The importance of direct fitness for helpers in advanced social societies

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Contents

Abstract

Social insect societies are characterized by a caste determined reproductive and non-reproductive division of labor. Usually, queens stay in the safe surrounding of the nest and produce offspring, while workers refrain from reproduction and care for the brood, forage or defend the nest. However, workers of many social insect species are principally capable of laying unfertilized eggs, which can develop into males. In cooperatively breeding birds or mammals, helpers forgo early reproduction to benefit from brood care experience or nest inheritance and thus, gain an increased direct fitness later in life. However, workers in highly social insect species as in many ants, bees and wasps, generally cannot inherit the nest and replace a fully fertile queen due to physiological restrictions, and the importance of direct fitness benefits for workers has long been neglected.

Referring to Hamilton (1964*b*), it is assumed that ant workers refrain from direct fitness (their own produced offspring) to benefit from indirect fitness (the offspring produced by the queen due to their help) and thereby, increase their inclusive fitness. Questions arose whether this is sufficient to explain a lifetime resignation from reproduction in social insect workers. Indeed, several studies report selfish behaviors like worker policing, egg dumping or the refusal of costly tasks, possibly to increase the chance for future direct fitness in individual workers.

Here, we studied the importance of direct fitness for workers in the monogynous, monandrous ant *Temnothorax crassispinus.* We examined the reproductive success of workers by ovary dissections and genotyping workers and males from natural queenless and queenright colonies (chapter 2), and monitored the survival and productivity of queenless and queenright colonies for four years in captivity (chapter 3). We compared fitness traits of queen- and worker-produced males under near-natural and standardized conditions (chapter 4) and investigated whether males produced by workers in the absence of a queen, are accepted in queenright colonies during colony reunification before hibernation (chapter 5). Moreover, we hypothesized that the reproductive potential of young workers might induce selfishness and limit behavioral flexibility under pathogen threat (chapter 6) and examined the effect of selfish reproduction on sanitary behavior

of the workers (chapter 7).

Our studies show, that queen presence or absence, respectively did not affect ovary development in workers and that around 30% of the genotyped males were not produced by the estimated queen in natural colonies. Most males that were not produced by the queen, were produced by workers not related to the colony. Accordingly, the reproductive success of workers related to the queen seemed to be comparably low in nature but increased under laboratory conditions. Workers seem to be capable of increasing their direct fitness by the expulsion or killing of the queen and a reinforced reproduction afterwards. Queenless colonies were highly productive and persistent and sperm traits of worker-produced males varied only little from that of queen-produced males. Furthermore, larvae produced in queenless colony fragments could be readily integrated in queenright laboratory colonies during colony reunification before hibernation, and contributed considerably to male production in queenright colonies. However, the prospect of future reproductive success did not affect altruistic self-removal in health compromised workers, but microbiota growth seemed to be encouraged in queenless colonies when sanitary behavior is neglected.

1 General introduction

An outstanding characteristic of life is the frequent interaction of single organisms within a complex inter- and intraspecific network. The complexity of these interactions varies strongly between species, and ranges from quorum sensing in single-celled bacteria (Miller and Bassler, 2001; Waters and Bassler, 2005) to the evolution of several languages in humans (Lieberman, 1984). Verbal and non-verbal communication between organisms contributed to the major transitions in the evolution of social societies (Szathmáry and Smith, 1995) and provides the basis of social behaviors in the context of a group.

Social behaviors can generally be classified according to the benefits and costs of fitness (i.e. the lifetime reproductive success) on the actor and the recipient. Mutually beneficial behaviors are characterized by a fitness benefit for both, and can be found in symbiotic interactions between members of different species or clades. In contrast, spiteful behavior causes fitness costs for both involved parties and often occurs between members of the same species. Finally, selfishness and altruism are intermediate behaviors, which cause either fitness benefits or costs on only one of the parties. Whereas, selfishness is only beneficial for the actor, altruism causes exclusively costs for the actor, but increases the fitness of the recipient. Usually, in complex social societies, benefits and costs eventually pay-off by alternating interactions between group members (Hamilton, 1964*a*,*b*, 1970; Trivers, 1971).

Cooperative and mutualistic behaviors can be frequently observed among groups in nature: fish collaboratively forage in groups (Foster, 1985; Pitcher et al., 1982), whales cooperatively raise calves in schools (Ohsumi, 1971) and antelopes elicit alarm calls to alert conspecifics to approaching predators (Tilson and Norton, 1981). Ultimately, mutualistic behaviors are not based on selfless intentions, but arise from the urge to increase individuals' own fitness (Connor, 1995). However, helping can reach extreme levels which can finally result in altruism. Outstanding altruistic behaviors, like a lifetime refrain from reproduction, can be observed rarely in vertebrates (Burda et al., 2000), but occur frequently in social insects such as bees, wasps and ants (Hamilton, 1972). Generations of sterile females (the workers) refrain from independent reproduction and help raise their brothers and sisters produced by the queen (Hölldobler and Wilson, 1990). However, the question why some individuals help others, even at a cost to themselves has long puzzled biologists.

Hamilton's theory of inclusive fitness (Hamilton, 1964*a*,*b*) provides a fundamental solution to this problem. According to this, the inclusive fitness of an individual consists of the sum of its direct fitness and indirect fitness: the number of their own offspring together with the number of offspring produced by relatives due to the helper's support, multiplied by the relatedness between the helper and the recipient (West et al., 2007).

Therefore, altruism and helping can be evolutionary stable if the number of the actor's genes that is spread by helping, is higher than through its direct reproduction. According to Hamilton's inequality ($c < B * r$), the cost of helping c (the number of not produced offspring) has to be smaller than its benefits B (offspring produced by the recipient due to the helper's efforts) weighted by the relatedness r between the helper and the recipient of the help (Hamilton, 1964*a*,*b*).

Accordingly, the degree of cooperation, and eventually the degree of altruism, depends on the relatedness between individuals. In cooperatively breeding birds and mammals for instance, helpers can initially increase their inclusive fitness by raising offspring produced by relatives, with whom they share a certain amount of genes (indirect fitness), and benefit from an increased direct fitness later in life, due to the heritage of the territory, an increased mating success and/or a gain in brood care experience (Clutton-Brock, 2009; Dickinson and Hatchwell, 2004; Russell, 2004). However, this seems not to sufficiently explain the evolution of extreme forms of altruism observed in other species. Although nest inheritance might be a crucial trait for direct fitness in some social insects (Almond et al., 2019; Leadbeater et al., 2011), workers in most highly eusocial species do not get the possibility to take over the colony and become a fully fertile queen and thus, it is assumed that direct fitness benefits are less important for workers in these species.

Colony organization, reproduction and relatedness in ants

Social insects can constitute up to 75% of a habitat's biomass and ants are an important factor for the entire terrestrial environment, as e.g. they ensure the soil quality and assist to obtain the ecological balance (Hölldobler and Wilson, 1990). Due to the wide distribution and a considerable number of species, ants were able to conquer a great diversity of habitats and occur in an extensive geographic range, e.g. from the Saharan desert (Wehner, 1983) to the arctic tundra (Coope, 1968). Colony sizes differ strongly between species and range from few dozen individuals as in some *Temnothorax* species (Strätz and Heinze, 2004), to several million ones as in the genus *Atta* (Beckers et al., 1989; Hölldobler and Wilson, 1990). All ant species are eusocial and consist predominantly of several generations of diploid females divided into different castes, i.e. many workers and one to few queens. In most species, queens and workers can be distinguished morphologically, as queens are e.g. usually initially winged, which results in a broadened thorax and a larger body size compared to workers. Furthermore, queens have a sperm storage organ (spermatheca), which workers of highly eusocial species are

lacking, and are therefore the only ones that are capable of fertilizing eggs and rearing female offspring (Hölldobler and Wilson, 1990; Trivers and Hare, 1976; Wilson, 1971). Accordingly, colony organization is usually based on a division of labor between queens and workers and also among workers. Whereas queens are generally responsible for reproduction and producing both, female (diploid) and male (haploid) offspring, workers usually conduct non-reproductive colony tasks such as brood care, foraging, and nest defense (Hölldobler and Wilson, 1990; Wilson, 1971).

However, in many social insect species, workers are principally capable of activating their ovaries and can lay haploid eggs which develop into males (Bourke, 1988*b*), but worker reproduction might be costly to the colony as reproductive workers refrain from colony tasks (Bocher et al., 2008; Bourke, 1988*b*; Dampney et al., 2004; Tsuji et al., 2012). Accordingly, queens suppress worker reproduction by queen pheromones (Alaux et al., 2004; Holman et al., 2010; Hoover et al., 2003; Keller and Nonacs, 1993) or policing behavior, as e.g. egg eating or attacking egg-laying workers (Bonckaert et al., 2011; Conte and Hefetz, 2008; Hoover et al., 2003; Wenseleers et al., 2004). However, frequent observations revealed that workers can nevertheless act selfishly and attempt to reproduce in order to gain direct fitness, at least under queenless conditions. Selfish worker policing (Brunner and Heinze, 2009; Stroeymeyt et al., 2007) is assumed to increase the prospective chance for direct fitness by destroying eggs laid by other workers (Stroeymeyt et al., 2007). In queenless colonies, workers establish reproductive rank orders by aggressive behaviors as antennal boxing and biting and the highest ranking workers start to reproduce (Bourke, 1988*a*; Cole, 1981; Heinze, 2010). Furthermore, the transition from inner to outer nest work strongly decreases the reproductive potential of workers (Dixon et al., 2014; Smeeton, 1982) and dominant workers selfishly refrain from costly tasks, like foraging and nest defense (Barth et al., 2010; Bourke, 1988*b*).

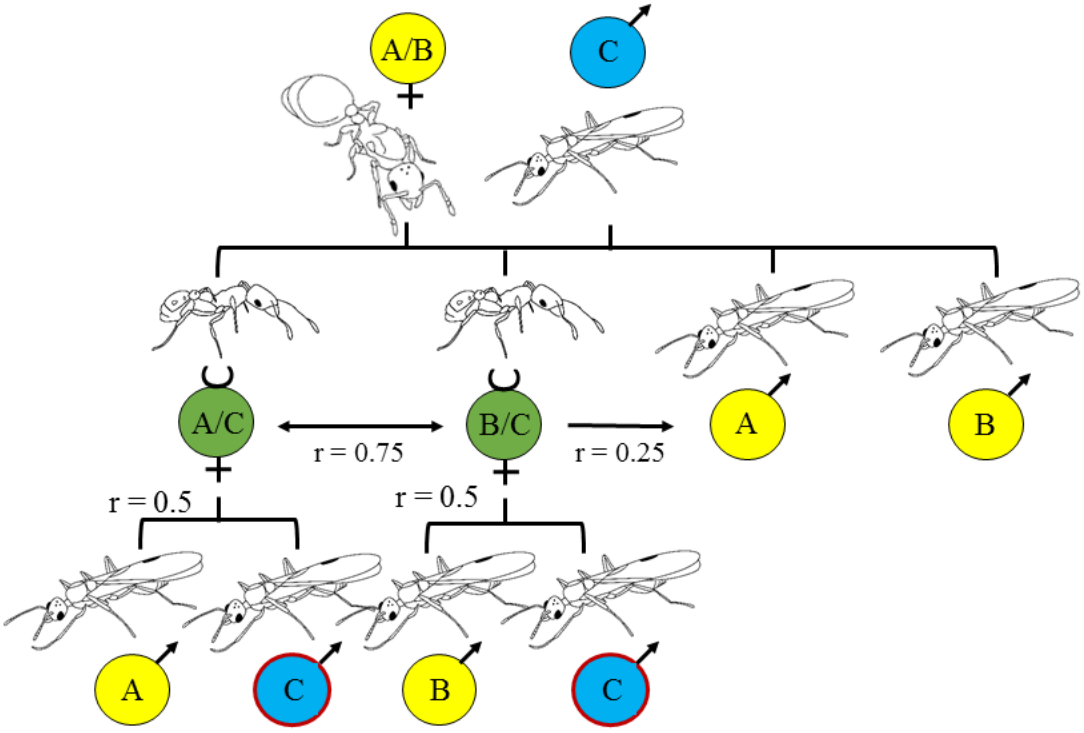

Figure 1.1: Schematic figure of the relatedness (r) in a monogynous, monandrous ant species. After the mating of a diploid queen (first row, yellow) and a haploid male (first row, blue), the diploid offspring (workers, second row green) inherits one of the queen's alleles and the paternal allele, whereas the haploid offspring (males, second row yellow) only inherit one of the queen's allele. Queens are thus equally related to their sons and daughters by 0.5, as both inherit 50% of their alleles. However, sisters are closely related by 0.75 ((100% paternal allele + 50% maternal alleles)/2), but less related to their brothers by 0.25 ((50% maternal alleles + 0% paternal allele)/2). In contrast, workers are highly related to their sons by 0.5 (they inherit 50% of their own alleles). Due to the haplodiploid sex determination mechanism (Trivers and Hare, 1976), one can only detect worker-produced males if they inherited the paternal allele (third row, blue with red circle), and thus only approximately 50% of the worker-produced males can be detected by genotyping workers and males. Here, workers represent both diploid offspring, i.e. workers and unmated virgin queens.

The conflict over the reproductive position in ant colonies arises from the relatedness asymmetry in social insects. Queens are equally related to their sons and daughters (1/2), but the haplodiploid sex determination mechanism (Trivers and Hare, 1976), in which unfertilized (haploid) eggs become males and fertilized eggs (diploid) become females, causes a relatedness asymmetry between brothers and sisters (see figure 1.1). Assuming monogynous, monandrous (single queen, singly mated) conditions, female offspring (workers and virgin queens) will inherit one of two queen alleles and the only

one available allele of the male, and thus are highly related to their sisters (3/4). In contrast, the relatedness of sisters to their brothers is reduced (1/4), as haploid males will only inherit one of the maternal alleles and no paternal allele. Therefore, workers are more closely related to their own offspring (1/2) with whom they share 50% of their own alleles, either their own maternal or paternal allele. The close relatedness between workers and their sons causes a potential conflict over the sex ratio and male parentage between workers and the queen. The queen favors an equal investment in males and females (1:1), whereas workers benefit most from a female based sex ratio in queen-produced offspring (2:1) and males produced by themselves or their sisters (3/8) (Queller and Strassmann, 1998). However, how successful ant workers reproduce in monogynous, monandrous colonies in nature has rarely been quantified (Bourke, 1988*b*; Helanterä and Sundström, 2007*b*; Walin et al., 1998).

Temnothorax crassispinus as a model system

Worker reproduction is well studied in monogynous honey bees (Bourke, 1988*b*; Oldroyd et al., 2001), as colonies can be easily maintained under near-natural as well as standardized conditions. In contrast, the colony size or distribution of some well-studied, monogynous ant species (Bourke, 1988*b*; Helanterä and Sundström, 2007*b*; Walin et al., 1998) aggravate simultaneous studies on life history traits in nature and the laboratory. However, there are also exceptions like for example the well-studied monogynous, monandrous ant species *Temnothorax crassispinus* (Karavaiev, 1926) (Seifert, 2007; Strätz and Heinze, 2004). *T. crassispinus* is a polydomous (inhabiting multiple nest sites) species (Strätz and Heinze, 2004) and colonies are widely spread across Europe (Seifert, 2007). The colonies are rather small with few dozen workers and live in ephemeral nest sites as e.g. in hollow twigs and acorns (Strätz and Heinze, 2004). Their small colony size and high abundance in deciduous forests across Europe, make *T. crassispinus* ants a well suited organism for the study of life history traits under natural conditions (figure 1.2).

Although colony founding is hard to observe under laboratory conditions, as mating of virgin queens and males occurs periodically during mating flights (Plateaux, 1971), recently collected *T. crassispinus* colonies can easily be kept under near-natural conditions outside the university building, but also under standardized conditions in the laboratory. Colonies can be split in queenless and queenright parts, and the small body size (ca. 2 - 3 mm) and low space requirements allows the long-term observation and efficient maintenance of several hundred colonies. The separation of workers from the queen

readily induces ovary activation and worker reproduction in queenless split colonies, and facilitates the observation of reproductive traits in related queen and workers from the same species. *T. crassispinus* workers do not have a spermatheca, but can rear males from unfertilized, haploid eggs (El-Shehaby et al., 2012). However, previous studies in laboratory reported that worker reproduction is presumably prevented by policing in queenright colonies, and is so far assumed to be mainly successful under queenless conditions (Brunner and Heinze, 2009; Stroeymeyt et al., 2007), but studies on natural colonies are rare.

Figure 1.2: *Temnothorax crassispinus* queen (center) with two workers surrounded by larvae. Queens can be determined by their broadened thorax and larger body size.

Aims of this thesis

The distinct social behavior and the extraordinary life history of ants make them an ideal study organism for sociobiological research. Ant colonies and their sophisticated network of single individuals are well suited for examinations associated with reproduction and colony fitness. The aim of this thesis was to assess the importance of direct fitness for workers and its effects on the fitness of the colony in a monogynous, monandrous ant species in nature and laboratory. Focusing on the reproductive success of *Temnothorax crassispinus* workers in natural (chapter 2) and laboratory colonies (chapter 3), associated with queen lifespan and colony survival, we investigated the quality of arising worker-produced males in comparison to queen-produced males (chapter 4). Furthermore, we tested whether polydomy can facilitate worker-reproduction (chapter 5) and examined the effects of future reproductive potential on selfishness in ant workers (chapter 6). Finally, we investigated whether the increased reproduction and productivity observed in queenless colonies (chapter 3), affects sanitary behavior (chapter 7) of the workers.

The manuscripts of two further studies on 1) the genetic structure of natural *T. crassispinus* colonies and 2) the effect of intercolonial exchange on colony productivity, are under preparation and not part of this thesis.

2 Substantial direct fitness gains of workers in a highly eusocial ant

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2.1 Abstract

Hamilton's theory of inclusive fitness suggests that helpers in animal societies gain fitness indirectly via increasing the reproductive performance of a related beneficiary of their support. Helpers in cooperatively breeding birds or mammals and also in primitively eusocial wasps may in addition obtain direct fitness, e.g., from producing own offspring after inheriting the nest or mating partner of the former recipient of their help. Here we show that also workers of a highly eusocial ant may achieve considerable direct fitness by producing males both in queenless and queenright colonies. We investigated the reproductive success of workers of the ant *Temnothorax crassispinus* in nature as well as under laboratory conditions by dissecting workers and determining the origin of males by microsatellite analysis. We show that *T. crassispinus* workers are capable of activating their ovaries and successfully raising males independent of the presence of a queen. Microsatellite genotypes revealed that across all queenright natural and laboratory colonies, at least one fifth of the reared males were not produced by the colony's queen. Most of the worker-produced males could be assigned to unrelated workers, suggesting egg laying by drifting workers as previously observed in bees

Keywords
Altruism; Worker reproduction; Direct fitness; Male production; Ants; *Temnothorax*

2.2 Introduction

Why some individuals help others even at a cost to themselves has long puzzled biologists. Hamilton's theory of inclusive fitness (Hamilton, 1964*b*) provides a fundamental solution to this problem. It states that helping and altruistic behavior can be stable in evolution, if through the helper's activities more copies of its genes are transmitted to the next generation indirectly via the offspring of the recipient of the help, than through own direct reproduction. The "inclusive fitness" of an individual is then the sum of its direct and its indirect fitness, i.e., the offspring it can produce without being helped by others, plus the additional offspring its relatives can produce because of the helper's efforts, multiplied by the relatedness between helper and the recipients of its help (West et al., 2007).

