and non-reproductive workers and on worker- and queen laid eggs in the ant *T. unifasciatus*.

Chapter 4 contributes to the discussion whether queen pheromones are honest signals or manipulative agents. By a comparative analysis between six *Temnothorax* species we try to estimate the evolutionary rate of queen pheromones regulating worker reproduction. While manipulative queen control might generate an arms race between queens and workers leading

to different chemical profiles between closely related species, honest signals are thought evolutionary stable with only minor random variations between species.

In **Chapter 5** we investigate whether colony productivity is influenced by worker reproduction in *T. crassispinus*. We checked whether experimentally increasing the brood size with either worker- or queen-produced brood will lead to higher colony productivity. Furthermore, we investigated correlations between colony size and per capita productivity, contributing to the ongoing discussion on the productivity effects of colony size.

Chapter 1

Worker dominance and policing in the ant *Temnothorax unifasciatus*

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Abstract

In many species of eusocial Hymenoptera conflict about the production of males is resolved through “policing.” Recent studies in wasps and the ant *Temnothorax unifasciatus* suggest that in these species policing workers are dominant themselves and selfishly increase their own chances of later becoming fertile. Policing may therefore to some extent be associated with dominance and selfishness, and dominance and policing behaviour are indeed difficult to distinguish and often not mutually exclusive. Moreover, selfish policing requires that workers form rank orders already in the presence of the queen. Here, we try to allocate aggressiveness by workers towards policing and/or dominance behaviour and investigate whether hierarchies based on subtle, non-aggressive interactions exist in queenright colonies of the ant *T. unifasciatus*. We either split colonies into a queenright and queenless halve or temporarily removed the queen from complete colonies, which in both cases allows a few dominant workers to lay eggs in the queenless colony. Reunification of colony halves and return of the queen to orphaned colonies led to aggression against those workers that had become fertile during the absence of the queen. Dominant workers in reunited, split colonies were much more severely attacked than those in orphaned colonies after return of the queen. Furthermore, we observed that workers, which later became dominant egg layers under queenless conditions, have more contact with the queen than other workers. Both results corroborate the existence of rank relationships among workers in queenright colonies and show that results from policing experiments may be affected by the disturbance of pre-existing hierarchies through colony splitting.

Keywords: Dominance hierarchy, Selfish worker policing, Queen policing, Kin conflict

Introduction

The societies of many group-living vertebrates are structured by fighting and ritualized dominance interactions, through which individuals attempt to increase their own share in reproduction and test each others' strength and motivation (Wilson 1975; Keller and Reeve 1994; Faulkes and Bennett 2001; Hart and Monnin 2006). Similar aggressive interactions are known from numerous species of social insects. For example, ant or wasp queens may struggle for the exclusive possession of a cooperatively founded new nest (e.g., Heinze 1993; Choe and Perlman 1997) or, in mature colonies, for reproductive monopoly after joint hibernation (Heinze and Smith 1990), and ant workers may fight for the chance of rearing sons from unfertilized eggs in colonies that have lost the queen (e.g., Bourke 1988; Heinze et al. 1997). In addition, workers may attack other workers, which develop their ovaries in the presence of a fertile queen or have begun to reproduce during the temporary absence of the queen. Whereas the first cases of aggression are easily explained within the framework of dominance and reflect an individual's aim to increase its own direct fitness, aggression against reproductive workers in queenright colonies appears to follow a different logic: here, workers aim at preventing other workers from selfishly laying eggs when this decreases the reproductive output of the colony as a whole and / or the average relatedness of workers to the colony's male offspring. Such "worker policing" serves the interest of the complete colony and increases the indirect fitness of the average colony member (Ratnieks 1988; Monnin and Ratnieks 2001).

Though all individuals benefit from policing, theoretical and empirical studies show that only a minority of the workers is actually doing so (Frank 1996; van Zweden et al. 2007), and in some species it appears that it is predominantly the strongest and most dominant individuals that take over this task (Saigo and Tsuchida 2004; Wenseleers et al. 2005; Wenseleers and Ratnieks 2006a; Stroeymeyt et al. 2007; but see Cuvillier-Hot et al. 2004). Successful policing may augment the probability of policers gaining reproductive rights in the future and in this way increases their own potential direct fitness. Policing may therefore to some extent be associated with dominance and selfishness, and dominance and policing behaviour are indeed difficult to distinguish and often not mutually exclusive (Monnin and Ratnieks 2001; Heinze 2004). Selfish policing in the presence of the queen indicates that workers form rank orders already in queenright colonies, even though aggressive interactions are usually restricted only to the period after queen loss.

Worker reproduction in the presence of a queen is uncommon in ants and studying the details of policing requires experimentally eliciting worker reproduction. This has frequently been done by splitting colonies into a queenright and a queenless part, which allows dominant workers to reproduce in the queenless part, and later reuniting the two parts again (Kikuta and Tsuji 1999; Liebig et al. 1999; Hartmann et al. 2003; Iwanishi et al. 2003; Stroeymeyt et al. 2007). Unfortunately, this manipulation does not only change the reproductive status of some workers but might also disrupt worker hierarchies that have existed in the colony before splitting. In this study we therefore aimed at separating policing and dominance behaviours in colonies of the ant *Temnothorax unifasciatus.*

In undisturbed, queenright colonies of this species no aggression or overt dominance behaviour can be observed (Heinze et al. 1997), and males are only produced by the queen (Heinze et al. 1997; Hammond and Keller 2004). However, when colonies are split, workers in the queenless colony fragment quickly establish near-linear hierarchies, in which the top-ranking workers soon begin to lay haploid eggs (Heinze et al. 1997). When colonies are reunited, these reproductive workers are attacked by individuals, which later become fertile themselves when the queen is removed from the reunited colony (Stroeymeyt et al. 2007).

We subjected colonies of *T. unifasciatus* to two different types of manipulation: a) standard splitting of colonies and b) temporary orphaning of otherwise unchanged colonies. Assuming that hierarchies exist in queenright colonies, temporary orphaning of colonies should affect the hierarchies to a lesser extent than splitting colonies into two halves. By splitting, some dominant workers may be left by chance in the queenright colony half and do not become fertile, whereas workers originally subordinate to them may begin to lay eggs in the queenless half. After reunification, this will result in a mismatch between social and reproductive status. In contrast, dominant individuals in orphaned colonies may only need to reassert their status when challenged by subordinate workers. Returning the queen to the orphaned colony will therefore elicit only worker policing against the top-ranking, fertile workers, while traditional splitting and reunification will result both in the disruption of the dominance hierarchy and in worker policing as above. We therefore expected aggression to be more pronounced after the reunification of split colonies than after the reconstitution of orphaned colonies.

Furthermore, we investigated if non-apparent hierarchies exist in the presence of the queen by examining whether those individuals that become dominant after queen removal show a distinct behavioural profile already in the presence of the queen.

Material and Methods

In March 2006 we collected complete colonies of the monogynous and monandrous ant *Temnothorax unifasciatus* (Latreille 1798) from their nests in small decaying wooden branches in a sun-exposed slope near Gargnano (Lago di Garda / Italy; 45° 41' N; 10° 39' E). Colonies were transferred into small plastic boxes (10 cm x 10 cm x 3 cm) with a regularly moistened plaster floor and kept in incubators under artificial climate conditions with the temperature gradually being raised from spring (10°C night / 20 °C day) to summer (17°C night / 28°C day) conditions (Buschinger 1974; Heinze and Ortius 1991). The colonies were provided with water, honey, and pieces of cockroaches twice per week.

Experiments were conducted between April and September 2006 after colonies had been transferred to summer condition. All workers from 16 colonies (23 to 63 workers per colony: X 44.7 ± SD 10.2) were individually marked by tying 0.025mm thin copper wire (courtesy of Elektrisola, Eckenhagen) around different body parts (Fig. 1.1).

Figure 1.1. *T. unifasciatus* worker, marked individually with copper wire.

The ants were allowed to adjust to the wire marking for at least four days before observations started. Colonies were then observed in 10-min sessions over a period of two to four weeks, resulting in a total observation time of 5 hours per colony. After this first observation period we either split colonies into two equal fragments ("split" colonies, S1-S8) or we removed only the queen and two foragers from the nest ("orphaned" colonies, O1-O8; Fig. 1.2). Larvae were divided equally between the two colony halves of split colonies. Separated queens of orphaned colonies were given five larvae, while the remaining larvae were left in the orphaned fragments. To be able to detect the onset of egg laying by workers in the queenless fragments of split colonies and in the orphaned colonies all eggs were given to the queenright fragments or to the separated queens, respectively. The absence of queen eggs in the queenless and orphaned colonies may also speed up egg laying by workers, as eggs may carry inhibitive queen signals (Endler et al. 2004). Queenless and queenright colony halves and orphaned colonies were again observed

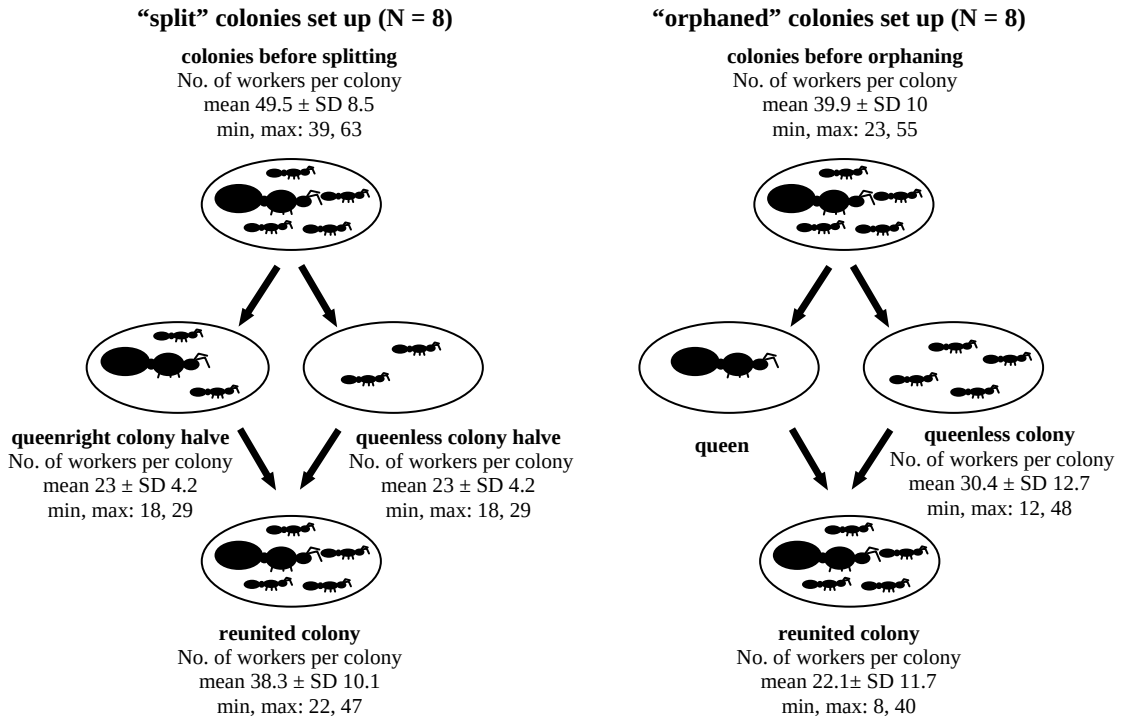

Figure 1.2. Colonies of *T. unifasciatus* were either split into two equal fragments ("split" colonies) or only the queen was removed from the colony ("orphaned" colonies). Two weeks after first worker-laid eggs had appeared in the queenless halve and in the orphaned colonies, split colony fragments were reunited and the queens returned to their colonies respectively.

for a total of 5 hours each, spread over a period of four to five weeks. Rank orders among workers were identified by aggressive interactions (antennal boxing and biting, e.g., Heinze et al. 1997; Stroeymeyt et al. 2007) among individuals in the queenless colony halves and in the orphaned colonies. Individuals initiating more than 1% of the aggressive acts per colony were classified as "dominant" workers and all other individuals from the queenless halves and from the orphaned colonies as "subordinate" workers. As overt aggression is absent or at least extremely rare in queenright colonies (Heinze et al. 1997), dominant individuals cannot be identified in the queenright colony halves, and all individuals are therefore referred to as "queenright" workers. The aggression level was estimated as aggressive acts per number of possible dyadic interactions [calculated with the equation (n/2) x (n-1), with n = colony size] in each colony to account for differences in colony size. Two weeks after first worker-laid eggs had appeared in the queenless halve and in the orphaned colonies, split colony fragments were reunited and the queens, without the two foragers, returned to their colonies, respectively (Fig. 1.2). All reunited colonies were again observed for a total of 5 hours spread over 10 days each.

After observations colonies were frozen and queens, all dominant and some subordinate workers were dissected to assess their ovary development (Buschinger and Alloway 1978). Workers containing elongated ovaries (> 1 mm) with viable, oval eggs similar in shape and colour to those found in the ovaries of queens were classified as "fertile."

All statistical analyses were performed using Statistica 6.0 and PAST 1.73 (Hammer et al. 2001). When necessary we combined the independent *p*-values from all colonies using Stouffer's method (Whitlock 2005) obtaining a single level of significance for the whole data-set.

Results

"Behaviour in queenright colonies"

In five of seven queenright colonies, those workers that became dominant after orphaning contacted the queen significantly more often by antennation, grooming, and trophallaxis than any other worker (Fig. 1.3). Queens actively interacted with workers only by antennation and in two of seven colonies antennated future dominant workers significantly more often than other workers (Fig. 1.3). The consensus *p*-value obtained from the meta-analysis confirmed that, overall, future dominant workers had more contact with the queen than any other worker (Stouffer's method; contact received by the queen from workers: $S = 6.193$, $p < 0.0001$; contact given from the queen to workers: $S = 2.194$, $p < 0.05$). Future dominants were also more frequently observed to engage in brood care than other workers (Mann-Whitney U-tests: O3, O6, O7 $p < 0.005$, O1 and O8 $p < 0.1$, O2 and O4 $p < 0.25$; Stouffer's method: $S = 5.029$, $p < 0.0001$).

"Behaviour in split and orphaned colonies"

In all queenless colony fragments of split colonies and all orphaned colonies, workers began to engage in aggressive antennal boxing and biting within one or two days after the experimental manipulation. Between two and nine workers per colony (median, quartiles: 5.5, 4, 8 workers) initiated more than 1% of the aggression (referred to as "dominant workers"). The number of these dominant workers did not significantly differ between split and orphaned

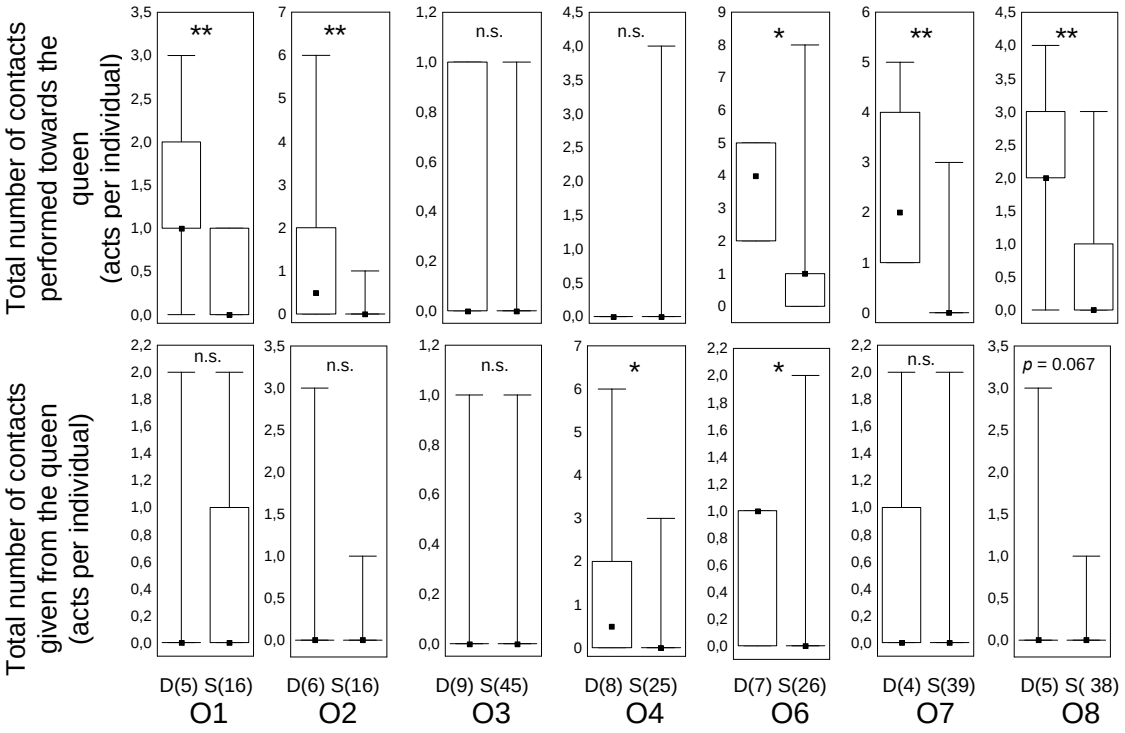

Figure 1.3. Total number of contacts (antennations, grooming, trophallaxis) per individual performed towards the queen from workers and given from the queen to workers before its removal from the "orphaned" colonies. Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dot*) are represented for each colony for future dominant individuals (D) on the left and subordinate workers (S) on the right. The number of workers in each category is given in parentheses. *P*-values are from two-sample permutation tests (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. nonsignificant).

colonies (split colonies: median, quartiles: 7, 4, 8; orphaned colonies: median, quartiles: 5, 4.5, 6.5; Mann-Whitney U-Test; $U = 23.5$, $p = 0.9$). First eggs appeared slightly, but not significantly earlier in the queenless fragments of split colonies (median 12.5 days; range 10 – 20 days) than in orphaned colonies (median 15.5 days; range 13-30 days, Mann-Whitney U-Test; $U = 14$, $p = 0.059$). Ovary dissection after observation confirmed that these eggs were most likely laid by the dominant workers (dominant workers: total $n = 60$, 16 with "fertile" ovaries, 44 with "non-fertile" ovaries; non-dominant workers from the queenless halve of split colonies and from the orphaned colonies: total $n = 60$: 1 with "fertile" ovaries, 59 with "non-fertile" ovaries; Yates corrected $\chi^2 = 13.43$, $p < 0.0005$). The queen of colony S8 and most workers of colony O5 died during the observation period and both colonies were therefore excluded from further analyses. Ovary dissection of queens from the remaining colonies showed that they were mated and fully fertile. None of the workers dissected from the queenright colony fragments had "fertile" ovaries ($n = 40$ workers).

The aggression level did not differ between the queenless halves of the split colonies and the orphaned colonies (Mann-Whitney U-Test: $U = 20$, $n_1 = 7$, $n_2 = 7$, $p = 0.565$). Different numbers of workers per colony, resulting from splitting colonies, did not influence the aggressive rates: colony size of orphaned and split colonies was not significantly correlated with the number of aggressive acts per individual (Spearman rank test, $n = 14$, $r_s = -0.144$, $p = 0.623$). In the queenright colony halves no aggression was observed.

"Behaviour after reunification and return of the queen"

The aggression level declined after reuniting the split colony fragments (see also Stroeymeyt et al. 2007) and after returning the queen to the orphaned colonies. While in colonies S5 and O4 no further aggression could be observed, in nine of the remaining twelve colonies aggression was predominantly directed towards former dominants (two-sample permutation test; S1, S3, S6, O3, O8: $p < 0.0005$; O1: $p < 0.01$; S4, S7, O6: $p < 0.05$; S2: $p = 0.07$; O2: $p = 0.49$; O7: $p = 0.995$). The consensus p-value obtained from the meta-analysis confirmed that, overall, aggression was higher towards formerly dominant workers than towards other workers (Stouffer's method; $S = 8.92$, $p < 0.0001$).

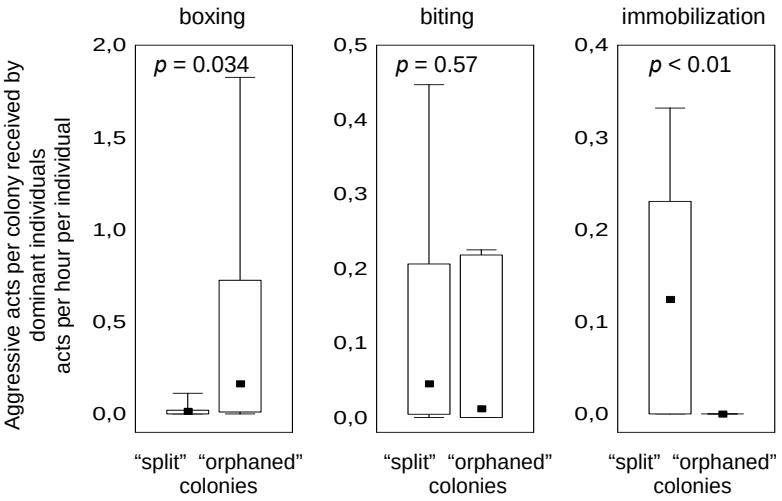

Figure 1.4. Aggressive acts per colony received by dominant workers of the ant *Temnothorax unifasciatus* after reunion of "split" colonies (N = 7 colonies, left) and return of the queen to "orphaned" colonies (N = 7 colonies, right). Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dot*) are given. *P*-values were obtained by two-sample permutation tests. After Bonferroni´s correction, *p*-values of < 0.017 are significant at the 0.05 level.

In reunited "orphaned" colonies, formerly dominant workers were attacked through antennal boxing and biting, and all attacked individuals survived until the end of the observations (Fig. 1.4). In contrast, in reunited "split" colonies former dominants were much more severely attacked through immobilization and being dragged through or out of the nest.

At least one of the formerly dominant workers in each colony died due to these attacks (median, quartiles: 1, 1, 6 killed workers per colony). Workers that were aggressive towards dominants in reunited "split" colonies were mostly from the queenright part, while in reunited "orphaned" colonies dominants mainly attacked each other (Fig. 1.5). Results were not statistically significant in those colonies in which only one or two dominant individuals were attacked (S2, S6, S7, O1, O2, O6, O7).

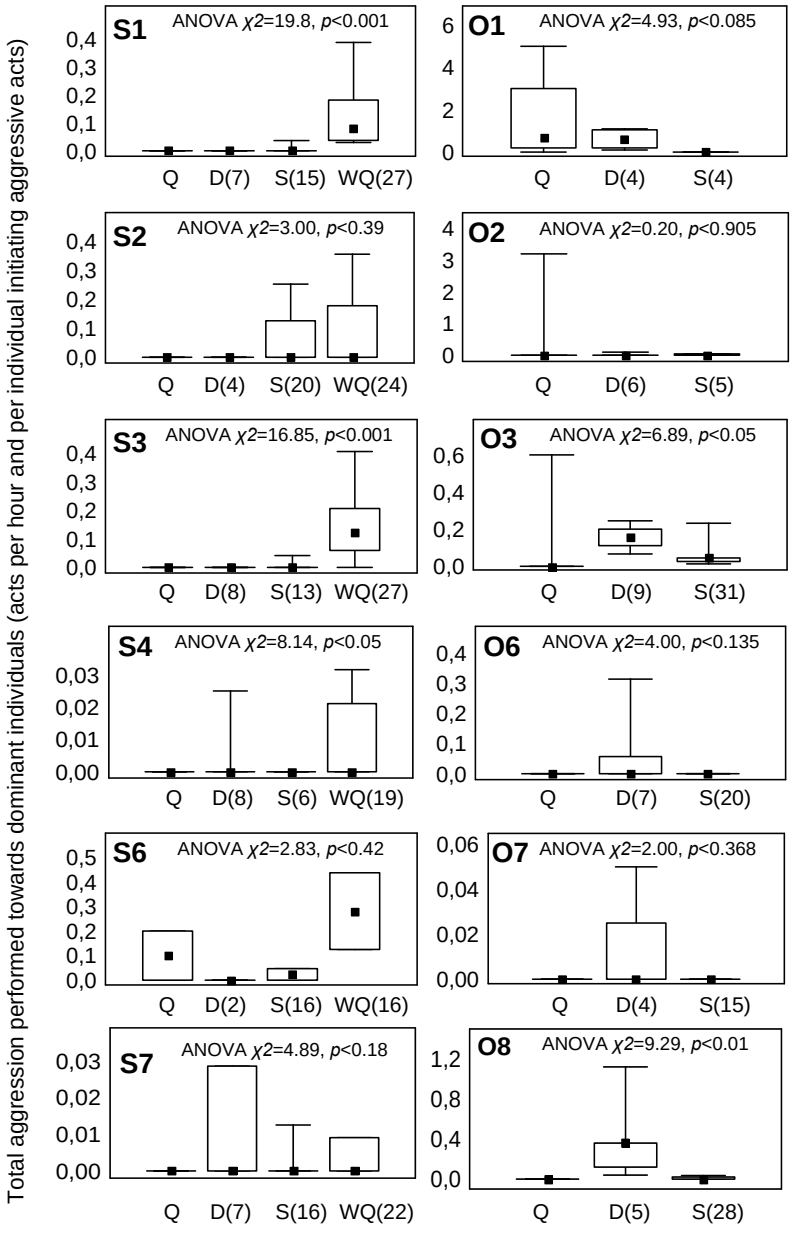

Figure 1.5. Total aggression performed towards dominant individuals from queens (Q), dominant workers (D), workers from queenright colony fragments (WQ), and subordinate workers from queenless colony fragments and orphaned colonies, respectively (S) after reunion of split colonies (left) and return of the queen to orphaned colonies (right). Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dots*) are shown. *P*-values are from Friedman ANOVAs for multiple dependant samples. No aggression could be observed in colonies S5 and O4.

Queens were rarely involved in aggression. The queen of S6 was observed attacking a dominant worker once, but was never aggressed by workers in any of the reunited "split" colonies. More aggression was observed in some of the orphaned colonies (Fig. 1.6). Formerly dominant workers usually tried to avoid the queen but exhibited a submissive crouching posture when attacked. Furthermore, workers occasionally attacked the returned queen in orphaned colonies (Fig. 1.6).