In cooperatively breeding birds and mammals, helpers in addition to indirect fitness benefits often gain substantial direct fitness, e. g., when they produce own offspring after inheriting the mating partner or territory of the former beneficiary of the help or when

they gain experience in brood care, which later benefits their own offspring (Clutton-Brock, 2009; Dickinson and Hatchwell, 2004; Russell, 2004). Similarly, direct fitness appears to play a considerable role in the societies of eusocial insects with limited caste diphenism, such as paper wasps or hover wasps. Here, helpers may mate and produce a few offspring in the presence of a dominant reproductive and in addition may take over the nest after the death of the latter (Field and Cant, 2007; West-Eberhard, 1978). Direct fitness may occasionally be the only component underlying helping behavior, e.g. when in paper wasps helpers and the recipient of the help are unrelated (Leadbeater et al., 2011; Queller et al., 2000). The possibility of inheriting an established nest has recently also been shown to be important for the maintenance of sociality in drywood termites (Korb and Schneider, 2007).

In contrast, direct fitness benefits have traditionally been thought to be much less important in the societies of highly eusocial ants and bees. However, workers of most species have retained functioning ovaries and are capable of laying unfertilized eggs. Though the hatchability of these eggs may be reduced (Khila and Abouheif, 2008), many studies show that at least in queenless colonies workers can produce their own sons from these unfertilized eggs (Bourke, 1988*b*). Numerous observations indicate that workers actively strive for direct fitness benefits through selfish egg laying. Workers of many species compete for egg laying rights and form social and reproductive rank orders by antennal boxing and biting (Bourke, 1988*a*; Cole, 1981; Heinze et al., 1997; Heinze, 2010). Dominant workers often refrain from costly and dangerous tasks, such as foraging or nest defense, and instead appear to "save their resources" for future reproduction (Barth et al., 2010; Bourke, 1988*a*; Charbonneau and Dornhaus, 2015). By attacking and eliminating rival egg-laying workers and destroying their eggs, workers appear to increase their own chances of becoming reproductive in the future ("selfish worker policing", Bonckaert et al. 2011; Saigo and Tsuchida 2004; Stroeymeyt et al. 2007; Zanette et al. 2012). Finally, drifting bee workers appear to selfishly dump their eggs into the nests of unrelated colonies (Alves et al., 2009; Beekman and Oldroyd, 2008; Lopez-Vaamonde et al., 2004).

Hence, workers of many eusocial insects appear to be under selection to increase their direct fitness, but how successful they are in doing so has rarely been quantified. Here, we examined the reproductive success of workers in the small Palearctic ant *Temnothorax crassispinus*. Previous studies have shown that colonies of *T. crassispinus* typically contain only a single, singly-mated queen and seasonally inhabit multiple nest sites (Strätz and Heinze, 2004; Tichá and Štys, 2002). We investigated the ovarian status of workers in queenright and queenless colonies both, in the field and under semi

natural conditions in captivity and quantified their contribution to the colonies' males by microsatellite genotyping. Our study shows that many workers in natural colonies have active ovaries and lay eggs regardless of whether a queen is present or not. The presence of the workers' paternal alleles in the genotypes of males revealed that many males in both queenless and queenright colonies are worker offspring. Furthermore, several male genotypes neither matched those of the present queen nor the queen-produced workers, suggesting a contribution from alien or no longer present individuals. In both queenless and queenright captive colonies workers readily produced males, indicating that they have a non-negligible chance of gaining direct fitness.

2.3 Material and methods

Study species and colony collection

Temnothorax crassispinus is a small (approximately 2 to 3 mm) monogynous, polydomous ant, whose colonies consist of up to 300 workers (Strätz and Heinze, 2004; Tichá and Štys, 2002). Previous genotyping had shown that, despite of monogyny and presumed monandry, many colonies consist of a mixture of different matrilines and patrilines (M. El-Shehaby, E. Schifelbein, unpublished data). This suggested a fluid colony and population structure with frequent queen replacement, seasonal polydomy, fusion of neighboring colonies, and the adoption of alien workers, similar to what has been described in its allopatric sibling species *T. nylanderi* (Foitzik and Heinze, 1998, 2000). We therefore complemented studies on the origin of males in natural colonies with an unknown history, with an analysis of male maternity in colonies kept in isolated, artificial nests under semi-natural conditions outside the university building.

In total we collected 682 colonies around Regensburg, Germany. Of these, 436 colonies were collected in calendar week 10 to 15 in early spring 2016. To avoid losing individuals in the field during collection we placed the whole nest site into sealable plastic bags. In the laboratory, we counted all adults and brood and transferred them from their natural nests into artificial nest boxes (10 x 10 x 3 cm^3). Colonies were kept outside the building under natural temperature and humidity conditions ("laboratory colonies"). They were fed twice per week with cockroaches and honey during spring and summer. Feeding frequency was decreased to once per week when day temperatures dropped below 15 °C and to once every two weeks at day temperatures below 5 °C. Colonies were covered with plant protection fleece when temperatures dropped below 0 °C.

Additional 246 colonies were collected in July 2016 as described above. Individuals and brood items were counted and subsequently stored in reaction tubes at -18 °C. Samples of frozen queens and workers were later dissected in a drop of ddH2O, using a Leica S8 Apo binocular microscope (80x). We counted the number of developing eggs in their ovaries.

Microsatellite analysis

To determine colony structure and the origin of males, we investigated the microsatellite genotypes (for details on microsatellite markers see below) of workers, males and, if present, the queen from each of 106 colonies (78 queenright (QR) colonies, 28 queenless (QL) colonies):

- 19 live laboratory colonies (originally collected in spring 2016):
  7 to 12 females (n = 19 colonies) and 7 to 12 males (n = 18 colonies)
  microsatellite markers 1-6
- 29 live laboratory colonies (originally collected in spring 2016 and reared under-near natural conditions for one year):
  10 to 12 females, 10 to 12 males
  microsatellite markers 1-6
- 58 frozen natural colonies (originally collected in summer 2016):
  - 24 QR colonies and 23 QL colonies: 12 workers, 12 males
    microsatellite markers 1-7
  - 6 QR and 5 QL colonies: all workers
    (median, Q1, Q3; QR: 106, 62, 162 workers; QL: 45, 29, 61 workers)
    worker and male 1-12: microsatellite markers 1-7
    other workers: microsatellite markers 3-5

DNA was extracted using a CTAB method modified from Sambrook and Russell (2001) (see supplement S2). In total, we used seven microsatellite markers: 1) GT1 (Bourke et al., 1997), 2) GT218 (Hamaguchi et al., 1993), 3) 2MS17 (Suefuji et al., 2011), 4) L5 (Foitzik et al., 1997), 5) L18 (Foitzik et al., 1997), 6) Ant3993 (Butler et al., 2014) and 7) Ant11893 (Butler et al., 2014). The 10 µl PCR reaction volume consisted of 5 µl Taq DNA polymerase, 3 µL ddH_2O, 0.5 µl unlabeled reverse primer, 0.5 µl labeled forward primer (HEX, FAM and TET) and 1 µL DNA. PCR consisted of initial denaturation at 94 °C (4 min), 33 cycles at 94 °C (denaturation, 45 sec), 57 °C (annealing, 80 sec) and 72 °C (elongation, 25 sec), and a final step at 72 °C (1 min). For 2MS17, the cycle number

was reduced to 32 cycles, denaturation time was extended to 75 sec, and annealing temperature was decreased to 54 °C.

We mixed 0.07 µl to 0.2 µL of the PCR product (depending on DNA quantity) with 25 µL formamid and 0.1 µL T486 size standard, and analyzed it in an ABI PRISM 310 Genetic Analyzer (PE Biosystems) after DNA denaturation at 90 °C (1 min). Allele sizes were determined using GENESCAN 3.1 software (PE Biosystems). Samples obtained from laboratory colonies collected in early spring 2016 were stored in 100% EtOH until analysis and microsatellite analysis was conducted the same way as described.

All seven loci were polymorphic and showed considerable variation with an average of 41 alleles in all samples (allele numbers; GT1: 41, GT218: 18, 2MS17: 50, L5: 58; L18: 58; Ant3993: 19; Ant18933: 36).

Data analysis

For each queenless colony, we reconstructed the most likely genotype of the queen (maternal alleles) from the genotypes of the majority of the colony's workers. Paternal alleles were determined by subtracting the maternal alleles from the worker genotypes. We used these data to estimate in each colony the minimal number of males that could not have been produced by the present queen (see detailed worker and male genotypes in supplement S1). Workers and males were defined as alien individuals when their alleles did not match the determined maternal or paternal alleles of the colony at at least two loci. This provides a conservative estimate of the presence of alien individuals, but makes it more unlikely that potential mismatches were caused by errors during sequencing or the determination of allele sizes. The ancestry of the males was analyzed by evaluating the queen and worker alleles and categorized as follows:

1. Sons of the queen, which showed at least one of the queen's alleles at at least six of the seven studied loci.
2. Grandsons of the queen, i.e. worker-produced males, which at two or more loci showed alleles that did not match the queen's alleles but were identical to the paternal allele as deduced from the genotypes of the majority of the workers.
3. Alien males, which had alleles, that did not match any allele in the colony, or alleles, which were otherwise present only in a minority of the colony's workers and not in the workers produced by the queen.

Half of the males produced by workers will at a given locus inherit the queen's allele. The likelihood that a worker-produced male by chance inherited the maternal allele at

all seven loci is $(1/2)^7$. Hence, the likelihood that worker-produced males were wrongly identified as queen-produced males is small.

Data were analyzed using R 3.2.3 software (R Development Core Team, 2008). Values are given as median and 1st (Q1) and 3rd (Q3) quantiles. As data were not normally distributed we used "lme4" package (Bates et al., 2015) for the generalized linear model. Kruskal-Wallis tests were used for group comparisons, Mann-Whitney U-tests for two-sample comparisons. All pairwise tests were corrected for multiple testing according to a false discovery rate (p adjust method: "fdr", Benjamini and Hochberg, 1995).

2.4 Results

Reproduction in natural colonies

About 30% of the collected natural colonies were queenless (QL) (spring 2016: 27%, 116/436; summer 2016: 33%, 80/246 colonies; $\chi^2 = 314$, p = 0.0076) and a minority of the queenright (QR) colonies contained two queens (early spring 2016: 12%, 37/320; summer 2016: 3%, 5/166; $\chi^2 = 8.08$, p = 0.004). Results from previous dissections (e.g. Strätz and Heinze, 2004) showed that unmated female sexuals may occasionally hibernate in their natal nests, but later are expelled. This might explain the decrease of the number of colonies with multiple queens from spring to summer.

Natural QL colonies contained on average fewer workers and brood than QR colonies in both spring and summer 2016 (table 2.1). Investment in castes and sexes varied between colonies: half of all natural colonies (50%, 123/246) invested in both female and male sexual offspring, approximately one fifth of the colonies each reared exclusively either male (17%, 43/246) or sexual female offspring (20%, 47/246), and 13% produced only workers (10%, 24/246) or no pupae at all (3%, 9/246). Regarding only those colonies that contained pupae, this investment was independent of queen presence ($\chi^2 = 6.2$, df = 3, p = 0.1).

Similarly, the number of total sexual offspring produced per colony in summer 2016 and the proportion of male offspring did not differ between QL and QR colonies (table 2.1). The proportion of males decreased significantly with increasing colony size in QR colonies (Spearman rank correlation: r_S = -0.21, p = 0.016), but not so in QL colonies (r_S = -0.04, p = 0.726).

Table 2.1: Worker number and offspring number in natural queenless and queenright *Temnothorax crassispinus* colonies collected in spring and summer 2016. Queenright colonies were larger and contained more brood items, independent of season, but the sex ratio did not differ between queenless and queenright colonies.

	Workers (median, quartiles)		**Brood (median, quartiles)**		**Sexuals (median, quartiles)**		**Males/total sexual offspring (median, quartiles)**	
	QL	QR	QL	QR	QL	QR	QL	QR
Spring	70 21, 120	160 100, 220	50 8, 250	150 80, 250				
	W = 8806 p < 0.0001		W = 6567 p < 0.0001					
Summer	33 20, 78	69 49, 114	87.5 39, 154	175 116, 252	21 7, 48	20 3, 47	0.44 0.13, 0.86	0.45 0.09, 0.93
	W = 3632 p < 0.0001		W = 3294 p < 0.0001		W = 6285 p = 0.618		W = 4497 p = 0.815	

The dissection of 36 queens and 12 workers from each of 64 natural colonies (27 QL and 37 QR) collected in summer 2016 showed, that workers are capable of activating their ovaries in the presence of a fertile queen. All dissected queens had well-developed ovaries with maturing eggs and yellow bodies, i.e., remnants of previously laid eggs. As above, QR colonies in this sample were larger than QL colonies (median, Q1, Q3; QL: 48, 24, 86 workers; QR: 85, 63, 151 workers; Mann-Whitney U-test: $W = 276$, $p = 0.002$). Half of the workers had active ovaries (median, Q1, Q3; QL: 50%, 29%, 58%; QR: 50%, 25%, 58%, figure 2.1 A) and neither the proportion of reproductive workers ($W = 491$, $p = 0.908$) nor the mean number of developing eggs in worker ovaries ($W = 507$, $p = 0.923$; median, Q1, Q3; QL: 0.92, 0.54, 1.3; QR: 1.0, 0.42, 1.25, figure 2.1 B) differed between QL and QR colonies. Furthermore, there was no difference in egg numbers ($W = 871$, $p = 0.563$) or in the proportion of workers with active ovaries ($W = 999$, $p = 0.628$) between queen-related workers and workers, which were not related to the queen (see below).

Dissections of all workers from additional 10 queenright and 11 queenless colonies from summer 2016 corroborated these findings: 258 of 530 workers from QL colonies and 382 of 803 workers from QR colonies had at least one egg in development ($\chi^2 = 0.16$, $p = 0.692$; see also El-Shehaby et al., 2012).

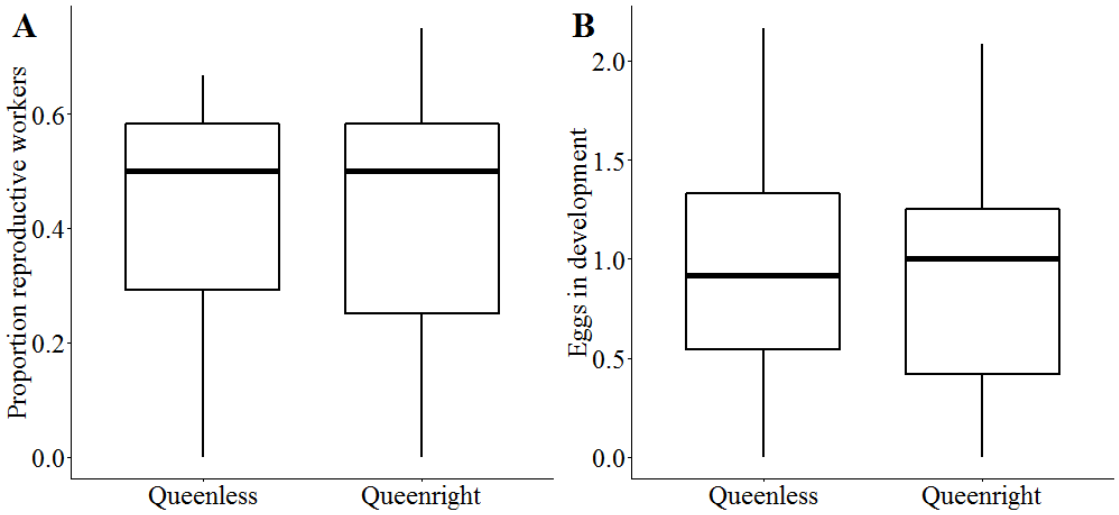

Figure 2.1: Number of reproductive workers (A) and number of eggs that are in development (B) in workers from queenless and queenright *Temnothorax crassispinus* colonies. Workers were equally capable of activating their ovaries in queenless and queenright colonies (median, Q1, Q3; QL: 50%, 29%, 58%; QR: 50%, 25%, 58%, W = 491, p = 0.908) and workers from both groups had on average one egg in development (median, Q1, Q3; QL: 0.92, 0.54, 1.3; QR: 1.0, 0.42, 1.25, W = 507, p = 0.923). Boxplots show median, 25 and 75 quartiles, 95% percentiles.

Individual worker fecundity (mean number of eggs per worker) increased with the proportion of reproductive workers independent of queen presence or worker number (generalized linear model: queen: estimate = -0.07, t-value = 13.72, p = 0.644; worker number: estimate = -0.04, t-value = -0.55, p = 0.581, proportion reproductive workers: estimate = -0.3, t-value = -4.30, $p < 0.0001$).

Genetic colony composition and maternity of males in natural colonies

Despite of monogyny and monandry, the genotypes of workers, queens, and males did not support the assumption that colonies are simple families. Instead, field colonies consisted of a mixture of different genetic lineages.

In 8 of 30 colonies (27%) from summer 2016, the genotypes of the analyzed queens were not compatible with those of the present workers. For example, in 38% of the QR laboratory colonies (spring 2016: 3/8 colonies) we found unrelated diploid larvae hibernating inside the colonies and eclosing in summer as virgin queens (median, quartiles 0%, 0%, 19%).

More than half of the QR colonies (spring 2016: 50%, 7/14; summer 2016: 57%, 17/30) contained workers (up to 92%) that definitively were not related with the present queen, or the majority of workers when queen alleles were not compatible with any worker alleles (median, quartiles, QR: spring 2016: median 4%, 0%, 21%; summer 2016: 12.5%, 0%, 25%). Similarly, two thirds of the QL colonies (summer 2016: 64%, 18/28) contained workers whose genotypes did not match those of the majority workers and their presumed queen. Such "alien workers" constituted up to 33% of the workforce (median, quartiles 17%, 0%, 25%).

Microsatellite analyses revealed that more than half of the QR colonies collected in spring (67%, 12/18) and summer 2016 (57%, 17/30, $\chi^2 = 0.47$, $p = 0.493$) reared males, which were not offspring of the resident queen. Across all QR colonies, about one fifth of the genotyped males had been produced by other females than the present queen (median, Q1, Q3; spring 2016: 8%, 0%, 33%; summer 2016: 17%, 0%, 58%). The proportion and absolute numbers of males not produced by the queen increased significantly with season (proportion: $W = 699$, $p = 0.03$; absolute numbers: spring 2016: 35/206 males; summer 2016: 112/360, $\chi^2 = 13.6$, $df = 1$, $p = 0.0002$). Only very few males shared the parental allele at each locus with workers related to the present queen, i.e., were presumably its grandsons (median, Q1, Q3; spring 2016: 0%, 0%, 6% per colony; min. 0%, max. 25%; summer 2016: 0%, 0%, 0% per colony; min. 0%, max. 42%; figure 2.2). The majority of those males, which had not been produced by the present queen, had genotypes that either matched those of alien workers found in the colony or did not match any of the studied females (median, Q1, Q3; spring 2016: 100%, 75%, 100% per colony; summer 2016: 100%, 100%, 100% per colony).

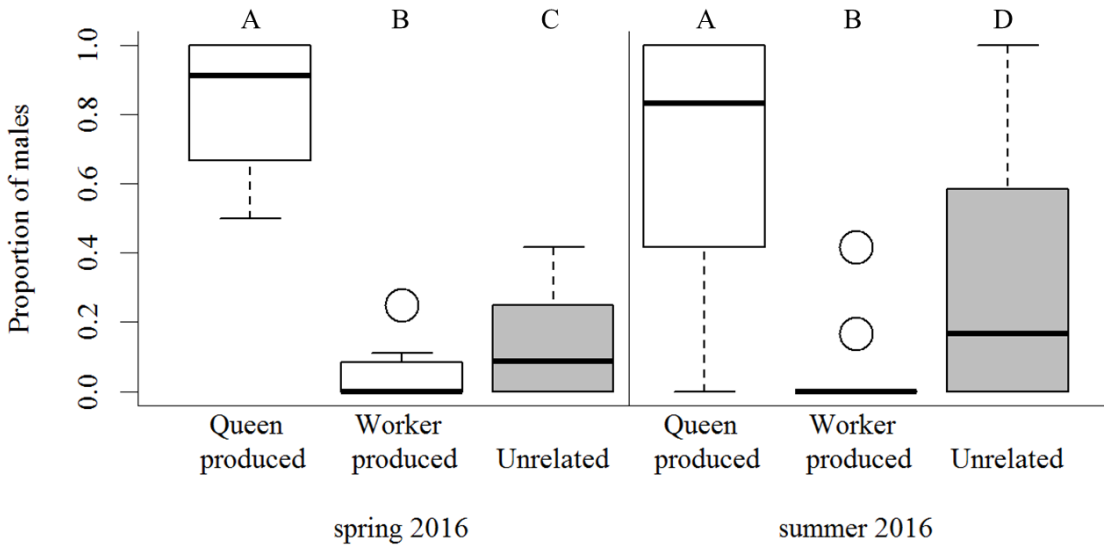

Figure 2.2: Proportion of males which were produced by the queen, by workers related to the queen or by unrelated workers in queenright *Temnothorax crassispinus* colonies collected in spring (left) and summer 2016 (right). Males produced by other females than the queen could be found frequently in natural colonies and their proportion inside the nests increased from spring to summer (median, Q1, Q3; spring 2016: 8%, 0%, 33% per colony; summer 2016: 17%, 0%, 58% per colony; $W = 699$, $p = 0.03$). Boxplots show median, 25 and 75 quartile and 95% percentiles and outliers. Pairwise comparisons (Mann-Whitney U-test) among seasons and groups significantly different at $p < 0.05$ (adjusted following Benjamini and Hochberg, 1995), are displayed by different letters.

Similarly, 89% (25/28) of the natural QL colonies collected in summer 2016 contained adult males, whose genotypes did not match those of the presumed queens. Across all queenless colonies, a median of 46% of the males (median, Q1, Q3; 46%, 23%, 58%) were definitively not offspring of the presumed queen. Of all males, only few (median, Q1, Q3; 0%, 0%, 13%; min. 0%, max. 50%) likely were grandsons of the queen, and a median of 25% (median, Q1, Q3; 25%, 15%, 52%) were offspring of mothers that did not share any alleles with the majority of workers and the presumed queen.

The number of alien males per present alien worker (2:1) was significantly higher (Kruskal-Wallis test: $\chi^2 = 80.06$, $df = 2$, $p < 0.0001$) than the number of males produced by the queen (0.86:1, $p = 0.022$) or per worker related to the queen (0.06:1; $p < 0.0001$; males produced by queen-related workers vs. queen-produced males $p < 0.0001$).

As genotyping only 12 workers per colony might have missed potential mothers of alien males we genotyped all the workers from eleven colonies. Of those males whose genotypes had not matched with the majority of the 12 previously genotyped workers,

23 males from eight colonies could be assigned to workers produced by the queen, and 20 males from two colonies to formerly not represented alien workers. Potential mothers made up 21% and 29% of the workers in these two colonies. Workers, whose genotypes matched those of the alien males but neither those of the queen nor its workers, were found in five of eleven colonies. Up to 29% of the colony's workers were such alien individuals (median, Q1, Q3 per colony: 16%, 8%, 21%). The number of different alleles found in alien workers ranged from one to five per colony and thus, alien workers could stem from up to four different colonies depending on loci (median: two possible source colonies of alien workers per loci). Furthermore, the genotypes of 38 of 132 (29%) analyzed males (median, Q1, Q3 per colony: 3, 2, 8) did not match any worker genotype on at least one of three analyzed loci in seven out of eleven colonies. Interestingly, all males shared one or two alleles and might therefore have been produced by a single alien mother.

Maternity of males in laboratory colonies

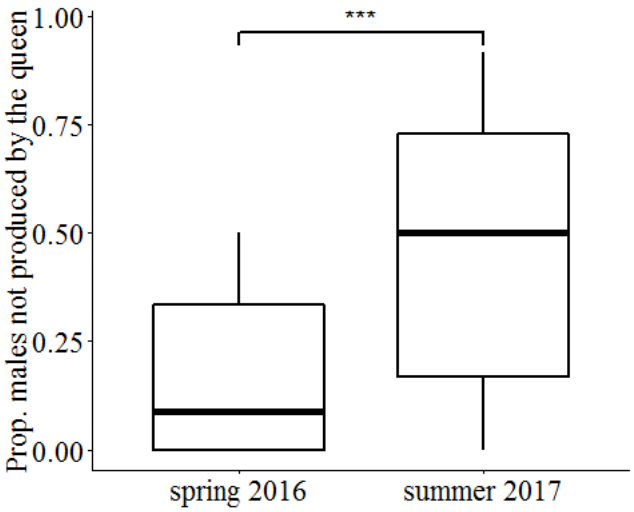

Figure 2.3: Proportion of males which are not produced by the resident queen, eclosed in 2016 and 2017 in queenright *Temnothorax crassispinus* colonies. The proportion of males produced by other females than the queen, increases significantly after one year in semi-natural conditions (median, Q1, Q3; summer 2017: 50%, 17%, 75%; spring 2016: 9%, 0%, 33%; W = 405, p = 0.0004). Boxplots show median, 25 and 75 quartile and 95% percentiles (*** $p < 0.001$).

Compared to natural colonies, workers appeared to increase their reproductive activities when colonies were kept under semi-natural conditions in artificial nests outside the university building. After one hibernation, almost all QR laboratory colonies contained males that had not been produced by the queen (summer 2017: 93%, 28/30, vs. spring 2016, $\chi^2 = 5.76$, df = 1, p = 0.016), and about half of the males (median, Q1, Q3; 50%, 17%, 73% per colony) were not sons of the present queen (figure 2.3). Whereas in natural colonies only a small percentage of males appeared to be offspring of workers related to the queen (see above), their share increased to a median of 33% when colonies were kept in experimental isolation (Q1, Q3: 8%, 67%; compared to a median of 0% in natural colonies, W = 354, p < 0.0001, figure 2.4). The proportion of males that did not share any allele with the queen and the majority of workers did not differ between laboratory and natural colonies (median, Q1, Q3; 0%, 0%, 17% compared to a median of 8% in natural colonies; W = 330, p = 0.178). Similarly, the proportion of alien workers did not change significantly during isolation in the laboratory (summer 2017: median, Q1, Q3; 0%, 0%, 12% vs. spring 2016: 0%, 0%, 18%; W = 138, p = 0.645)

The increased worker reproduction in laboratory colonies caused a shift in the proportion of males among all produced sexuals (Kruskal-Wallis test: $\chi^2 = 32.1$, df = 2, p < 0.0001). While in 2016, the proportion of males did not differ between QR colonies in the laboratory (median, Q1, Q3; 51%, 1%, 73%) and the field (median, Q1, Q3; 40%, 0%, 87%, p = 0.92), QR laboratory colonies produced significantly more males in 2017 (median, Q1, Q3; 88%, 74%, 95%; vs. natural colonies 2016: p < 0.0001, vs. laboratory colonies 2016: p < 0.0001).

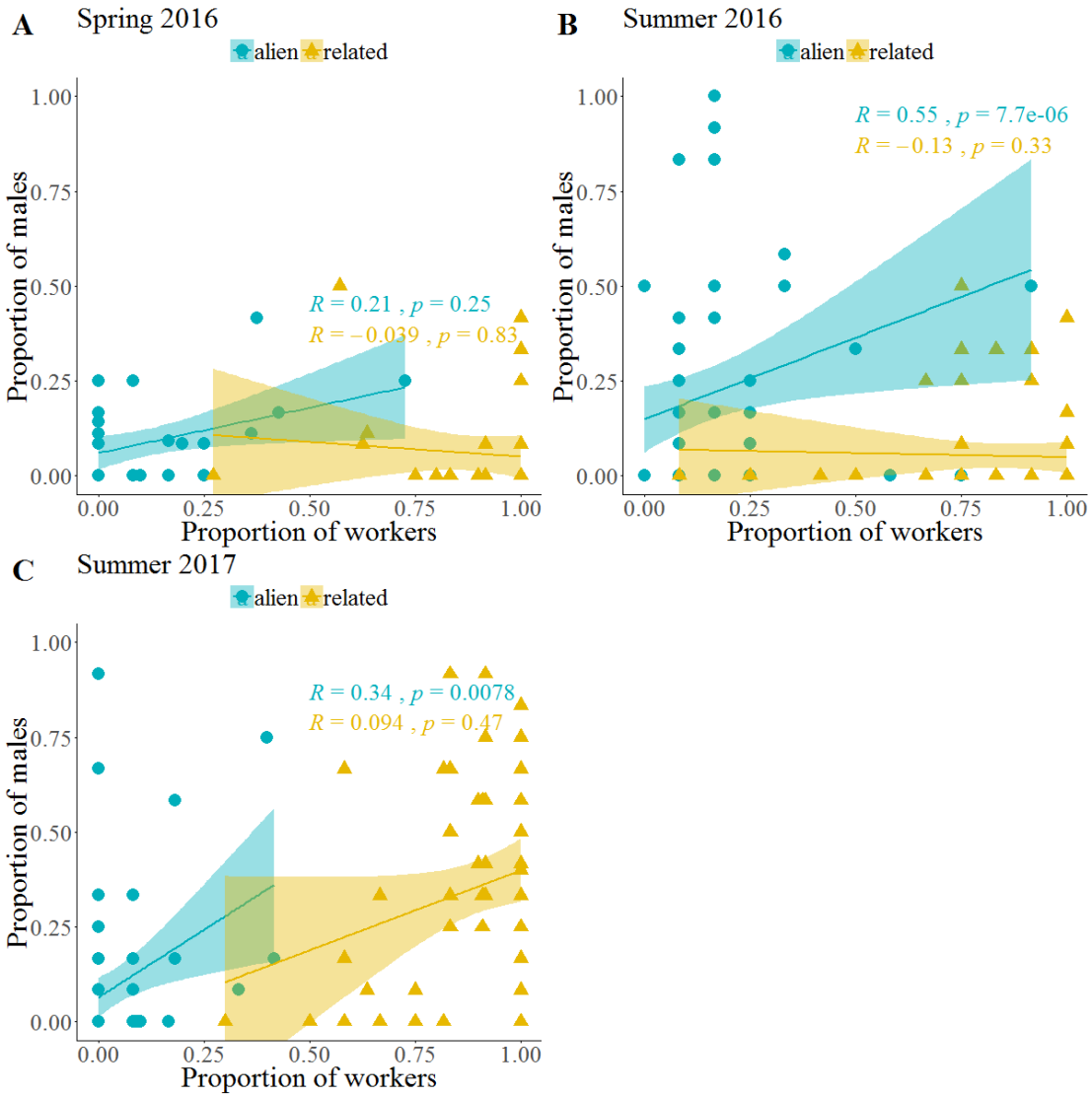

Figure 2.4: Correlation between the proportion of *Temnothorax crassispinus* males and workers that are either related (yellow) or not related (blue, "alien") with the queen (queenright colonies) or the majority of workers (queenless colonies). The number of males that are produced by workers which are related with the queen increases under laboratory conditions in summer 2017 (see results).

2.5 Discussion

Workers of most species of social ants, bees, and wasps are in principle capable of gaining direct fitness at least by producing sons from unfertilized eggs (Bourke, 1988*b*; Choe, 1988), but how successful they do so has rarely been quantified (e.g., Walin et al., 1998).

While workers readily lay eggs in the absence of the queen (Cao, 2013; Debout et al., 2007; Partridge et al., 1997), in queenright colonies worker reproduction is assumed to be prevented by worker and queen policing and queen pheromones (Brunner and Heinze, 2009; Helanterä and Sundström, 2007*a*; Oldroyd et al., 2001; Ratnieks, 1988*a*; Stroeymeyt et al., 2007; Walter et al., 2011). Genotyping males, queens, and workers from queenless and queenright colonies of the ant *Temnothorax crassispinus* indicates that in this species a surprisingly large fraction of males are not sons of the queen but either its grandsons, i.e., offspring of the queen's workers, or produced by unrelated individuals. Our study therefore reveals considerable direct fitness benefits for ant workers, which appears to be even more pronounced in the case of unrelated, reproducing workers.

The genotypes of queens and workers from queenright colonies suggested a rather instable structure of the studied *T. crassispinus* populations, with frequent queen turnovers, colony usurpations, and fusions, similar to what has been observed in its sibling species *T. nylanderi* (Foitzik and Heinze, 1998, 2000; Foitzik et al., 2007; Strätz and Heinze, 2004). Furthermore, natural queenless colonies might have lost their queen or be fragments of queenright colonies (polydomy, Cao, 2013; Debout et al., 2007; Smith et al., 2012; Strätz and Heinze, 2004). This population fluidity appears to result from very high nest densities, the fragility of nest sites, and presumably also a nestmate recognition system strongly based on environmental cues (Heinze, Foitzik, Hippert and Hölldobler, 1996; Foitzik et al., 2007). In natural colonies, all these factors may have contributed to the frequent occurrence of males, which were not sons of the resident queen. For example, males may have been produced by workers under queenless conditions, either in the case of seasonal polydomy or when a queenright colony merged with an unrelated colony that had lost its own queen. At least in the laboratory, brood produced by workers in the absence of a queen is readily accepted when fragments of experimentally polydomous colonies merge for hibernation (chapter 5).

Queen turn-over and the merging of two queenright colonies might also explain the presence of workers and queen-produced males with aberrant genotypes. However, as *Temnothorax* workers can live for several years (see chapter 3, Plateaux, 1986; Sendova-Franks and Franks, 1995), this cannot explain the presence of alien males without related workers. In such cases, males with aberrant genotypes might be offspring of drifting alien workers that were adopted by the colony or simply dumped their eggs into neighboring nests, as occurs in bees (Beekman and Oldroyd, 2008; O'Connor et al., 2013; Pearson et al., 1997; Tallamy, 2005). Finally, brood raiding, as observed in related social parasites (Buschinger et al., 1980) but not yet substantiated for non-parasitic *Temnothorax*, might also lead to the presence of alien workers and sexuals in a colony. Hence, this all makes

it difficult to exactly determine the origin of those males that were not offspring of the queen present in a natural colony.

Even though a fraction of these males may thus have been produced by unknown queens or by workers from queenless colony fragments, indirect evidence strongly supports our view that workers significantly contribute to the production of males also in the presence of the queen. First, many workers in natural colonies had active ovaries independent of queen presence and relatedness to the colony (see also El-Shehaby et al., 2012). Second, the origin of males in colonies that were kept isolated from other colonies clearly showed that also workers related to the present queen readily lay eggs (chapter 3).

This reveals a high reproductive potential of *T. crassispinus* workers compared to other social insect species (ants: Helanterä and Sundström, 2007*b*; Smith et al., 2012; Walin et al., 1998; honey bees and wasps: Ratnieks, 1993; Foster et al., 2001) including many other congeneric species, in which the development of worker-produced eggs is prevented by worker policing or queen pheromones (Helanterä and Sundström, 2007*a*; Stroeymeyt et al., 2007; Walin et al., 1998; Walter et al., 2011). The fluid colony structure in two of the study populations, with a relatedness among workers considerably lower than the 0.75 expected for full sisters (population 1) 0.640, 2) 0.651 and 3) 0.716 (n. sign.), J. Giehr, in preparation; but see Strätz and Heinze, 2004, for high relatedness in another population) might reduce the efficiency of worker policing (Wenseleers and Ratnieks, 2006). At the same time, frequent queenlessness might select for worker reproduction (e.g., Nanork et al., 2007). Balanced sex ratios in natural colonies indicate that worker reproduction in nature is not associated with high costs, but the strong increase of male-bias under laboratory conditions might reveal negative effects under artificial conditions: egg-laying workers are known to work less and to refrain from costly tasks (e.g., Bourke, 1988*a*; Barth et al., 2010; Charbonneau and Dornhaus, 2015). Of particular interest is the apparent higher direct fitness of alien workers. In bees, drifted workers engage less in brood care (Pfeiffer and Crailsheim, 1999) and have a higher reproductive potential by escaping queen control and worker policing (Birmingham et al., 2004; Lopez-Vaamonde et al., 2004; Nanork et al., 2005, 2007; O'Connor et al., 2013).

In summary, our data show that *T. crassispinus* workers are capable of raising own sons and gaining considerable direct fitness in natural colonies. Assuming that approximately 25-30% of the males might be offspring of the workers and that a *T. crassispinus* colony consists on average of a few dozens to hundreds of workers, the contribution of an average worker to the male population is about 1% in workers related to the queen and even more in the case of alien workers. The non-negligible chance of

obtaining direct fitness might explain why workers of many ant species form dominance hierarchies and selfishly police eggs laid by other workers (e.g., Cole, 1981; Franks and Scovell, 1983; Bourke, 1988*a*; Heinze et al., 1997; Stroeymeyt et al., 2007).

Declarations

Ethics approval

Temnothorax crassispinus is an unprotected ant species. All experiments comply with European laws.

Availability of data and material

The datasets of the article are available in supplement S1.

Competing interest

The author(s) declare(s) that they have no competing interests.

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Author contributions

JG and JH designed the study; JG, JW, LS, KR and TK performed the experiments; JG analyzed the data; JG and JH interpreted the data and wrote the manuscript.

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3 Off with her head - queen expulsion and execution increases direct fitness of workers in a monogynous ant

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3.1 Abstract

Workers in many social insect species are not completely sterile but principally capable of laying unfertilized eggs, which can develop into males. This causes a potential conflict about male parentage between queen and workers, as the relatedness asymmetry causes a disequilibrium in the relatedness between workers and brothers and their own sons. Usually, worker reproduction is suppressed by queen pheromones or policing, and in the latter, reproductive attempts are punished physically. However, in some species, arising conflicts can even result in matricide, i.e. the killing of the colony founding queen by daughters. In monogynous species, killing the only queen is assumed to be very costly due to the loss of further diploid offspring, and matricide has so far only been observed in polygynous or annual species at the end of the reproductive period. Here, we report for the first time queen expulsion and matricide in a monogynous, monandrous ant. Workers aggressively expelled related as well as unrelated queens from the nest, several weeks after colony collection in early spring. Initially, matricide caused a significant decrease of work force and brood items, but eventually increased the direct and inclusive fitness of workers by significant male production. Long-term observations revealed a rather short lifespan of queens, while workers in orphaned colonies survived for several years and produced comparable amounts of offspring as queenright colonies.