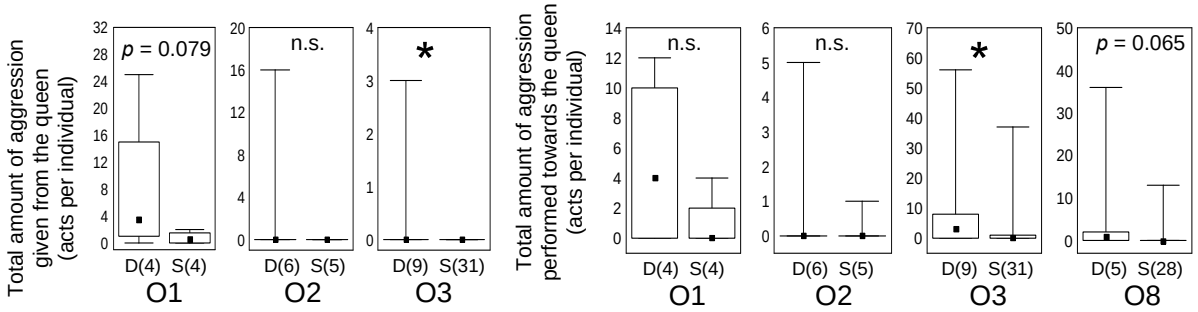

Figure 1.6. Total amount of aggression (antennal boxing and biting) per individual given from the queen to workers and performed towards the queen from workers in reunited orphaned colonies. Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dots*) are represented for dominant workers (D) on the left and for subordinate workers (S) on the right. The number of workers in each category is given in parentheses. *P*-values are from two-sample permutation tests (*$p < 0.05$; n.s. nonsignificant). In colonies O4 to O8 the queen was not aggressive and in colonies O4 to O7 it did not receive any aggression.

Discussion

Our study on dominance behaviour and worker policing in the ant *Temnothorax unifasciatus* reveals two novel phenomena. First, workers that show a higher level of interactions with the queen achieve top ranks in the dominance hierarchies resulting from worker aggression after queen removal. Second, the level of aggressiveness of workers towards nestmates, which have become fertile during the temporary absence of the queen, differs between orphaning and colony splitting experiments. Workers, which had laid eggs in temporarily orphaned colonies, were attacked by antennation and biting after the return of the queen, and all of them survived the attacks by their nestmates. In contrast, workers, which had laid eggs in a queenless colony fragment, were more fiercely attacked, and some were even killed after reunification of the two colony halves.

Both the uneven distribution of worker-queen interactions and the difference in aggression between split and orphaned colonies after the return of the queen suggest the

existence of worker rank orders in the presence of the queen, even though they are not expressed in aggression and egg laying. Previously reported worker hierarchies in queenright colonies are associated with both worker aggression and egg laying in the presence of the queen (Cole 1981; Franks and Scovell 1983). Aggressive dominance behaviour is energetically costly (Cole 1986), which may outweigh the benefits for "hopeful reproductive" workers in those species, in which the queen monopolizes reproduction. Instead, hierarchies based on more subtle interactions, associated with a short phase of hierarchy testing after queen removal but rarely resulting in prolonged fighting, might allow a rather cost-efficient and rapid division of reproductive labour after queen loss. Being in close contact to the queen and the brood might help high ranking workers to monitor the queen's reproductive status and to prepare for queen death and replacement.

Rank orders existing in the presence of the queen will often be disrupted by random experimental splitting but left untouched by orphaning. Reunification of split colonies led to violent dominance interactions and policing mostly from non-fertile, presumably high-ranking individuals from the queenright colony halve. They may in this way "punish" individuals, which formerly had been subordinate to them, for having become fertile. In a previous study we could indeed demonstrate that those workers that attack egg laying nestmates later become fertile themselves when the queen is removed from the colony again (Stroeymeyt et al. 2007). In addition, the number of workers fighting for reproductive rights in queenless colonies is positively correlated with the total number of workers per colony (Heinze 2008), and high-ranking workers in reunited "split" colonies might eliminate excess dominant individuals. The large between-colony variation in the incidence of immobilization and killing might reflect the fact that we split colonies randomly without taking existing hierarchies into account: in some split colonies, the dominants may have ended up in the queenless colony, i.e., dominance relationships would have remained untouched. This might also explain the lack of casualties in an earlier study (Stroeymeyt et al. 2007), but as these colonies came from another population the difference between the two studies may also reflect geographical differences.

In three reunited "orphaned" colonies the queens were aggressive and predominantly attacked fertile workers. Active queen policing, usually restricted to small colonies, is either expressed through the selective removal of worker-laid eggs by the queen (e.g., bees: Michener and Brothers 1974; Koedam et al. 2001; wasps: Saigo and Tsuchida 2004; Liebig et al. 2005; Wenseleers et al. 2005; ants: Bourke 1991; Nakata and Tsuji 1996; Monnin and Peeters 1997; Kikuta and Tsuji 1999) and/or through aggression or food begging directed

towards fertile workers (Franks and Scovell 1983; Kikuta and Tsuji 1999; Wenseleers et al. 2005). In *T. unifasciatus,* queen policing occurred after the return of queens into orphaned colonies, but never in reunited "split" colonies. As policing is costly, queen policing may be unnecessary when other dominant workers already engage in policing, as in the case of reunited "split" colonies. Queens of *T. unifasciatus* seem to contact future dominant workers more often than other workers, but this may either be due to dominant workers seeking their proximity or queens recognizing them as dominant. In four reunited "orphaned" colonies, aggressive workers also attacked the queen. Worker aggression towards queens and queen-killing is known from annual social insects at the end of a breeding season (e.g., Bourke 1994; Strassmann et al. 2003), but ovary dissections of queens in our study confirmed that they were fully fertile. We therefore cannot exclude a change of colony odour during the separation period.

To conclude, worker aggression in experimentally manipulated colonies of *T. unifasciatus* may result from the disruption of pre-existing hierarchies and selfish policing. As hierarchies are subtle and not expressed in aggression in the presence of a reproductive, their existence may always have to be taken into account in policing experiments that involve splitting and reunification of colonies. More careful analyses of worker-worker and worker-queen interactions in queenright colonies are therefore needed to fully understand the results from studies on policing and dominance in social insects.

Acknowledgements

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Chapter 2

Policing and dominance behaviour in the parthenogenetic ant *Platythyrea punctata*

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* Authors contributed equally

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Abstract

In the parthenogenetic ant *Platythyrea punctata* policing behaviour is not expected on relatedness grounds as workers are normally clonemates and thus equally related to all offspring in the colony. Nevertheless, colonies usually contain only a single reproductive and other workers that begin to lay eggs are attacked by their nestmates ('policing'). We found that those individuals that most actively engaged in policing later themselves had activated ovaries when the old reproductive was removed from the colonies. This suggests that police workers, through attacking and eliminating others, increase their own chances of becoming reproductive themselves. Because regular parthenogenesis leads to a clonal colony structure, individuals are not expected to invest energy in dominance and policing. On the assumption that physical dominance reflects an individual's reproductive potential, aggression among workers might ensure that the most fecund individual becomes the next reproductive, which would benefit the colony as a whole. Furthermore, aggression among nestmates may be maintained in this species despite predominant clonality, because infrequent sex, recombination or the adoption of alien workers may introduce genetic heterogeneity into the colony.

Keywords: ant**,** dominance hierarchy, *Platythyrea punctata*, reproductive conflict, thelytokous parthenogenesis, worker policing

Introduction

The societies of eusocial Hymenoptera (ants, bees, and wasps) give the appearance of harmoniously cooperating groups, in which most individuals refrain from producing their own offspring and instead help rear the progeny of one or a few reproductives in the colony. Nevertheless, conflicts of interest occur in such societies, and several mechanisms, such as dominance, punishment and policing, have evolved to resolve such conflicts and to prevent the breakdown of the society (Ratnieks 1988; Heinze et al. 1994; Monnin und Ratnieks 2001; Heinze 2004; Ratnieks et al. 2006). For example, in the presence of a fertile queen workers may prevent other workers from reproducing by policing, that is, they attack egg-laying workers or eat their eggs. Policing is expected to evolve when workers are less closely related to the male offspring of other workers than to the sons of the queen(s), for example when colonies have multiple related queens or when queens mate multiply (Woyciechowski und Łomnicki 1987; Pamilo 1991; Ratnieks et al. 2006). Furthermore, even when workers are more closely related to worker-produced males than to queen-produced males as in colonies with a single, singly mated queen, policing may be beneficial if the costs associated with worker reproduction or the benefits of policing to colony efficiency are high (Frank 1996; Ratnieks et al. 2006).

Assuming policing behaviour to be costly and the availability of resources to vary among individuals, models predict that policing is performed particularly by the strongest group members (Frank 1996). Indeed, not all individuals engage randomly in policing, but a subset of workers within the colony performs aggressive behaviour towards uncooperative individuals (van Zweden et al. 2007). Recent studies suggest such policing workers are the most dominant workers (Saigo und Tsuchida 2004; Wenseleers et al. 2005; Wenseleers und Ratnieks 2006a; Stroeymeyt et al. 2007), which lay eggs themselves when the colony's queen is removed (Stroeymeyt et al. 2007). Policing may therefore serve to increase not only the average inclusive fitness of all group members but also the potential direct fitness of the police worker. In this case of 'selfish policing' (Saigo und Tsuchida 2004; Wenseleers et al. 2005; Ratnieks et al. 2006; Stroeymeyt et al. 2007), the concept of policing (individuals attack egg-laying workers or eat their eggs to increase the indirect fitness gains of themselves and others, Ratnieks 1988; Monnin und Ratnieks 2001) partly overlaps with the concept of dominance (high-ranking individuals attack others to increase their own direct fitness, Clutton-Brock und Parker 1995; Monnin und Ratnieks 2001).

Workers of a few species of social Hymenoptera are capable of producing female offspring from unfertilized eggs by thelytokous parthenogenesis. As recombination is extremely rare (Schilder et al. 1999a), thelytokous mothers produce genetically identical offspring, and colonies are essentially clones (Onions 1912; Heinze und Hölldobler 1995). Conflicts and mechanisms of conflict resolution based on genetic grounds are not expected to occur in such clonal societies, as workers then are equally related to offspring produced by themselves or any other nestmate (Greeff 1996). However, in the thelytokous Cape honeybee, *Apis mellifera capensis*, worker-laid eggs are selectively removed (Pirk et al. 2003; but see Moritz et al. 1999; Beekman el al. 2002). Similarly, in the thelytokous ant *Platythyrea punctata*, workers that begin to lay eggs in the presence of an established reproductive are attacked and their eggs are selectively removed (Hartmann et al. 2003). Decreased colony-level performance caused by a mismatch between egg layers and nurses might be the driving force of policing in such species (Ratnieks 1988; Hartmann et al. 2003).

Here, we show a correlation between aggressive policing behaviour and the future reproductive success of workers in thelytokous societies of *P. punctata*. Those workers that had been most aggressive towards new reproductive individuals had activated ovaries after the established reproductive had been removed from the colony. It thus appears that individuals fight for reproductive rights, even though they do not gain direct fitness benefits because of the clonality of colonies.

Material and Methods

Set-up and Housing of Colonies

We used six colonies of *P. punctata* (K1, K2, K3, K6, K7, K8; containing 45 to 59 individuals; $X \pm SD = 50.5 \pm 5.8$) that were collected in Puerto Rico in 2003 and 2005. Colonies from the Puerto Rico population are queenless and headed by a single worker or in rare cases by two workers which monopolize reproduction (Schilder et al. 1999a,b). Since these workers are not mated, and because of the mechanism of thelytoky, colonies show a clonal structure, with all colony members being identical (Schilder et al. 1999a). Colonies used in this experiment were genotyped beforehand and showed no intracolonial variation

(K1, K2, K3, K6, K8 analysed in Hartmann et al. 2005; K7 analysed by K. Kellner, unpublished data).

During the experiment, colonies were housed in plastic boxes (20 x 10 cm and 6 cm high) with plaster floors. A preformed cavity in the plaster (7 x 5 cm and 0.5 cm high), covered with a glass plate and red foil, served as a nest. Colonies were kept under near natural conditions (27 °C, 60% humidity, 12:12 h light:dark cycle) and fed ad libitum a mixed diet of honey and pieces of crickets, cockroaches and *Drosophila* flies. The plaster was regularly moistened and a tube with water and cotton served as a permanent water supply.

Behavioural Observations and Experimental Design

Behavioural observations were carried out under a stereomicroscope. All ants were marked individually with dots of enamel paint. Colonies were observed by opportunistic sampling in each session of 10 min, three to seven sessions per day. We recorded the location of individuals (inside/outside the nest, sitting on brood) and the occurrence of brood care, foraging, allogrooming behaviour and aggression (ritualized aggression: antennal boxing; overt aggression: biting, biting and dragging, gaster flexing, stinging attempts).

Since reproductive individuals do not have a distinct morphology, we identified them before the experiment by directly observing egg laying or other traits typical of reproductives (see Hartmann et al. 2003). To corroborate our predictions, individuals suspected to be the reproductive were then separated from the colony overnight (ca. 12 h) and we checked for freshly laid eggs the next morning.

To induce policing behaviour, we applied a standard experimental design following Hartmann et al. (2003). By splitting the colonies first into two fragments (Phase 1) we induced the establishment of a second reproductive individual; then by reuniting the two parts (Phase 2), we caused aggression to arise because of the presence of a supernumerary reproductive. By expanding the experiment with a third phase (the two reproductives were removed, and the establishment of a further reproductive was induced), we could determine whether individuals that engaged in aggression in Phase 2 became reproductive in Phase 3. This association might clarify whether aggression has a selfish component. An overview of the different phases of the experiment is given in Fig. 3.1.

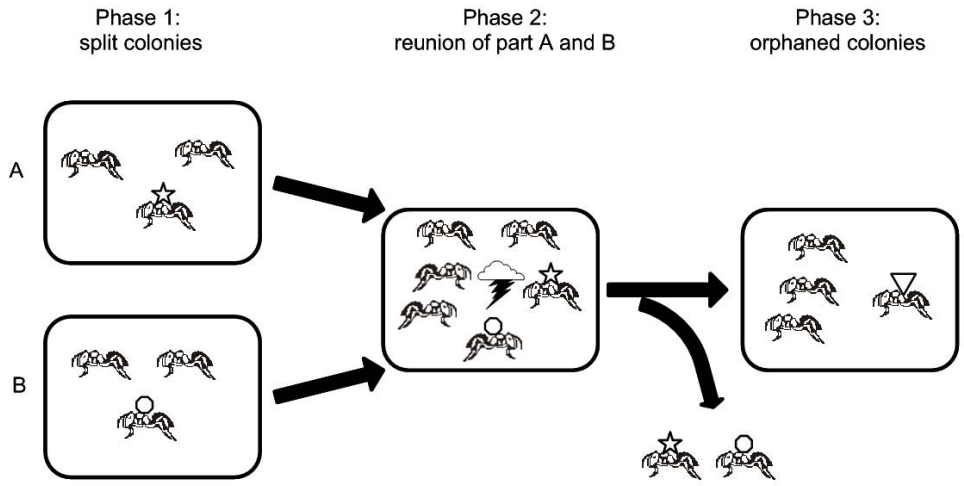

Figure 3.1. Experimental design. Prior to the experiment, the reproductive individual (marked with a star) was determined by directly observing egg laying. In Phase 1, colonies were split into two parts 'A' and 'B', with part 'A' containing the reproductive. In part 'B', a new reproductive individual (marked with a circle) quickly became established. In Phase 2, both parts were reunited and aggression between the two reproductives and other individuals was recorded. The two reproductives were then removed from the nest and, in Phase 3, the orphaned colonies were observed until a new reproductive individual (marked with a triangle) was established. After the observations, all individuals were dissected to record the state of ovary activation.

Phase 1: splitting of colonies

Colonies were split into two fragments with one fragment containing the established reproductive individual (fragment 'A') and one fragment without a reproductive individual (fragment 'B'). Nonreproductive workers were distributed randomly between the two colony halves. Both colony fragments were observed; colony fragments without the reproductive individual were observed until new reproductive individuals had been established and new eggs appeared. Consistent with former observations (Hartmann et al. 2003) this generally occurred after a period of 5-15 days with 30-53 observation sessions of 10 min each (300 – 530 min total observation time; X = 441.7min). To confirm reproductive status, we separated individuals for 1 night (ca.12 h) if they were not directly observed laying eggs.

Phase 2: reunion of colonies

Thereafter, both fragments were reunited. After reuniting the colonies in Phase 2, we observed policing behaviour by aggression against the old and new reproductive individuals (OR and NR respectively), as shown before by Hartmann et al. (2003). The presence of a supernumerary reproductive caused aggression between the two reproductives and other colony members. The colonies were observed over several days until aggression had ceased (10-35 days, 35-52 sessions of 10 min each; 350-520 min total observation time; X = 436.6 min).

Phase 3: orphaning the colonies

We removed all old and the new reproductives plus all eggs and kept them frozen for further analyses. Colonies were then observed until new eggs were discovered (which indicates the establishment of a new reproductive individual) in the colonies and aggression had ceased (observation time: 4-5 days, 16-17 sessions of 10 min each; 160-170 min total observation time; X = 165 min; first eggs appeared 2-3 days after removal of the former reproductives, which suggests that policing workers had already begun to activate their ovaries during Phase 2). To test whether aggressive behaviour has a selfish component, we investigated whether this new reproductive had been engaged in aggression in Phase 2. If aggression serves selfishness, the individual that becomes reproductive in Phase 3 should have been among the aggressive individuals in Phase 2.

Ovarian Activation

After observations, all individuals were frozen and dissected under a stereomicroscope to assess the activation of their ovaries. As described in Hartmann et al. (2003), individuals were categorized by their state of ovarian activity: Status I: undeveloped ovaries (ovary length: $X \pm SD$ = 1.40 ± 0.88 mm), Status II: developed ovaries, but no yellow bodies (ovary length: $X \pm SD$ = 4.76 ± 1.45 mm) and Status III: fully developed, mature ovaries with oocytes and yellow bodies suggesting former egg-laying activity (ovary length: $X \pm SD$ = 8.44 ± 2.43 mm). We determined quantitatively that our assignment of categories indeed corresponded to ovary length, since ovary length differed significantly between all three categories (ANOVA:

$F_{2,134} = 121.9$, $p < 0.0001$). Specifically, we found that ants with ovary Status III had the longest ovaries and ants with Status I, the shortest (post hoc Scheffé's test: Status I-II: $p < 0.0001$; Status I-III: $p < 0.0001$; Status II-III: $p < 0.01$).

All analyses were conducted with Statistica version 6.1 (Statsoft , Tulsa, OK, U.S.A.). For comparing the number of aggressive attacks received by reproductives and nonreproductives (calculated by aggressive acts/individual per 10 min), two-sample permutation tests (two-tailed) were performed with PAST version 1.82b (Hammer et al. 2001).

Results

Whereas in natural, unmanipulated colonies only infrequent ritualized aggression (antennal boxing) was observed (observations before colony splitting), in reunited colonies workers acted much more violently by stinging, biting, dragging and immobilizing the opponent. ORs and NRs were observed fighting each other, showing sting-smearing behaviour, again as previously observed (Hartmann et al. 2003). Sting smearing appears to cause nonreproductives to join the fight and to attack the ORs and NRs. Generally, nonreproductives did not show any preference for either of the reproductives (see Table 3.1). An overview of the number of attacks reproductive individuals received from other reproductives and nonreproductives is given in Table 3.1. In four of six colonies, reproductives (OR or NR) were significantly more frequently attacked than nonreproductive individuals (two-sample permutation test; Fig. 3.2).

Colony	Reproductives	Number of attacks received by non-reproductives	Number of attacks received by reproductives	χ^2	*P*
K1	OR	0	0	15	<0.001
	NR	7	8		
K2	OR	3	1		
	NR	1	1		
K3	OR	15	6	21	<0.001
	NR	0	0		
K6	OR	15	36		
	NR1	11	22	0.04	0.84
	NR2	10	6		
K7	OR	0	0		
	NR	1	1		
K8	OR	1	2		
	NR	1	1		

Table 3.1. Number of attacks old and new reproductives (OR, NR) received from non-reproductives and other reproductive individuals in colonies of the parthenogenetic ant *Platythyrea punctata* after the re-unification of colony fragments (for details see text, Chi square tests, χ^2 and *P* values are given in the table).

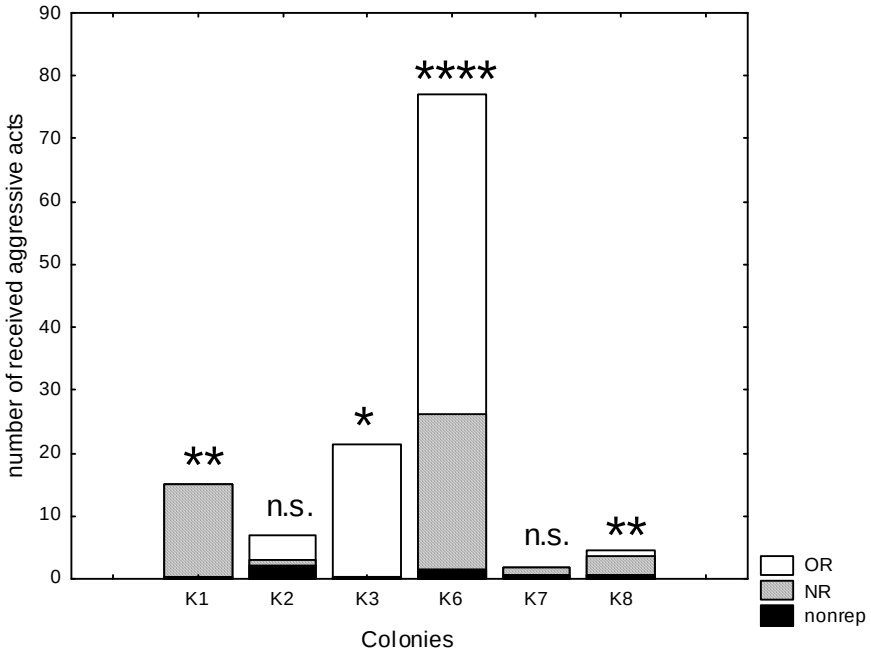

Figure 3.2. Number of attacks received by old reproductives (OR), new reproductives (NR) and nonreproductive workers (nonrep) during the policing phase (Phase 2) in colonies of the clonal ant *Platythyrea punctata* (calculated by aggressive acts/individual/10 min). Number of individuals and total time of observation in each colony: K1: 42 individuals, 350 min observation; K2: 35 individuals, 520 min; K3: 31 individuals, 440 min; K6: 41 individuals, 430 min; K7: 22 individuals, 450 min; K8: 14 individuals, 430 min. *P* values denote the significant differences between the number of received attacks by ORs and NRs together against nonreproductive individuals (two-sample permutation test: $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$).

In the policing phase (Phase 2), individuals were not equally likely to initiate aggressive acts. In all six colonies, only a minority of workers exhibited at least one aggressive act, and not all workers attacked one or both reproductives (Table 3.2). The number of attacks expected from a Poisson distribution differed significantly from the observed number of attacks (Chi-square test: $\lambda = 1.676$, $X^2_2 = 149.94$, $p < 0.00001$; Fig. 3.3). Some of these attackers had been in the colony fragment with the OR, some in the fragment with the NR, and in three colonies the attackers were freshly born individuals, which eclosed during Phase 2 and therefore had not been present during the splitting process. Table 3.2 gives an overview of the origin of aggressive individuals and shows that aggressive behaviour against reproductive individuals was not influenced by a change of colony odours during Phase 1.

How often individuals behaved aggressively during the policing phase (Phase 2) was clearly connected with the reproductive status they later obtained in the orphaned colonies (in Phase 3). When dissected after the experiment, individuals that were most aggressive during

Colony	Total number of non-reproductives	Total number of non-reproductives being aggressive	Total number of non-reproductives attacking reproductives	Reproductives	Origin of nonreproductive attackers
K1	40	9	7	OR	-
				NR	1 'A'; 3 'B'; 3 callows
K2	33	24	4	OR	2 'A'; 1 'B'
				NR	1 'B'
K3	29	19	15	OR	6 'A'; 6 'B'; 3 callows
				NR	-
K6	38	23	20	OR	6 'A'; 6 'B'; 3 callows
				NR1	7 'A'; 1 'B'; 3 callows
				NR2	7 'A'; 1 'B'; 2 callows
K7	20	9	1	OR	-
				NR	1 'A'
K8	12	5	2	OR	1 'A'
				NR	1 'B'

In the splitting phase (Phase1), old and new reproductives (OR and NR) had been separated with non-reproductives into fragments 'A' and 'B'. After new reproductives were established, both fragments were reunited.

Table 3.2. Distribution of aggression and origin of non-reproductive individuals attacking reproductives after the re-unification of two colony fragments of *Platythyrea punctata*. Not all non-reproductive individuals were aggressive, and not all of the aggressive individuals attacked the reproductives. In the splitting phase (phase1), old and new reproductives (OR and NR) had been separated with non-reproductives in two colony fragments 'A' and 'B'. After reuniting both fragments, ORs and NRs were attacked similarly by non-reproductives from both fragments. Freshly eclosed workers (callows), which were born after reunion, were engaged in aggression in five colonies.

Phase 2 had ovaries with the highest status of activation (Status III) after we orphaned the colonies again in Phase 3 (ANOVA: $F_{2, 144} = 3.92$, $p' < 0.05$ after Bonferroni's correction for 3 comparisons; Fig. 3.4).

In four of six colonies, aggression (number of aggressive acts/individual per 10 min) decreased significantly after we orphaned the colonies in Phase 3 (Wilcoxon signed-ranks test: K1: $Z = 0.228$, $n = 49$, $p = 0.820$; K2: $Z = 4.432$, $n = 35$, $p < 0.0001$; K3: $Z = 3.823$, $n = 36$, $p < 0.0001$; K6: $Z = 4.457$, $n = 41$, $p < 0.0001$; K7: $Z = 2.366$, $n = 17$, $p = 0.018$; K8: $Z = 0.731$, $n = 14$, $p = 0.465$). Aggression decreased rapidly within a few days, and the appearance of new eggs after 2-3 days suggests that a new egg layer had very quickly become established.

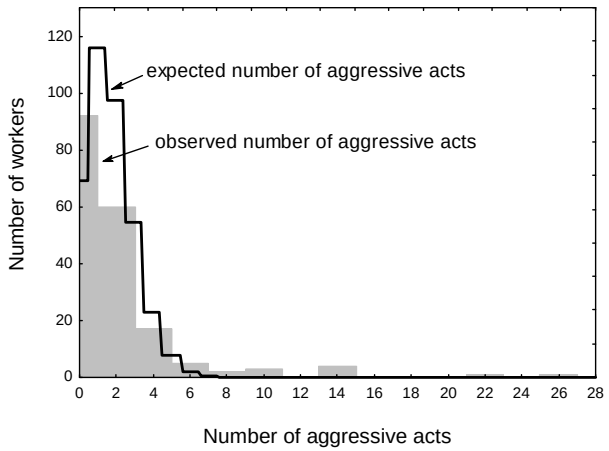

Figure 3.3. Frequency distribution of worker aggression during the policing phase (Phase 2) in colonies of *Platythyrea punctata*. The number of aggressive acts per individual workers is highly skewed, with only few workers initiating most attacks (Chi-Square test (expected versus observed) of the number of aggressive acts from a Poisson distribution, $\lambda = 1.676$, $X^2 = 149.94$, $df = 2$ (adjusted), $P < 0.00001$).

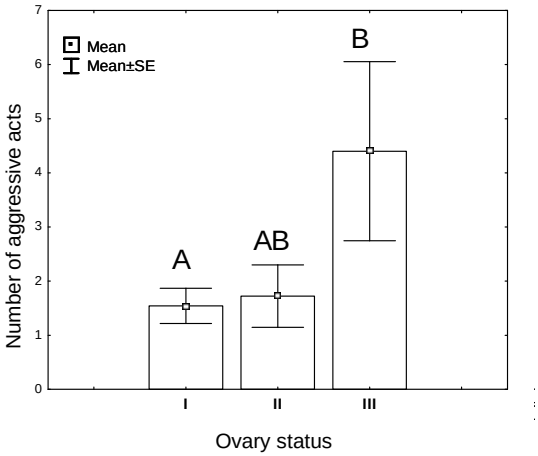

Figure 3.4. Aggressive acts initiated during phase 2 of the experiment by workers of *Platythyrea punctata* with different ovarian status at the end of the experiment. Workers with developed ovaries in orphaned colonies (Phase 3) were most aggressive during the policing phase (Phase 2) (Scheffé's test, $df = 144$). Significant differences are indicated by different letters. Data were square-root transformed prior to statistical analysis but are here depicted untransformed.

Discussion

Our study demonstrates that aggression in *P. punctata* is associated with an increase in the likelihood of an individual becoming a reproductive in the future. As predicted by policing models (Frank 1996), only the most dominant individuals of a society attacked new reproductives. They were also most likely to become the future reproductives when all established reproductives were removed from the nest. Both freshly eclosed workers, which soon after eclosion establish their position in the hierarchy of unmanipulated colonies (Heinze und Hölldobler 1995; K. Kellner, unpublished data), and a few older workers behaved aggressively. By our manipulation of splitting and later reuniting the colonies, we presumably disturbed the natural rank orders, and individuals that attacked the new reproductives tried to re-establish or improve their social status. When we removed all reproductives and eggs from the colonies, it took only 2-3 days until new eggs were found within the colonies, and only 4-5 days until aggression declined. This suggests that police workers not only behaved aggressively during the policing phase, but also at least prepared for egg laying at the same

time, probably in response to the changed social structure of the nest. Another explanation could be that successful aggression reinforces the dominance status of high-ranking workers, which may result in the activation of ovaries so that, as soon as the colony becomes orphaned, a new reproductive becomes established and ensures egg production without delay. This might explain the right-skewed distribution of aggression in Fig. 3.3.

Why do workers of *P. punctata* aggressively compete for egg-laying rights and dominance, even though they are usually all members of the same clone? Policing and dominance might maximize the reproductive output of the whole group. By attacking surplus reproductives or destroying their eggs, workers maintain a fine-tuned numerical balance between reproductives and nonreproductives, which maximizes the total output of the colony and thus maximizes inclusive fitness (Hartmann et al. 2003). Furthermore, workers, even in clonal societies, might vary in their reproductive potential. If physical strength were a reliable indicator of an individual's reproductive potential (but see Ortius und Heinze 1999 for a case in which it is not), aggressive policing and dominance might ensure that the most fecund individual becomes the next reproductive. This would increase the average inclusive fitness of all nestmates, while simultaneously minimizing the costs of reduced group productivity. Policing in *P. punctata* might therefore be maintained because it serves the interests of the group, but we doubt that it evolved as a group-level adaptation. Policing is widespread in ponerine ants, whereas thelytoky in *P. punctata* is a derived trait (e.g. Hartmann et al. 2005). Policing, therefore, was presumably present already in genetically heterogeneous ancestors of thelytokous *P. punctata* as an adaptation at the individual level, because, as shown by Gardner & Grafen (2009), mechanisms of conflict resolution cannot be regarded as group-level adaptations.

Rather, we feel that policing in *P. punctata* originally had a selfish component, as recently shown also in other social insects (Saigo und Tsuchida 2004; Wenseleers et al. 2005; Wenseleers und Ratnieks 2006a; Stroeymeyt et al. 2007). Genetic data document that a considerable percentage of colonies are genetically heterogeneous because of the adoption of unrelated workers, colony fusion (K. Kellner, unpublished data) or occasional sexual reproduction (Hartmann et al. 2005). Furthermore, in the thelytokous ant *Pristomyrmex punctatus* selfish clones exploit other clones by monopolizing reproduction (Dobata et al. 2009). In the case of fusion or usurpation, aggressive competition among workers of *P. punctata* may therefore be considered as selfish policing, as it would benefit some but not all members of the society.

Disentangling the various causes of the maintenance of policing in clonal colonies of *P. punctata* requires more detailed studies on group productivity, the frequency of adoption and fusion, and the likelihood that adopted individuals or usurpers start to reproduce.

Acknowledgments

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Chapter 3

Chemical correlates of reproduction and worker policing in a myrmicine ant

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Abstract

In a number of wasps, bees, and in particular ponerine ants, quantitative and qualitative variation in the profile of cuticular hydrocarbons is associated with variation in fecundity and is likely to serve for communicating the reproductive status of an individual. Here, we demonstrate that the chemical profile on the cuticle of fertile workers and queens of the myrmicine ant *Temnothorax unifasciatus* is different from that of non-reproductive workers. Fertility and apparently also cuticular signatures are reversible under the influence of policing by worker aggression. Though no policing by egg-eating occurs in this species, queen and worker laid eggs also differed in their chemical profile.

Keywords: Cuticular hydrocarbons, Worker policing, Fertility signalling, Fertility reversion

Introduction

The observation of fighting, domination, and punishment among nestmates in the colonies of ants, bees, and wasps has challenged the conception of insect societies as harmonious and conflict-free superorganisms. Instead, their structure often appears to rely on mutual control and policing, through which individual selfishness that counteracts the joint interests of the whole group is minimized.

One of the predominant causes of disunity in the society is the unequal kinship of a worker to the male offspring produced by itself, other workers, and the queen (Hamilton 1964; Trivers and Hare 1976; Ratnieks 1988). Workers of most species of ants, bees, and wasps have retained their ovaries in evolution and in principle are capable of producing males from unfertilized eggs (Bourke 1988a). Excluding the few species with clonal societies, workers are always most closely related to their own sons. Furthermore, in a society with a single, singly-mated queen (monogyny and monandry) they are also more closely related to the sons of other workers than to the sons of the queen (Ratnieks 1988; Heinze 2004; Ratnieks et al. 2006). Worker reproduction in monogynous, monandrous societies therefore increases the relatedness between workers and the male sexuals they take care of. Nevertheless, worker reproduction is surprisingly rare in the presence of the queen (Hammond and Keller 2004; Heinze 2004; but see Wenseelers and Ratnieks 2006a). This is probably because worker selfishness negatively affects the productivity of the colony as a whole (Cole 1986). Workers may then always benefit from policing reproductive workers by aggression (e.g., Ratnieks 1988; Visscher and Dukas 1995; Kikuta and Tsuji 1999; Liebig et al. 1999; Iwanishi et al. 2003; Cuvillier-Hot et al. 2005; Stroeymeyt et al. 2007) or the selective removal of eggs laid by workers (e.g., Kikuta and Tsuji 1999; d´Ettorre et al. 2004; Endler et al. 2004; Ratnieks and Visscher 1989; Foster and Ratnieks 2001b; Saigo and Tsuchida 2004; Wenseelers et al. 2005; Liebig et al. 2005).

Worker policing implies that workers are capable of assessing the fertility of individuals and / or of discriminating between queen- and worker-laid eggs (Nonacs and Carlin 1990; Boomsma et al. 2003). Cuticular hydrocarbons (CHCs) have long been known to play a role in species and nestmate recognition (Singer 1998; Hefetz 2007). They often vary also in quantity and quality with reproductive status of individuals and may convey information about the origin or parentage of freshly laid eggs. Queens and fertile workers posses a chemical bouquet different from that of non-fertile workers (reviewed in Monnin

2006), and queen-laid eggs differ from worker-laid eggs (Vander Meer and Morel 1995; Monnin and Peeters 1997; Endler et al. 2004; d´Ettorre et al. 2004; Dietemann et al. 2005).

With the exception of the honey bee, most previous research on the variation in chemical compounds associated with fecundity has focussed on taxa with a limited caste dimorphism, such as polistine wasps and the ant subfamily Ponerinae (Monnin 2006). Social insects with more pronounced differences between workers and queens, including the Formicinae and the largest ant subfamily, the Myrmicinae, have been less well covered. It was even concluded from the lack of distinct profiles in fertile workers in the formicine *Camponotus floridanus* that workers of such species are not able of producing a queen-like signal due to their reduced reproductive abilities (Endler et al. 2007).

The species-rich myrmicine genus *Temnothorax* has long been established as a model system in investigations of colony odour (Stuart 1988; Provost et al. 1993; Heinze et al. 1996; Trabalon et al. 2000; Tentschert et al. 2002), kin conflict, and policing (Cole 1981; Franks and Scovell 1983; Bourke 1988b; Heinze and Smith 1990; Heinze et al. 1997; Stroeymeyt et al. 2007). Here, we demonstrate that the chemical profiles of queens and fertile workers of the monogynous and monandrous ant *Temnothorax unifasciatus* differ from those of non-fertile workers. Furthermore, queen-laid eggs differ from eggs laid by workers, though policing by egg eating does not occur (Stroeymeyt et al. 2007). Finally, we provide data suggesting that, under prolonged policing, fertile workers stop reproducing and also revert to chemical profiles similar to those of non-laying workers.

Material and Methods

Ant collecting, maintenance, and behavioural observation

In March 2006 we collected complete colonies of *Temnothorax unifasciatus* (Latreille 1798) from their nests in rotting sticks in a sun-exposed slope near Gargnano (Lago di Garda / Italy; 45° 41’ N; 10° 39’ E). Colonies were transferred into small plastic boxes (10 cm x 10 cm x 3 cm) with a regularly moistened plaster floor and kept in incubators under artificial climate conditions with the temperature gradually being raised from spring (10°C night / 20 °C day) to summer (17°C night / 28°C day) conditions (Buschinger 1974; Heinze and Ortius 1991). The colonies were provided with water, honey, and pieces of cockroaches twice per week.

Experiments were conducted in May and June 2006, i.e., four to eight weeks after colonies had been transferred to summer condition (group *a*, ten colonies, A1 – A10), and in August, i.e., 13 weeks after transfer to summer conditions (group *b*, six colonies, B1 – B6). All workers (23 to 63 workers per colony: X 44.7 ± SD 10.2) were individually marked by tying 0.025mm thin copper wire (courtesy of Elektrisola, Eckenhagen) around different body parts. Two to four weeks after marking, each colony was split into a queenright and queenless colony fragment. Larvae were equally apportioned to the two colony halves, while the eggs were given only to the queenright fragment. Dominant workers were identified by observing aggressive interactions (antennal boxing and biting) among individuals in the queenless colony fragments. Individuals initiating more than 20% of the aggressive acts per colony were classified as "dominants 1" (D1), less aggressive workers initiating between 1 and 20 % were classified as "dominants 2" (D2). All other workers from the queenless fragment were classified as "subordinates" (S), and workers from the queenright colony fragments (QW) were used as control. All colonies were observed in 10-min sessions over a period of four to five weeks, resulting in a total observation time of 5 hours per colony. Two weeks after first worker-laid eggs had appeared in the queenless half, colony fragments were reunited and observed again for a total of 5 hours over 10 days.

For comparison of aggressions directed towards dominant and subordinate workers after reunion of colonies, we used two-sample permutation tests computed by the program PAST (PAleaentological STatistics, ver. 1.73, Hammer et al. 2001). We combined the independent *p* values from all colonies using Stouffer's method (Whitlock 2005) to obtain a single level of significance for the whole data-set.

Before splitting the colonies we collected ten to 15 presumably queen-laid eggs (QE) each from nine colonies (A1-A5, B1, B2, B4, B6) and ten to 15 worker-laid eggs (WE) each from nine queenless colony fragments (A1-A4, B1, B2, B4-B6). Eggs from each colony were pooled in one vial each and stored at -20°C. Colonies were killed by freezing 5 to 8 weeks (group *a*) and 1½ to 2½ weeks (group *b*) after reunification and stored for further analyses. After solvent extraction (see below) workers and queens were dissected to assess the development of their ovaries (Buschinger and Alloway 1978). The ovaries of workers were classified into four stages of development: stage I = short (<0.5mm), undeveloped ovarioles; stage II = slightly elongated (> 0.5mm) ovarioles without eggs; stage III = slightly elongated (> 0.5mm) ovarioles with non-viable eggs (roundish, with large vacuole), stage IV = strongly elongated (> 1mm) ovarioles with viable, oval eggs similar to those found in the ovaries of queens (Fig. 2.1).

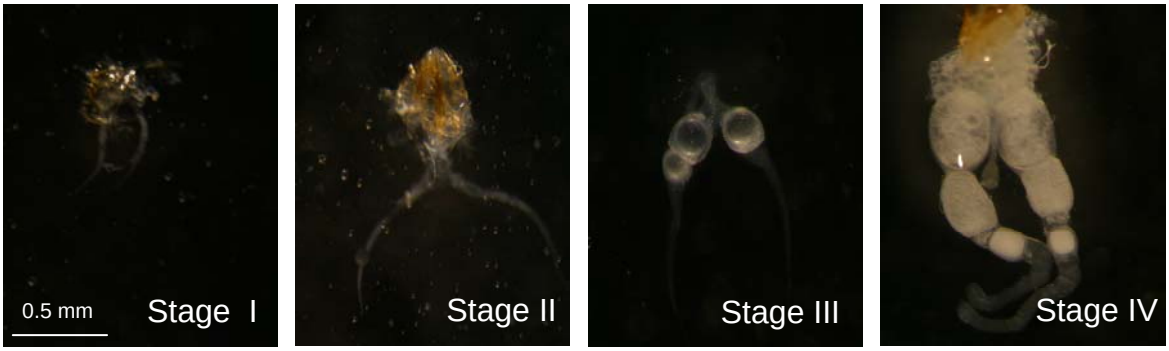

Figure 2.1. Four different stages of ovarian development in *T. unifasciatus* workers. Stage I represents short undeveloped ovarioles, stage II slightly elongated ovarioles without eggs, stage III slightly elongated ovarioles with non-viable eggs and stage IV strongly elongated ovarioles with viable eggs.

Chemical Analysis

Hydrocarbons were extracted from all dominant workers (D1, D2) surviving the attacks, several subordinate individuals (S), workers from the queenright colony fragments (QW) and the queen (Q) from each colony by individually immersing them for 10 min in 20 µl pentane. After evaporation of the solvent, the residues were re-dissolved in 15 µl pentane, of which 2 µl were injected into an Agilent Technologies 6890N gas chromatograph equipped with a flame ionisation detector and a HP-5 capillary column (30 m x 0.32 mm x 0.25 µm, J&W Scientific, USA). Hydrocarbons of eggs were obtained by soaking pooled eggs from each colony for 1 min in 15 µl of pentane, of which 2 µl were injected. The injector was split / splitless and the carrying gas was helium at 1 ml/min. Temperature was held at 70°C for 1 min, increased from 70°C to 180°C at 30°C/min, from 180°C to 310°C at 5°C/min, and held constant at 310°C for 5 min.

Gas chromatography gave consistently 39 peaks, of which 30 peaks could be identified using combined gas chromatography and mass spectrometry (GC-MS) of the pooled extracts of 30 workers. The remaining peaks could not yet be identified because of their very low abundance. The GC (Agilent Technologies 6890N) was equipped with a RH-5ms+ fused silica capillary column (30 m × 0.25 mm x 0.25 µm, J&W Scientific, USA). The injector was split/splitless (250°C) with the purge valve opened after 60 sec and the carrying gas was helium at 1 ml/min. Temperature was held constant for 1 min at 60°C, increased from 60°C to 300°C at 5°C / min and held constant for 10 min at 300°C. The electron impact mass spectra (EI-MS; Agilent 5973 inert mass selective detector) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. We

identified *n*-alkanes by comparing mass spectra with data from a commercial MS library (NIST, Gaithersburg, MD, USA). Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices by the method of Carlson et al. (1998). MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA) for Windows was used for data acquisition.

For statistical analysis of the chemical profiles we included only peaks with a relative area of more than 0.7 % that were present in at least 50 % of the samples of a given group (Q, D1, D2, S, QW, QE, WE) (Liebig et al. 2000). Standardized peak areas were transformed by using the formula: $Zij = \log[Xi,j/g(Xj)]$, with Xi,j being the standardized peak area i for the sample j, and $g(Xj)$ the geometric mean of all peaks of the sample j (Reyment 1989). For multivariate analyses the number of variables was reduced by principle component analysis (PCA). The factor scores obtained by PCA were used in a subsequent discriminant analyses (DA) to determine whether predefined groups could be distinguished on the basis of their cuticular profiles and to assess the degree of similarity between groups. Groups were also compared by calculating the squared Mahalanobis distances between the group centroids. Wilks´ λ significance and the percentage of correct assignments were used to evaluate the validity of the discriminant function. We used Mann-Whitney U-tests to compare percentages of single compounds between the different categories (Q, D1, S, QE, WE). In each category individuals of groups *a* and *b* were analysed together. *p*-values were adjusted for multiple comparisons using Bonferroni's method. Statistical analysis was performed using Statistica 6.0.

Results

Behavioural observations and ovary dissection

As previously reported for colonies of this and other species of *Temnothorax* (e.g., Heinze et al. 1997; Stroeymeyt et al. 2007), one or two days after colony splitting a few workers began to behave aggressively in the queenless colony fragment (details not shown). First eggs appeared in the queenless colony fragments between 10 and 30 days after splitting (median, quartiles 15, 12.5, 20 days). In each colony, one or two individuals initiated more than 20% of the aggressive acts (D1) and up to eight additional workers exhibited aggressive behaviour at

a much lower rate (D2; median, quartiles 4, 2.5, 6 workers). The queen of colony B3 and most workers of colony A10 died during the observation period. These colonies were therefore excluded from further analyses.

After reunification of the colony fragments, we could observe aggression in 12 of 14 colonies. In five colonies, the aggressive acts were predominantly directed towards formerly aggressive individuals (D1 and D2; colonies A1 and B1, $p < 0.0001$; A6, A8 and B6, $p < 0.05$). In the other colonies, aggressions were too infrequent for meaningful statistical analysis (two-sample permutation tests: A7, $p = 0.806$; A9, $p = 0.449$; B2, $p = 0.696$; B4, $p = 0.582$; B5, $p = 0.164$; no statistical analysis possible in A3 and A4). The consensus *p*-value obtained from the meta-analysis confirmed that, over all, aggression was more frequently directed against dominant than subordinate workers (Stouffer's method; $S = 4.834$, $p < 0.0001$). In group *a* (frozen 5 to 8 weeks after reunion) four of nine D1-workers and in group *b* (frozen 1½ to 2½ weeks after reunion) nine of ten D1-workers had stage IV ovaries with viable eggs (Yates corrected $\chi^2 = 2.690$, $p = 0.101$; Table 2.1).

Classification of workers	No. total workers	No. workers stage I	No. workers stage II	No. workers stage III	No. workers stage IV
D1*a*	9	0	3	2	4
D2*a*	28	1	11	16	0
S*a*	32	10	12	10	0
WQ*a*	22	3	7	12	0
D1*b*	10	0	1	0	9
D2*b*	13	2	1	7	3
S*b*	28	13	5	9	1
WQ*b*	18	4	6	8	0

Table 2.1. Ovary development of high-ranking workers "dominants 1" (D1), "dominants 2" (D2), subordinate workers (S), and workers from the queenright colony fragments (QW) of both groups *a* (9 colonies, analysed 5 to 8 weeks after reunification of colony fragments in June 2006) and *b* (5 colonies, analysed 1 ½ to 2 ½ weeks after reunification in August 2006) of *Temnothorax unifasciatus*. For stages of ovary development see Figure 2.1.

Chemical analysis

The profiles of both adult ants and their eggs were predominantly characterized by the linear alkanes *n*-C_{27} and *n*-C_{29} (Fig. 2.2). Cuticular profiles of workers appeared to by colony-specific and it was possible to correctly assign 83.5% (81 of 97) of all workers from group *a*

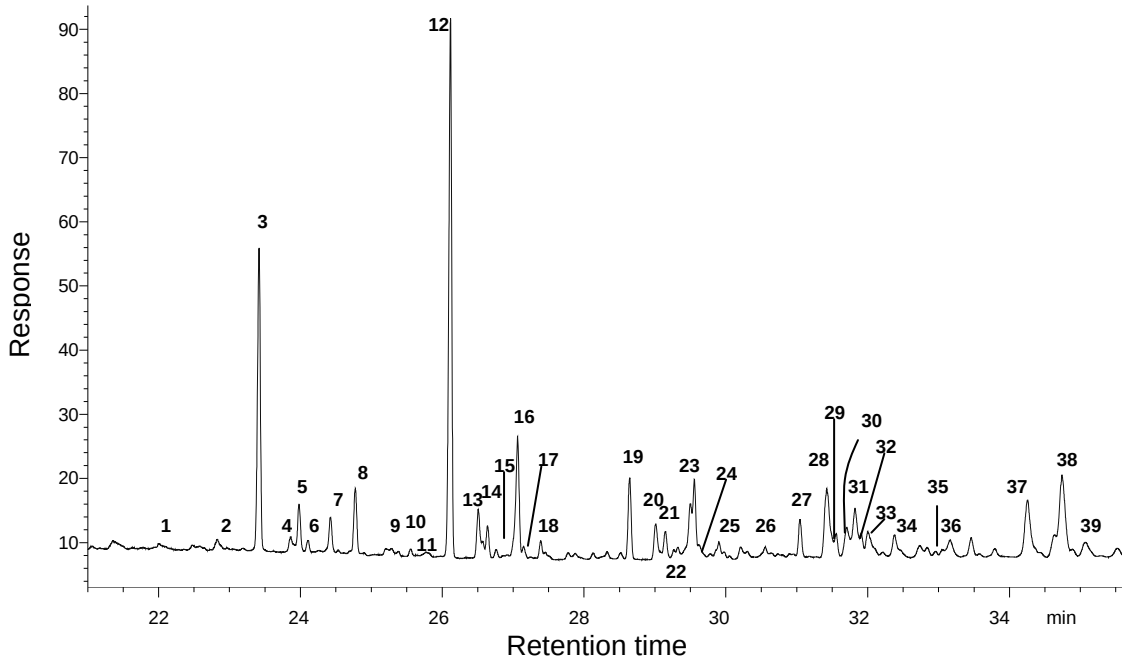

Figure 2.2. Representative gas chromatogram of a *Temnothorax unifasciatus* queen. Peaks used for the statistic analysis are marked with numbers. Identification of peaks is given in Table 2.5.

and 88.6% (62 of 70) of all workers from group *b* to their colony of origin. Variation among colonies in group *a* was explained by six significant functions, with the first three functions each explaining more than 10% of the total variation (Wilks' $\lambda = 0.007$, $F_{64.473} = 10.185$, $p < 0.0001$), and among colonies in group *b* by four significant functions, again with the first three functions each explaining more than 10% of the total variation (Wilks' $\lambda = 0.028$, $F_{32.215} = 11.074$, $p < 0.0001$).

In colonies of group *a*, studied early in the reproductive season, queens (Q*a*), aggressive workers (D1*a* and D2*a*), subordinate workers (S*a*), and workers from the queenright colony fragment (WQ*a*) were clearly separated from each other (Wilks' $\lambda = 0.249$, $F_{36.350} = 4.364$, $p < 0.0001$). Squared Mahalanobis distances among the five groups were all statistically significant at the 0.05-level except of that between D1*a* and D2*a* (Table 2.2). Between 44.4 and 88.9% of the individuals were correctly classified, with mismatches in particular among the different types of workers (Table 2.3). In colonies of series *b*, analysed approximately two months later, queens (Q*b*) were much less well separated from high-ranking (D1*b*) and subordinate workers (S*b*) (Wilks' $\lambda = 0.409$, $F_{32.233} = 2.005$, $p < 0.005$). The squared Mahalanobis distance between D1*b* and S*b* was still significant at the 0.05-level (Table 2.2). Mismatches involved all categories of animals, e.g., queens were misclassified as D1, D2, S and WQ (Table 2.3). In a third discriminant analysis including queens and workers

from both groups *a* and *b* (Wilks' $\lambda = 0.251$ $F_{72.101} = 3.568$, $p < 0.0001$), the pairs of aggressive workers (D1*a* / D1*b*, D2*a* / D2*b*) and subordinate workers (S*a* / S*b*) were significantly different, while the differences between queens (Q*a* / Q*b*) and between workers from the queenright parts (WQ*a* / WQ*b*) were only marginally significant ($p < 0.06$; Table 2.4; Fig. 2.3).