Keywords
Altruism; Matricide; Queen execution; Worker reproduction; Ants; *Temnothorax*

3.2 Introduction

Social insect colonies are based on a highly structured, well-organized system which is generally arranged by centralized and decentralized control. Colonies mainly consist of female individuals, which are divided into different castes according to their reproductive and non-reproductive tasks: the queen stays inside the nest and produces female and male offspring, while the workers refrain from reproduction but care for the brood, forage or defend the nest. However, social insect colonies are not free of conflicts, although caste determination is irreversible in highly eusocial species (Hölldobler and Wilson, 1990; Oster and Wilson, 1978). One potential conflict, the conflict over male parentage, is caused by the relatedness asymmetry in monogynous, monandrous species (Aron et al., 1994; Bourke, 1988*a*; Hammond and Keller, 2004; Heinze, 2010; Owen and Plowright, 1982; Paxton et al., 2001; Zanette et al., 2012). Despite physiological constraints, as the lack of a spermatheca and the inability to mate, workers of many social insect species are

capable of laying unfertilized eggs and can increase their direct fitness by independent male production (Bourke, 1988*b*; Bloch and Hefetz, 1999; Cole, 1986; El-Shehaby et al., 2012; Heinze et al., 1997; Helanterä and Sundström, 2007*b*; Imperatriz-Fonseca and de Matos Peixoto Kleinert, 1998; Oldroyd et al., 2001; Visscher, 1989). Whereas queens are closely related to their sons (1/2), workers should favor rearing their own sons (1/2) and also their nephews (3/8) over their brothers (1/4) to maximize direct fitness (Hamilton, 1964*b*, 1987; Trivers and Hare, 1976). However, worker reproduction can be costly for the colony, as reproducing workers contribute less to colony tasks, (Bocher et al., 2008; Bourke, 1988*a*; Dampney et al., 2004; Tsuji et al., 2012) and queens try to prevent workers from reproducing by queen pheromones (Conte and Hefetz, 2008; D'Ettorre et al., 2004; Endler et al., 2004; Holman et al., 2010; Hoover et al., 2003; Keller and Nonacs, 1993; Van Oystaeyen et al., 2014; Vargo and Robert, 1998) or policing, as e.g. actively preventing workers from oviposition or eating worker-produced eggs (Kikuta and Tsuji, 1999; Oster and Wilson, 1978; Ratnieks, 1988*b*).

Especially in small colonies, queen policing behavior can resolve the reproductive conflict (Ratnieks, 1988*b*), which can otherwise result in matricide (Bourke, 1994). The killing of the maternal queen, causes the loss of prospective female offspring, but can be a successful strategy to increase the inclusive fitness of the colony in annual species, when the queen's fecundity is decreasing at the end of the reproductive season (Bourke, 1994; Loope, 2015). There, matricide should only occur when the future reproductive success of the queen is low (Bourke, 1994) and workers can increase their direct fitness and the inclusive fitness of the colony by independent reproduction (Almond et al., 2019; West et al., 2007). However, in monogynous ants, queens usually live for several years (Keller and Genoud, 1997; Keller, 1998; Plateaux, 1971) and do not suffer from a decreased fecundity with increasing age (Heinze and Schrempf, 2012). Accordingly, killing the only fully fertile queen is supposed to be extremely costly and has so far only been reported for surplus queens in polygynous ant species (Balas, 2005; Inoue et al., 2015; Keller et al., 1989; Reuter et al., 2001).

Here, we report frequent matricides for the first time in a monogynous ant species (Bourke, 1994). We took a long-term approach to examine worker reproductive success in originally queenright *Temnothorax crassispinus* colonies and in colonies that actively expelled and/or killed their queen (orphaned colonies). Queens of the monogynous, monandrous species *T. crassispinus* can live up to several years (Plateaux, 1971), but previous studies indicate high turn-over rates in queens and frequent colony usurpations and fusions (Strätz and Heinze, 2004, see also *T. nylanderi* Foitzik and Heinze, 1998, 2000). Worker reproductive potential is high in natural queenright and queenless colonies

(El-Shehaby et al., 2012, and see chapter 2) and also queenless colonies are highly productive. We compared worker fecundity and quantified worker male production in recently orphaned colonies with that of still queenright colonies. Furthermore, we monitored colony fitness and development, i.e. number of male offspring and colony survival, for four years in captivity. Our study shows, that orphaned colonies produced a comparable amount of offspring and are not necessarily disadvantaged by queenloss, as queenright colonies also strongly declined in the number of workers and brood items. Similarly to queenright colonies, about half of the orphaned colonies survived until the fourth year and could therefore significantly contribute to male production in nature as queen lifespan seems to be rather short in general.

3.3 Material and methods

Study species and collection of colonies

Temnothorax crassispinus is a small (approximately 2 to 3 mm) monogynous ant, whose colonies consist of up to 300 workers and live in acorns or rotting twigs (Mitrus, 2015; Strätz and Heinze, 2004; Tichá and Štys, 2002). In total, we collected 436 colonies around Regensburg, Germany. Colonies were collected in calendar week 10 to 15 in early spring 2016. To avoid losing individuals in the field during collection, we placed the whole nest into sealable plastic bags. In the laboratory, we counted all adults and brood, transferred them from their natural nests into artificial nest boxes (10 x 10 x 3 cm^3), and kept them outside the building under natural temperature.

One to several workers from about 1/3 of the queenright colonies started to behave aggressively towards the queen one to six weeks after colony collection (starting calendar week 16). Aggressive behaviors ranged from mandible threatening, to the removal of the queen from the nest or even the killing of the queen and lasted for several days. Expelled queens were actively hindered from re-entering the nest and colonies were checked for queen survival several times per week for the first three months after colony collection. Worker number and brood items were additionally counted approximately five weeks, ten weeks and twenty weeks after colony collection during the first year (2016). In the following years (2017, 2018, 2019), queen survival, worker number and the number of brood items was noted once per year in summer, when the caste and sex of pupae was identifiable. Colonies were fed twice per week with cockroaches and honey during spring and summer. Feeding frequency was decreased to once per week when day temperatures dropped below 15 °C and to once every two weeks at day temperatures below 5 °C.

Colonies were covered with plant protection fleece when temperatures dropped below 0 °C.

Microsatellite analysis

To determine colony structure and the origin of males, we investigated the microsatellite genotypes of workers, males and, if present, the queen from each of 54 colonies that expelled their queen:

1. 23 live laboratory colonies 2016 (originally collected in spring 2016):
   23 colonies: 7 to 12 females; 15 colonies: 7 to 12 males
2. 31 live laboratory colonies 2017 (originally collected in spring 2016):
   9 to 12 females, 10 to 12 males

DNA was extracted using a CTAB method modified from Sambrook and Russell (2001) (see supplement S2). We used six highly polymorphic (see chapter 2) microsatellite markers: GT218 (Hamaguchi et al., 1993), 2MS17 (Suefuji et al., 2011), L5 (Foitzik et al., 1997), L18 (Foitzik et al., 1997), Ant3993 (Butler et al., 2014) and GT1 (Bourke et al., 1997). The 10 µl PCR reaction volume consisted of 5 µl Taq DNA polymerase, 3 µL ddH_2O, 0.5 µl unlabeled reverse primer, 0.5 µl labeled forward primer (HEX, FAM and TET) and 1 µL DNA. PCR consisted of initial denaturation at 94 °C (4 min), 33 cycles at 94 °C (denaturation, 45 sec), 57 °C (annealing, 80 sec) and 72 °C (elongation, 25 sec), and a final step at 72 °C (1 min). For 2MS17, the cycle number was reduced to 32 cycles, denaturation time was extended to 75 sec, and annealing temperature was decreased to 54 °C.

We mixed 0.07 µl to 0.2 µL of the PCR product (depending on DNA quantity) with 25 µL formamid and 0.1 µL T486 size standard, and analyzed it in an ABI PRISM 310 Genetic Analyzer (PE Biosystems) after DNA denaturation at 90 °C (1 min). Allele sizes were determined using GENESCAN 3.1 software (PE Biosystems). Samples were stored in 100% EtOH until analysis.

Data analysis

In some colonies, the queen was not available due to further analyses by Nathalie Stroeymeyt (University of Fribourg, see figure S3.1) or an advanced decay and we reconstructed the likely genotype of the queen (maternal alleles) from the genotypes of the majority of workers within each colony. Paternal alleles were determined by

subtracting the maternal alleles from the worker genotype. We used these data to estimate in each colony the minimal number of males not produced by the present queen. Workers and males were defined as alien individuals when their alleles did not match the determined maternal or paternal alleles of the colony at at least two loci. This provides a conservative estimate of the presence of alien individuals, but makes it more unlikely that potential mismatches were caused by errors during sequencing or the determination of allele sizes. The ancestry of the males was analyzed by evaluating the queen and worker alleles.

We had three categories:

1. Queen-produced males, which showed at least one of the queen's alleles at at least five of the six studied loci.
2. Worker-produced males, which at two or more loci showed alleles that did not match the queen's alleles but were identical to the paternal allele as deduced from the genotypes of the majority of the workers.
3. Alien males, which had alleles, which did not match any allele in the colony, or alleles, which were otherwise present only in a minority of the colony's workers.

Half of the males produced by workers will at a given locus inherit the queen's allele. The likelihood that a worker-produced male by chance inherited the maternal allele at all six loci is $(1/2)^6$. Hence, the likelihood that worker-produced males were wrongly identified as queen-produced males is small.

Data were analyzed using R 3.2.3 software (R Development Core Team, 2008). Values are given as median and 1st (Q1) and 3rd (Q3) quantiles. As data were not normally distributed we used Mann-Whitney U-test (independent samples) and Wilcoxon signed rank-tests (dependent samples) for two-sample comparisons. Multiple comparisons were corrected for a false discovery rate using "fdr" (Benjamini and Hochberg, 1995).

3.4 Results

Reproduction and queen survival in laboratory colonies

A high proportion of the collected colonies was queenright (320/436 colonies; 73%, see table 3.1), but almost half of the queens (48%, 152/320), which after collection in their natural colonies in spring 2016 had been kept at the university, died before the first hibernation in 2016. A large fraction of these queens (78%, 119/152) left the nest

because they were attacked or were actively killed by workers one to six weeks after colony collection (starting calendar week 16). In the aggressive colonies, one or several workers could be observed to bite on to the queen's legs and to even cut off limbs or the head. The genotypes of 12/27 attacked and genetically analyzed queens (44%, 22 colonies, four colonies contained two or three queens), did not match those of the majority of the workers, but 11/27 queens could be determined as the foundress of the colony and 4/27 queens were presumably sisters of the present workers (table S3.1).

Table 3.1: Yearly progress of colony and queen numbers in queenright colonies, colonies that expelled their queen and queenless colonies of the monogynous ant *Temnothorax crassispinus* collected in early spring 2016. Colony numbers were noted once per year in summer for four years.

	Number still existing colonies					Queens alive				
	collection	2016	2017	2018	2019	collection	2016	2017	2018	2019
Queenright	320	186	151	138	107	320	168	125	61	29
Queen expelled		114	93	85	60					
Queenless	116	56	45	35	15					
Total	436	356	289	258	182					

Further queens died in the following years: in 2017, 43 of the remaining 168 queens (26%) vanished or died, in 2018 64 of the remaining 125 queens (51%) died and in 2019 32 of the remaining 61 queens (52%) died, i.e., only 9% of the initially collected queens survived until summer 2019 (29/320) (see figure 3.1). Finally, 53% (107/201) of the initially peaceful queenright colonies, 50% (60/119) of the colonies that expelled their queen and 13% (15/117) of the queenless colonies still contained workers and survived until summer 2019 ($\chi^2 = 54.88$, df = 2, $p < 0.0001$).

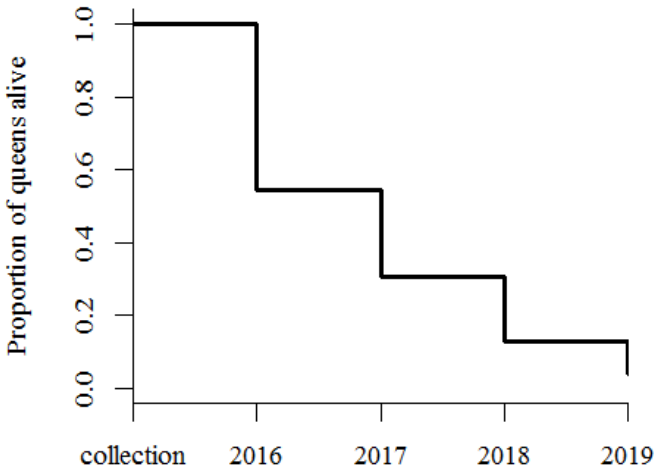

Figure 3.1: Lifespan of *T. crassispinus* queens during a four year observation period starting after collection in early spring 2016. Only 9% of the collected queens survived until the fourth year in captitivity (summer 2019).

Colonies that expelled their queens were significantly larger than other queenright colonies (W= 9448, p = 0.002, for details see table 3.2). After the expulsion and killing of queens in laboratory colonies, worker number (n = 119 colonies, W = 13177, p = 0.024) and brood number (W = 10236, p = 0.001) decreased significantly over the next four weeks compared to still queenright colonies. Colonies which had expelled their queen, initially contained more larvae (W = 7237, p = 0.011) and eventually produced more eggs (W = 8497, p < 0.0001) in summer 2016. This resulted in a higher number of larvae in fall 2016 (W = 7956, p = 0.0002) than in queenright colonies.

Because sexual larvae typically hibernate, the death or killing of the queen did not affect the sex ratio produced in the same year (males/all sexuals, W = 5278, p = 0.727) or the proportion of males that were not produced by the queen (median, Q1, Q3; queen expelled: 9%, 0%, 33%; compared to queenright: 17%, 8%, 38%; W = 160, p = 0.388; see chapter 2). In the year following collection (2017), the number of workers (W = 9998, p = 0.393) and the number of larvae (W = 6758, p = 0.300) did not differ between queenright colonies and colonies that expelled their queen. Most of the males produced in colonies in which the queen had been expelled or killed were offspring of workers related to the former queen (median, Q1, Q3; 33%, 17%, 56%), but unrelated workers were still present in 44% of the colonies (8/18; worker 0%, 0%, 22%) after hibernation and contributed to the colonies' males (median, Q1, Q3; 8%, 0%, 25%).

In the third year (2018) and fourth year (2019) under isolated laboratory conditions, colonies that had killed their queen produced the same amount of offspring as queenright

colonies (2018: W = 1966, p = 0.051; 2019: W = 826, p = 0.176) although they could produce only males and their sex ratio (here: all males/all females; as colonies barely produced virgin queens) differed from queenright colonies (2018: W = 4430, p < 0.0001; 2019: W = 1608, p < 0.0001). Surprisingly, although colony size generally decreased strongly under laboratory conditions (Wilcoxon signed rank-tests: 2016 vs. 2017: V = 60601, p < 0.0001; 2017 vs. 2018: V = 31058, p < 0.0001; 2018 vs. 2019: V = 30202, p < 0.0001), worker number in colonies that expelled their queen and still queenright colonies did not differ in the third year (W = 3680, p = 0.634) but only in the fourth year (W = 361, p < 0.0001).

Figure 3.2: Summary of colony data (median, Q1, Q3) collected over a four year period in queenright *Temnothorax crassispinus* colonies and in colonies that expelled their queen. Significant differences (Mann-Whitney U-test, p < 0.05) between both groups were marked in bold (for details see results).

	Queenright			Queen expelled		
	median	Q1	Q3	median	Q1	Q3
Worker number at collection	**150**	**100**	**213**	**180**	**128**	**230**
Proportion of workers left four weeks after queen expulsion	**0.73**	**0.60**	**0.96**	**0.69**	**0.53**	**0.86**
Worker number summer 2017	47	27	72	50	28	81
Worker number summer 2018	15	6	28	12	7	23
Worker number summer 2019	**16**	**12**	**28**	**6**	**2**	**9**
Amount of larvae at collection	**122**	**80**	**220**	**180**	**100**	**270**
Proportion of larvae left four weeks after queen expulsion	**0.91**	**0.60**	**1.40**	**0.69**	**0.47**	**1.02**
Eggs produced summer 2016	**53**	**12**	**120**	**100**	**40**	**240**
Larvae produced summer 2016	**250**	**136**	**395**	**368**	**198**	**615**
Larvae produced summer 2017	73	30	160	72	38	178
Offspring produced 2018	30	16	62	19	6.75	51
Offspring produced 2019	45	25	70	35	13	66
Sex ratio summer 2016	0.54	0.08	0.96	0.50	0.15	0.88
Sex ratio summer 2018	**0.78**	**0.62**	**0.91**	**1.00**	**1.00**	**1.00**
Sex ratio summer 2019	**0.68**	**0.20**	**0.90**	**1.00**	**1.00**	**1.00**

3.5 Discussion

Workers in several social insect species are not completely sterile and are capable of gaining direct fitness by independent reproduction. So far, it is assumed that worker reproduction is surpressed in the presence of a queen, and workers mainly reproduce after the death of the queen. Previous studies revealed alternative reproductive strategies for social insect workers, as reproduction in unrelated or orphaned colonies (Beekman and Oldroyd, 2008; Chapman et al., 2009; Holmes et al., 2013) or even active matricide (Bourke, 1994). However, the latter was so far mainly observed in polygynous (Balas, 2005; Inoue et al., 2015; Keller et al., 1989; Reuter et al., 2001) or annual species (Bourke, 1994), where the loss of the queen does not impair the inclusive fitness (Hamilton, 1964*a*,*b*) of the colony. Here we report, for the first time, the selfish expulsion and killing of queens in a monogynous, monandrous ant species. Workers of *T. crassispinus* ants could be observed killing single and surplus queens, or removing them from the nest and hindering them from re-entering until the queen's death. Although worker number in orphaned colonies decreased during the observational period, workers produced a significant amount of males during the following years and thus, gained considerable fitness.

Workers started to expel or to actively kill the queens one to six weeks after colony collection in early spring. Contrary to the previous assumptions and observations in wasps and bees (Bourke, 1994; Loope, 2015, 2016), queen executions in ants seem to be independent of relatedness, fecundity or trophic status of the queens (Keller et al., 1989; Reuter et al., 2001). However, queen executions have so far only been reported for polygynous ant species and may ultimately serve to increase relatedness within supercolonies (Inoue et al., 2015). In our study, expulsions and executions applied to unrelated and related queens of a monogynous ant species, where workers are not easily capable of rearing surrogate queens and queens can live for several years (Keller and Genoud, 1997; Keller, 1998), in contrast to annual bees and wasps (Bourke, 1994).

Queen expulsion was probably not caused by a decreased fecundity in queens, as worker aggressiveness started right after hibernation before the first eggs were laid, and the sex ratio of offspring eclosed in summer did not indicate a limited queen fecundity, at least the year before. Furthermore, we do not expect that queen removal is based on the queen's health, as we could not verify lethal pathogen infections in expelled queens (n = 21 analyzed queens; N. Stroeymeyt, personal comment, see figure S3.1) and also previous studies did not reveal an effect of queen health on worker aggressiveness (Giehr and Heinze, 2018). However, we cannot completely exclude that queen executions are

an artifact arising from colony collection, although we could not observe this behavior in colonies collected for other studies in summer (n > 80 colonies, J. Giehr, personal comment).

The presence of unrelated queens (see also chapter 2) indicate frequent colony takeovers in natural *T. crassispinus* colonies, as already reported for its sister species *T. nylanderi* (Foitzik and Heinze, 1998, 2000, 2001), and killing the queen to maximize own fitness before her natural death and/or colony usurpation might be an important reproductive strategy for workers. *T. crassispinus* queens and the entire colonies seemed to be rather short-lived (see also chapter 2) and polydomy (Strätz and Heinze, 2004), migration and usurpation (Foitzik and Heinze, 1998, 2000, 2001) might cause frequent turn-overs of queens in nature. Orphaned colonies produced a high amount of males and the direct fitness gained by early, independent reproduction (Bourke, 1988*b*) might compensate for the decreased indirect fitness after the queen's death. Although queenless colonies cannot lay diploid eggs and thus, cannot rear a surrogate queen, the survival of the colony could be eventually assured by the adoption of new queens, as supported by the presence of unrelated queens in nature (chapter 2). In honey bees, workers can increase their direct fitness by successfully reproducing during the period between queen exchange (Holmes et al., 2013) and also in our colonies, egg laying by queen related workers and the number of larvae strongly increased after the queen's death. This matches observations in natural colonies (chapter 2), where a minority of unrelated workers, which mighht stem from a former, deceased queen, produced most of the males that were not produced by the present queen. Generally, orphaned workers were highly productive over several years and although worker number decreased significantly in the orphaned colonies, they still produced similar amounts of offspring as queenright colonies. However, it still needs to be investigated why worker number also decreased in queenright colonies under laboratory conditions.

Here we report that matricide can lead to a substantial direct fitness gain for workers of the monogynous ant *T. crassispinus.* Most of the queens (91%) died within the four year observation period and queens suffering from an unnatural death were expelled or killed independent of their relatedness to other colony members. However, the cost of queenloss was presumably low, as orphaned colonies survived for several years and produced a considerable amount of males. Therefore, queenless colonies might contribute significantly to male production after the death of the queen in nature.

Declarations

Ethics approval

Temnothorax crassispinus is an unprotected ant species. All experiments comply with European laws.

Availability of data and material

The datasets of the article are available in supplement S1.

Competing interest

The author(s) declare(s) that they have no competing interests.

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Author contributions

JG and JH designed the study; JG performed the experiments and analyzed the data; JG and JH interpreted the data and wrote the manuscript.

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4 Body size and sperm quality in queen- and worker-produced ant males

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To be submitted

4.1 Abstract

Workers of many ant species have functional ovaries and are capable of laying haploid, unfertilized eggs, at least in the absence of a queen. Previous studies in monogynous *Temnothorax* ants revealed a high proportion of worker-produced males in natural and laboratory colonies, and thereby arising males may considerably contribute to the direct fitness of workers. Social insect queens can live for several years and their reproductive success depends strongly on the quality of the sperm stored in their spermatheca. However, fitness traits as body size and sperm quality of worker-produced males are so far unstudied in ants. Here we examine whether queen- and worker-produced males of the monogynous ant species *Temnothorax crassispinus* differ in morphology or sperm length and viability. We either split queenright colonies in queenright and queenless halves, or removed the queen from a part of the queenright colonies, and allowed the colonies to reproduce until the eclosion of the males. Male quality traits vary mainly among colonies, especially in the presence of a queen, but differed generally only slightly between queen- and worker-produced males. Worker-produced males outnumbered and outlived queen-produced males, but the latter can have more viable sperm, depending on rearing conditions. Since sperm viability was generally rather high, our results indicate an enhanced selection pressure on viability, which might cause a trade-off between sperm length homogeneity and sperm viability.

Keywords
Male quality; Worker reproduction; Sperm quality; Ants; *Temnothorax*

4.2 Introduction

Social insects are well known for their prolonged reproductive phase and extraordinary lifespan compared to solitary insects (Keller and Genoud, 1997; Keller, 1998). Reproductive ant females can live for several decades and maintain their fecundity by livelong sperm preservation after a mating flight at early age (Fjerdingstad and Boomsma, 1998; Hölldobler and Wilson, 1990). Contrary to this, males have only a very short lifespan and usually die after swarming and mating (Hölldobler and Wilson, 1990). Thus, a male's fitness is mainly determined by its competitiveness and it's fertility, which are among others specified by morphology and sperm quality (Gomendio and Roldan, 1991; García-González and Simmons, 2005; Heinze et al., 1998; Hunter and Birkhead, 2002; Koffler et al., 2016; Stürup et al., 2013).

After the successful copulation, queens store preferably living sperm in their

spermatheca (Stürup et al., 2013), which is then used for livelong egg production. Furthermore, spermatheca capacity is limited and cryptic female choice seems to select for sperm length by storing either longer or shorter sperm cells, apparently depending on colony size and life expectancy (Baer et al., 2003; Boomsma et al., 2005; Fitzpatrick and Baer, 2011). Variation in sperm size and viability within and between males is therefore strongly affected by selection pressure (Fitzpatrick and Baer, 2011; Gomendio and Roldan, 1991; Hunter and Birkhead, 2002; Koffler et al., 2016). In general, sperm morphology might be crucial to sustain the queen with a lifelong sperm supply and to enable a sufficient colony growth (in *Atta colombica* millions of produced offspring during a lifespan of more than ten years) (Fjerdingstad and Boomsma, 1998). Hence, sperm length might be a trade-off between the male's mating strategy and the queen's storage capacity (Boomsma et al., 2005).

Furthermore, previous studies showed a trade-off between sperm traits and body structures (Simmons and Emlen, 2006), probably indicating energetic constraints between them. Indeed, energy requirements for sperm production increase with increasing sperm length (LaMunyon and Ward, 1998; Pitnick, 1996), but investment might pay off in terms of sperm motility (Gomendio and Roldan, 1991), competition (LaMunyon and Ward, 1998) and survival (Ball and Parker, 1996; Parker, 1993). However, rearing male offspring is generally energy consuming (Hrassnigg and Craislheim, 2005) and male quality might differ between colonies according to work force and nutritional status (Fitzpatrick and Lüpold, 2014).

In many social insect species, workers are capable of producing male offspring at least in the absence of a queen. However, since reproducing workers engage less in colony tasks, worker reproduction can be costly to the colony (Bourke, 1988*b*; Dampney et al., 2004; Tsuji et al., 2012) and thus, nutrition might be limited due to arising aggressions (Bocher et al., 2008; Brunner and Heinze, 2009; Stroeymeyt et al., 2007), a decreased immunity (Keiser et al., 2018) or a generally reduced worker activity (Gobin et al., 2003). Indeed, studies in honey bees revealed that worker-produced males suffer from reproductive restrictions, as they are smaller and less heavy than queen-produced males (Berg et al., 1997; Gençer and Çetin Firatli, 2005), but studies on worker-produced ant males are rare.

Here we examine, whether queen- and worker-produced males of the monogynous ant species *Temnothorax crassispinus* (Strätz and Heinze, 2004), differ in physiological traits under near-natural as well as standardized, climatic conditions. Although reproduction is mainly occupied by the queen in queenright ant colonies, *Temnothorax* workers are capable of laying unfertilized, haploid eggs (El-Shehaby et al., 2012). Under queenless

conditions workers establish reproductive rank orders by antennal boxing and biting and the highest ranking workers start to reproduce (Bourke, 1988*b*; Brunner and Heinze, 2009; Cole, 1981; El-Shehaby et al., 2012; Heinze et al., 1997; Stroeymeyt et al., 2007). Worker-produced males might therefore contribute significantly to the fitness of workers and the entire colony. We either split natural queenright colonies into queenright and queenless parts, or removed the queen from a fraction of recently collected colonies. Colonies were allowed to reproduce until the eclosion of the first males. Furthermore, we investigated, whether worker-produced males differ in survival or morphology. We compared sperm viability and length of males from queenless or queenright colonies, either from the same original colony or from unrelated colonies.

The results of our study show, that workers produce highly viable males. Queen- and worker-produced males were of similar quality, but sperm traits differed strongly among colonies in general. Worker-produced males lived longer than males from queenright colonies but had less viable sperm under near-natural conditions. Interestingly, male size and motility were independent of origin.

4.3 Material and methods

Temnothorax crassispinus is a small (approximately 2 to 3 mm) monogynous, monandrous ant species, whose colonies consist of up to 300 workers (Strätz and Heinze, 2004). Like in other *Temnothorax* ants, workers are capable of activating their ovaries and producing haploid offspring (El-Shehaby et al., 2012; Pusch et al., 2006).

We investigated whether males produced in queenright colonies – presumably mostly queen offspring – differed from males produced in queenless colonies – presumably mostly worker offspring. To do so, we compared male traits between two different series of experimental queenright (QR) and queenless (QL) colonies. Not all traits could be studied in both series.

For series 1, we used 19 queenright colonies that were collected in March 2016 near Regensburg, Germany. Colonies were put into sealable plastic bags together with their natural nests (decaying twigs etc.) and brought back to the university. After counting all adults and brood we transferred the colonies into artificial nest boxes (10 x 10 x 3 cm^3). The queens were removed before the appearance of the first eggs (four to five weeks after collection) from ten colonies to obtain queenless colonies, but otherwise all colonies retained their natural composition. Colonies were kept outside the building under natural climatic conditions. They were fed two times per weeks with honey and cockroaches or

Drosophila flies in summer and fall, and once every two weeks in winter. To obtain adult males of standardized age, we removed 10 workers and 20 male pupae of comparable developmental stages from nine different queenright and ten different queenless colonies and kept them separate from the rest of the colony in July 2017. These groups were checked daily until the first males hatched. The experiment started when more than three males had eclosed on the same day and in the same colony. All additional males were removed from the colony to prevent age differences affecting the comparisons. In total, 68 queenright and 69 queenless males were used for the analysis of male longevity (32 QR, 35 QL), male motility (23 QR, 18 QL), and sperm traits (13 QR, 16 QL).

For series 2, we set up six standardized queenright and queenless split colonies from eight collected colonies in September 2017. Each colony consisted of 25 workers, 25 larvae, and a queen in queenright colonies. Colonies were not further manipulated until the eclosion of the first males in July 2019. Colonies were initially kept in incubators at artificial summer conditions (12 h 26 °C / 12 h 23 °C, day / night cycle) to support egg laying. Five weeks later, after the originally present larvae had hatched and newly laid eggs had developed into small larvae, the temperature was gradually reduced to 6 °C / 4 °C for hibernation, which started in December 2017 and lasted for approximately four months. Thereafter, the temperature was gradually increased and again reached summer conditions at the end of June 2018. The seasonal cycles were repeated until the first males had eclosed in July 2019. Sperm viability and length were analyzed in five day old males as described above. In total, we analyzed sperm cells of 30 males from the queenless and 17 males from the queenright colony fragments.

Male developmental time and longevity

We analyzed the longevity of 32 adult males from five queenright colonies and 35 males from six queenless colonies from series 1. Colonies were checked daily until the last male had died. Only verifiably dead males were used for survival analysis. In series 2, we analyzed the developmental time of male larvae from hibernation to the first adult eclosion (six queenless and four queenright colonies; two queenright colonies did not produce males).

Male motility

We videotaped the motion of 23 males from six queenright colonies and 18 males from four queenless colonies males from series 1 for 10 minutes each. The motility of males was

videotaped 12 days after eclosure in a circular arena (diameter 1.1 cm). The recorded videos were analyzed by EthoVision XT 10 software (Noldus, Wageningen).

Sperm viability and length

We dissected the seminal vesicles of 13 males from five queenright colonies and 16 males from four queenless colonies from series 1 and 17 males from queenright and 30 males from queenless fragments from series 2 four to five days after the eclosure. We opened the seminal vesicles and accessory glands in Beadle solution (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM CaCl2, Ephrussi and Beadle, 1936) and mixed the fluid with 10 µl SYBR-Green (Thermo Fisher). After 10 minutes we added 2 µl of propidium iodide and incubated the sample for 7 minutes before analyzing the preparations under a fluorescence microscope (Zeiss, magnification 40x). Dead sperm cells fluoresced red, living sperm cells green. After the samples had dried completely, they were rinsed with 70% ethanol to fixate sperm and store them for later measurements. In total 40 sperm cells per male were measured using the software Digvision Pro 20.10.100. To estimate the general measuring error, one sperm was measured ten times in a row.

Dead males from series 1 were stored at -20 °C for subsequent body size measurements using a Keyence VHX-500FD digital microscope.

Data analysis

Data were analyzed using R 3.5.1 (R Development Core Team, 2008). Male survival was analyzed using "survival" package (Therneau and Grambsch, 2000). Non-parametric data (Shapiro-Wilk test $p < 0.05$) were analyzed using Mann-Whitney U-test, Kruskal-Wallis test, PerMANOVA ("vegan" package, Oksanen et al., 2017) and generalized linear models (lme4 package, Bates et al., 2015). Parametric data (Shapiro-Wilk test $p > 0.05$) were analyzed using t-test, ANOVA and linear mixed models ("nlme" package, Pinheiro et al., 2018). Pairwise Mann-Whitney U-tests and Wilcoxon signed rank-tests were corrected for a false discovery rate ("fdr", Benjamini and Hochberg, 1995) . Coefficient of variation was calculated as $cv = \frac{\sigma}{\mu}$ (σ: standard deviation, µ: mean).

4.4 Results

Colony productivity, male longevity, and motility

Workers of *T. crassispinus* do not mate and therefore cannot produce female offspring but readily produce males from unfertilized eggs at least in queenless conditions. Queenless fragments in series 2 produced significantly more males (max. male number median, Q1, Q3: 32, 16, 52) than queenright fragments of the same source colony (2, 0.8, 4; Wilcoxon signed rank-test: V = 34, p = 0.02). Queenright fragments instead focused on worker production (max. worker pupae: 57, 35, 79, only two colonies produced one virgin queen respectively). Males of both groups developed equally fast and the developmental time from hibernated larvae to the first adult males did not differ between related queenless (series 2, days until hatching median, Q1, Q3; 57, 55, 58) and queenright colonies (61, 56, 63: Mann-Whitney U-test: W = 12, p = 0.354). Males from queenless (n =15, four colonies) and queenright colonies (n = 9, four colonies) from series 1 did not differ in size (thorax width, mean±sd, queenless: 540±42 µm; queenright: 544±42 µm; t-test: t = -0.26, df = 17.78, p = 0.796).

In queenless colonies from series 1, males lived longer (lifespan (days) median, Q1, Q3: 18, 13, 26) than males from queenright colonies (13, 10, 19; series 1 Kaplan-Meier survival analysis: $\chi^2 = 4.3$, df = 1, p = 0.04, figure 4.1). The activity patterns did not differ between queenless and queenright males from series 1 (total distance covered: Mann-Whitney U-test: W = 227, p = 0.612; time spent immobile: W = 188, p = 0.631).

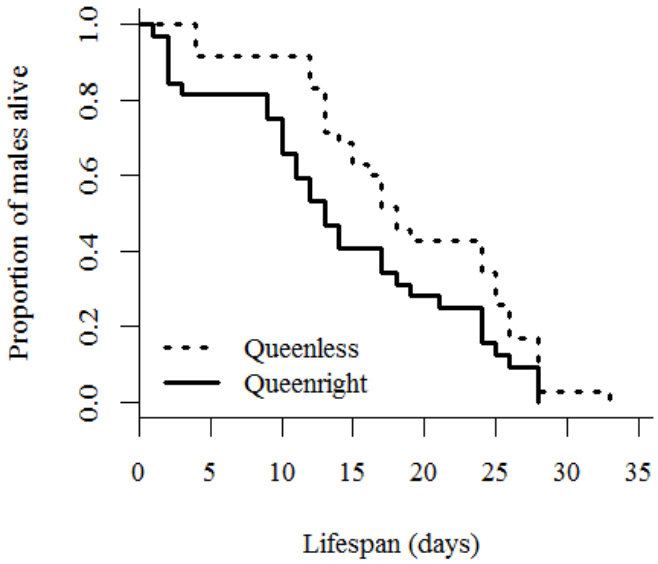

Figure 4.1: Lifespan of *T. crassispinus* males produced in queenless and queenright colonies. Males from queenless colonies lived significantly longer than males from queenright colonies.

Sperm length and viability

Mean sperm lengths and the variance of sperm length differed considerably across the studied males. Sperm length varied among colonies in the four groups (series 1 QL, series 1 QR, series 2 QL, series 2 QR) (Two factorial ANOVA, group: $F = 1.61$, $df = 3$, $p = 0.198$; colony: $F = 4.18$, $df = 12$, $p = 0.0001$; interaction group and colony: $F = 5.13$, $df = 5$, $p = 0.0006$; figure 4.2 A). When we controlled for the colony effect, the effects of series and queen presence on sperm length disappeared (linear mixed model: queen presence: $df = 63$, t-value = -0.14, $p = 0.900$; series: $df = 13$, t-value = -1.73, $p = 0.108$; worker number: $df = 13$, t-value = -1.62, $p = 0.128$, colony included as random factor, figure 4.3).

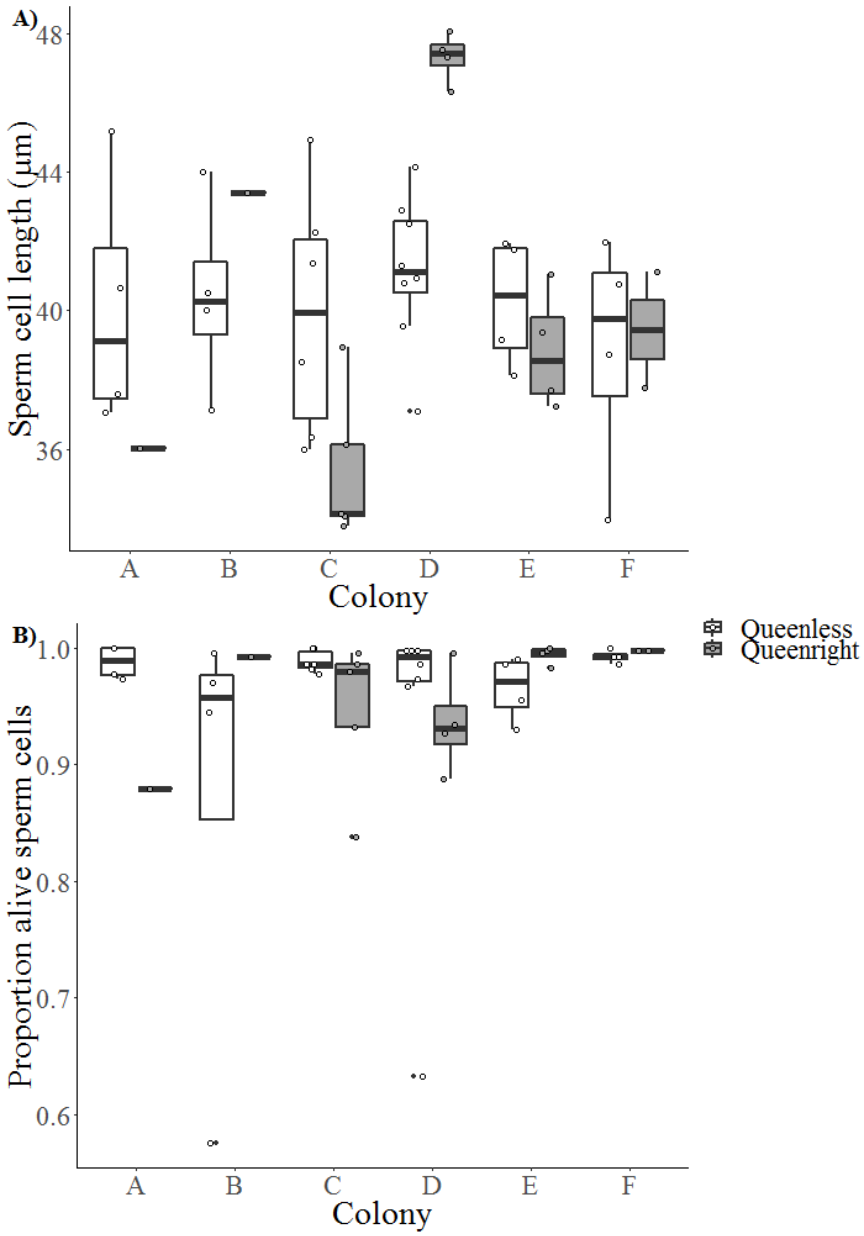

Figure 4.2: Sperm length (µm, A) and proportion of live sperm cells (B) in males produced in pairs of queenless (white) and queenright (grey) colony fragments of the ant *Temnothorax crassispinus*.