Category	D1*a*	D2*a*	S*a*	WQ*a*
Q*a*	5.44 *p*<0.05	11.26 ***p*<0.0001**	13.78 ***p*<0.0001**	8.95 ***p*<0.0001**
D1*a*		2.34 *p*=0.118	5.28 ***p*<0.001**	3.74 *p*<0.05
D2*a*			2.89 ***p*<0.0001**	1.65 *p*<0.05
S*a*				3.65 ***p*<0.0001**

Category	D1*b*	D2*b*	S*b*	WQ*b*
Q*b*	2.48 *p*=0.497	2.82 *p*=0.346	3.71 *p*=0.099	5.49 *p*<0.05
D1*b*		1.95 *p*=0.290	3.73 *p*<0.01	4.79 ***p*<0.005**
D2*b*			1.47 *p*=0.189	3.02 *p*<0.05
S*b*				1.43 *p*=0.089

Table 2.2. Squared Mahalanobis Distances and corresponding *p*-values between the categories queens (Q), high-ranking workers (D1), low-ranking workers (D2), subordinate workers (S) and workers from the queenright colony fragments (WQ) of *Temnothorax unifasciatus*, based on discriminant analyses of cuticular hydrocarbon profiles. Group *a* was analysed 5 to 8 weeks after reunification of colony fragments in June 2006 (left side), group *b* 1 ½ to 2 ½ weeks after reunification in August 2006 (right side). Figures in bold indicate *p*-values that are significant at the 5% probability after Bonferroni's correction ($p' < 0.005$).

	total No.	Q*a*	D1*a*	D2*a*	S*a*	WQ*a*	% correct assignment
Q*a*	9	**8**	0	0	0	1	88.9
D1*a*	9	0	**4**	2	1	2	44.4
D2*a*	28	0	1	**19**	3	5	67.9
S*a*	34	2	0	4	**24**	4	70.6
WQ*a*	26	0	0	4	5	**17**	65.9

	total No.	Q*b*	D1*b*	D2*b*	S*b*	WQ*b*	% correct assignment
Q*b*	5	**1**	1	1	1	1	20
D1*b*	10	0	**6**	2	0	2	60
D2*b*	13	0	1	**4**	5	3	30.8
S*b*	28	0	1	4	**21**	2	75
WQ*b*	19	0	0	0	6	**13**	68.4

Table 2.3. Percentage of correct assignments of individuals of queens and workers of *Temnothorax unifasciatus* to their predefined groups. Rows: classification based on behaviour and morphology (queens Q, high-ranking workers D1, lower-ranking workers D2, subordinate workers S, workers from the queenright colony fragments WQ). Columns: classification predicted by DA based on cuticular hydrocarbon profiles. Group *a* was analysed 5 to 8 weeks after reunification of colony fragments in June 2006 (above), group *b* 1 ½ to 2 ½ weeks after reunification in August 2006 (below).

Pairs of categories	Q*a* / Q*b*	D1*a* / D1*b*	D2*a* / D2*b*	S*a* / S*b*	WQ*a* / WQ*b*
Mahalanobis Distance	5.076	6.194	3.293	4.928	1.489
p-values	$p = 0.055$	$\mathbf{P < 0.001}$	$\mathbf{p < 0.001}$	$\mathbf{p < 0.0001}$	$p = 0.055$

Table 2.4. Squared Mahalanobis Distances and corresponding *p*-values between the similar types of individuals of *Temnothorax unifasciatus* (queens Q, high-ranking workers D1, lower-ranking workers D2, subordinate workers S, workers from the queenright colony fragments WQ) analysed either 5 to 8 weeks after reunification of colony fragments in June 2006 (group *a*) or 1 ½ to 2 ½ weeks after reunification in August 2006 (group *b*). Figures in bold indicate *p*-values that are significant at the 5% probability after Bonferroni's correction ($p' < 0.001$).

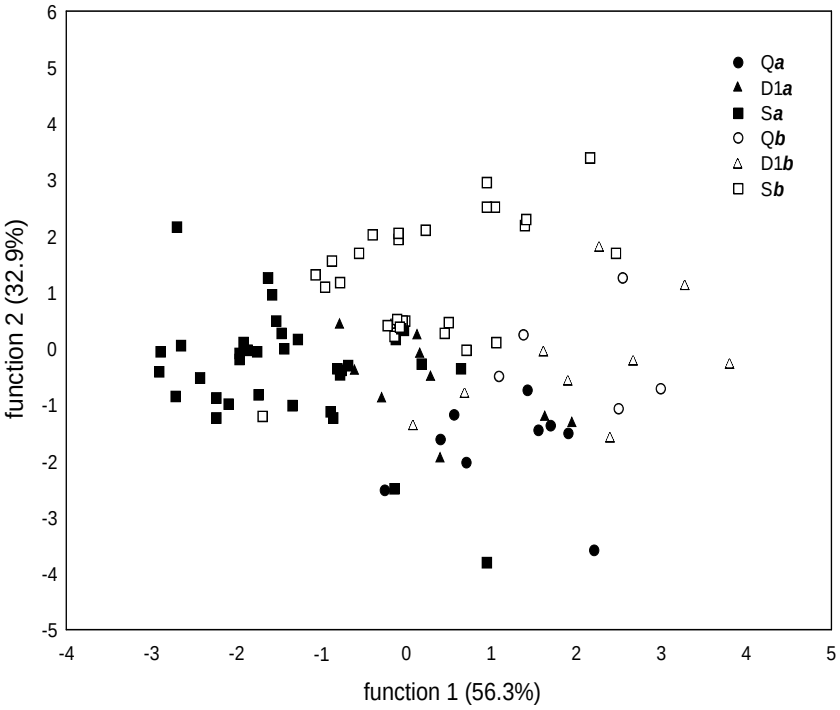

Figure 2.3. Discriminant analysis based on the cuticular hydrocarbons profiles of *Temnothorax unifasciatus* queens (Q*a*, $n = 9$; Q*b*; $n = 5$), dominant workers (D1*a*; $n = 9$; D1*b*; $n = 10$) and subordinate workers (S*a*, $n = 34$; S*b*, $n = 28$) from groups *a* (analysed 5 to 8 weeks after reunification of colony fragments in June 2006) and *b* (analysed 1 ½ to 2 ½ weeks after reunification in August 2006). Workers that exhibited aggressive behaviour with a lower frequency (D2) and workers from queenright colonies (WQ) are not shown for clarity of the figure. The percentages of variance explained by the two main discriminant functions are given in parenthesis (for details see text).

The cuticular blend of high-ranking workers and queens was characterized by significantly higher proportions of *n*-C_{29} and showed also higher percentages of two unidentified substances of low volatility (peaks No. 36 and 39; Fig. 2.4; Table 2.5). High-ranking workers were further characterized by significantly higher proportions of *n*-C_{27} compared to subordinate workers, while several other substances had a significantly higher relative abundance in subordinate workers (Table 2.5).

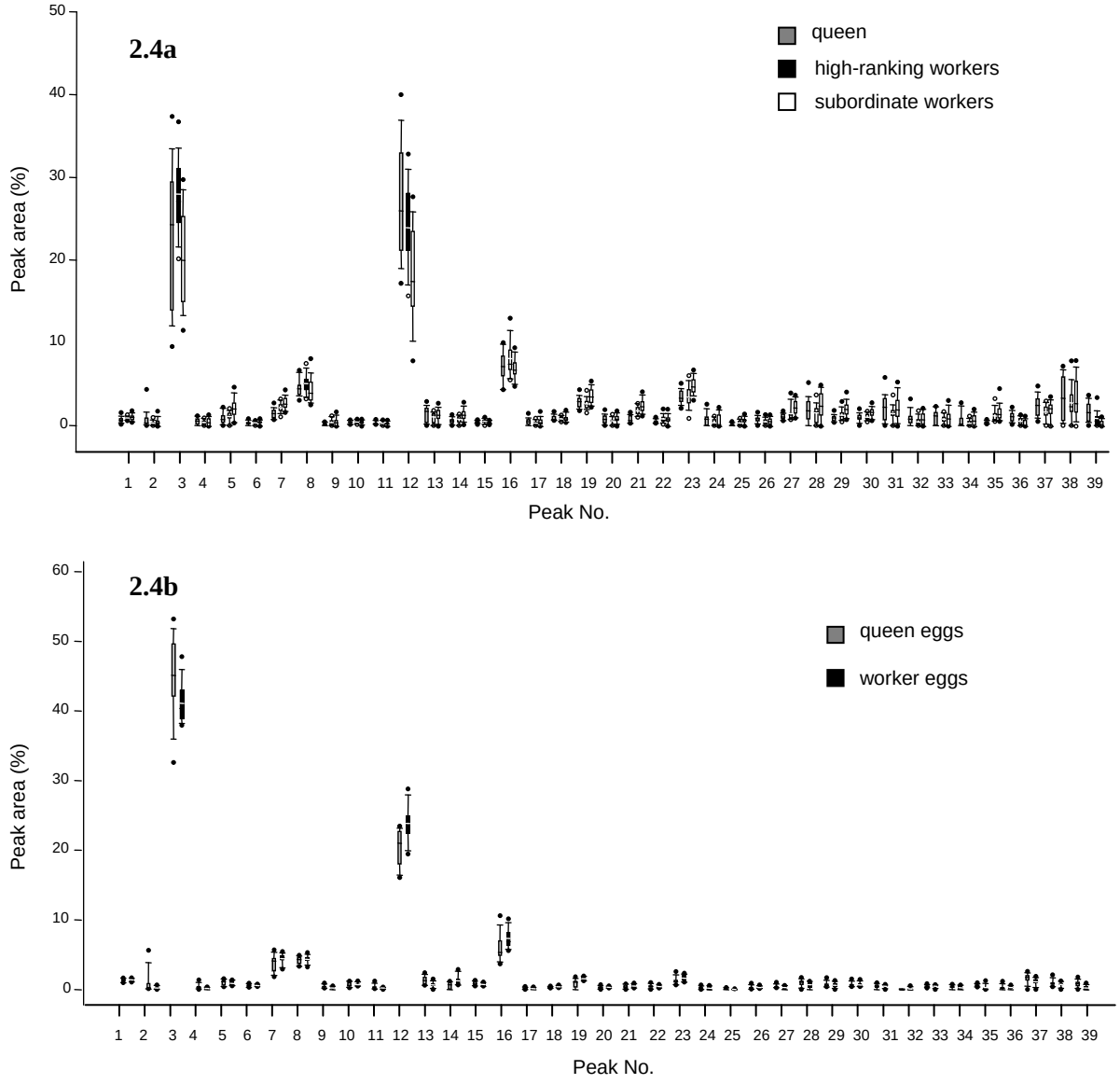

Fig. 2.4. Proportions (%) of peak areas in chromatograms from cuticular hydrocarbon extracts of queens, high-ranking workers (D1) and subordinate workers of *Temnothorax unifasciatus* (2.4a) and for queen- and worker eggs (2.4b). Box plots show medians and 25% and 75% quartiles. Whiskers depict the range of 90% of all cases. Extreme outliers are denoted by circles. *p*-values of substances differing significantly between the various groups are given in Table 2.5.

Worker-laid eggs were statistically significantly separated from queen-laid eggs ($p < 0.005$) (Wilks'λ 0.056, $F_{30.112} = 6.219$, $p < 0.0001$). All queen-laid eggs (QE) and seven of nine samples of worker-laid eggs (WE) were correctly classified. Queen-laid and worker-laid eggs differed mainly in peaks that constituted very low percentages of the whole cuticular profile (Table 2.5).

Peak No. (Fig.2.2)	Compound	Difference between Q(14) and D1(14)	Difference between Q(14) and S(62)	Difference between D1(14) and S(62)	Difference between QE(9) and WE(9)
1	*n*-C_{26}	$p < 0.05$	$p < 0.01$	*n.s.*	*n.s.*
2	4-me C_{26}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
3	*n*-C_{27}	*n.s.*	*n.s.*	**$p < 0.0001$**	*n.s.*
4	9-me C_{27} + 11-me C_{27} + 13-me C_{27}	*n.s.*	*n.s.*	*n.s.*	$p < 0.05$
5	7-me C_{27}	*n.s.*	**$p < 0.001$**	**$p < 0.001$**	*n.s.*
6	5-me C_{27}	*n.s.*	$p < 0.05$	$p < 0.05$	*n.s.*
7	3-me C_{27}	$p < 0.01$	**$p < 0.0001$**	$p < 0.05$	*n.s.*
8	*n*-C_{28}	*n.s.*	*n.s.*	$p < 0.05$	*n.s.*
9	8-me C_{28}	*n.s.*	$p < 0.01$	*n.s.*	$p < 0.05$
10	4-me C_{28}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
11	3-me C_{28}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
12	*n*-C_{29}	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	$p < 0.05$
13	9-me C_{29} + 11-me C_{29} + 13-me C_{29} + 15-me C_{29}	$p < 0.05$	*n.s.*	*n.s.*	$p < 0.05$
14	7-me C_{29}	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	*n.s.*
15	5-me C_{29}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
16	3-me C_{29}	*n.s.*	*n.s.*	$p < 0.05$	*n.s.*
17	5,x di-me C_{29}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
18	C_{30}	*n.s.*	*n.s.*	*n.s.*	$p < 0.05$
19	C_{31}	*n.s.*	$p < 0.01$	**$p < 0.0001$**	$p < 0.01$
20	9-me C_{31} + 11-me C_{31} + 13-me C_{31} + 15-me C_{31}	*n.s.*	*n.s.*	$p < 0.01$	*n.s.*
21	7-me C_{31}	**$p < 0.0001$**	**$p < 0.0001$**	**$p < 0.001$**	*n.s.*
22	13,17 di-me C_{31}	$p < 0.05$	$p < 0.01$	*n.s.*	*n.s.*
23	3-me C_{31}	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	*n.s.*
24	5,x di-me C_{31}	*n.s.*	*n.s.*		*n.s.*
25	unidentified	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	$p < 0.05$
26	unidentified	$p < 0.05$	*n.s.*	*n.s.*	*n.s.*
27	C_{33}	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	*n.s.*
28	9-me C_{31} + 11-me C_{31} + 13-me C_{31} + 15-me C_{33}	*n.s.*	*n.s.*	$p < 0.05$	*n.s.*
29	7-me C_{33}	*n.s.*	**$p < 0.0001$**	**$p < 0.001$**	*n.s.*
30	unidentified	*n.s.*	**$p < 0.001$**	**$p < 0.0001$**	*n.s.*
31	13,x di-me C_{33}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
32	unidentified	$p < 0.05$	*n.s.*	$p < 0.01$	*n.s.*
33	3-me C_{33}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
34	unidentified	*n.s.*	*n.s.*	$p < 0.01$	*n.s.*
35	unidentified	**$p < 0.0001$**	**$p < 0.0001$**	$p < 0.01$	$p < 0.01$
36	unidentified	$p < 0.01$	**$p < 0.0001$**	*n.s.*	*n.s.*
37	unidentified	*n.s.*	*n.s.*	*n.s.*	*n.s.*
38	x-me C_{36}	*n.s.*	*n.s.*	*n.s.*	*n.s.*
39	unidentified	$p < 0.05$	**$p < 0.0001$**	$p < 0.05$	$p < 0.05$

Table 2.5. Identification of cuticular compounds and corresponding significant *p*- values of differences of their relative amounts between pairs of categories of queens (Q), high-ranking (D1) and subordinate workers (S) and between queen-laid eggs (QE) and worker-laid eggs (WE) of *Temnothorax unifasciatus*. The number of samples in each category is given in parentheses. Peak numbers correspond with numbers in Fig. 2.2. Directions of differences are shown in Fig. 2.4. Figures in bold indicate *p*-values from Mann-Whitney U-tests that are significant at the 5% probability after Bonferroni's correction for 39 comparisons ($p' < 0.001$); *n.s* = nonsignificant.

Discussion

Workers of *Temnothorax unifasciatus*, which have began to lay eggs during a temporary separation from the queen, quickly become target of aggression from other workers after re-introduction into the queenright colony (Stroeymeyt et al. 2007). In this study we show that those workers differ in their chemical profiles from non-laying workers and are capable of producing a queen-like signature, which might provide the basis of worker policing. Despite of the rather pronounced size difference between queens and workers, *T. unifasciatus* in this respect resembles previously studied ponerine ants (e.g., Monnin, 2006). It differs from the formicine *Camponotus floridanus*, where egg-laying and infertile workers have very similar hydrocarbon profiles and fertile workers are not policed by physical aggression (Endler et al. 2007).

Direct evidence that certain cuticular substances communicate fecundity in social insects is as yet lacking and most studies remain correlative. This is also the case for our study. Individuals of different reproductive status differed consistently in their hydrocarbon profiles. In addition to a few minor peaks, which could not yet be identified, the most conspicuous differences involved linear alkanes, in particular n-C_{27} and n-C_{29}. Linear alkanes are also over-represented in egg-layers of other species (Monnin 2006). However, it is unclear whether n-alkanes are involved in communication (e.g. Dani et al. 2001; Monnin 2006; but see Akino et al. 2004; Lorenzi et al. 2004). Instead, branched alkanes or alkenes have mostly been associated with differences in fecundity, though a common pattern is as yet not clearly evident (reviewed by Monnin 2006). In addition, like in the honeybee (Slessor et al. 2005), pheromones originating in various glands might also be involved in signalling the fertility status.

Worker policing by aggression serves to prevent or stop selfish workers from laying eggs (Monnin and Ratnieks 2001). It clearly achieves this aim when formerly fertile workers are killed or expelled from the nest, as occasionally occurs also in *T. unifasciatus* (unpublished). A comparison between colonies of groups *a* and *b* indicates that policing might occasionally also lead to the reversal of reproductive status, as previously shown in workers of bumblebees (Alaux et al. 2007) and honeybees (Malka et al. 2007). In colonies of groups *a* and *b*, extractions of cuticular hydrocarbons and dissections of workers were performed at different times of summer. The difference among queens and workers from groups *a* and *b* in their chemical profiles might in part reflect temporal changes of hydrocarbon profiles. However, the changes in the cuticular profiles of queens and workers from queenright halves

appear to be less distinct than those in workers from the queenless halves. Differences in the hydrocarbon profile of high-ranking workers between groups *a* and *b* might therefore be affected also by the different duration of exposure to worker policing and reflect different stages of ovary degeneration. After two weeks of policing, the ovaries of nine of ten workers still contained viable eggs, while 5 to 8 weeks after reunion only half of the dominants were still reproductive. This, albeit not statistically significant, is a trend in the expected direction. Similarly, high-ranking workers and queens could not be separated on the basis of their chemical profiles two weeks after reunion, but were clearly different after 5 to 8 weeks. We therefore propose that under the influence of policing high-ranking workers appear to "switch off" their ovaries and to change their chemical profile. This matches previous findings that workers, which had reproduced in queenless colony fragments, were no longer observed to lay eggs after several days of reunification with the queenright half. Though such workers laid stained eggs in queenless fragments after being fed fat-soluble dyes (e.g., Stroeymeyt et al. 2007), no stained eggs were found after reunification of the colony fragments (unpublished data). The potential reversibility of cuticular profiles might also have contributed to the comparatively low percentage of correct assignments in the discriminant analyses. In addition, social and reproductive hierarchies form a continuum, which we artificially split into discrete categories in the discriminant analysis. Whether the assumed reversal of reproduction in *T. unifasciatus* workers is due to self-restraint (Keller and Nonacs 1993), induced by the presence of the queen and the imposed cost by being policed (Wenseleers et al. 2004a, b), or whether the reproductive workers are physically suppressed by other workers and / or the queen remains unclear.

We have previously shown that worker policing by egg eating does not occur in *T. unifasciatus* (Stroeymeyt et al. 2007). Nevertheless, eggs laid by queens and workers could be statistically separated on the basis of their chemical profiles. This finding is in contrast to other species in which worker-laid eggs differ chemically from queen-laid eggs and are selectively removed (Monnin and Peeters 1997; Endler et al. 2004; d´Ettorre et al. 2004; but see Dietemann et al. 2005). In *T. unifasciatus*, queen- and worker-laid eggs differed only in a few minor peaks and, although statistically separated from each other, workers might not be capable of distinguishing between the two. Compounds that aid statistical differentiation may not be used as recognition cues (Breed 1998). Furthermore, genetic data indicate that male production by workers is very rare in natural, queenright colonies (Heinze et al. 1997; Hammond and Keller 2004). Workers may therefore usually never encounter viable eggs laid by other workers and not selected to police such eggs. Dissection data revealed that the

ovaries of many workers from queenright colonies were to some extent elongated and contained roundish eggs with large yolk vacuoles. Trophic eggs are regularly produced by workers of other *Temnothorax* species (Wesson 1940; Le Masne 1953; Dejean and Passera 1974), but as trophic egg laying has only occasionally been observed in *T. unifasciatus* (B. Walter, unpublished), such eggs may perhaps be usually reabsorbed.

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Chapter 4

Queen Pheromones in *Temnothorax* ant species: Queen Control or Honest Signals?

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Abstract

The division of reproductive labor among group members in insect societies is regulated by "queen pheromones." However, it remains controversial whether they actively suppress worker reproduction or honestly signal the fertility status of the queen, to which workers react in their own interest by refraining from laying eggs. Manipulative queen control is thought to lead to an evolutionary arms race between queens and workers, resulting in complex queen bouquets that diverge strongly among different populations and species. In contrast, honest signals would evolve more slowly and might therefore differ less strongly within and among species. In this study, we compared the composition of worker and queen cuticular hydrocarbons among different species of the ant genus *Temnothorax*. The chemical profiles of two sibling species were more similar to each other than the profiles of more distantly related species. Furthermore, in mixed-species colonies, queens were not able to fully suppress egg-laying by workers of more distantly related species, while workers did not reproduce under the influence of a queen from a sibling species. These results suggest that pheromones are not fully conserved in evolution but evolve at a slow rate, in contrast to what would be expected under the queen control hypothesis.

Keywords: Queen pheromones, worker reproduction, honest signal, *Temnothorax*

Introduction

The efficiency and integrity of the societies of ants, bees, and wasps relies on a well-controlled division of reproduction (Hölldobler and Wilson 2008; Wilson 1971). Workers rarely lay eggs in the presence of fertile queens (Hammond and Keller 2004; Heinze 2004). This is surprising, given that workers are more closely related to their own sons (r = 0.5) than the sons of the queens (r = 0.25) and in most species are well capable of producing their own male offspring from unfertilized eggs when the queen has been removed (Bourke 1988; Choe 1988).

Complete worker sterility benefits the queen, which should be selected to inhibit worker reproduction. However, overt aggression by the queen is very rare and appears restricted to very small colonies (Brunner and Heinze 2009; Franks and Scovell 1983; Kikuta and Tsuji 1999; Wenseleers et al. 2005). This suggests the involvement of chemical cues in the regulation of reproduction, and indeed, the composition of gland secretions and / or cuticular waxes varies with fecundity (Heinze and d'Ettorre 2009; Monnin 2006).

Queen-specific chemicals might act as primer pheromones that actively suppress the ovaries of workers (Hölldobler and Wilson 1983; Kaatz et al. 1992). However, it has been argued that inhibitive queen control were instable in evolution if acting against the fitness interests of the workers (Keller and Nonacs 1993). Mutations that rendered workers insensitive to queen pheromones would spread in the population, again changing the selection pressures on queens and favoring queen mutations that qualitatively or quantitatively changed their manipulative agents. The resulting arms race between queens and workers would eventually lead to more and more complex pheromone mixtures. Alternatively, pheromones produced by the queens might honestly signal their level of fertility to which workers react in their own interest, e.g., to avoid being attacked by the queen or other workers (Heinze and d'Ettorre 2009; Keller and Nonacs 1993; Ratnieks 1988; Wenseleers et al. 2004a,b). Honest signaling requires that the quantity or quality of queen pheromones is strictly associated with their level of fertility and mating status (Keller and Nonacs 1993). This, however, is often not the case. For example, unmated reproductives may produce a similar pheromonal bouquet as mated reproductives (d' Ettorre et al. 2004; Heinze and d'Ettorre 2009). Furthermore, the hypothesis of manipulative regulation of reproduction appears to gain renewed support. Worker altruism has recently been interpreted as "enforced", in that workers refrain from

laying eggs because of effective policing mechanisms (Ratnieks and Wenseleers 2008; Wenseleers et al. 2004b; Wenseleers and Ratnieks 2006a,b).

Distinguishing between queen control and honest signaling is difficult without detailed knowledge about the molecular and cellular mechanisms involved. However, both mechanisms likely leave different traces in evolution (Heinze and d'Ettorre 2009). The series of manipulation and counter-manipulation associated with the scenario of queen control results in a rapid evolution of queen compounds. Queen pheromones therefore likely differ even between related species. In contrast, honest signals are expected to be more stable in evolution and to evolve more slowly. Unfortunately, little is known about the variation of fertility-associated chemical compounds among related species. In this study, we compared the composition of worker and queen cuticular hydrocarbons among different species of the ant genus *Temnothorax*. In addition, we investigated whether workers begin laying eggs in the presence of a queen from another, closely or distantly related species. We hypothesized that with queen control and rapid evolution, queens should be less efficient in suppressing ovary development by workers from another species. Furthermore, we expected the chemical bouquet of queens to differ more between species than those of workers. In contrast, in the case of honest signaling, workers would react to fertility signals by queens from another species, and the bouquets of queens from different species would be not more different than those of workers.