The variance of sperm length differed between males from queenless and queenright colonies (samples pooled per group; series 1: Levene's test: $F = 8.93$, $df = 1$, $p = 0.006$; series 2: Levene's test: $F = 4.32$, $df = 1$, $p = 0.040$; p values corrected for a false discovery rate) and also among colonies (samples pooled per colony; series 1: Levene's

test: F = 4.97, df = 8, p < 0.0001; series 2: Levene's test: F = 5.19, df = 6, p < 0.0001; p values corrected for a false discovery rate). The coefficient of variation in sperm length calculated for each male differed between series (linear mixed model: queen presence: df = 57, t-value = 1.35, p = 0.182; series: df = 14, t-value = -2.45, p = 0.028; sperm length: df = 57, t-value = -4.18, p = 0.0001; see table 4.1). It decreased with increasing sperm length (Spearman's rank correlation; series 1: r_S = -0.51, p = 0.006; series 2: r_S = -0.44, p = 0.002), i.e., males producing longer sperm also produced sperm of a more homogeneous length.

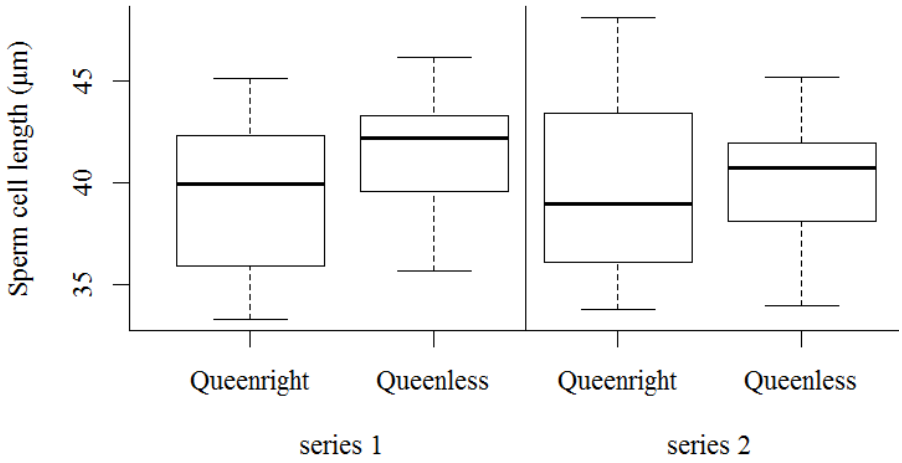

Figure 4.3: Sperm length (µm) of males from queenright and queenless colonies of the ant *Temnothorax crassispinus*. Lengths did not differ with queen presence and experimental series (see results for details). Boxplots show medians, 25 and 75 quartiles, and 95% percentiles.

Sperm viability varied with queen presence and series (Kruskal-Wallis test: $\chi^2 = 14.9$, df = 3, p = 0.002; figure 4.4). Males from queenright colonies reared under near-natural conditions (series 1) had a higher sperm viability than similarly reared males from queenless colonies (pairwise Mann-Whitney U-test: p = 0.007). Sperm viability of males from queenright colonies from series 1 was also higher compared to that of males from queenright (p = 0.002) and queenless (p = 0.002) colonies from series 2. Interestingly, though sperm viability varied across colonies in series 1 (Kruskal-Wallis test: $\chi^2 = 20.24$, df = 8, p = 0.009) it did not differ between colonies in series 2 (PerMANOVA: queen: F = 0.09, df = 1, p = 0.442; colony: F = 0.97, df = 5, p = 0.442; interaction group*colony: F = 0.63, df = 5, p = 0.544; figure 4.2 B).

Table 4.1: Sperm cell length, coefficient of variation and proportion of alive sperm in queenright and queenless *Temnothorax crassispinus* males from series 1 and series 2 (for details see results)

	series 1		series 2	
	Queenright	Queenless	Queenright	Queenless
sperm cell length (µm) mean±sd	39.8±3.7	41.7±3.1	39.9±4.9	40.7±2.8
coefficient of variation median, Q1, Q3	19.8, 19.3, 21.6	16.3, 13.7, 20.2	15.2, 12.7, 20.8	14.4, 12.6, 18.2
proportion alive sperm median, Q1, Q3	1.00, 1.00, 1.00	0.975, 0.945, 1.00	0.986, 0.932, 0.996	0.986, 0.973, 0.998

Across the whole sample, sperm viability was affected by queen presence and sperm length (generalized linear mixed model: queen presence: estimate = 0.21, z-value = 5.37, $p < 0.0001$; series: estimate = 0.29, z-value = 0.38, $p = 0.708$; worker number: estimate = 0.57, z-value = 0.87, $p = 0.386$; sperm length: estimate = 0.19, z-value = 5.25, $p < 0.0001$; colony included as random factor; see figure S4.1). Sperm viability was also positively correlated with male thorax width (series 1, Spearman's rank correlation: $S = 1266.7$, $r_S = 0.45$, $p = 0.028$).

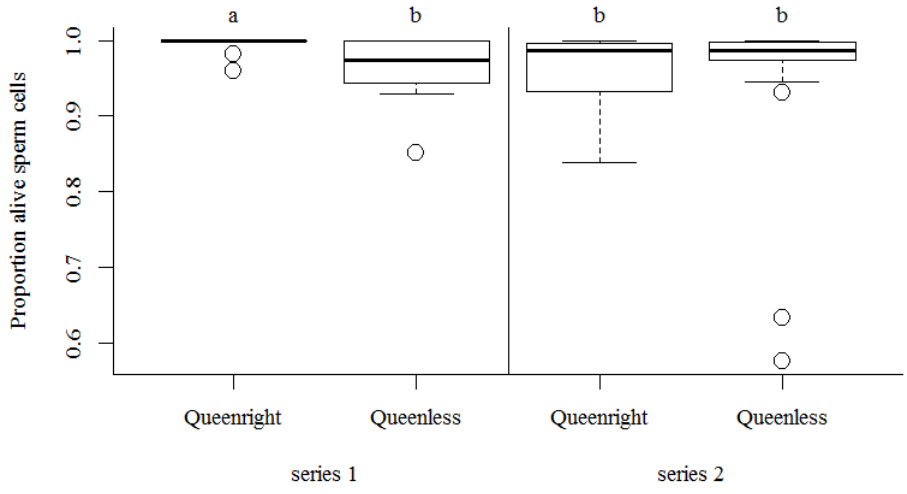

Figure 4.4: Proportion of live sperm cells in queenright and queenless colonies of the ant *Temnothorax crassispinus*. Viability was significantly higher in males from queenright colonies kept under near-natural conditions (series 1). Boxplots show medians, 25 and 75 quartiles, and 95% percentiles and outliers.

4.5 Discussion

Workers in many social insect species can increase their direct fitness by independent reproduction, at least in absence of a reproductive queen (Bourke, 1988*b*; Choe, 1988; Cole, 1986; El-Shehaby et al., 2012; Heinze et al., 1997; Helanterä and Sundström, 2007*b*; Holmes et al., 2013; Nanork et al., 2007; O'Connor et al., 2013; Oldroyd et al., 2001; Visscher, 1989). However, whether worker-produced males can compete with queen-produced males remains unknown in ants and the estimation of fitness traits in worker-produced males will contribute considerably to the quantification of worker direct fitness in social insects. Here we document, that queen- and worker-produced males are of comparable quality in *Temnothorax crassispinus* ants and that variations are often rather determined by genetic traits than by queen presence.

Queenless *T. crassispinus* colonies produced significantly more males than queenright colonies, but males from both colonies developed equally well and eclosed simultaneously. Queen- and worker-produced males did neither differ in body size nor in motility, which are important traits for the male's mating success (Abell et al., 1999; Berg, 1991; Berg et al., 1997; Davidson, 1982; Gençer and Çetin Firatli, 2005; Gençer and Kahya, 2011; Wiernasz et al., 2001) as they determine a male's flight capability (Stone et al., 1995) and reflect its nutritional status and quality (Fitzpatrick and Lüpold, 2014). Males in species with small colonies, as it is the case in *T. crassispinus,* are usually attracted by female calling behavior and follow cues from secretions of the female poison gland (Franks et al., 1991; Oberstadt and Heinze, 2003). Females are apparently capable of assessing a male's quality by physiological cues and mate selectively (see *Leptothorax gredleri,* Oberstadt and Heinze, 2003), and thus a male's morphology might be crucial for its mating success. Furthermore, the prolonged lifespan of worker-produced males and their high abundance might increase their mating success, as they might benefit from less competition at the end of the reproductive season (see also honey bees Gençer and Kahya, 2011; Page and Erickson, 1988).

Sperm length did not differ between queen- and worker-produced males per se, but sperm length in males produced in the queenright part differed tremendously between colonies, while the sperm size of related males in queenless parts was relatively homogenous. Although studies on the most advantageous sperm length are ambiguous, and the targeted size is probably depending on the mating system or the social environment (Baer et al., 2003; Gomendio and Roldan, 1991; García-González and Simmons, 2007; Immler et al., 2011; Simmons and Fitzpatrick, 2012; Tourmente et al., 2015), most studies agree that quality is characterized by little variation within males

(Parker, 1993; Simmons and Fitzpatrick, 2012). Sperm competition is presumably rather low in singly mated *T. crassispinus* ants (Strätz and Heinze, 2004), and the quality of certain sperm traits might be under low selection pressure in contrast to polyandrous species (den Boer et al., 2010; Fitzpatrick and Baer, 2011; Simmons and Fitzpatrick, 2012). Sperm characteristics as velocity (Baer et al., 2003; Gomendio and Roldan, 1991) or the rapid filling of the spermatheca (Boomsma et al., 2005), might be negligible in monandrous species, which might be reflected by the observed strong variation in sperm length (e.g. compared to polygynous honey bees, Woyke, 1983) among colonies and especially among queen-produced males. Variations in sperm size produced by one male or males with the same genetic background are elusive as haploid males produce only clonal sperm (Fitzpatrick and Baer, 2011). Nevertheless, half of the worker-produced males will inherit the parental allele from the workers, which might cause the observed deviation from queenright fragment. Finally, the observed strong variation in sperm length, which decreases with increasing sperm size, indicates that there is only little investment in sperm quality, probably due to absent sperm competition (Fitzpatrick and Baer, 2011; García-González and Simmons, 2007; Kleven et al., 2008; Parker, 1982; Simmons and Fitzpatrick, 2012).

However, sperm length seems to be crucial for sperm viability and especially in queenright colonies, an intermediate sperm length was apparently most advantageous for sperm viability. A minimum size of sperm cells might be required to sufficiently supply sperm with energy (Fitzpatrick and Lüpold, 2014), but the production of long sperm cells is costly and might cause a trade-off with viability (Birkhead et al., 2005; Evans, 2011; Fitzpatrick and Lüpold, 2014; Simmons and Moore, 2009), as reported for sperm length and number (LaMunyon and Ward, 1998; Parker, 1982; Pitnick, 1996). However, larger males had presumably less difficulties to cope with a high energy demand, probably due to a better nutritional condition (Fitzpatrick and Lüpold, 2014) and produced more viable sperm (see also frogs Dziminski et al. 2010).

In our study, sperm viability did not differ between males from related queenless and queenright colonies, but was highest in queenright colonies, reared under natural conditions. This indicates, that on the one hand viability might be determined by the genetic background of the individuals (Evans, 2011; Fitzpatrick and Lüpold, 2014; Simmons and Moore, 2009), but on the other hand might probably also be affected by social or environmental conditions. Studies in honey bees for instance, showed that warmer temperatures have negative effects on male fecundity (Stürup et al., 2013). Natural temperature cycles (series 1) might therefore be more beneficial for sperm development and storage than standardized laboratory conditions (series 2), which

might result in the observed decreased sperm viability in laboratory queenright colonies. However, this would not explain the difference in sperm viability observed between queenless and similarly kept queenright colonies from series 1, and might rather indicate a genetic effect on viability. Finally, workers are generally highly productive in laboratory (see chapter 3) and we cannot exclude that parts of the males in either of the queenright colonies were produced by workers.

In general, the overall high viability but strong size variation observed in all samples indicates a selection on viability, assuming that sperm length is rather negligible in monandrous ant species (Fitzpatrick and Baer, 2011; García-González and Simmons, 2007; Gomendio and Roldan, 1991; Parker, 1982, 1993; Simmons and Fitzpatrick, 2012), and that sperm viability and size are genetically correlated as reported for other species (Evans, 2011; Simmons and Moore, 2009). Sperm viability is an important fitness trait (Simmons and Fitzpatrick, 2012) and especially under strong selection in polyandrous insect species (Hunter and Birkhead, 2002). However, in social insects, viability of the stored sperm is generally important for the reproductive success of both, the male and the females, as queens mate only once during their early life and store the transferred sperm in their spermatheca for several years or even decades (Boomsma et al., 2005; Zareie et al., 2013).

In summary, our study shows that worker-produced males are apparently capable of contributing to the direct fitness of workers in a monogynous social insect. The high reproductive success in queenless colonies might contribute to a high proportion of males in nature. Worker-produced males live longer than queen-produced males which might be an important trait at the end of the reproductive season. Sperm traits vary only little between queen- and worker-produced males but might rather be under genetic control. The overall high viability might correspond to an increased storage requirement in social insects where sperm viability might determine the lifetime reproductive success of males and females (Boomsma et al., 2005; Zareie et al., 2013).

Declarations

Ethics approval

Temnothorax crassispinus is an unprotected ant species. All experiments comply with European laws.

Availability of data and material

The datasets of the article are available in supplement S1.

Competing interest

The author(s) declare(s) that they have no competing interests.

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Author contributions

JG and JH designed the study; JG, TK and JW performed the experiments; JG analyzed the data; JG and JH interpreted the data and wrote the manuscript.

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5 Fitness away from home: ant workers produce males in queenless parts of multi-nest colonies

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5.1 Abstract

Workers of several social insects are capable of gaining direct fitness by laying unfertilized eggs, which then develop into males. However, under queenright conditions, direct reproduction of workers is usually prevented by queen-induced regulatory mechanisms. In nature, some ant colonies inhabit multiple nests sites (polydomy). This might allow workers to escape queen control and to reproduce. However, whether worker-produced brood survives after colony reunion in seasonally polydomous species remains unclear. So far, it is known that worker-produced eggs are selectively destroyed in queenright colonies and that sex ratio is influenced by the destruction of brood items according to sex independent of origin. Here, we test whether workers discriminate between queen- and worker-produced larvae during colony reunion. We examined the reproductive success of workers in queenless colony fragments of our study species *Temnothorax crassispinus*. Our results show that queen-produced brood items did not inhibit worker reproduction, but had a positive effect on worker lifespan. Larvae produced by workers were readily integrated into queenright colony fragments during colony reunion and these larvae successfully developed into adult males.

Keywords
Altruism; Worker reproduction; Polydomy; Direct fitness; Ants; *Temnothorax*

5.2 Introduction

Hamilton's theory of inclusive fitness elegantly explains the altruistic behavior of workers in social wasps, bees, and ants by gains in indirect fitness (Hamilton, 1964*b*). Through helping a related egg-layer, workers can greatly increase the relative's reproductive output and in this way can transmit more copies of their genes indirectly than directly by producing own offspring. In addition to indirect fitness benefits, workers might also attempt to obtain direct fitness. In most species of social Hymenoptera, workers are capable of laying at least unfertilized eggs, which can develop into males. Worker reproduction is usually impaired in the presence of a queen through its pheromones or aggressive policing (Hammond and Keller, 2004; Ratnieks, 1988*b*; Wenseleers et al., 2004), but in queenless colonies workers may readily produce sons (Bourke, 1988*b*; Choe, 1988).

Colonies of several ant species seasonally inhabit multiple nest sites, probably because of spatial constraints or because it allows them to forage more efficiently and to better exploit and defend a territory (polydomy, Debout et al., 2007; Ellis and Robinson,

2014). In addition, polydomy might allow workers to escape queen control (Fletcher and Ross, 1985; Herbers, 1983; Snyder and Herbers, 1991). Indeed, sex ratios are often more male-biased in multi-nest than in single-nest colonies (Rosengren et al., 1986; Walin et al., 2001). However, polydomy is often associated with the presence of multiple queens and colony founding by budding, which both also might lead to male bias (Debout et al., 2007). Furthermore, whether worker-produced brood survives when colony fragments of a seasonally polydomous species fuse for hibernation remains unclear. Workers of several ants and wasps are known to discriminate at least between queen- and worker-produced eggs and eat the latter (D'Ettorre et al., 2004; Endler et al., 2004; Kikuta and Tsuji, 1999), and male brood is selectively destroyed regardless of origin in other species (Aron et al., 1994; Sundström et al., 1996). Previous studies in *Temnothorax unifasciatus* ants revealed, that manually introduced queen- or worker-produced eggs had been integrated independent of origin in queenless and queenright split colonies (Stroeymeyt et al., 2007). Nevertheless, it remains unclear whether the reunion of reproductive workers with the queenright fragment affects the survival of the brood.

Here we examine, whether polydomy can increase worker direct fitness in the monogynous ant *Temnothorax crassispinus* (Karavaiev, 1926). Colonies of *Temnothorax* nest in spatially limited cavities in rock cracks, rotting branches, or hollow acorns, and in summer often space out to multiple nests (Alloway et al., 1982; Cao, 2013; Foitzik and Heinze, 2001; Herbers and Grieco, 1994; Herbers and Foitzik, 2002; Partridge et al., 1997; Roberts et al., 1999; Strätz and Heinze, 2004). In queenless conditions, *Temnothorax* workers form rank orders by antennal boxing and biting and only the highest ranking workers lay unfertilized, male-destined eggs (Brunner and Heinze, 2009; Cole, 1981; El-Shehaby et al., 2012; Heinze et al., 1997; Stroeymeyt et al., 2007). Genetic analyses revealed that in both queenless and queenright colonies of *T. crassispinus* a large fraction of males are worker offspring (Krüger, 2019; Wallner, 2017). We split colonies into a queenright and queenless part for ten weeks and then allowed the colony fragments to reunite again. We investigated, whether worker-produced male larvae from queenless fragments are accepted by workers from the queenright colonies during and after colony fusion. We compared the reproductive output of queenless and queenright split fragments and checked for possible inhibitory effects of queen-produced larvae on worker egg laying and for discrimination against larvae from the queenless fragment.

The results of our study show that *T. crassispinus* workers successfully reproduce under queenless conditions. Queen-produced larvae delay, but do not inhibit worker reproduction in queenless split fragments but in contrast might have a beneficial effect on worker survival. Workers from queenright colonies accept adult workers and larvae from

the queenless fragment and worker-produced larvae were reared to adulthood. Behavioral observations show that workers preferentially cared for brood items that originated from their own colony fragment, but queenless and queenright larvae were equally cared for.