Material and Methods

In spring 2005 and 2006 we collected complete colonies of six different *Temnothorax* species: *T. nylanderi* (Förster, 1850) and *T. affinis* (Mayr, 1855) in Sommerhausen (Würzburg, Germany), *T. crassispinus* (Karavejev, 1926) in Unterisling (Regensburg, Germany), *T. unifasciatus* (Latreille, 1798) in Waldenhausen (Germany) and Gargnano (Lago di Garda, Italy) and *T. recedens* (Nylander, 1856) and *T. lichtensteini* (Bondroit, 1918) Gargnano (Lago di Garda, Italy). While *T. nylanderi* and *T. crassispinus* are close related sibling species (Seifert 1995) the other four species are phylogenetically more distantly related (Fig. S1; see appendix).

Temnothorax colonies were collected from their nests in decaying branches on the ground and, in *T. unifasciatus*, *T. lichtensteini* and *T. recedens*, also from crevices in stone

walls. The colonies were transferred into small plastic boxes (10 cm x 10 cm x 3 cm) with a regularly moistened plaster floor and kept in incubators under artificial climate conditions with the temperature gradually being raised from spring (10°C night / 20 °C day) to summer (17°C night / 28°C day) conditions (Buschinger 1974; Heinze and Ortius 1991). Twice per week, colonies were provided with water, honey, and pieces of cockroaches.

Mixed-species colony set up

In 2005, colonies of *T. nylanderi*, *T. crassispinus* and *T. unifasciatus*, with a sufficient amount of larvae in each colony, were chosen for the mixed-species experiment (N = 45 colonies; Table 4.1). In 2006, the same mixed-species colonies were set up with additional mixed colonies plus *T. recedens* and colonies without a queen (N = 160 colonies; Table 4.1). The number of worker pupae in *T. recedens* colonies was restricted; therefore, only five mixed colonies with *T. recedens* worker pupae were set up (Table 4.1). Mixed colonies were set up in early summer, when most larvae had developed into pupae. We transferred 50 worker pupae of the same species into a nest with either a queen from a different species in mixed colonies or a con-specific queen in control colonies (Table 4.1). To obtain the required sample size of 50 pupae, worker pupae were taken from five different con-specific colonies. No larvae or eggs were added to the colonies. To allow worker pupae to fully develop we placed 30 marked adult workers from the colony of queen origin into each nest and removed them four weeks later after most of the pupae had developed into adult workers.

Worker ovary activation

In 2005, worker ovary activation was investigated in all colony set ups. In 2006, we investigated worker ovary activation in all colonies without a queen, all colonies with *T. recedens* workers and a queen from a different species, and five randomly chosen colonies of each of the remaining colony set ups (total N = 145 colonies; Table 4.1). The colonies were frozen six weeks after the transferred worker pupae had developed into adult worker, and workers and queens were dissected to assess their ovary activation (Buschinger and Alloway 1978). Workers having elongated ovaries (> 1 mm) with viable, oval eggs similar in shape and color to those found in the ovaries of queens were classified as “fertile”.

Colony composition		Colony name	Colonies set up in 2005	Colonies set up in 2006	No. total colonies	No. colonies used for the assessment of ovary activation	No. colonies used for the assessment of worker male-production
Queen species	Worker species						
T. nylanderi (control)		NN	NN1 – NN5	NN6 – NN15	15	10	5
T. crassispinus		CN	CN1 – CN5	CN6 – CN15	15	10	5
T. unifasciatus	*T. nylanderi*	UN	UN1 – UN5	UN6 – UN15	15	10	5
T. recedens		RN		RN1 – RN10	10	5	5
no queen		ØN		ØN1 – ØN5	5	5	-
T. crassispinus (control)		CC	CC1 – CC5	CC6 – CC15	15	10	5
T. nylanderi		NC	NC1–NC5	NC6–NC15	15	10	5
T. unifasciatus	*T. crassispinus*	UC	UC1 – UN5	UC6 – UN15	15	10	5
T. recedens		RC		RC1 – RC10	10	5	5
no queen		ØC		ØC1 – ØC5	5	5	-
T. unifasciatus (control)		UU	UU1 – UU5	UU6 – UU15	15	10	5
T. nylanderi		NU	NU1–NU5	NU6–NU15	15	10	5
T. crassispinus	*T. unifasciatus*	CU	CU1 – CU5	CU6 – CU15	15	10	5
T. recedens		RU		RU1 – RU10	10	5	5
no queen		ØU		ØU1 – ØU5	5	5	-
T. recedens (control)		RR		RR1 – RR5	5	5	-
T. nylanderi		NR		NR1 – NR5	5	5	-
T. crassispinus	*T. recedens*	CR		CR1 – CR5	5	5	-
T. unifasciatus		UR		UR1 – UR5	5	5	-
no queen		ØR		ØR1 – ØR5	5	5	-
Total no. of colonies:			45	160	205	145	60

Table 4.1. Mixed-species colony set ups composed of four different *Temnothorax* species. Colonies were set up in the years 2005 and 2006. The number of colonies randomly chosen for the assessment of ovary activation and for the assessment of worker male-production is given. The first letter in colony denominations indicates the species of the queen (Ø for queenless), the second the species of the workers.

For statistical analyses, two sample permutation tests were used to assess the difference of numbers of fertile workers per colony between groups of control colonies and mixed colonies and between control colonies and colonies without a queen.

Male-production by workers

The remaining 60 colonies of the 2006 set up were kept in incubators (gradual decrease of temperature to 0°C night / 10°C day for 15 weeks and gradual increase again to 17°C night / 28°C day thereafter) until hibernated brood had developed in 2007 (Table 4.1). From May to August 2007 all freshly enclosed adult males were collected and frozen at -20°C for further analyses. After all male pupae had enclosed, all colonies were frozen and queens were dissected to determine their ovarian status.

T. nylanderi, *T. crassispinus* and *T. unifasciatus* males are of dark brown pigmentation. *T. recedens* males have a pale pigmentation and could easily be distinguished from males of the other three species by inspecting their coloration (Fig. 4.1). *T. nylanderi*, *T.*

crassispinus and *T. unifasciatus* males are morphologically similar and thus were distinguished by electrophoresis of the glucose-6-phosoate isomerase (GPI; Seifert 1995; Pusch et al. 2006) or sequencing the mitochondrial cytochrome b (Cyt b) gene.

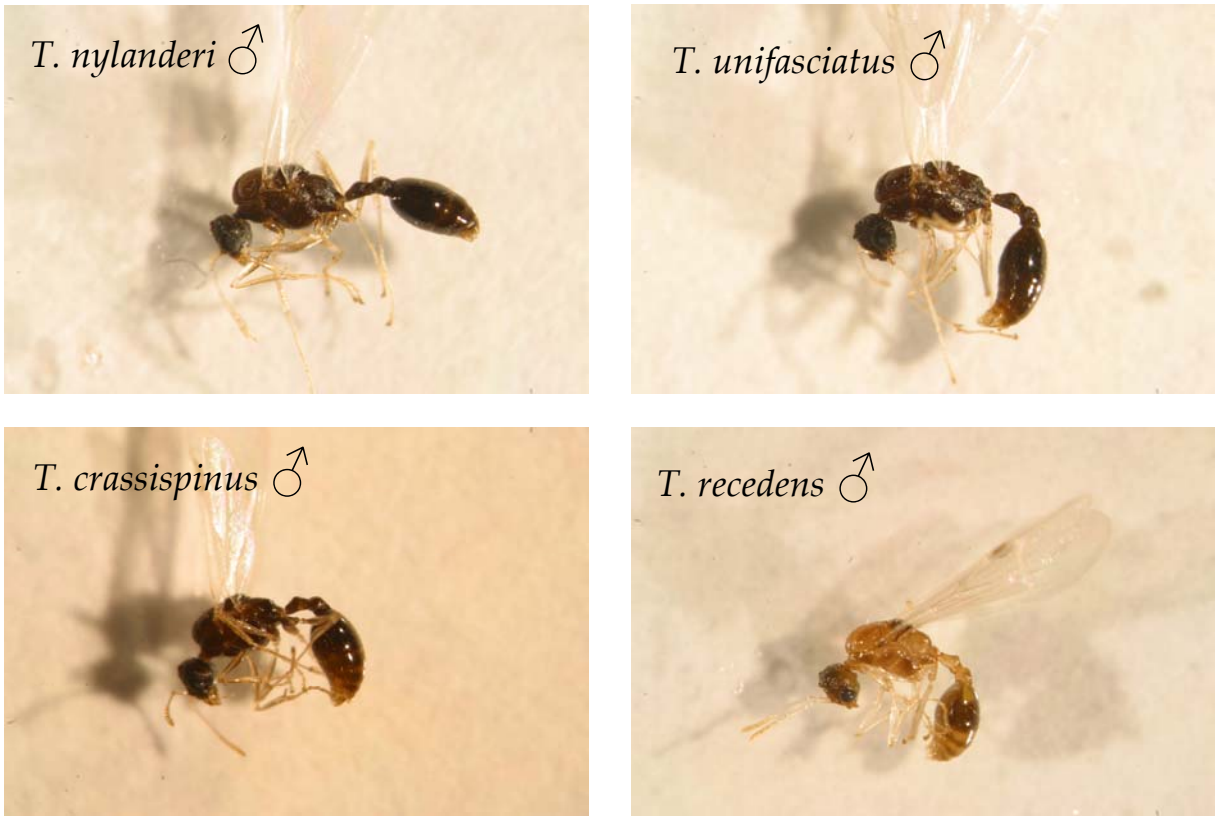

Figure 4.1. Males from the four *Temnothorax* species used for the mixed-species colonies.

Allozyme analyses

Electrophoresis of glucose-6-phosoate isomerase for *Temnothorax* ants has been described previously (Pusch et al. 2006). Electromorphs were named according to their migration velocities in the gel (fast *f*; medium *m*; slow *s*). *T. crassispinus* and *T. nylanderi* are fixed almost completely for the electromorphs *m* and *f*, respectively (Pusch et al. 2006; Seifert 1995) and *T. nylanderi* occasionally exhibits the electromorph *s* (Pusch et al. 2006). In *T. unifasciatus*, 32 of 36 workers from 20 colonies were homozygous for the electromorph *s* and 4 were heterozygous with electromorph genotype *sm* (see also Heinze et al. 1997). Queens were analyzed when necessary.

The gasters of individual workers and queens were homogenized in 20 µl Tris-EDTA pH 7.0 buffer. Proteins were separated by 90min electrophoresis at 10V/cm and 20mA on 10cm x 8 cm x 0.75mm 7.5% polyacrylamide slab gels using a Tris-glycine pH 8.3 buffer. The enzyme was stained using standard histochemical techniques (Murphy et al. 1996).

Mitochondrial analyses

When males could not be distinguished by electrophoresis, we in addition sequenced the cytochrome b (Cyt b) gene. DNA was extracted from the gasters of males using the CTAB method (1%) as previously described (Sambrook and Russell 2001). The mitochondrial cytochrome b (Cyt b) gene was analyzed using the primers CbI (CB-J-10933) and 16Sar (LR-N-13398) (Simon et al. 1994). The 20µl PCR reaction mixture consisted of 1µl DNA, 0.125 mM dNTPs, 0,25 µM of each primer, 11.1µl dd H_2O, 2µl 10x PCR buffer (MBI), 2.5 mM $MgCl_2$ and 1 µl of 1 unit /µl Taq Polymerase. Genes were amplified at an annealing temperature of 48°C with 38 cycles. PCR products were separated by electrophoresis on a 1% ethidiumbromide-stained agarose gel (TAE buffer) for 30 min at 100 mA and then purified with High Pure PCR cleanup Micro Kit (Roche). Cycle sequencing was carried out with 3µl of purified PCR-Product using ABI-Cycle sequencing Kit Version 1.1. Single-stranded PCR products were sequenced using an ABI PRISM 310 automatic sequencer (Perkin-Elmer, Applied Biosystems). The first 450 base pairs of the Sequences representing the Cyt b gene were read and aligned with Sequencing Analysis Software version 3.4 (Perkin-Elmer, Applied Biosystems).

Cuticular hydrocarbons of queens and workers from different species of Temnothorax ants

To estimate the chemical distances between different species of *Temnothorax* ants, queens and workers of the four species used for the mixed colony set ups, *T. nylanderi*, *T. crassispinus*, *T. unifasciatus*, and *T. recedens,* and queens and workers from two additional species, *T. affinis* and *T. lichtensteini,* were analyzed. From each species the queens of 5 to 10 colonies plus 1 to 3 workers from each of the colonies were chemically analyzed. All colonies were collected in spring 2006 (see above). *T. unifasciatus* colonies were used only from the population in Italy.

Chemical Analysis

Hydrocarbons were extracted four to five weeks after colonies had been subjected to artificial summer condition (17°C night / 28°C day; see above). Workers were frozen and hydrocarbons were obtained through solvent extraction by individually immersing each worker for 10 min in 20 µl pentane. After evaporation of the solvent, the residues were re-dissolved in 15 µl pentane, of which 2 µl were injected into an Agilent Technologies 6890N gas chromatograph. Hydrocarbons of queens were obtained through SPME (Solid Phase Micro Extraction) which gives qualitative and quantitative similar results (Tentschert et al. 2002). A 30µm polydimethylsiloxane fiber was gently rubbed for 10 min against the gaster of the immobilized queen and injected into the injection port of the same gas chromatograph as above. The gas chromatograph was equipped with a flame ionization detector and a HP-5 capillary column (30 m x 0.32 mm x 0.25 µm, J&W Scientific, USA). The injector was split / splitless and the carrying gas was helium at 1 ml/min. The same temperature program was used for the solvent and the solid phase micro extraction with the temperature initially held at 70°C for 1 min, increased from 70°C to 180°C at 30°C/min, from 180°C to 310°C at 5°C/min, and held constant at 310°C for 5 min.

For identification of the peaks, the pooled extracts of 30 workers of each species were injected into a combined gas chromatography and mass spectrometry (GC-MS; Agilent Technologies 6890N) equipped with a RH- 5ms+ fused silica capillary column (30 m × 0.25 mm x 0.25 µm, J&W Scientific, USA). The injector was split/splitless (250°C) with the purge valve opened after 60 sec and the carrying gas was helium at 1 ml/min. Temperature was held constant for 1 min at 60°C, increased from 60°C to 300°C at 5°C / min and held constant for 10 min at 300°C. The electron impact mass spectra (EI-MS; Agilent 5973 inert mass selective detector) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. We identified *n*-alkanes by comparing mass spectra with data from a commercial MS library (NIST, Gaithersburg, MD, USA). Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices by the method of Carlson et al. (1998). MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA) for Windows was used for data acquisition.

For statistical analysis of the chemical distance between species, we only included peaks consistently present in queens and workers of every species. Standardized peak areas were transformed by using the formula: $Zij = \log[Xi,j/g(Xj)]$, with Xi,j being the standardized

peak area *i* for the sample *j*, and g(X*j)* the geometric mean of all peaks of the sample *j* (Reyment 1989). For multivariate analyses, the number of variables was reduced by principle component analysis (PCA). The factor scores obtained by PCA were used in a subsequent discriminant analyses (DA) to determine whether groups could be distinguished on the basis of their cuticular profiles and to assess the degree of similarity between groups. Groups were also compared by calculating the squared Mahalanobis distances between the group centroids. Wilks´ λ significance and the percentage of correct assignments were used to evaluate the validity of the discriminant function. We used Mann-Whitney U-tests to compare percentages of single compounds between groups and adjusted *p*-values for multiple comparisons using Bonferroni's method. Statistical analysis was performed using Statistica 6.0.

Results

Worker ovary activation and male-production by workers

In some colonies the transferred worker pupae did not develop into adults. In 2005, 27 of 45 colonies and in 2006, 92 of 100 colonies chosen for the assessment of ovary activation in workers remained. In these colonies, all workers and the queen were dissected. All queens had elongated ovaries with numerous yellow bodies and maturing eggs, i.e, they were fully fertile. None of the workers in the control colonies had activated ovaries (colony groups NN, CC, UU, and RR; Fig. 4.2a-d). Though in a previous study (unpublished data) we observed sporadic worker egg-laying in queenright colonies of *T. recedens*, dissection data did not corroborate this result for the presently studied colonies.

In all queenless colonies the ovaries of several workers were activated (Fig. 4.2a-d). This indicates that workers are capable of activating their ovaries within six weeks after removal of the queen. Fertile workers were also found in some of the mixed-species colonies, such as RC (Fig. 4.2b), CU (Fig. 4.2c), NR and CR (Fig 4.2d; for colony abbreviation see Table 4.1).

In 2007, a total of 752 males were collected from 60 colonies (Table 4.2). Five colonies, in which the queen or most of the workers had died, were excluded from further analyses. In all colonies, except RC8, queens had fully developed and activated ovaries with yellow bodies and maturing eggs.

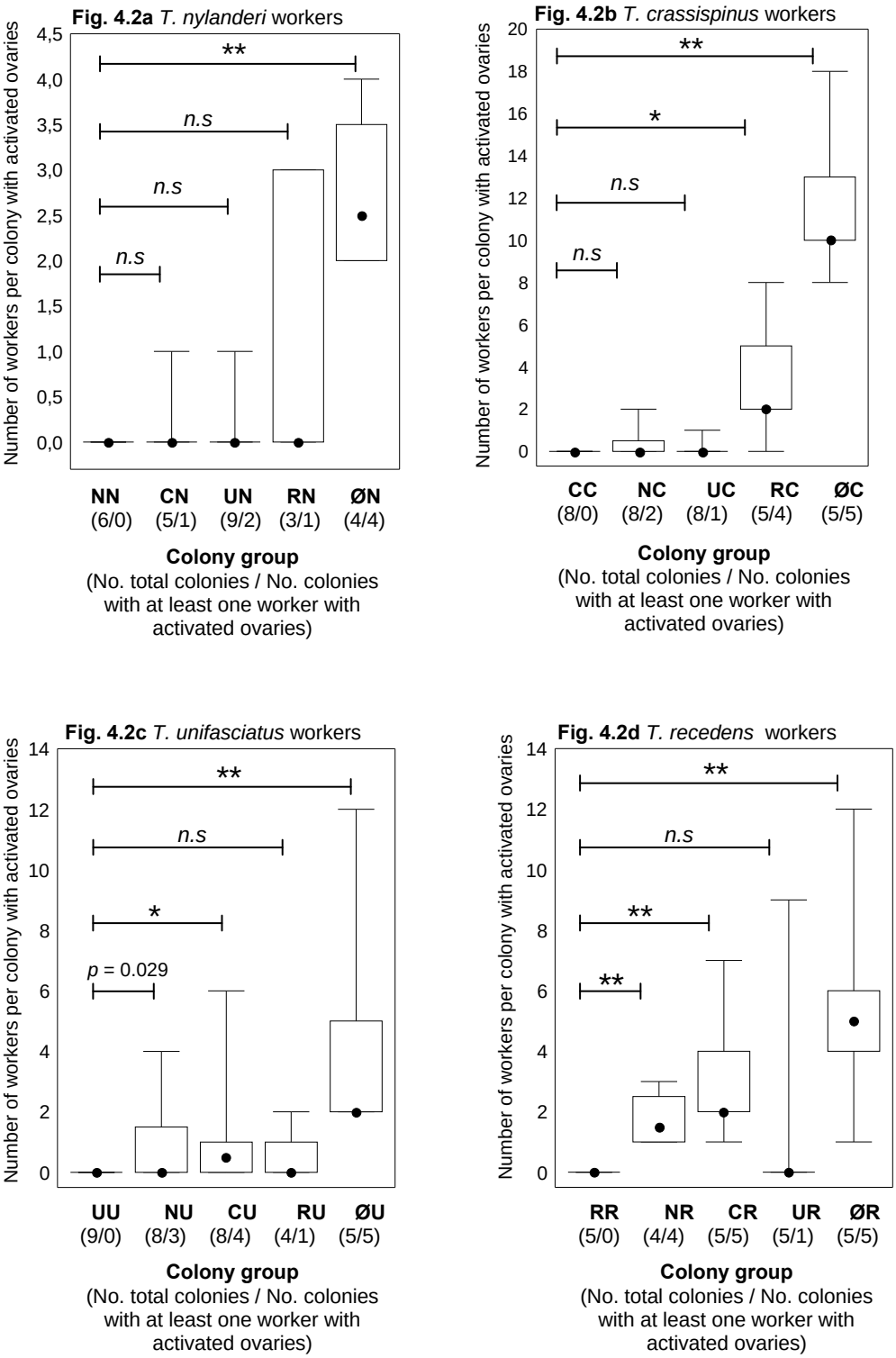

Figure 4.2a-d. Number of workers per colony with activated ovaries. Minimum and maximum (horizontal lines), first and third quartiles (rectangle), and the median (dot) are shown. The total number of colonies and the number of colonies with at least one worker with activated ovaries in each group is given in parentheses. *P*-values are from two-sample permutation tests (**p* < 0.01; ***p* < 0.0001; n.s. nonsignificant). After Bonferroni´s correction, *p*-values of < 0.01 are significant at the 0.05 level. Colony composition and colony names are explained in Table 4.1.

Colony	No. total males produced per colony	No. males *Allozyme electromorph*	No. males produced by the queen	No. males produced by workers
NN11, NN13, NN14, NN15	none			
CN7, CN12	none			
CN8	15	15 *f*	-	15
CN13	3	3 *m*	3	-
UN7, UN11, UN12, UN14, UN15	none			
RN4	3		3	-
RN5	20		11	9
RN6	20		15	5
RN8	5		5	-
CC12, CC13, CC14, CC15	none			
NC11, NC12, NC13, NC14, NC15	none			
UC7, UC13, UC15	none			
UC6	3	1*s*, 2*m*	1	2
UC8	1	1*m*	-	1
RC3	6		6	-
RC4	2		2	-
RC6	1		1	-
RC8	54		1	53*
UU6	1		1	-
UU8	2		2	-
UU9	1		1	-
UU10	5		5	-
UU12	29		29	-
NU10	8	8*s*	-	8
NU12	74	42*s*, 19*m*, 13*f*	13	61
NU13	37	28*s*, 3*m*, 6*f*	6	31
NU14	13	6*s*, 7*f*	7	6
NU15	59	59*s*	-	59
CU6	8	8*s*	-	8
CU9	2	2*s*	-	2
CU12	14	6*s*, 8*m*	-	14
CU15	44	26*s*, 18*m*	18	26
RU7	31		31	-
RU8	95		27	68
RU9	105		105	-

*In colony RC8 the ovaries of the queen were not fully developed.

Table 4.2. Number of males produced per colony in control and mixed-species colonies composed of different *Temnothorax* species. Numbers of males produced by the queen or by workers are shown, respectively. Colony composition and colony names are explained in Table 4.1.

Allozyme analyses revealed that males in mixed-species colonies were produced by the queen (e.g., CN13), by the workers (e.g., CN8) or both (e.g., RN5, NU12, CU15, Table 4.2). Queens of colonies UC6 and UC8 were homozygous for the *s* allele and *m*-males were therefore offspring of *T. crassispinus* workers, while the one *s*-male allele was son of a *T. unifasciatus* queen. All queens of colonies NU10 to NU15 were homozygous *ff*, and the *s*- and *m*-males were therefore offspring of *T. unifasciatus* workers.

In the CU colonies, *s*-males were presumably produced by *T. unifasciatus* workers, but *m*-males could in principle be sons of a *T. crassispinus* queen or *T. unifasciatus* workers. Mitochondrial DNA analysis revealed that the eight males in colony CU12 were offspring of *T. unifasciatus* workers, while the 18 males from the colony CU15 were produced by the *T. crassispinus* queen.

Figure 4.3 depicts the influence of *Temnothorax* queens on heterospecific workers. Though in one colony *T. nylanderi* workers produced males in the presence of a *T. crassispinus* queen, the results clearly suggest that the control mechanism is more efficient between the two closely related species than between more distantly related species.

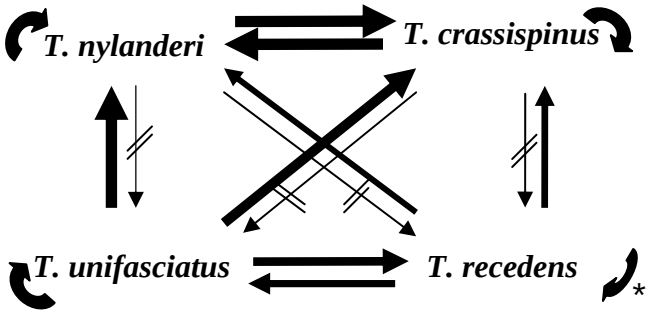

Figure 4.3. Influence of *Temnothorax* queens on ovary development and male production by workers from another *Temnothorax* species in mixed-species colonies in which workers were exposed to a queen from a different species. The thickness of the bar represents the strength of the influence. * *T. recedens* workers occasionally produce males in queenright conspecifics colonies (unpublished data).

Cuticular hydrocarbons of queens and workers from different species of Temnothorax ants

The cuticular profile of queens and workers of the six *Temnothorax* species consistently consisted of 40 peaks of which 35 could be identified in almost all species by GC-MS [see appendix: Fig. S2. Representative chromatography profiles from a queen of each species; Fig. S3. Proportions (%) of peak areas form cuticular hydrocarbon extracts of queens and workers

of each species; Table S1. Identification of cuticular compounds and differences of their relative amounts between queens (Q) and workers (W) of each species; see also Brunner et al. 2009 for identification of compounds in *T. unifasciatus*]. The profiles of queens and workers were predominantly characterized by the linear alkane *n*-C_{27} (Fig. S2, S3), while individuals of *T. recedens* were characterized by several longer chained hydrocarbons. Due to their very low abundance, peaks 36 to 40 and a few other peaks marked in Table S1 with an asterisk could not be identified. However, the latter peaks had exactly the same retention time as peaks in other species, which could be identified and are therefore assumed to be chemically identical to these compounds.

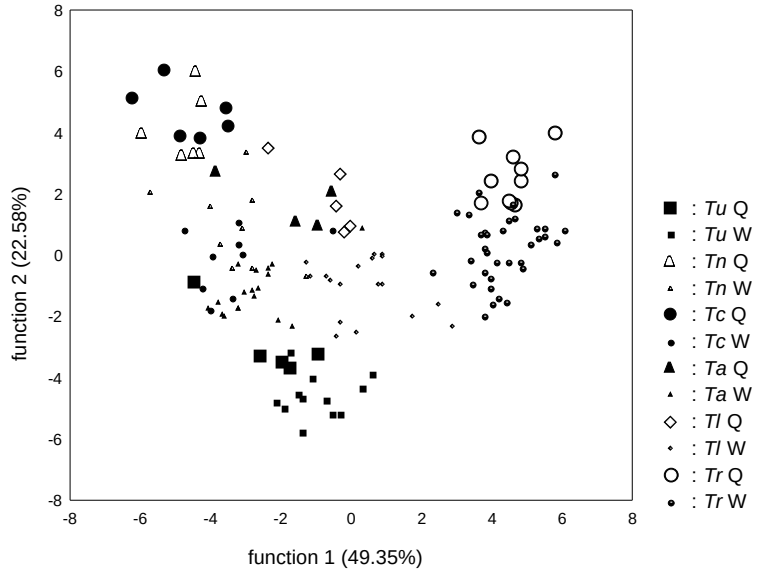

Figure 4.4. Discriminant analysis based on the cuticular hydrocarbons profiles of queens (Q) and workers (W) from six different species of *Temnothorax* ants: *T. unifasciatus* (*Tu* Q, *n* = 5; *Tu* W, *n* = 12), *T. nylanderi* (*Tn* Q, *n* = 6; *Tn* W; *n* = 9), *T. crassispinus* (*Tc* Q, *n* = 6; *Tc* W, *n* = 9), *T. affinis* (*Ta* Q, *n* = 4; *Ta* W, *n* = 18), *T. lichtensteini* (*Tl* Q, *n* = 5; *Tl* W, *n* = 18), *T. recedens* (*Tr* Q, *n* = 10; *Tr* W; *n* = 34). The percentages of variance explained by the two main discriminant functions are given in parentheses.

Queens	*Tc* Q	*Tu* Q	*Tr* Q	*Ta* Q	*Tl* Q
Tn Q	**10.53** *p*=0.0048	65.6 *	96.79 *	26 *	34.58 *
Tc Q		69.68 *	103.29 *	51.74 *	40.48 *
Tu Q			88.39 *	52.61 *	49.99 *
Tr Q				62.3 *	47.01 *
Ta Q					**16.44** *p*=0.001

Workers	*Tc* W	*Tu* W	*Tr* W	*Ta* W	*Tl* W
Tn W	**15.49** *	55.2 *	69.78 *	**19.44** *	40.68 *
Tc W		36.58 *	67.68 *	23.27 *	42.57 *
Tu W			56.36 *	29.66 *	43.22 *
Tr W				57.64 *	31.21 *
Ta W					**18.83** *

Table 4.3. Squared Mahalanobis Distances and corresponding *p*-values between the groups of queens (Q; left side) and workers (W; right side) of five species of *Temnothorax* ants (*Tn* = *T. nylanderi*, *Tc* = *T. crassispinus*, *Tu* = *T. unifasciatus*, *Tr* = *T. recedens*, *Ta* = *T. affinis* and *Tl* = *T. lichtensteini*) based on a discriminant analyses of their cuticular hydrocarbon profiles. * indicates *p*-values significant at the 5% probability after Bonferroni's correction (*p'* < 0.0007). Figures in bold indicate a close Mahalanobis Distance between a pair of groups in contrast to the other compared pairs of groups.

All groups of queens and workers from the six *Temnothorax* species were clearly separated from each other (Fig. 4.4; Wilks' $\lambda = 0.0002$, $F_{121.911} = 14.659$, $p < 0.0001$). Squared Mahalanobis distances among the groups were all statistically significant at the 0.05-level except between the groups of queens of *T. nylanderi* and *T. crassispinus* and the groups of queens of *T. affinis* and *T. lichtensteini* (Table 4.3). The groups of *T. recedens* queens and workers were chemically most distant from the groups of the other five *Temnothorax* species (Fig. 4.4; Table 4.3). Cuticular profiles of workers and queens appeared to be colony-specific as most individuals were classified correctly, with only single mismatches between the groups of *T. affinis* and *T. lichtensteini* and between the groups of workers of *T. nylanderi* and *T. crassispinus* (Table 4.4). Furthermore, several mismatches appeared between the groups of *T. recedens* queens and workers (Table 4.4). Most similarities of the comparison of percentages of single compounds between groups of queens and workers within a species were found among the two species *T. nylanderi* and *T. crassispinus* (Table 4.5). Interestingly, the Mahalanobis distance among queens was higher than that among workers in 11 of 15 pairwise comparisons.

Group	No. individuals per group	% correct classification	No. individuals (incorrect assignment)
Tu Q	5	100	
Tu W	12	100	
Tn Q	6	100	
Tn W	9	100	
Tc Q	6	100	
Tc W	9	88.89	1 (*Tn* W)
Ta Q	4	75	1 (*Tl* Q)
Ta W	18	94.44	1 (*Tl* W)
Tl Q	5	100	
Tl W	18	94.44	1 (*Ta* W)
Tr Q	10	70	3 (*Tr* W)
Tr W	34	97.06	1 (*Tr* Q)

Table 4.4. Percentage of correct classification of individuals from predefined groups of queens and workers from six different *Temnothorax* species predicted by DA based on cuticular hydrocarbon profiles. Group abbreviations are explained in Table 4.3.

	T. nylanderi	*T. crassispinus*	*T. recedens*	*T. lichtensteini*	*T. affinis*
T. unifasciatus	6	6	4	3	2
T. nylanderi		**16**	4	5	3
T. crassispinus			5	3	2
T. recedens				6	6
T. lichtensteini					7

Table 4.5. Number of cuticular compounds, which in pairwise comparisons among *Temnothorax* species exhibit similar differences between queens and workers. For example, among the substances differing between conspecific queens and workers, six show similar patterns of reproduction-specific variation in *T. unifasciatus* and *T. nylanderi.*

Discussion

Our study about the cross-specificity of the chemical compounds used in the regulation of reproduction in colonies of *Temnothorax* ants reveals a promising new approach to answering the question of whether queen pheromones are manipulative or honest signals. Queen pheromones appear to be less active across species-borders than within species. No worker reproduction was observed in single-species colonies and rarely in mixed colonies with workers and queens from the two sibling species *T. nylanderi* and *T. crassispinus*. In contrast, queens were not able to fully prevent ovary development and male-production by workers from a more distantly related species. On the one hand, this indicates that queen pheromones are not fully conserved in evolution. On the other hand, the analysis with *T. nylanderi* and *T. crassispinus*, which probably have diverged several hundred thousand years ago (Pusch et al. 2006), speaks against the rapid evolution of queen pheromones expected from the queen control hypothesis. These behavioral results are reflected in the chemical profiles of queens and workers, which in most pair wise comparisons differed more between queens than between workers from different species but not tremendously so. This might suggest that queen bouquets diverge slightly more quickly than those of workers, but not at an extremely rapid speed. A much higher tempo of evolution has been suggested by studies on the paper wasp *Polistes dominulus*, in which the cuticular hydrocarbons that differed between egg layers and non-reproductives varied even between different populations (Dapporto et al. 2004). Whether there is similar variation among conspecifics populations of *Temnothorax* remains to be investigated.

Our data do not reveal a clear trend in the chemistry of those hydrocarbons that differentiate queens and workers among the six investigated species. This matches the heterogeneous picture found in other, less closely related ants, where reproductives are characterized by particular long-chained hydrocarbons in some species but shorter or branched hydrocarbons in others (Endler et al., 2004; de Biseau et al., 2004; Monnin, 2006; van Zweden et al., 2009). However, it needs to be pointed out that it is usually not known, which of the large number of substances that differ between queens and workers are biologically active and which are mere side-products of the hydrocarbon metabolism without a function in communication (Heinze and d'Ettorre 2009). The similar direction of the differences between queens and workers in the two sibling species *T. nylanderi* and *T. crassispinus* might help identifying the active compounds.

Our study reveals a number of species idiosyncrasies that do not match phylogenetic relationships and are difficult to explain in the light of hypotheses about the nature of queen pheromones. For example, *T. nylanderi* and *T. crassispinus* workers seem to respond to the presence of a *T. unifasciatus* queen, while *T. unifasciatus* workers readily develop their ovaries in the presence of a *T. nylanderi* or *T. crassispinus* queen. This resembles the situation in honeybees, in which the presence of a heterospecific queen increases the rate of worker ovary activation strongly in *Apis cerena* but only slightly in *Apis mellifera* (Tan et al. 2009). In contrast, workers of the bumblebee *Bombus terrestris* develop their ovaries in queenright colonies of the phylogenetically related *B. lapidarius* at a similar rate as under queenless conditions, but not in queenright homospecific colonies (Alaux et al. 2006). The results in these studies might have been affected by the presence of workers belonging to the species of the queen. These might have an interest in preventing allospecific workers from reproducing through aggression or egg eating. Worker nepotism might explain the common absence of reproduction by host workers in socially parasitic ants. *Temnothorax* workers are often parasitized by queens of slave-making species, such as *Chalepoxenus*, *Myrmoxenus* or *Protomognathus*. Though these genera are less closely related to *Temnothorax* than the pairs of species used in our study (e.g., Beibl et al. 2005), enslaved workers rarely produce males (e.g., Foitzik and Herbers 2001; Heinze 1996a,b). Either slave-making queens have evolved particularly manipulative queen pheromones, which are active across large phylogenetic distances, or their reproductive monopoly is additionally enforced by aggression. Indeed, both queens and workers of slave-making ants have been observed to attack host workers in a way resembling the dominance interactions among the slave-makers themselves (Franks and Scovell 1983; Heinze 1996b).

Though our results do not allow drawing final conclusions about the speed of queen pheromone evolution, comparisons of their cross-species activity might help to learn more about the nature of such pheromones and how quickly they diverge among species.

Acknowledgements

Tina Wanke and Sabine Frohschammer kindly helped in the field. We hereby declare that the experiments comply with the current laws of Germany. This work was supported by Deutsche Forschungsgemeinschaft (He 1623/17).

Chapter 5

Cost of worker reproduction in the ant *Temnothorax crassispinus*

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Manuscript, submitted to Evolutionary Ecology

Abstract

Reduced colony productivity may be one of the reasons maintaining the altruistic behavior of workers in most social insect societies. Additional brood laid by workers could be detrimental for the colony as a whole and reduce the inclusive fitness of each individual. By experimentally increasing the brood size in colonies of a given size of the ant *T. crassispinus*, we checked whether surplus worker- or queen-produced brood led to higher colony productivity compared to colonies with normal amount of brood. While the addition of surplus worker-produced brood lead to a significant increase in the reproductive output, the addition of queen-produced brood only lead to a marginal increase. Hence, colonies of *T. crassispinus* appear to be able to raise additional male brood. Furthermore, in non-manipulated colonies, colony size was found negatively correlated with per capita productivity of workers, and positively with the per capita productivity of sexuals.

Keywords: Cost of worker reproduction, colony productivity, *Temnothorax*

Introduction

One of the key factors of the success of ants, bees and wasps is their highly efficient division of reproductive labor (Wilson 1971; Hölldobler and Wilson 2008). In most species, the queen produces the offspring of the colony, while workers engage in brood care, foraging, and colony maintenance. This is surprising, given that in colonies with a single, singly-mated queen (monogyny, monandry) workers are more closely related to their own sons (r = 0.5) and also the sons of other workers (r = 0.375) than to the sons of the queen (r = 0.25). Workers therefore should benefit most if they themselves or at least other workers produced the male offspring of the colony (Ratnieks 1988; Heinze et al. 1994; Bourke and Franks 1995). Workers are indeed capable of producing haploid males from unfertilized eggs (Bourke 1988; Choe 1988), but rarely do so in the presence of a queen (Hammond and Keller 2004; Heinze 2004). Workers, which in an experimental situation lay eggs in queenright colonies, are attacked by other workers and their eggs are eaten (worker policing; Ratnieks 1988) despite of relatedness benefits.

What explains worker self-restraint in the presence of the queen and policing against egg laying workers despite of the high relatedness between workers and worker-produced males? One hypothesis suggests that reproductive activities of workers are detrimental to the colony as a whole and lead to lower colony performance (Ratnieks 1988; Monnin and Ratnieks 2001; Hammond and Keller 2004; Wenseleers et al. 2004b). Reproductive workers work less and waste energy in dominance interactions (Cole 1986; Dampney et al. 2004). Furthermore, the monopoly of brood production by a single queen might reflect an optimized balance between the numbers of brood items and workers, which might be shifted to a suboptimal ratio through the presence of additional egg layers (Ratnieks and Reeve 1992; Hartmann et al. 2003). Worker reproduction might result in a ‘tragedy of the commons’ (Hardin 1968), in which colonies simply cannot rear all the additional worker-produced brood. Selection acting on the colony level would then stabilize the reproductive monopoly of the queen.

As yet, the hypothesis of productivity costs arising from the presence of additional reproductives has rarely been tested. As expected, the experimental addition of surplus brood did not lead to increased colony productivity in the parthenogenetic ant *Platythyrea punctata* (Hartmann et al. 2003). However, due to the peculiar life history of this ant, this finding cannot be generalized.

In this study, we investigated whether worker reproduction is costly at the colony level by manipulating the ratio between colony size and amount of brood items in colonies of the monogynous, monandrous ant *Temnothorax crassispinus* (Strätz and Heinze 2004). Though expected on relatedness grounds, workers do not reproduce in queenright colonies. As the correlation between worker number and the reproductive output of insect colonies appears to vary tremendously among species (Michener 1964; Jeanne and Nordheim 1996; Karsai and Wenzel 1998), we first determined the association between colony- and brood-size by counting eclosing adults in colonies of different size. For the manipulation of brood size, we set up colonies of a standardized size, which either received the average amount of unrelated brood found in the field or the double amount of either worker- or queen-produced, unrelated brood. We then monitored the brood development until brood eclosure the following reproductive season. If worker reproduction were selected against because it is associated with efficiency costs at the level of the colony, the experimental increase in the number of brood items would not simultaneously lead to higher productivity of the colony.

Material and Methods

Colony productivity

Colonies of *Temnothorax crassispinus* (Karavejev, 1926) nest in small decaying wooden branches in Eastern Central Europe (Seifert 2007). Though colonies are usually monogynous and monandrous, the decay of nest sites during summer occasionally leads to colony fusion and subsequent genetic heterogeneity in late summer. As this affects sex allocation ratios (Strätz and Heinze 2004), we collected colonies in early summer when most contain workers from a single matriline.

In May 2005, 220 complete colonies of *T. crassispinus* were collected in a deciduous forest in Unterisling (Regensburg; Germany). The colonies were transferred into small plastic boxes (10 cm x 10 cm x 3 cm) with a regularly moistened plaster floor and kept in incubators under artificial climate conditions (17°C night / 28°C day; Buschinger 1974; Heinze and Ortius 1991). Twice per week colonies were provided with standardized quantities of water, diluted honey (150 µl), and pieces of cockroaches of a standardized size. We used only the second and third pairs of legs with all segments from large cockroaches.

In November 2005, just before hibernation, workers were counted in each colony. Colonies were then transferred into artificial hibernation, with the temperature being gradually decreased (3 weeks at 15°C night / 25°C day, 4 weeks at 10°C night / 20°C day, 15 weeks at 0°C night/ 10°C day), and gradually increased again thereafter (6 weeks at 10°C night / 20°C day, 3 weeks at 15°C night/ 25°C day and eventually 17°C night / 28°C day). After hibernation, sexuals and callow workers produced in each colony were counted in May 2006.

In queenless colonies of *T. crassispinus* resources are allocated more into sexuals and sex investment ratios are more female-biased (Strätz and Heinze 2004). Therefore, colonies in which queens died during hibernation were not included in the analyses.

Brood manipulation / Colony set up

For the experimental manipulation of worker and brood ratio, additional *T. crassispinus* colonies were collected in June 2006 and 2007. The variance of brood size of the colonies collected in May 2005 was lowest in colonies with a size of 30 to 40 workers, producing an average of 110 brood items. Therefore, 60 colonies with a similar size were chosen and adjusted to 40 workers per colony. All brood items were removed from the colonies, mixed, and redistributed to randomly chosen 40 colonies. Mixing brood items should minimize the potential risk of nepotistic brood rearing. Twenty colonies used as controls received the normal amount of brood (n = 110) and the other 20 colonies received the double amount of brood (n = 220). Brood composition was arranged accordingly to the stage of the present brood development in the colonies: 21 eggs, 16 small larvae, 33 large larvae and 40 pre-pupae. In 2007, additional 20 colonies of similar size were chosen and adjusted to 40 workers per colony. Again all brood was removed, mixed, and redistributed to all colonies, in which each colony received 110 brood items plus the same amount of worker-produced brood items (total n = 220). Brood was composed as in the previous year. Worker-produced brood was obtained from additional queenless colonies in which the queen had been removed the previous year.

Brood manipulation / Colony observation

In both years, brood items and eclosing brood were counted every week for four weeks, and thereafter every three weeks for a total of 15 weeks before hibernation. Colonies were then

transferred into artificial hibernation with a gradual decrease of temperature and gradual increase again thereafter (see above). The remaining eclosing adults were counted after hibernation until all brood items that had been added to the colonies had developed. Eclosing sexuals and callow workers were removed when counted.

Statistics

Deviations of the distribution of the amount of developing brood from normality were tested using the Kolmogorov-Smirnov test. Data sets with no deviation from normality were analysed using Student's t-test. Data sets significantly deviating from normality were analysed using non-parametric statistics (Mann-Whitney U-test, Kruskal-Wallis test). *P*-Values were adjusted for multiple comparisons using Bonferroni's method. Correlation between colony size and colony productivity was analysed by Spearman Rank correlations. Statistical analysis was performed using Statistica 6.0.

Results

Colony productivity

In 52 of the 220 colonies collected, the queen died during hibernation and these colonies were excluded from the analyses. Colony size was found to be significantly negatively correlated with the number of per capita produced workers and significantly positively correlated with the per capita productivity of sexuals (Figure 5.1). Colony size did not affect the ratio of per capita produced female sexuals to males (Spearman's rank test, $n = 153$, $r_S = 0.122$, $p = 0.134$). Total productivity (produced workers plus sexuals) did not significantly correlate with colony size (Spearman's rank test, $n = 169$, $r_S = -0.134$, $p = 0.082$). The findings are corroborated when dividing colonies into small, medium and large size colonies. Similar to the sibling species *Temnothorax nylanderi* (Foitzik und Heinze 2000), small colonies ($n = 7$; worker number: mean ± SD 32.4 ± 14.9) produced mainly workers, medium-sized colonies ($n = 28$; worker number: mean 47.1 ± 33.0) produced workers and females or workers and males, and large colonies ($n = 134$; worker number: mean 90.3 ± 43.2) produced workers, females and males (Kruskal-Wallis test; $n = 169$, $df = 3$; $H = 35.32$, $p = 0.0001$).

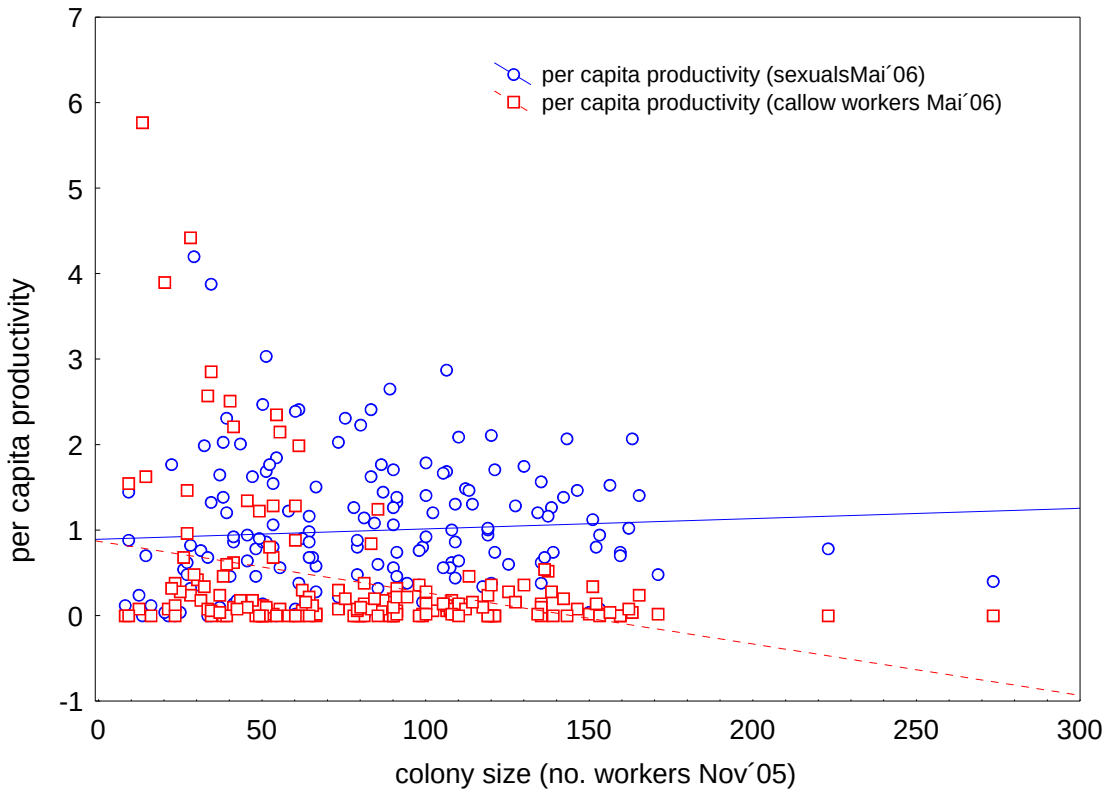

Figure 5.1. Correlation between colony size (no. workers counted in Nov´05 in each colony) and per capita productivity (callow workers and sexuals eclosing in each colony during the next reproductive season) in the ant *Temnothorax crassispinus*. Circles and the continuous line denote the per capita productivity of sexuals in each colony and correlation to colony size respectively. Squares and the dotted line denote the per capita productivity of callow workers in each colony and correlation to colony size respectively. Spearman's rank test; per capita productivity of callow workers, $n = 169$, $r_S = -0.262$, $p < 0.001$; per capita productivity of sexuals, $n = 169$, $r_S = 0.186$, $p < 0.05$.

Brood manipulation

Several experimental colonies, in which the queen died during the experiment, were excluded from further analyses (for *n* colonies see Table 5.1). In all three types of colonies, significantly less brood than was originally added to the colonies developed into adults (Table 5.1). Irrespective of whether normal amount or additional queen- or worker produced brood were added to the colonies, a significantly small proportion of the originally added brood developed into adults. The decline of the number of brood items most probably stems from high mortality rates of larvae and pupae during brood development (Martin et al. 1995) and may simultaneously be due to handling of the brood items during the colony set up.

	n colonies	*n* added brood	*n* developed brood *mean* ± *SD*	t-test for dependant samples *t*; *p*-level	Kolmogorov-Smirnov *d*; *p*-level
Control colonies	13	110	86.69 ± 19.46	-4.319; $p < 0.001$	0.171; $p > 0.2$
Colonies with additional queen brood	15	220	114.80 ± 45.96	-8.865; $p < 0.00001$	0.132; $p > 0.2$
Colonies with additional worker brood	16	220	154.56 ± 36.93	- 7.087; $p < 0.00001$	0.122; $p > 0.2$

Table 5.1. Total amount of brood that developed into adulthood in *Temnothorax crassispinus* colonies with normal amount of queen brood added (control colonies) and additional amount of queen or worker brood added.

In colonies with additional worker brood, significantly more brood developed into adults than in colonies with additional queen brood (Table 5.1; t-test for independent samples; $t = 2.66$, $p < 0.05$). A few workers died during the experiment, and brood size per capita was calculated in each observation. For the average number of workers per colony, see Figure 5.3. In colonies with additional queen brood, the average number of brood items, including eggs, quickly declined to that found in control colonies (Fig. 5.2a; Table 5.2a), while she slower decline of brood size in colonies with additional worker brood was due to higher egg laying rates, presumably by the queen, between the second and sixth week (Fig. 5.2c; Table 5.2c), which simultaneously led to more larvae after the ninth week of observation (Fig. 5.2b; Table 5.2b). After four weeks of observation, in both groups with additional brood items, the amount of larvae had declined to the same level as found in control colonies (Fig. 5.2b; Table 5.2b). While the decline in both groups mainly resulted from larvae and pupae developing into adults (Fig. 5.2d; Table 5.2d), in colonies with additional queen brood, several abandoned larvae and pupae were left outside the nest during the first three weeks of observation. In colonies with additional queen brood, mainly callow workers eclosed before hibernation; in colonies with additional worker brood, callow workers and sexuals eclosed (Fig. 5.3b,c).

Overall, while the addition of worker-produced brood led to a significant increase in the total reproductive output of the colonies, the addition of queen-produced brood only led to a marginal increase (Fig. 5.4). As expected, compared to the other colony set ups, colonies with additional worker brood significantly produced more males than females or workers (Fig. 5.4). Control colonies and colonies with additional queen brood produced more callow

workers than males or females (Fig. 5.4). In all three colony set-ups, more males than female sexuals were produced (Mann-Whitney U-test; control colonies, $U = 47.50$, $p = 0.058$; colonies with additional queen brood, $U = 52.50$, $p < 0.05$; colonies with additional worker brood, $U = 0.00$, $p < 0.00001$).