5.3 Material and methods

Temnothorax crassispinus is a small monogynous, monandrous ant species (a single, singly-mated queen per colony), which lives in small colonies of up to 300 individuals in hollow acorns or twigs throughout Eastern Central Europe (El-Shehaby et al., 2012; Seifert, 2007; Strätz and Heinze, 2004; Pusch et al., 2006). Previous field studies had suggested that *T. crassispinus* is seasonally polydomous (Strätz and Heinze, 2004), similar to its sibling species *T. nylanderi* (Foitzik and Heinze, 2000, 2001). Colonies were collected in August 2017 in deciduous forests around Regensburg, Germany. From 30 colonies we set up 30 queenright and queenless experimental colonies each, resulting in 60 experimental colonies (for a schematic figure see figure S5.1). All 30 queenright split colonies and 10 of the queenless split colonies received 25 workers and 25 larvae from the initial colony. The remaining 20 queenless split colonies consisted of 25 workers without larvae to investigate a possible influence of queen-produced larvae on reproduction as reported, e.g., for *Novomessor* ants (Ebie et al., 2015). To be later capable of distinguishing between workers from queenright and queenless experimental colonies, workers in queenright colonies were marked by clipping the right tarsae of the middle leg, workers of queenless colonies by clipping the left tarsae.

Each split colony was reared in a separate box (9.6 x 9.6 x 3 cm^3) containing a nest composed of a plastic frame sandwiched between two microscope slides (1.2 x 5 x 0.3 cm^3) with a narrow entrance (0.3 x 1 x 0.3 cm^3). Colonies were fed twice per week with cockroaches and honey. Honey was colored with commercially available food dyes to allow to distinguish between larvae reared in queenless and queenright split fragments (red: Allura red AC, E129, 12.5% pure color, 2% aluminum; blue: Brilliant blue FCF, E133, 9.26% pure color, 3.6% aluminum, carrying agent sulfate/chloride, RBV Birkmann GmbH & Co; 4g per liter of solution). Red and blue food coloring were used for queenright and queenless colonies, respectively, as in previous experiments (Bernadou et al., 2018; Czaczkes et al., 2015). The color of food dyes did not have an influence on brood care or larval survival rate (Stroeymeyt et al., 2007 and see results).

Colonies were kept in incubators at artificial summer conditions (12 h 26 °C / 12 h 23 °C day / night cycle) to support egg laying and after five weeks was gradually reduced to 18 °C / 13 °C (2 °C every three weeks for nine weeks), to simulate pre-hibernation

conditions and facilitate the natural merging of experimental colonies before hibernation (see Partridge et al., 1997; Roberts et al., 1999). Workers and brood items were counted once per week to monitor colony productivity and survival.

The queenright and queenless split colonies from 17 of 30 stock colonies were transferred after ten weeks into a larger arena (nine queenless colonies which initially received queen-deprived brood and eight queenless colonies that did not receive brood items at the beginning; 13 pairs of split colonies were not allowed to fuse and used for later experiments). The arena (diameter 13.5 cm) contained uncolored honey, cockroaches/*Drosophila* flies, and water. We placed the nests at the opposite sides of the arena and observed the arena for 20 minutes. After the initial observation phase colonies were observed for 10 minutes every hour on the first day (total 60 minutes per colony) and 10 minutes every two hours on the second, third and fourth day (40 minutes per day per colony). We measured the time until reunification of the colonies and noted interactions among workers and with brood items. Non-antagonistic interactions with brood items (feeding, grooming and carrying inside the nest) were counted as brood care. Observations were conducted blindly, i.e., the observing person did not have information about the experimental setup, the meaning of the colored food, or the origin of the marked individuals.

The staining of the larvae vanished quickly when workers were fed with uncolored honey in the shared arena, thus items and workers could be counted for a maximum of seven days. Subsequently, temperature was gradually decreased to 6 °C / 2 °C during the following six weeks to provide hibernating conditions. The males needed in total 19 months to develop from eggs to adults and eclosed after the second hibernation (3.5 months, minimum 6 °C / 2 °C). This might come from the late start of egg production at the end of the natural reproductive season (middle of October) and the shortened hibernation period (2 months). In total, colonies were kept for more than 19 months to monitor worker and offspring development. Worker and brood number decreased strongly in four reunited colonies (less than five workers and/or less than ten larvae) within 19 months and these colonies had to be excluded from the analyses of male offspring. One queen died during hibernation and the colony was excluded from the analyses of brood items and offspring numbers after 16 months.

Data are given as median, first quartile (Q1) and third quartile (Q3) and were analyzed with R 3.2.3 software (R Development Core Team, 2008). We used Kruskal-Wallis χ^2-tests for independent and Friedman tests and Wilcoxon tests for dependent data, as data were not normally distributed (Shapiro-Wilk test $p < 0.05$). Survival of the workers was compared with Kaplan-Meier survival analysis ("survival" package,

Therneau and Grambsch, 2000) and survdiff pairwise comparisons ("survminer" package, Kassambara and Kosinski, 2017) for group comparisons. We only included verifiably dead individuals in the survival analysis.

5.4 Results

Brood development in queenright and queenless colony fragments

First eggs were produced five to six weeks after colony splitting. The treatment groups (1) queenright, 2) queenless with queen-derived larvae and 3) queenless without queen-derived larvae) differed in the onset of reproduction (Kruskal-Wallis test: $\chi^2 = 20.70$, df = 2, $p < 0.0001$; for details see table 5.1).

Table 5.1: Measures of reproductive performance in queenright and queenless (with and without brood) colonies of the ant *Temnothorax crassispinus*. Values shown are median, first quartile (Q1) and third quartile (Q3).

(median, Q1, Q3)	Queenright	Queenless with brood	Queenless without brood
weeks to first egg	6.0, 6.0, 6.9	6.0, 5.0, 6.0	5.0, 5.0, 5.0
time until reproductive peak (weeks)	8.0, 6.0, 8.0	7.0, 7.0, 7.8	7.0, 6.0, 7.0
maximum egg number	46.0, 28.5, 70.0	108.5, 97.25, 120.75	71.5, 44.0, 92.25
weeks to first larvae	3.0, 2.0, 3.0	5.0, 4.0, 5.0	5.0, 4.0, 5.0
relation larvae to eggs after two weeks (%)	82.0, 55.1, 115.0	46.8, 37.3, 62.6	73.5, 49.2, 91.3
maximum larvae number	77.0, 57.0, 98.5	112.50, 84.8, 149.0	78.5, 42.8, 115.5

Queenless colonies without brood laid their first eggs on average one week earlier than queenright colonies ($p < 0.0001$) or queenless colonies with larvae ($p = 0.008$). Colonies reached their reproductive peak (highest egg number inside the nest) after seven to eight weeks (Kruskal-Wallis test: $\chi^2 = 1.59$, df = 2, $p = 0.452$). Furthermore, queenless colonies with brood reached higher maximal numbers of eggs than colonies without brood and contained more than twice as many eggs as queenright colonies

(Kruskal-Wallis test: $\chi^2 = 12.31$, df = 2, p = 0.002; QL with brood vs. QL: p = 0.019; QL with brood vs. QR: p = 0.002; QR vs. QL: p = 0.169; figure 5.1). The developmental time from egg to larva was extended by approximately two to three weeks in queenless compared to queenright colonies (Kruskal-Wallis test: $\chi^2 = 21.62$, df = 2, $p < 0.0001$, QL with brood vs. QL: p = 0.806, QL with brood vs. QR p = 0.0008, QR vs. QL: p = 0.0002) but the eggs developed equally well into larvae (ratio eggs and larvae two weeks later: Kruskal-Wallis test: $\chi^2 = 4.90$, df = 2, p = 0.086). The maximum number of larvae produced within ten weeks did not differ among the three groups (Kruskal-Wallis test: $\chi^2 = 3.90$, df = 2, p = 0.142).

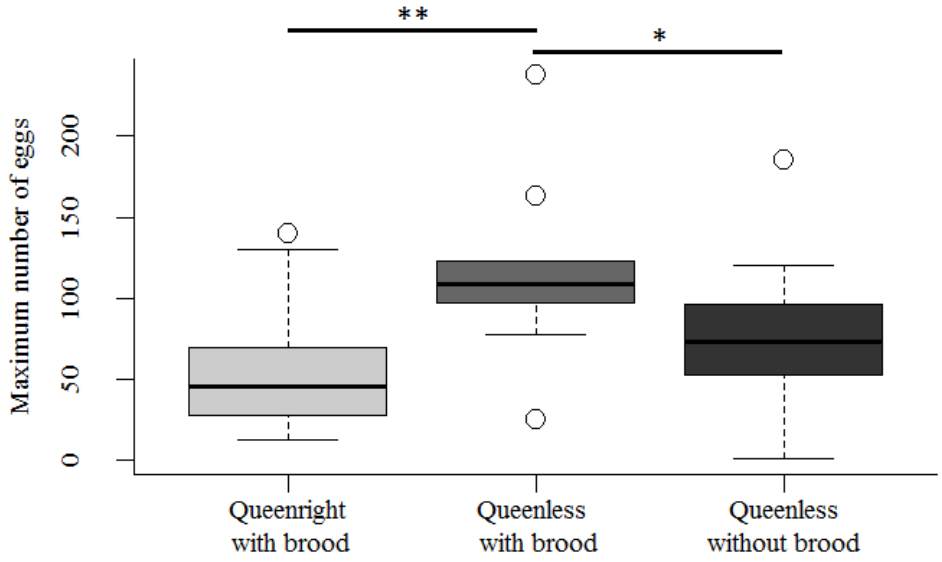

Figure 5.1: Maximum egg number in queenright and queenless fragments of *T. crassispinus* colonies with brood and queenless fragments without brood. Queenless colonies that had received brood items from the stock colony at the beginning of the experiment produced more eggs than colonies from the other two groups. Boxplots show medians, 25 and 75 quartiles, and 95% percentiles (* $p < 0.05$, ** $p < 0.01$ corrected for a false discovery rate according to Benjamini and Hochberg (1995)).

The absence of brood items decreased worker survival

Worker survival differed significantly among the treatment groups (Kaplan-Meier survival analysis: $\chi^2 = 13.2$, df = 2, p = 0.001, figure 5.2). Survival was significantly decreased in

queenless colonies that had not received brood items (dead workers: 5, 1.25, 6) compared to queenright colonies (dead workers median, Q1, Q3: 2.5, 1, 5; $p = 0.0015$) and probably also queenless colonies with brood (dead workers: 1, 0, 2.75; $p = 0.057$, queenright vs. queenless with brood: $p = 0.627$).

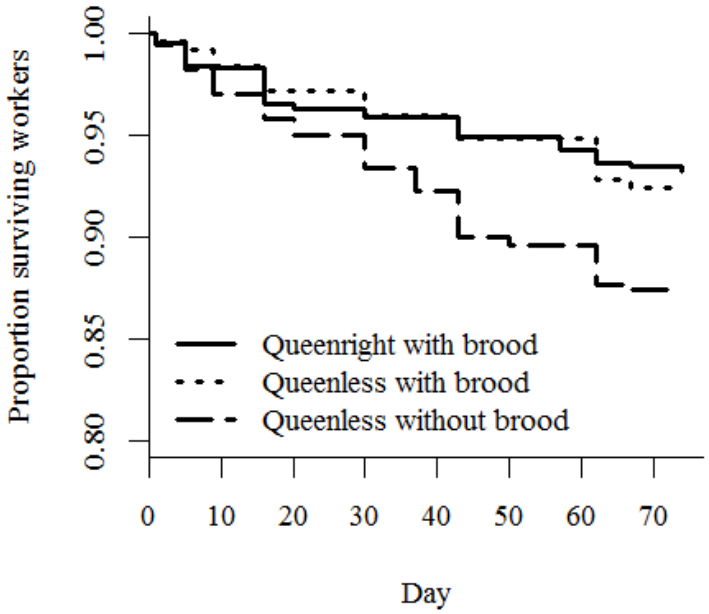

Figure 5.2: Survival of *T. crassispinus* workers in queenright colonies and colonies that had received brood from the stock colony (Queenless with brood) or not (Queenless without brood). Significantly more workers died in colonies that had not received larvae at the beginning of the experiment compared to queenless colonies with brood and queenright colonies.

Workers did not selectively reject queen- or worker-produced larvae during colony reunion

The three groups did not differ in the number of larvae before we allowed the colonies to reunite after ten weeks (Kruskal-Wallis test: $\chi^2 = 4.82$, df = 2, $p = 0.090$; for details see table 5.2).

Table 5.2: Changes in the number of larvae during seven days after the reunification of queenless and queenright fragments of colonies of the ant *Temnothorax crassispinus*. The number of larvae did not decrease significantly in queenright or queenless colonies over a seven day period and the mortality of larvae after seven days was similar for larvae originally produced in the queenright and queenless fragment. Values are given as median, first quartile and third quartile.

	Before reunion	1st day after reunion	7th day after reunion	Change in the number of larvae
Reunited colonies				
Queenright	57, 30, 80	56, 28, 71	30, 26, 51	-12, -4, -33
Queenless	51, 38, 70	38, 30, 47	30, 28, 47	-12, -8, -27
Not reunited colonies				
Queenright	57, 32, 70	56, 30, 68	45, 40, 51	-8, -4, -19
Queenless	50, 36, 61	36, 29, 47	25, 24, 31	-9, -7, -24

When placed into a common arena, 13 of the 17 colonies reunited within 29 hours (mean±sd: 20.8±8.6 hours). Four pairs of fragments did not reunite within the observation period. Eight of the colonies fused in the queenright nest and five reunited in the queenless nest. In nine observed cases (five colonies), workers from the same split fragment transported larvae to the new nest, and only in one observation, a worker carried a larva from the other split fragment. Adults were carried by members from the same colony fragment (three observations) as well as from the other fragment (three observations). We could not observe attacks of workers or killed workers within the seven day observation period and did not find any difference in behavior or colony composition between parts that merged and those that did not.

In the reunited colonies, larvae from queenright as well as queenless fragments were accepted after fusion, and there was no significant decrease in the number of either type of larvae during the seven days following fusion, during which the larvae from queenright and queenless parts of the colony parts could still be distinguished because of their coloration (queenright larvae: 7th day after reunion, median Q1, Q3: 30, 26, 51; Friedman test before reunion, 1st day after reunion, 7th day after reunion: $\chi^2 = 0.275$, df = 2, p = 0.871; queenless larvae: 30, 28, 47; $\chi^2 = 0.039$, df = 2, p = 0.981). Similarly,

in the colonies that refused to reunite there was no change in the number of larvae (queenright larvae 7th day median Q1, Q3: 45, 40, 51; Friedman test before reunion, 1st day after reunion, 7th day after reunion: $\chi^2 = 0.50$, df = 2, p = 0.779; queenless larvae: 25, 24, 31; $\chi^2 = 1.29$, df = 2, p = 0.526). There was also no significant difference in the decrease of numbers between red or blue larvae within the seven days after fusion (reunited colonies: Mann-Whitney U-test: W 73.5, p = 0.589, not reunited colonies: W = 9.5, p = 0.772). Workers in reunited colonies generally seemed to preferentially care for brood from their original split colony (instances of carrying, grooming, and feeding observed during 120 min, Wilcoxon test: V = 66, p = 0.004, own larvae median, Q1, Q3: 14, 11.5, 24; alien larvae: 11, 7.5, 16.5). Larvae from formerly queenless and queenright fragments received equal attention (V = 29, p = 0.754, queenless larvae median, Q1, Q3: 14, 10.5, 19.5; queenright larvae: 11, 10, 20.5). This also indicates that color did not affect brood care.

Colony reunion affects colony growth and offspring sex-ratio

Worker number differed between the colonies 16 months after colony reunion (Kruskal-Wallis test: $\chi^2 = 13.258$, df = 3, p = 0.004). At this time, queenright colonies that experimentally had been kept separate from queenless fragments had more workers (n = 8; worker number median, Q1, Q3: 36.5, 32, 71) than reunited colonies (n = 13; p = 0.03; worker number: 22, 5, 26), not reunited colonies (n = 4; p = 0.01; worker number: 10.5, 7.75, 14.75) or queenless split fragments (n = 9; p = 0.009; worker number: 15, 12, 20; reunited colonies vs. not reunited colonies and separated queenless fragments $p > 0.05$). However, before the first prepupae developed and the males eclosed, the colonies did not differ in the number of larvae (Kruskal-Wallis test: $\chi^2 = 5.21$, df = 3, p = 0.157; median, Q1, Q3: reunited colonies: 38, 12, 67; not reunited colonies: 25.5, 22.25, 31.00; QR colonies: 69, 47, 127.25; QL colonies: 33, 22, 59). Colonies that had not reunited produced less offspring (males+workers; no female sexuals produced; N = 4; median, Q1, Q3: 5.5, 1.5, 9; Kruskal-Wallis test: $\chi^2 = 10.66$, df = 3, p = 0.014) than queenright colonies (N = 8: 46, 33.3, 81; p = 0.025) and queenless colonies (N = 9; 27, 16, 51; p = 0.025; reunited colonies: N = 9; 39, 17, 51; p = 0.075). However, sex ratios were significantly more male-biased in reunited colonies than in still separated queenright colonies, which rarely produced males but focused on worker production (Mann-Whitney U-test: W = 7, p = 0.006; males/total offspring median, Q1, Q3; reunited colonies: 0.41, 0.33, 0.88; queenright: 0.03, 0.007, 0.08, figure 5.3; not reunited produced too few sexuals for a meaningful statistical analysis).

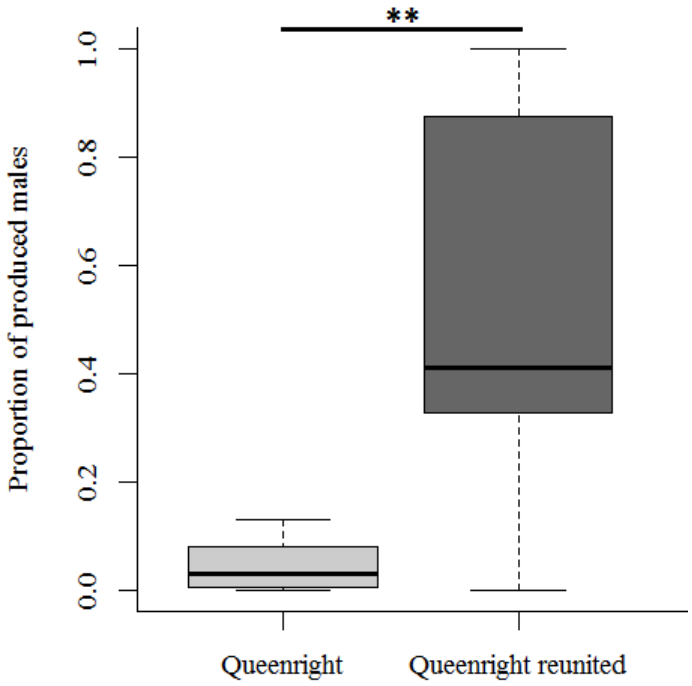

Figure 5.3: Proportion of produced males (males/all offspring) in still separated queenright colonies ("Queenright") and reunited colonies ("Queenright reunited"). Queenright colonies that did not reunite with their queenless split fragment produced significantly more males than already reunited colonies. Boxplots show medians, 25 and 75 quartiles, and 95% percentiles (** $p < 0.01$ corrected for a false discovery rate according to Benjamini and Hochberg (1995))

5.5 Discussion

Seasonal polydomy has been suggested to allow workers to escape queen and worker policing and to thus achieve direct fitness by producing own male-destined larvae from unfertilized eggs (Debout et al., 2007; Fletcher and Ross, 1985). However, whether worker-derived brood survives when the colonies fuse for joint hibernation remained unclear. Here we document that males produced by workers in queenless colony fragments survive colony reunion and lead to male-biased sex ratios in the seasonally polydomous, monogynous ant *Temnothorax crassispinus*.