5.2a. brood items including eggs plus eclosing adults

	week 1	week 2	week 3	week 4	week 6	week 9	week 12	week 15
C versus Q	$U = 0.00$ **$p < 0.00001$**	$U = 16.00$ **$p < 0.001$**	$U = 90.00$ *n.s.*	$U = 89.00$ *n.s.*	$U = 86.50$ *n.s.*	$U = 81.00$ *n.s.*	$U = 75.00$ *n.s.*	$U = 85.00$ *n.s.*
C versus W	$U = 1.00$ **$p < 0.00001$**	$U = 9.50$ **$p < 0.0001$**	$U = 1.00$ **$p < 0.00001$**	$U = 18.00$ **$p < 0.001$**	$U = 0.00$ **$p < 0.00001$**	$U = 3.00$ **$p < 0.00001$**	$U = 13.00$ **$p < 0.0001$**	$U = 12.00$ **$p < 0.0001$**
Q versus W	$U = 114.00$ *n.s.*	$U = 98.50$ *n.s.*	$U = 0.00$ **$p < 0.00001$**	$U = 12.50$ **$p < 0.0001$**	$U = 1.00$ **$p < 0.00001$**	$U = 9.00$ **$p < 0.0001$**	$U = 22..5$ **$p < 0.001$**	$U = 17.00$ **$p < 0.0001$**

5.2b. brood items excluding eggs plus eclosing adults

	week 1	week 2	week 3	week 4	week 6	week 9	week 12	week 15
C versus Q	$U = 0.00$ **$p < 0.00001$**	$U = 0.00$ **$p < 0.00001$**	$U = 28.50$ **$p < 0.002$**	$U = 52.50$ $p < 0.05$	$U = 80.00$ *n.s.*	$U = 81.00$ *n.s.*	$U = 75.00$ *n.s.*	$U = 85.00$ *n.s.*
C versus W	$U = 0.00$ **$p < 0.00001$**	$U = 0.00$ **$p < 0.00001$**	$U = 2.00$ **$p < 0.00001$**	$U = 83.50$ *n.s.*	$U = 81.00$ *n.s.*	$U = 3.00$ **$p < 0.0001$**	$U = 13.00$ **$p < 0.0001$**	$U = 12.00$ **$p < 0.0001$**
Q versus W	$U = 99.00$ *n.s.*	$U = 73.50$ *n.s.*	$U = 32.00$ **$p < 0.001$**	$U = 85.00$ *n.s.*	$U = 117.50$ *n.s.*	$U = 9.00$ **$p < 0.0001$**	$U = 22.5$ **$p < 0.002$**	$U = 17.00$ **$p < 0.001$**

5.2c. eggs only

	week 1	week 2	week 3	week 4	week 6	week 9	week 12	week 15
C versus Q	$U = 43.00$ $p < 0.05$	$U = 87.00$ *n.s.*	$U = 35.50$ $p < 0.01$	$U = 31.00$ $p < 0.01$	$U = 74.00$ *n.s.*	-	-	-
C versus W	$U = 35.00$ $p < 0.01$	$U = 27.00$ **$p < 0.001$**	$U = 17.00$ **$p < 0.001$**	$U = 13.50$ **$p < 0.0001$**	$U = 0.00$ **$p < 0.00001$**	-	-	-
Q versus W	$U = 119.50$ *n.s.*	$U = 24.00$ **$p < 0.001$**	$U = 0.00$ **$p < 0.00001$**	$U = 2.00$ **$p < 0.00001$**	$U = 0.00$ **$p < 0.00001$**	-	-	-

5.2d. eclosing adults only

	week 1	week 2	week 3	week 4	week 6	week 9	week 12	week 15	next year
C versus Q	$U = 97.50$ *n.s.*	$U = 19.50$ **$p < 0.001$**	$U = 70.00$ *n.s.*	$U = 61.00$ *n.s.*	$U = 27.00$ **$p < 0.002$**	-	-	-	$U = 46.00$ $p < 0.05$
C versus W	$U = 104.00$ *n.s.*	$U = 104.00$ *n.s.*	$U = 97.5$ *n.s.*	$U = 1.50$ **$p < 0.00001$**	$U = 38.00$ $p < 0.01$	-	-	-	$U = 32.00$ **$p < 0.002$**
Q versus W	$U = 120.00$ *n.s.*	$U = 24.00$ **$p < 0.001$**	$U = 92.00$ *n.s.*	$U = 5.00$ **$p < 0.00001$**	$U = 9.50$ **$p < 0.0001$**	-	-	-	$U = 93.00$ *n.s.*

Table 5.2. Difference between total amount of brood items plus eclosing adults per worker and per colony, counted at different stages, in control colonies (C), colonies with additional queen brood (Q) and colonies with additional worker brood (W). Directions of differences are shown in figure 2a-d. Figures in bold indicate *p*-values from Mann-Whitney U-tests that are significant at the 5% probability after Bonferroni's correction for 24 comparisons in 2a-c and for 27 comparisons in 2d ($p' < 0.002$); *n.s* = nonsignificant.

Fig. 5.2a: brood items including eggs plus eclosing adults

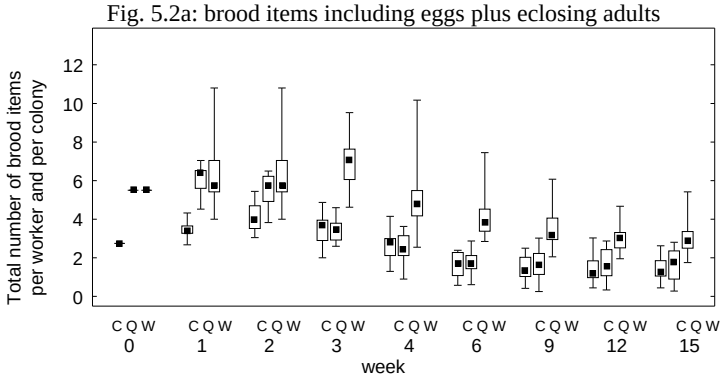

Fig. 5.2b: brood items excluding eggs plus eclosing adults

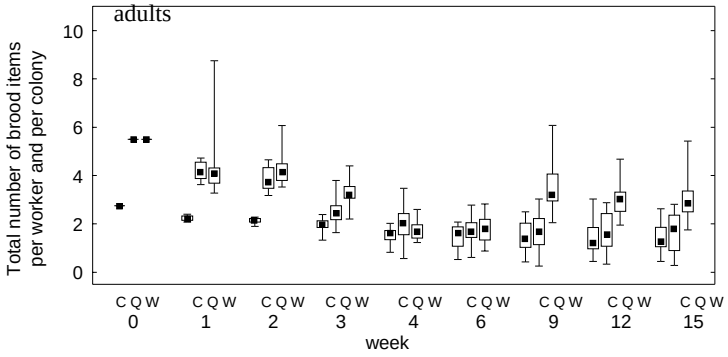

Fig. 5.2c: eggs only

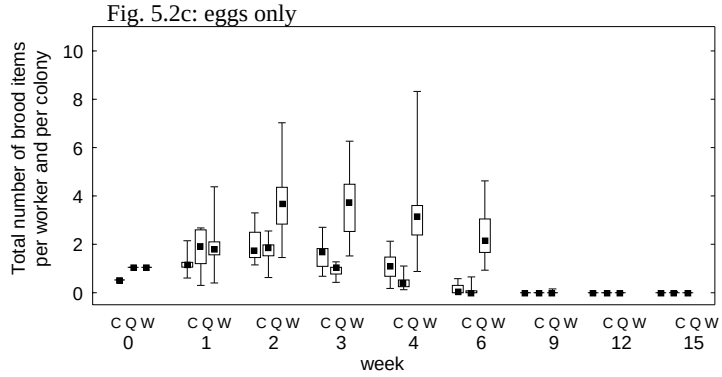

Fig. 5.2d: eclosing adults only

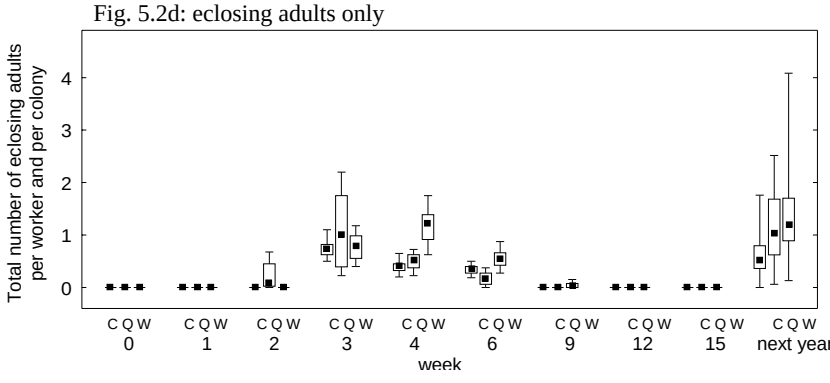

Figure 5.2. Difference between total amount of brood items and eclosing adults per worker and per colony, counted at different stages. Figure "5.2a" gives values for the total amount of undeveloped brood (eggs, larvae, pupae) and eclosing adults (callow worker, males, females), figure "5.2b" gives values for the total amount (as above) minus eggs, figure "5.2c" gives values for the total amount of eggs produced only and figure "5.2d" gives values for the amount of eclosing adults in control colonies with normal amount of brood (C), colonies with additional queen brood (Q) and colonies with additional worker brood (W). Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dot*) are represented for each observation. Corresponding *p*-values are given in Table 5.2.

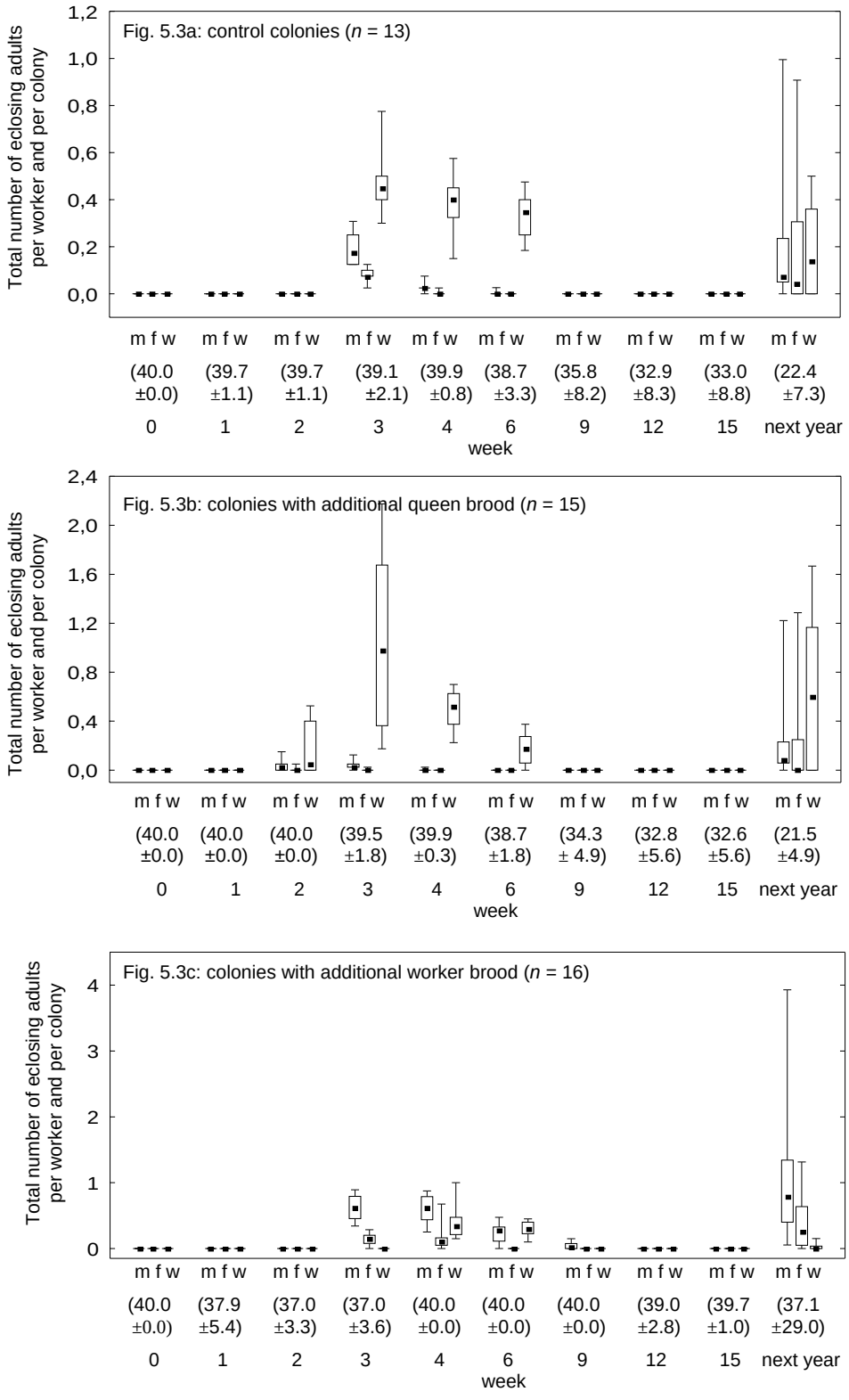

Figure 5.3. Total number of eclosing males (m), females (f) and callow workers (w) per colony and per individual in a) control colonies, b) colonies with additional queen brood and c) colonies with additional worker brood. Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dot*) are represented for each observation. The mean number (± standard deviation) of workers per colony is given in parentheses.

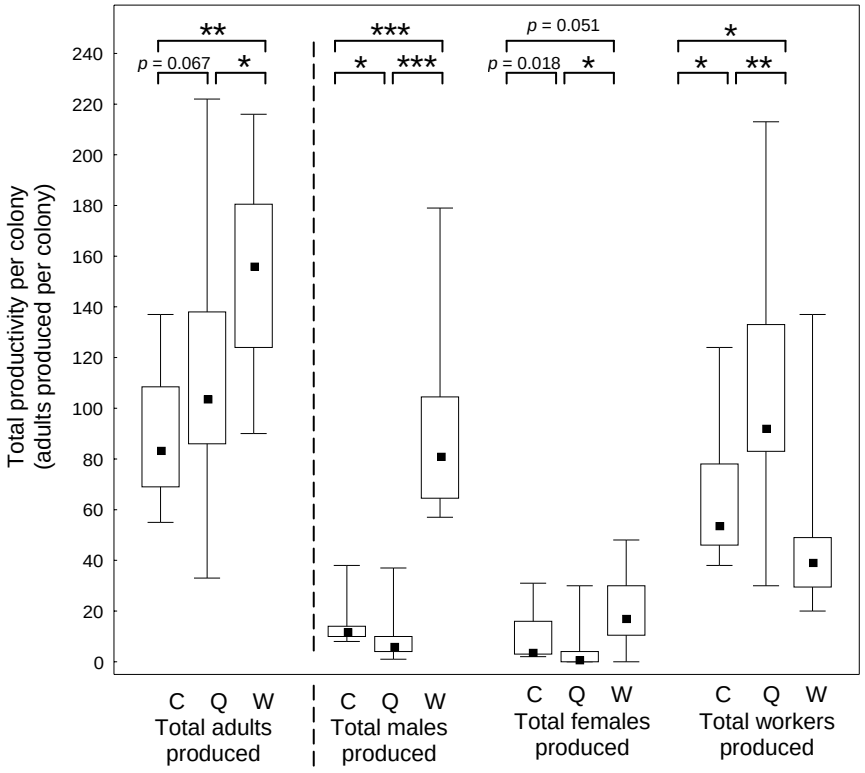

Figure 5.4. Total productivity per colony in control colonies with normal amount of brood (C), in colonies with additional queen brood (Q) and in colonies with additional worker brood (W). The graph shows the total amount of adults (left side), and the total amount of males, females and workers (right side) produced per colony. Minimum and maximum (*whiskers*), first and third quartiles (*bars*), and the median (*dot*) are represented for each observation. * $p < 0.017$; ** $p < 0.001$; *** $p < 0.0001$ (Mann Whitney-U test). After Bonferroni´s correction, *p*-values of < 0.017 are significant at the 0.05 level.

Discussion

Colony size in the ant *Temnothorax crassispinus* was negatively correlated with per capita productivity of new workers, and positively correlated with the per capita productivity of sexuals. This matches previous observations that small colonies invest in colony growth ("ergonomic phase"), medium size and large colonies invest mainly in reproduction and colony growth ("reproductive phase", Oster and Wilson 1979; Foitzik and Heinze 2000; Poitrineau et al. 2009). Brood size in *T. crassispinus* was least variable at a medium colony size of 40 workers. Such colonies invested simultaneously in sexuals and workers. We chose colonies of this size to investigate whether the addition of brood leads to a decreased

reproductive output, as expected from the hypothesis that worker reproduction unbalances an optimal brood/worker ratio and thus is associated with costs (e.g., Hartmann et al. 2003). Surprisingly, the experimental addition of queen-produced brood led to a marginal increase compared to control colonies, while the addition of worker-produced brood led to a significant increase of the reproductive output in particular through the production of males. It therefore appears that nursing worker-produced brood does not constitute a significant cost at the given colony size and under standardized laboratory conditions. On the contrary: a few weeks after surplus worker brood had been added, colonies contained more eggs than other types of colonies. Queens might recognize worker-produced brood by its chemical profile (e.g., Brunner et al. 2009a) and perhaps attempt to compensate the ratio between queen- and worker-laid eggs by producing more eggs.

Colonies with additional queen-produced brood reared less sexuals than control colonies and colonies with additional worker-produced brood. On the one hand, this decline of productivity might reflect costs arising from surplus female larvae. Workers appeared to be well capable of raising males from added male larvae but did not succeed in rearing the much larger and more costly female sexuals (Strätz and Heinze 2004) from the added female brood. On the other hand, our result matches the still unexplained finding that in *T. nylanderi*, the Western sibling species of *T. crassispinus*, colony fusion results in strongly male-biased sex ratios (Foitzik et al. 2003). As in our study, both control and experimental colonies similarly received mixtures of larvae from several unrelated colonies, heterogeneity of brood odor or incompatibilities between workers and unrelated brood cannot explain this result.

In contrast to results from a study in the clonal ant *Platythyrea punctata* (Hartmann et al. 2003), our experiment therefore refutes the hypothesis that in *T. crassispinus* worker self-restraint in the presence of the queen and aggressive policing against reproductive workers serves to maintain an optimal brood / worker ratio. Other factors might therefore contribute to colony-level costs of worker reproduction in *T. crassispinus*. For example, physical constraints may make egg-laying more costly to workers than to queens. For example, while the ovaries of queens consist of 2 x 4 ovarioles, worker ovaries usually have only 2 x 1 ovariole. Furthermore, egg-laying by workers in queenright colonies is usually associated with aggressive dominance behavior (Cole 1981; Franks and Scovell 1983; Heinze et al. 1997). Energy invested in dominance and policing activities due to worker reproduction may constitute the actual cost leading to lower colony performance (Cole 1986, but see Lopez-Vaamonde et al. 2003).

Acknowledgments

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III. Conclusion and Perspectives

Aim of this thesis was to investigate the role of kinship in the occurrence and resolution of reproductive conflicts in eusocial insect societies. Varying relatedness among group members are usually used to explain, whether workers reproduce in the presence of a queen or not. In colonies, where workers are closer related to their brothers than to their nephews, they are expected to inhibit each other from reproducing through aggression towards nestmates attempting to reproduce or eating worker-laid eggs (worker policing, Ratnieks 1988), whereas no such policing is expected in colonies, in which workers are closer related to their nephews than to their brothers (Ratnieks 1988; Ratnieks and Reeve 1992). In recent years, results of a constantly growing number of studies on worker reproduction and conflict resolution in insect societies do not meet predictions based on kin theory alone. Power held by each individual to enforce its own interest, informational constraints and colony-level performance may be additional factors responsible for the maintenance of altruistic behaviour. By using model systems within the genus *Temnothorax*, and the parthenogenetic ponerine ant *P. punctata*, we examined how these additional factors may influence the regulation of worker reproduction in these species. In *Temnothorax* societies, in which, according to kin theory, workers are predicted to reproduce, males are solely produced by the queen (Heinze et al. 1997; Hammond and Keller 2004), and in the clonal ant *P. punctata*, though not expected on relatedness grounds, workers police individuals attempting to reproduce (Hartmann et al. 2003).

In both species, *T. unifasciatus* and *P. punctata* we could demonstrate a strong link between policing and dominance behaviour (Stroeymeyt et al. 2007; Brunner et al. 2009). Policing behaviour is assumed to benefit the colony as a whole, in which all colony members are expected to police selfish individuals, and in that way increase their indirect fitness gains (Ratnieks 1988). Dominant behaviour, in which individuals fight for reproductive rights, is performed by one or a few high ranking individuals towards individuals lower in the hierarchy (Heinze et al. 1997). As dominance behaviour only benefits the actor by increasing its own direct fitness gains (Monnin and Ratnieks 2001), it is expected to occur independent on relatedness structures within the colony. Policing and dominance behaviour are difficult to distinguish, and policing individuals may often pursue their own interests.

Selfish policing suggests that dominant hierarchies exist even in the presence of a queen. In *T. unifasciatus* we could demonstrate that hierarchies, though based only on more subtle interactions, already exist before the queen was experimentally removed from the colony (Brunner and Heinze 2009). Existing hierarchies most likely influence policing behavior when the colony loses its queen, therefore, observed policing behaviour may often be associated with dominance behaviour, occurring independent of colony kin structure. Furthermore, as dominance hierarchies in the presence of the queen are most likely disturbed by worker reproduction, existing rank relationships among workers may be one important reason for the absence of worker reproduction in queenright colonies.

In *P. punctata* policing by dominant individuals follows a different logic. Because regular parthenogenesis leads to a clonal colony structure, individuals are equally related to all offspring in the colony and are not expected to invest energy in dominance and policing behaviour (Greeff 1996). Policing individuals are often the strongest individuals in the colony (Saigo und Tsuchida 2004; Wenseleers et al. 2005; Wenseleers and Ratnieks 2006a; Stroeymeyt 2007). Assuming that physical dominance reflects an individual's reproductive potential, aggression among workers might ensure that the most fecund individual becomes the next reproductive, which would benefit the colony as a whole.

Results in Chapter 3 further contribute to the important role of cuticular hydrocarbons in the regulation of worker reproduction. We found the chemical profile on the cuticle of fertile workers and queens of *T. unifasciatus* different from that of non-reproductive workers. Though, this is no direct evidence that certain chemical substances communicate fertility it goes in the expected direction that fertility is honestly signalled through cuticular hydrocarbons. Furthermore, queen- and worker-laid eggs differed in their chemical profile. Physiological constraints may make it impossible for workers to camouflage their fertility status and their eggs. Studies have shown a tight link between yolk production by fat bodies and the activity of oenocytes, in which cuticular hydrocarbons are synthesized (Fan et al. 2003; Jensen and Børgesen 2000). The worker's abilities to recognize fertile nestmates and to distinguish between worker- and queen-laid eggs are an important pre-requisite of effective policing behaviour. Effective policing may lower the incentive for workers to selfishly reproduce in the first place (Wenseleers et al. 2004a). Cuticular Hydrocarbons, honestly signalling the state of fertility and origin of eggs, may be an important reason why workers do not reproduce in queenright colonies.

Queens probably play the most important role in the regulation of worker reproduction. Independent of the kin structure within the colony, queens always are selected

to inhibit workers from reproducing. In *T. unifasciatus*, we observed queens attacking fertile workers (Brunner and Heinze 2009). Physical control may no longer be efficient in large colonies, and queen pheromones are involved in the regulation of worker reproduction. Results from our cross-species study with several *Temnothorax* species suggest that queen pheromones are most likely honestly communicate the queen's fertility status to which workers could react in their own interest. We have found that workers no longer respond to signals of a queen of distantly related *Temnothorax* species, while the signals employed by queens of the sibling species *T. crassispinus* and *T. nylanderi* appear to be active across species boundaries. The results do not fully confirm the honest signal hypothesis, that is, signals are conserved between species, and do not fully refute the manipulative agent hypothesis either.
Further studies on the evolutionary rate of queen pheromones are needed to clarify the precise role of queen pheromones in the regulation of worker reproduction (see below).

In *T. crassispinus*, colonies appear to be able to raise additional male brood. At least in *T. crassispinus* lower productivity by additional worker brood may not be the reason for the absence of worker reproduction. Instead, energy invested in dominance and policing activities due to worker reproduction may constitute the actual cost leading to lower colony performance.

Overall, kinship seems less important in the maintenance of social life than is originally assumed for social insect societies. The strong link between policing and dominance behavior suggests that individuals pursue selfish interests, independent of colony kin structures. Reproductive hierarchies in queenright colonies and therefore dominant workers play an important role in the regulation of worker reproduction. However, queens probably play the most important role, as they are always selected to inhibit workers from reproducing, independent of relatedness structures within the colony.

Perspecitve

Conflicts between queens and workers over sex allocation ratios, the differential investment in female and male sexuals produced within a population, are similarly expected to vary with differential kin structures resulting in unequal sex-ratio optima for workers and queens

(Trivers and Hare 1976; Boomsma and Grafen 1991). In monogynous and monandrous colonies, workers prefer a threefold investment in female sexuals (worker-sister: r = 0.75) compared to males (worker-brother: r = 0.25), whereas the queen's optimum would be an equal investment in both sexes (queen-offspring: r = 0.5). When the queen is mated to more than one male (monogyny and polyandry), the worker's relatedness towards their sisters approaches the value of 0.25. In this case the workers and queen optima converge to one (Boomsma and Grafen 1991). Evidence for sex allocation to vary with kin structure comes from *Formica* ants (Sundström 1994). Apparent agreement with kin theory was found in *T. nylanderi*, where homogenous colonies produce more female-biased ratios and heterogeneous colonies more male-biased ratios and less sexuals (Foitzik and Heinze 2000; Foitzik et al. 2003). However, in *T. nylanderi*, lower nestmate results from colony fusion or the usurpation of established colonies by young founding queens, followed by the aggressive replacement of the old queen (Foitzik et al. 2003; Strätz et al. 2002). As a consequence, workers in these colonies are unrelated to the queen's offspring and should not prefer a particular sex ratio. Hence, in *T. nylanderi* sex allocation decisions seem independent of predictions made by kin theory. Instead, inefficient cooperation between workers in genetically heterogeneous colonies may result in the observed male-based ratio and less production of sexuals. This hypothesis is based on observations that artificially mixed colonies of *Leptothorax longispinosus* had a lower productivity than homogenous colonies (Trampus 2001). To determine whether and how reproductive output in *T. nylanderi* depends on genetic colony composition, brood production, sex allocation and colony performance could be compared in colonies composed of different amounts of genetic lineages. In a preliminary project I composed colonies with one worker lineage (r = 0.75) as control colonies, and heterogeneous colonies with two (r = 0.375), three (r = 0.245) and five (r = 0.15) worker lineages. I then monitored colony performances by observing and measuring several traits: foraging efficiency, quantity and quality of worker interactions, brood care, distribution of workers in the nest, moving to a new site, reaction to introduced strangers in the colony, survival rates of adult queens and workers and brood development. Unfortunately the data obtained gave no clear results that could be interpreted and further investigation of the data and/or repeating of some of the experimental set ups may be necessary to complete this project.

A comparison to the close related species *Temnothorax recedens*, in which workers produce males in queenright colonies, though only in minor quantities (Julia Scharrer, Diplomathesis) and to species of the closely related genus *Cardiocondyla*, in which workers

completely lack ovaries, may provide further insights into the role of queen pheromones in the regulation of worker reproduction. In our study on queen pheromones (Chapter 4) we observed *T. recedens* developing their ovaries in the presence of queens from related species, simultaneously *T. recedens* queens were not able to fully prevent worker reproduction in these species. The genetic and social structure of *T. recedens* is very similar to the other four species in this study and the difference concerning worker reproduction may result from the loss of an active queen fertility signal or the loss of worker sensitivity to such a signal. Investigations of queen pheromones among different populations of *T. recedens* may clarify this. If no active queen fertility signal regulates worker reproduction in this species, we would expect no high variation of queen pheromones among different populations. However, if workers and queens are involved in an arms race in which workers are ahead, chemical compounds related to fertility may even differ between populations as found in paper wasps (Dapporto et al. 2004). Studies on monogynous species within the related genus *Cardiocondyla* (e.g. *C. elegans, C. batesii, C. nigra*) might further clarify whether queen pheromones are honest or manipulative agents. Workers in this species have no functional ovaries, and queens do not need to suppress worker reproduction. Therefore, chemical profiles of queens should be stable in evolution and variations between different species within the gender should be rather low.

IV. Summary

Aim of this thesis was to investigate the role of kinship in the occurrence and resolution of reproductive conflicts in eusocial insect societies. Kin theory predicts different strategies on the regulation of worker reproduction in genetically heterogeneous than in homogenous societies. However, a growing number of studies do not meet predictions made by kin theory. While kinship indisputably plays a major role in the evolution of eusocial insect societies, for organisational traits, such as sex allocation, partitioning of reproduction and conflict resolutions, for example punishment or worker reproduction (worker policing), it may be less important. Power held by each individual to enforce its own interest, informational constraints and colony-level performance may be additional factors responsible for the maintenance of social life. By using the model systems of several *Temnothorax* species and the parthenogenetic ant *Platythyrea punctata* I investigated these additional factors in the context of worker reproduction. Though predicted by kin theory workers in most *Temnothorax* species refrain from reproducing in the presence of the queen, but readily produce males from unfertilized eggs in orphaned colonies.

In *T. unifasciatus*, rank relationships exist among workers in queenright colonies. Existing hierarchies influence policing behavior when the colony loses its queen, and observed policing behaviour may often be dominance behavior independent on colony kin structure. As dominance hierarchies are likely disturbed by worker reproduction, existing rank relationships among workers and costly aggressive interactions may be important reasons for the absence of worker reproduction in the presence of a queen. In the clonal ant *P. punctata* in which policing and dominance behavior is not expected on relatedness grounds, policing individuals that selfishly increase their chance of becoming reproductives later themselves, are most likely the strongest and simultaneously the most fecund individual which would benefit the colony as a whole.

Cuticular Hydrocarbons, honestly signalling the state of the worker's fertility may be an additional reason why workers do not reproduce in queenright colonies. Being recognized and effectively policed by nestmates would lower the incentive for workers to selfishly reproduce in the first place. Queen pheromones, involved in the regulation of worker reproduction may similarly be an honest signal. Workers no longer respond to signals of a queen from distantly related species but respond to queens from a sibling species, which

speaks against the rapid evolution of queen pheromones expected from the queen control hypothesis.

Colonies appear to be able to raise additional male brood, therefore energy invested in dominance and policing activities due to worker reproduction may constitute the actual cost leading to lower colony performance and presumably explains why workers of most *Temnothorax* species refrain from reproducing.

Kinship theory, so far considered to be of great importance in the occurrence of reproductive conflict and their resolution, appears to play a minor role in the regulation of worker reproduction in ants.

V. Zusammenfassung

Ziel dieser Dissertation ist es, die Rolle von Verwandtschaften für die Entstehung und Lösung reproduktiver Konflikte innerhalb sozialer Insektenstaaten zu untersuchen. Die Theorie der Verwandtenselektion besagt, dass in genetisch heterogenen Insektenstaaten, andere Strategien zur Regulierung reproduktiver Konflikte verfolgt werden, als in genetisch homogenen. Dem entgegen werden die Aussagen dieser Theorie in einer zunehmenden Anzahl von Studien nicht gestützt. Während Verwandtschaftsverhältnisse unbestreitbar eine wichtige Rolle in der Evolution sozialer Insektenstaaten spielen, erscheinen sie für die Organisation des Staates weniger wichtig. Die Aufzucht von weiblichen oder männlichen Geschlechtstieren, die Aufteilung der Reproduktion, und die Lösung reproduktiver Konflikte, insbesondere der Bestrafung reproduzierender Arbeiterinnen („worker policing“), scheinen unabhängig von Verwandtschaftsverhältnissen zu sein. Die Fähigkeiten einzelner Individuen ihre eigenen Interessen durchzusetzen, nicht ausreichende Informationen, und die Produktivität der Kolonie, können weitere Faktoren für die Aufrechterhaltung altruistischen Verhaltens sein. Diese Faktoren wurden anhand der Modell-Systeme, sowohl mehrerer *Temnothorax* Arten als auch der parthenogenetischen Ameise *Platythyrea punctata*, in Bezug auf Arbeiterreproduktion untersucht. Entgegen der Theorie der Verwandtenselektion, reproduzieren die Arbeiterinnen der meisten *Temnothorax* Arten im Beisein der Königin nicht. In Kolonien ohne Königin jedoch, produzieren Arbeiterinnen Männchen von unbefruchteten Eiern.

In Anwesenheit der Königin existieren in *T. unifasciatus*-Kolonien zwischen den Arbeiterinnen Rangordnungen. Nach dem Verlust der Königin beeinflussen bestehende Hierarchien das „policing“-Verhalten. Dies kann jedoch oft auch Dominanzverhalten sein, welches unabhängig von Verwandtschaftsverhältnissen auftritt. Da Dominanzhierarchien durch Arbeiterreproduktion beeinflusst werden, sind bestehende Rangverhältnissse und kostspielige aggressive Interaktionen zwischen Arbeiterinnen wahrscheinlich wichtige Gründe für die mangelnde Arbeiterreproduktion. Bei der klonalen Ameise *P. punctata*, kann „Policing“- und Dominanzverhalten aufgrund der Verwandtschaftsverhältnisse nicht erwartet werden. Individuen, welche ihre Chance sich später selbst reproduzieren zu können, durch „worker policing“ erhöhen, sind mit hoher Wahrscheinlichkeit gleichzeitig auch die stärksten und fruchtbarsten Individuen. Dies wiederum ist zum Nutzen der gesamten Kolonie.

Kutikulare Wasserstoffe, welche zuverlässig die Fertilität der Arbeiterinnen signalisieren, können ebenfalls Gründe sein, warum Arbeiterinnen im Beisein der Königin nicht reproduzieren. Der Anreiz zur Reproduktion, wird durch das Risiko von anderen Arbeiterinnen erkannt und dementsprechend bestraft zu werden, bereits im Voraus verringert.

Pheromone der Königinnen, welche in der Regulation der Arbeiterreproduktion eine Rolle spielen, sind wahrscheinlich ebenfalls ehrliche Signale. Arbeiterinnen reagieren nicht auf Signale von Königinnen weniger verwandter *Temnothorax*- Arten, reagieren aber auf Signale von Königinnen einer Schwesternart. All dies spricht gegen eine rapide Evolution der Königinnenpheromone, welche unter der Annahme, dass Signale der Königinnen manipulativ sind, zu erwarten wäre.

Zugefügte, von Arbeiterinnen produzierte Brut hat keinen negativen Einfluss auf die Gesamtproduktion der Kolonie. Die durch „Policing“ - und Dominanzverhalten entstehenden Kosten, sind vermutlich eher der Grund, warum Arbeiterinnen der meisten *Temnothorax* Arten keine Eier legen.

Die Theorie der Verwandtenselektion, welche bislang als sehr wichtig für die Entstehung von reproduktiven Konflikten und deren Lösung angesehen wurde, scheint eine eher untergeordnete Rolle zu spielen.

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VII. Appendix

Appendix 1

Supporting Information for Chapter 4:

Queen Pheromones in *Temnothorax* ant species: Queen Control or Honest Signals?

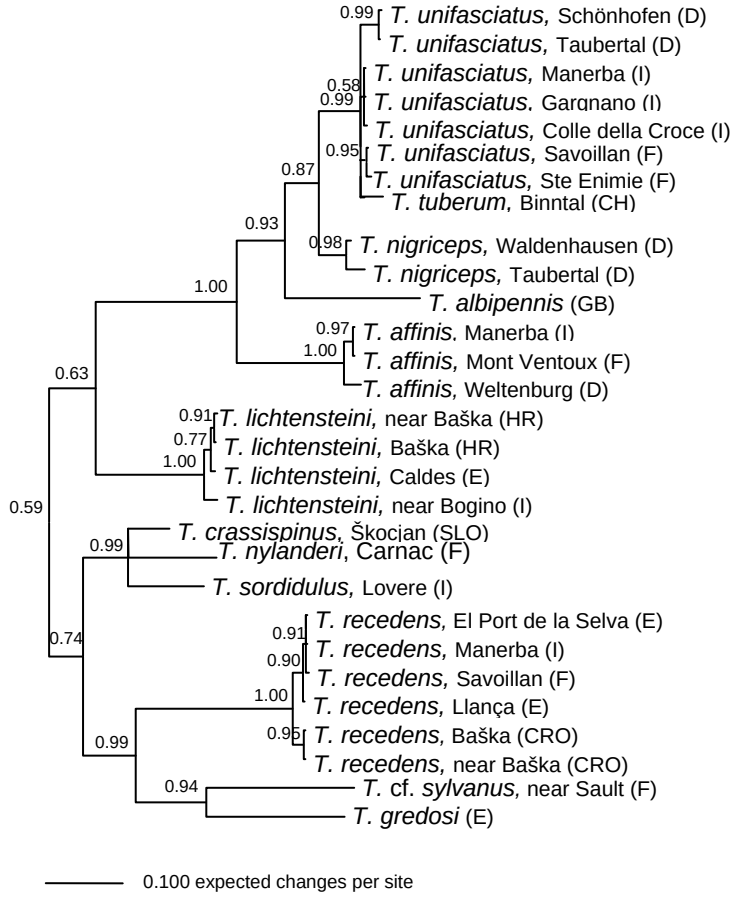

Figure S1. Phylogenetic tree of *Temnothorax* species. Majority rule consensus tree recovered in a Bayesian analysis (4,000,000 generations) with the GTR + I + G model. The tree is based on 651 base pairs of the mitochondrial CO I gene and numbers represent clade credibility values (J. Beibl, pers. comm.).

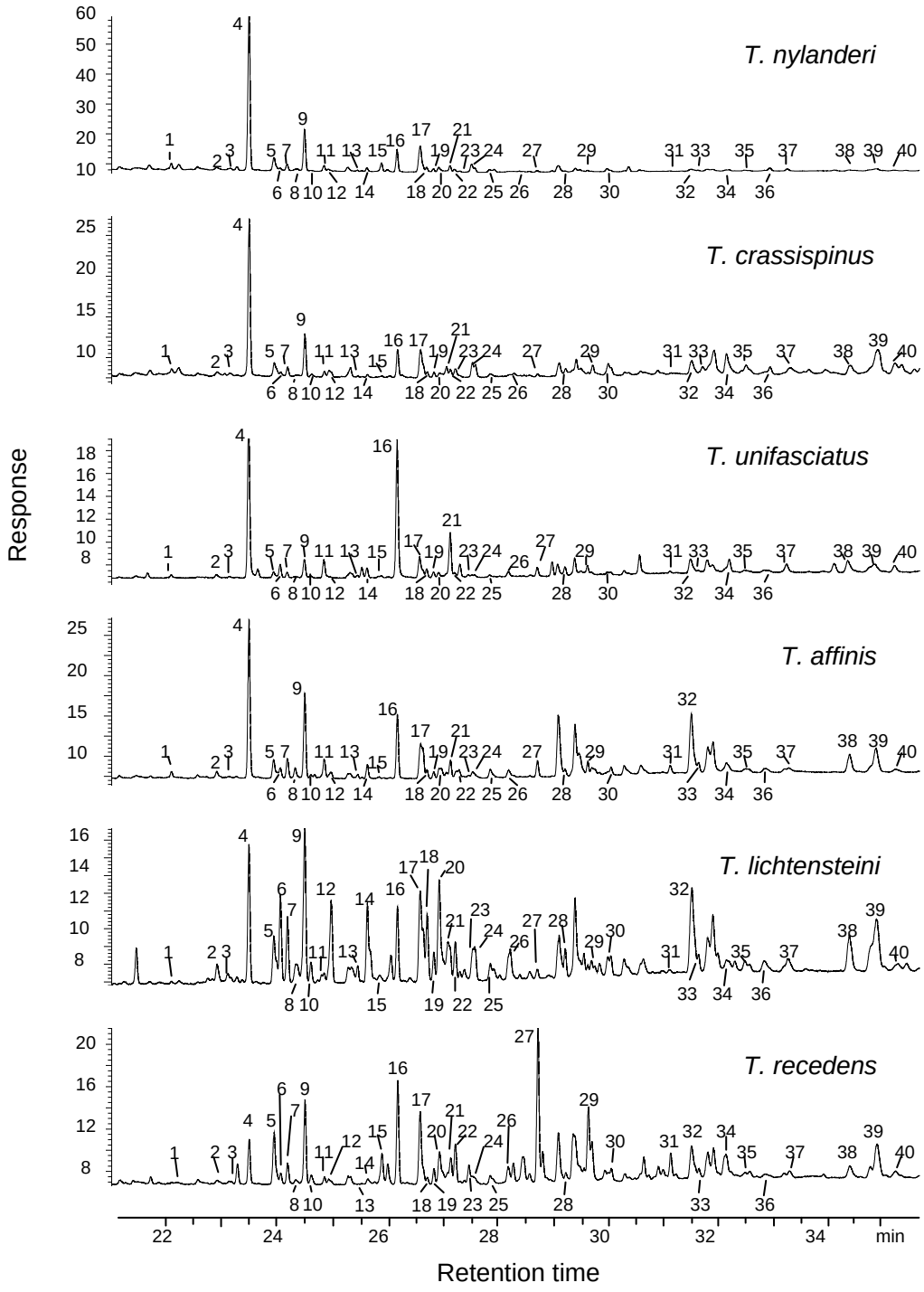

Figure S2. Representative gas chromatography profiles of queens from six *Temnothorax* species. Peaks used for the statistical analysis are marked with numbers. Identification of peaks is given in Table S1.

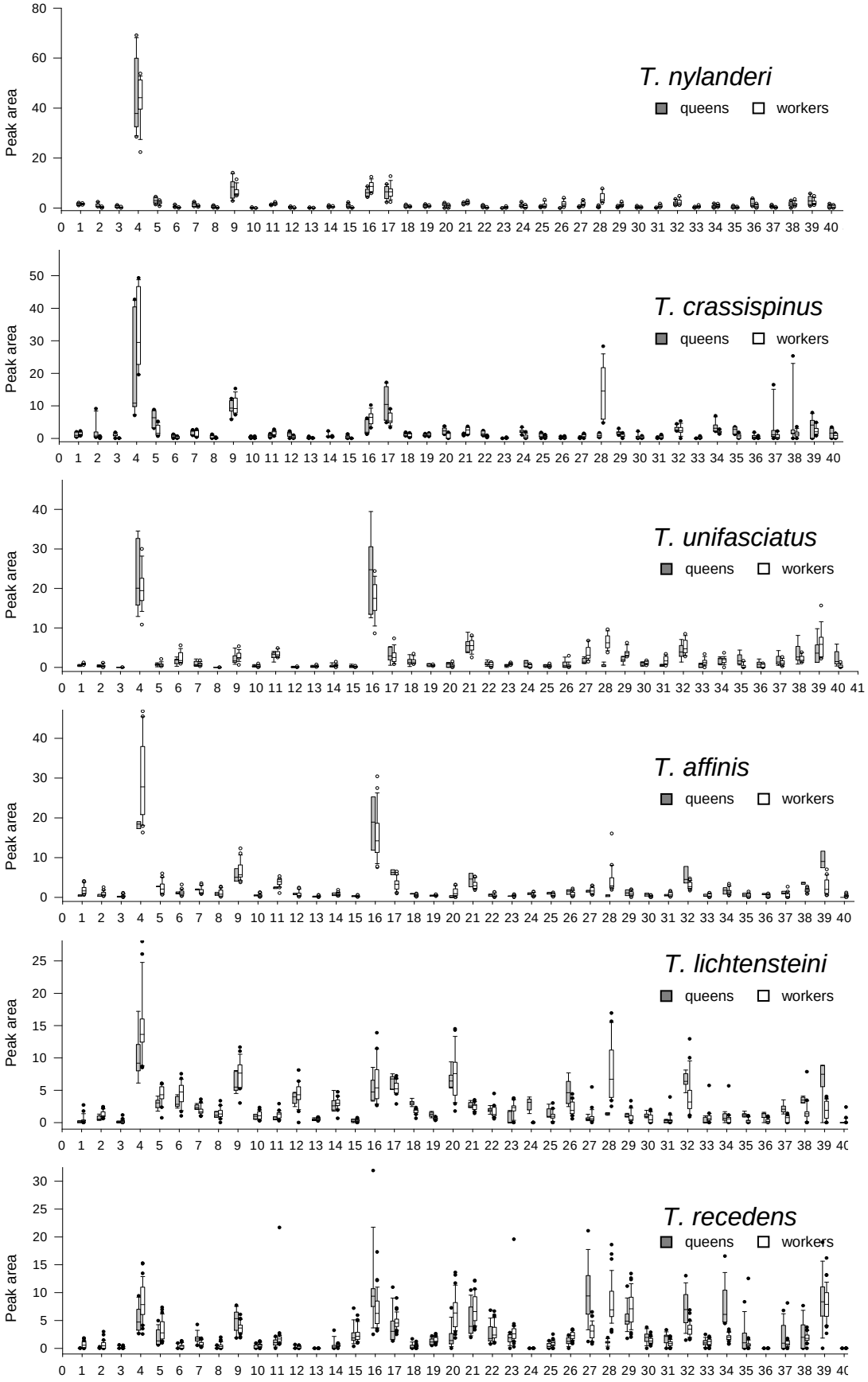

Figure S3. Proportions (%) of peak areas in chromatograms from cuticular hydrocarbon extracts of queens and workers in six *Temnothorax* species. Box plots show medians and 25% and 75% quartiles. Whiskers depict the range of 90% of all cases. Extreme outliers are denoted by circles. *p*-values of substances differing significantly between the various groups are given in Table S1.

Peak No. (Fig. S2)	Compound	Difference between *T.unifasciatus* Q (5) and W (12)	Difference between *T. nylanderi* Q (6) and W (9)	Difference between *T. crassispinus* Q (6) and W (9)	Difference between *T. recedens* Q(4) and W (18)	Difference between *T. lichtensteini* Q(5) and W (18)	Difference between *T. affinis* Q (10) and W (34)
1	*n*-C_{26}	$p < 0.01$	*n.s.*	*n.s.*	$p < 0.01$	*n.s.*	$p < 0.01$
2	4-me C_{26}	*n.s.*	$p < 0.01$	*n.s.*	$p < 0.05$	$p < 0.05$	*n.s.*
3	3-me C_{26}	$p < 0.05$	$p < 0.01$	$p < 0.01$	*n.s.*	*n.s.*	*n.s.*
4	*n*-C_{27}	*n.s.*	*n.s.*	*n.s.*	$p < 0.05$	$p=0.053$	$p < 0.05$
5	9-me C_{27} +11-me C_{27} +13-me C_{27}	*n.s.*	*n.s.*	$p < 0.05$	$p < 0.05$	*n.s.*	*n.s.*
6	7-me C_{27}	*n.s.*	$p < 0.05$	$p=0.059$	*n.s.*	*n.s.*	*n.s.*
7	5-me C_{27}	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$	$p < 0.05$	*n.s.*
8	11,15 di-me C_{27}	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	*n.s.*	*n.s.*
9	3-me C_{27}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
10	5,x di-me C_{27}	*n.s.*	$p < 0.05$	$p < 0.05$	$p < 0.05$	*n.s.*	*n.s.*
11	*n*-C_{28}	*n.s.*	$p < 0.05$	$p < 0.05$	$p < 0.05$	*n.s.*	$p < 0.05$
12	3,7 di-me C_{27}	*n.s.*	$p=0.059$	$p < 0.05$	*n.s.*	*n.s.*	*n.s.*
13	6-me C_{28}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
14	4-me C_{28}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
15	3-me C_{28}	*n.s.*	$p < 0.01$	$p < 0.05$	*n.s.*	*n.s.*	*n.s.*
16	*n*-C_{29}	*n.s.*	$p=0.059$	$p < 0.05$	$p < 0.05$	*n.s.*	*n.s.*
17	9-me C_{29} +11-me C_{29} +13-me C_{29} +15-me C_{29}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$
18	7-me C_{29}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$\mathbf{p < 0.001}$	$p < 0.01$
19	5-me C_{29}	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$	*n.s.*
20	x,y di-me C_{29}	*n.s.*	*n.s.*	$p < 0.05$	$\mathbf{p < 0.001}$	*n.s.*	$p < 0.05$
21	3-me C_{29}	*n.s.*	*n.s.*	$p < 0.05$	$p < 0.05$	*n.s.*	*n.s.*
22	5,x di-me C_{29}	*n.s.*	$p < 0.01$	$p < 0.01$	*n.s.*	*n.s.*	*n.s.*
23	*n*-C_{30}	$p < 0.01$	$p < 0.01$	$p < 0.01$*	$p < 0.05$	$p < 0.01$	*n.s.*
24	3,x di-me C_{29}	*n.s.*	$p < 0.05$	$p < 0.01$	*n.s.*	$\mathbf{p < 0.001}$	*n.s.*
25	x-me C_{30}	*n.s.*	$p < 0.05$	*n.s.**	$p < 0.05$	*n.s.**	$p < 0.05$
26	4-me C_{30}	*n.s.*	$p < 0.01$*	$p < 0.05$*	$p < 0.01$	$p < 0.05$	*n.s.*
27	*n*-C_{31}	$p < 0.05$	$p < 0.01$	$p < 0.01$	$\mathbf{p < 0.001}$	*n.s.*	*n.s.*
28	7-me C_{31}	$p < 0.01$	$p < 0.01$*	$p < 0.01$*	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$	$p < 0.01$
29	3-me C_{31}	$p < 0.01$	$p < 0.01$	$p < 0.01$	*n.s.*	*n.s.*	*n.s.*
30	3,7 di-me C_{31}	*n.s.*	*n.s.**	*n.s.**	$p=0.057$	*n.s.*	*n.s.*
31	*n*-C_{33}	$p < 0.01$	$p < 0.01$	$p < 0.01$*	$p < 0.05$	*n.s.*	*n.s.*
32	9-me C_{33}+11-me C_{33} +13-me C_{33} +15-me C_{33}	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$	$p < 0.05$	$p < 0.05$
33	7-me C_{33}	$p < 0.05$	$p=0.059$	$p < 0.01$*	*n.s.*	*n.s.*	*n.s.*
34	3-me C_{33}	*n.s.*	*n.s.*	$p < 0.01$*	$\mathbf{p < 0.001}$	*n.s.*	*n.s.*
35	3,x di-me C_{33}	$p < 0.05$	*n.s.**	$p < 0.01$*	*n.s.**	$p < 0.01$*	*n.s.*
36	unidentified	*n.s.*	$p < 0.05$	*n.s.*	*n.s.*	$p < 0.05$	$p < 0.05$
37	unidentified	*n.s.*	$p < 0.01$	*n.s.*	*n.s.*	$\mathbf{p < 0.001}$	*n.s.*
38	unidentified	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$	$p < 0.01$
39	unidentified	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$p < 0.05$	$p < 0.01$
40	unidentified	$p < 0.05$	*n.s.*	*n.s.*	*n.s.*	*n.s.*	$p < 0.01$

Table S1. Identification of cuticular compounds and differences of their relative amounts between queens (Q) and workers (W) in five *Temnothorax* species. The number of samples of queens and workers is given in parentheses. Peak numbers correspond with numbers in Figures S2 and S3. Directions of difference are shown in Figure S3. Bold *p*-values from Mann-Whitney U-tests are significant at the 5% probability after Bonferroni's correction for 40 comparisons ($p' < 0.001$); *n.s* = not significant. *Due to very low abundance, peaks marked with a star could not be identified. They had exactly the same retention time in GC as peaks in other species, which could be identified. We therefore assume these compounds to be chemically identical.

Appendix 2

Posters and talks at conferences

Brunner E, Heinze J, Stroeymeyt N: Reproductive patterns in insect societies.
- 2005: Verhandlungen der Gesellschaft für Ökologie 35, Regensburg, p 28

Brunner E, Stroeymeyt N, Heinze J: Conflict resolution in insect societies.
- 2005: Proceedings 3rd European Congress IUSSI, St. Petersburg, p 104

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