Worker-produced larvae did not differentially disappear after the queenless and queenright colony fragments fused again, and reunited colonies reared a significantly higher percentage of males than queenright colonies, which had not reunited with their queenless fragment. This suggests that larvae produced by workers in queenless fragments

readily survive and are cared for in the presence of the queen and after hibernation contribute to the males reared by queenright colonies. Genetic data on the maternity of males from natural colonies corroborate this finding and suggest that workers of *T. crassispinus* obtain direct fitness (Wallner, 2017).

We did not find any evidence for queen-produced brood inhibiting worker reproduction, in contrast to what has been reported previously for honey bees (Maisonnasse et al., 2009; Traynor et al., 2014) and ants (Ebie et al., 2015; Heinze, Trunzer, Oliveira and Hölldobler, 1996; Mamsch, 1967). Instead, queenless fragments, which contained larvae from the original colony, produced larger numbers of eggs than queenright colony fragments or queenless fragments without brood. This positive effect of larvae on worker egg laying matches observations by Smeeton (1982) and might suggest that larval inhibition depends on larval stage and brood/worker ratio. Similarly, we did not observe any negative effects of larvae on the life expectancy of workers, in contrast to what has been found in honey bees (Amdam et al., 2009; Smedal et al., 2009). Workers that had to care for larvae appeared to live slightly longer than workers from colonies that initially contained no brood. Such workers might have spent more time inactively than workers, which had to engage in brood care. Inactivity may negatively affect worker survival (Kohlmeier et al., 2017, but see Charbonneau et al., 2017). Furthermore, the presence of larvae may affect the nutritional balance of the colony. Workers in colonies with larvae generally consume more food with a higher protein to carbohydrate ratio to provide larvae with proteins (Dussutour and Simpson, 2009). Although workers can barely digest proteins directly, pre-digestion by larvae and the uptake of larval gland secretion may enable them to uptake protein-based nutrients, which might positively affect their nutritional status and lifespan (Dussutour and Simpson, 2009; Petralia et al., 1980; Went et al., 1972).

The absence of the queen appeared to delay egg development: eggs produced in queenless colonies needed more time before they hatched into larvae than eggs in queenright colonies. This is presumably not due to the ploidy of eggs, as the development times of haploid and diploid eggs in ants and other Hymenoptera appear to differ only marginally if at all (Bulmer, 1983; O'Neal and Markin, 1975; Kureck et al., 2013). The observed difference might rather stem from intra-colonial conflicts, in that egg eating associated with dominance behavior and worker policing might have caused a frequent exchange of newly produced eggs during the initial phase (Brunner and Heinze, 2009; Stroeymeyt et al., 2007).

In contrast to what has been shown for worker-laid eggs in other social insects (Endler et al., 2004; Kikuta and Tsuji, 1999; Saigo and Tsuchida, 2004), our behavioral observations did not indicate a categorical rejection or destruction of worker-produced

larvae following colony reunion (for *T. unifasciatus* see Stroeymeyt et al., 2007). Reunited queenright colonies produced a significantly more male-biased sex ratio than queenright colony fragments, which had not fused with their queenless counterpart. This matches observations in natural queenright colonies (Strätz and Heinze, 2004) and indicates that worker-produced larvae survived colony fusion and hibernation.

Workers preferentially interacted with larvae that had been produced in their original colony fragment. This indicates that workers are capable or recognizing the origin of the larvae. Environmental cues are important in nestmate discrimination in *T. crassispinus* (Foitzik et al., 2007). In our experiment, colony fragments were kept under identical conditions and were treated equally, except for the addition of different food colorants. Although food dyes do not appear to affect egg treatment (Stroeymeyt et al., 2007), egg staining may have changed different larval odor and workers might have preferred to care for larvae with a familiar scent.

In the long run, colony fusion with reproductive workers appears to have a negative impact on colony fitness, as reunited colonies contained significantly fewer workers after one year than still separated queenright colonies. Although workers did not attack others during reunification (see also Stroeymeyt et al., 2007), we cannot completely rule out later aggression among workers associated with reproductive status. Long-term conflict might have had negative effects on worker survival and colony growth. Furthermore, stress and changing colony structures might lead to task shifts in workers (Amdam, 2011). Task allocation is highly flexible in social insect workers and shifts can cause physiological changes, which again affect worker lifespan (Amdam, 2011; Amdam et al., 2005; Kuszewska and Woyciechowski, 2013; Rüppell et al., 2007). However, under natural conditions, *T. crassispinus* workers can migrate between nests (Foitzik et al., 2007; Krüger, 2019; Wallner, 2017), which might enable them to avoid conflict and negative effects of long-term colony fusions. Furthermore, frequent intercolonial fusions and queen usurpation (Foitzik and Heinze, 1998, 2001; Krüger, 2019; Wallner, 2017) might outbalance possible energetic costs resulting from worker reproduction (Cole, 1986; Tsuji et al., 2012).

In summary, our data show that *T. crassispinus* workers are capable of gaining direct fitness in queenless nests of polydomous colonies. Queenless colony fragments were highly productive. Workers from queenless split colonies were not prevented from entering the queenright nest and could bring their larvae into the joint nest. After colony reunion, workers appeared to be capable of differentiating between the brood items but all brood items were equally cared for. Surprisingly, queenright colonies barely produced males, indicating that workers might contribute to male production also in nature.

Declarations

Ethics approval

Temnothorax crassispinus is an unprotected ant species. All experiments comply with European laws.

Availability of data and material

The datasets of the article are available in supplement S1.

Competing interest

The author(s) declare(s) that they have no competing interests.

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Author contributions

JG designed and coordinated the study and analyzed the data. JG, LS and KR performed the experiments. JG and JH wrote the manuscript and interpreted the data. All authors read and approved the final manuscript.

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6 Queens stay, workers leave: caste-specific responses to fatal infections in an ant

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6.1 Abstract

Background

The intense interactions among closely related individuals in animal societies provide perfect conditions for the spread of pathogens. Social insects have therefore evolved counter-measures on the cellular, individual, and social level to reduce the infection risk. One striking example is altruistic self-removal, i.e., lethally infected workers leave the nest and die in isolation to prevent the spread of a contagious disease to their nestmates. Because reproductive queens and egg-laying workers behave less altruistically than non-laying workers, e.g., when it comes to colony defense, we wondered whether moribund egg-layers would show the same self-removal as non-reproductive workers. Furthermore, we investigated how a lethal infection affects reproduction and studied if queens and egg-laying workers intensify their reproductive efforts when their residual reproductive value decreases ("terminal investment").

Results

We treated queens, egg-laying workers from queenless colonies, and non-laying workers from queenright colonies of the monogynous (single-queened) ant *Temnothorax crassispinus* either with a control solution or a solution containing spores of the entomopathogenic fungus *Metarhizium brunneum*. Lethally infected workers left the nest and died away from it, regardless of their reproductive status. In contrast, infected queens never left the nest and were removed by workers only after they had died. The reproductive investment of queens strongly decreased after the treatment with both, the control solution and the *Metarhizium brunneum* suspension. The egg laying rate in queenless colonies was initially reduced in infected colonies but not in control colonies. Egg number increased again with decreasing number of infected workers.

Conclusions

Queens and workers of the ant *Temnothorax crassispinus* differ in their reaction to an infection risk and a reduced life expectancy. Workers isolate themselves to prevent contagion inside the colony, whereas queens stay in the nest. We did not find terminal investment; instead it appeared that egg-layers completely shut down egg production in response to the lethal infection. Workers in queenless colonies resumed reproduction only after all infected individuals had died, probably again to minimize the risk of infecting the offspring.

Keywords

Altruism; Self-removal; Lethal infection; Disease management; Terminal investment; Ants; *Temnothorax*

6.2 Background

Social insects, such as honey bees, ants, and termites, provide the prime examples of altruistic, self-sacrificing behavior in nature (West et al., 2006), but not all members of the insect society are equally prone to sacrifice themselves. Workers typically do not produce offspring and increase their fitness indirectly through increasing the reproductive output of the queen. They readily engage in costly or dangerous tasks, such as foraging and defense, while the queens focus on laying eggs in the safety of their nests (Hölldobler and Wilson, 1990; Korb and Heinze, 2016; Wilson, 1971). In terms of inclusive fitness (Bourke, 2011), the life of an individual worker therefore counts less for the colony than that of the egg-laying queen. This is illustrated, for example, by honey bee workers killing themselves when defending the hive, because their barbed stingers cannot be withdrawn from mammal skin. In contrast, honey bee queens have a smoother stinger with smaller barbs and in principle can use it repeatedly (Winston, 1987). Similarly, soldiers of *Globitermes* termites and workers of *Colobopsis saundersi* ants "explode" and cover attackers with sticky secretions (Laciny et al., 2018; Shorter and Rüppell, 2012), but such "autothysis" has never been observed in queens. Finally, individuals likely to take over reproduction and gain direct fitness in the future avoid risky tasks, such as colony defense (Barth et al., 2010; Bourke, 1988*a*; Cant and Field, 2001; Monnin and Ratnieks, 1999).

Here, we examine whether queens and workers also differ in the readiness for altruism in their response to lethal infection. Insect societies, in which closely related individuals constantly interact with one another in the confined space of a shared nest, provide ideal conditions for the transmission of pathogens (Schmid-Hempel, 1998). Therefore, social insects have evolved powerful mechanisms to counteract the transmission of pathogens, including the destruction of infected brood, intense allogrooming, avoidance, killing, and even the walling-in of diseased nestmates (Baracchi et al., 2012; Pull et al., 2018; Rosengaus et al., 1998).

One particularly striking behavior is altruistic self-removal: moribund workers of several social insects leave their nests to die outside in isolation, probably preventing the spread of a potentially dangerous pathogen to their nestmates (Bos et al., 2012; Heinze and Walter, 2010; Rüppell et al., 2010). In contrast, dying queens have been observed to stay in the nest, where their corpses may be groomed by workers for days or even weeks (e.g., *Atta mexicana* López-Riquelme and Fanjul-Moles, 2013, *Solenopsis invicta* Williams et al., 1981, *Pogonomyrmex badius* Wilson et al., 1958). As the survival of the colony depends on the queen, it might more strongly invest into its immune defense

than workers (Graeff et al., 2007; Koch et al., 2013; von Wyschetzki et al., 2016) and try to overcome an infection. Alternatively, fatally infected queens might turn all available resources into reproduction in the nest, resulting in terminal investment (Clutton-Brock, 1984; Duffield et al., 2017). Indeed, *Cardiocondyla obscurior* ant queens dying from an infection increased their egg laying before imminent death (Giehr et al., 2017), whereas a non-lethal injury led to the upregulation of immune genes and a temporary decline of egg laying rate (von Wyschetzki et al., 2016).

Unfortunately, the behavior of dying queens and workers has never been studied in the same species, which makes it difficult to determine whether the response to pathogen stress and impending death is caste-specific or merely varies among species. Here, we infected workers and queens of the monogynous (single-queened) ant *Temnothorax crassispinus* with spores of the entomopathogenic fungus *Metarhizium brunneum*. We investigated the behavior of conspecific dying queens and workers in queenright colonies and also queenless colony fragments. This set up enabled us to examine the specific effects of both caste (queens vs. workers) and reproductive status (reproductive vs. non-reproductive individuals), as in queenless colonies a small number of socially dominant *T. crassispinus* workers may lay eggs (Heinze et al., 1997; El-Shehaby et al., 2012). From the above-cited observations (Bos et al., 2012; Heinze and Walter, 2010; López-Riquelme and Fanjul-Moles, 2013; Rüppell et al., 2010; Williams et al., 1981; Wilson et al., 1958) we expected fatally infected, non-reproductive workers from both queenless and queenright colonies to die in isolation outside the nest but queens and egg-laying workers to die inside.

The results of our study indicate caste-specific behavior of queens and workers dying from an infection: all moribund queens remained in the nest and all infected workers, both reproductive and non-reproductive, left the nest and died in isolation. Furthermore, in contrast to our expectation that egg-laying rates increase after exposure to a lethal pathogen (see *Cardiocondyla obscurior* queens Giehr et al., 2017), egg-laying by queens and workers stopped after *Metarhizium* treatment and egg numbers slowly began to increase again in queenless colonies only after all or at least most of the infected nestmates had died.

6.3 Methods

Temnothorax crassispinus is a small monogynous, monandrous ant species (a single, singly-mated queen per colony), which lives in small colonies of up to 300 individuals in hollow acorns or twigs throughout Eastern Central Europe (El-Shehaby et al., 2012;

Pusch et al., 2006; Seifert, 2007; Strätz and Heinze, 2004). Colonies were collected in July 2017 in deciduous forests around Regensburg, Germany. We split 19 colonies into queenright (QR) and queenless (QL) parts consisting each of 18 old (darkly colored) and 18 young (lightly colored) workers or soon to emerge worker pupae and, in QR colonies, one queen. After queen loss, young workers engage in dominance interactions and one or a few dominant workers begin to produce male offspring from unfertilized eggs after one to four weeks (Brunner and Heinze, 2009; Heinze et al., 1997). Old workers are needed for the successful establishment of split colonies in the laboratory as they conduct non-reproductive tasks before young workers are old enough to take over.

To distinguish old workers from aging young workers, we marked all old workers by clipping the tarsae of the middle leg. Young workers remained unharmed. Colonies were reared in small plastic boxes (10 x 10 x 3 cm^3) with a moistened plaster floor in incubators under 23 °C / 15 °C day / night cycles. They were fed twice per week with cockroaches and sugar solution. Dead individuals were removed but not replaced as this would have disturbed the established dominance hierarchies (Brunner and Heinze, 2009). Hence, worker numbers varied slightly among colonies.

Four weeks after the experimental colonies were set up we counted and removed all brood. We then noted the egg number daily for ten days to estimate the reproductive rate of each colony before the treatment. Thereafter, all individuals were censused again and all brood was removed. To investigate whether queens and / or young, egg-laying workers adjust their behavior and reproductive efforts to pathogen stress, we treated the queen / all young workers with either a control solution (0.05% Triton X) or a *Metarhizium brunneum* spore suspension ($1x10^8$ spores / ml 0.05% Triton X). Preliminary tests had shown that 71% of *T. crassispinus* workers develop a lethal infection within 10 days after being dipped for 1 sec into 500 µl of a spore suspension of this concentration. *M. brunneum* is an obligate-killing entomopathogenic fungus that penetrates the host cuticle within 48 hours after exposure (Bidochka et al., 2005; Hajek and Leger, 1994). Subsequent hyphal growth and the release of toxins result in the death of the host. The fungus completes its life cycle by producing infectious conidiospores on the host surface (Bidochka et al., 2005; Hajek and Leger, 1994; Hughes et al., 2004; Schrank and Vainstein, 2010).

Our experimental setup consisted of five different treatments:

1. queenless control (n = 9): all young workers treated with Triton X;
2. queenless infected (n = 10): all young workers treated with spore solution;
3. queenright control (n = 6): all young workers and queen treated with Triton X;
4. queenright, workers infected (n = 5): all young workers treated with spore solution, queen treated with Triton X;
5. queenright, queen infected (n = 8): all young workers treated with 0.05% Triton X, queen treated with spore solution.

We treated all young workers and the queen if present with either the control or spore solution. Note that in most colonies a few young workers disappeared from the nest or died between set-up and treatment so that the number of these individuals is typically lower than 18 per colony (see results).

The marked old workers remained completely untreated. After exposure all individuals were placed on sterilized filter papers to remove surplus fluid and thereafter were kept in groups isolated from their colonies for 36 to 40 hours to inhibit immediate transmission of spores to their uninfected and untreated nestmates. This short period of isolation is not long enough to change colony odor or the dominance hierarchy in the colony, and none of the returned workers or queens were attacked after being placed back into the nest. After return to their colonies, we noted the position and condition of queens and workers every morning and counted the numbers of eggs and dead individuals once every 24 hours for the first ten days and subsequently three times a week. Sampling was conducted blindly regarding the control and experimental groups.

All dead individuals were removed. To verify that the ants had died of an infection with *M. brunneum* their corpses were immersed for 5 sec in 70% EtOH, rinsed with distilled water, and surface-sterilized for 1 min in 1% NaClO. Subsequently, they were again cleaned with distilled water and dried on filter paper. The gaster was removed with sterile forceps and frozen at -20 °C for ovary dissections (see below). The head and thorax were placed into a sterilized Petri dish containing a moist cotton ball and lined with moist filter paper. After covering the Petri dish with a lid, the dish was sealed with Parafilm to prevent the loss of humidity. Samples were checked regularly until spore growth was visible on the ant surface or for a maximum of three weeks. The reproductive status of workers from both queenless and queenright colonies was determined by dissecting the ovaries of 70 workers that had died outside the nest and whose corpses had been frozen within $\leq$ 24 hours after death (41 infected and 9 uninfected young workers, 20 uninfected old workers).

As only few eggs were laid by the colonies during the first weeks after treatment, we increased the temperature in the incubators to 26 °C / 22 °C (day / night) on day 39 to accelerate egg production. Individuals that died later were similarly sterilized and checked for spore growth. Four control workers could not be used for surface sterilization due to an advanced decay. Hence, the sample size differs in this case from that of the survival analysis. The final analysis of death rate was conducted after 75 days if not stated otherwise. For egg number comparisons day zero was defined as the day of the return of the treated individuals to their colonies, while for survival analysis day zero was the day of the treatment.

Data (supplement S1) were analyzed with R 3.2.3 software (R Development Core Team, 2008) using packages "vegan" for PerMANOVA (Oksanen et al., 2017) and "survival" (Therneau and Grambsch, 2000) for the Kaplan–Meier survival analysis and graph. Pairwise survival comparisons were conducted using the package "survminer" (Kassambara and Kosinski, 2017). In addition to the treatment group as predictor we included the colony as a random effect ("frailty") in the Cox survival analysis model (Therneau and Grambsch, 2000) of the young workers to control for survival differences between colonies. Data from surviving individuals were included as censored. Kruskal-Wallis tests were used for group comparisons, Mann-Whitney U-test (unpaired) and Wilcoxon signed-rank test (paired) for two-sample comparisons. All pairwise tests were corrected for multiple testing according to a false discovery rate (p adjust method: "fdr") (Benjamini and Hochberg, 1995).

6.4 Results

Survival rate of treated workers and queens

At the day of the treatment each colony fragment contained 12.5±2.7 (mean±sd) young workers and 10.5±3.3 (mean±sd) old workers. Direct spore contact strongly reduced the lifespan of both queens and workers: 158 of 196 young workers (median percentage dead workers per colony 85%; Q1 67%; Q3 96%) and six of eight queens died within 75 days after treatment with spore solution, in contrast to 33 of 266 young control workers (median percentage dead workers per colony 9%; Q1 7%; Q3 14%; Mann-Whitney U-test: $U = 330$, $p < 0.0001$) and one of 11 queens (Fisher's exact test, $p < 0.0063$) exposed to Triton X and 91 of 398 old workers (median percentage dead workers per colony 18%; Q1 10%; Q3 33%) that had not been treated at all. Of the 158 dead, spore-treated workers, 23 (14.5%, eight QL and one QR colony) did not produce any *M. brunneum*

spores after surface sterilization. Four of them did not show any pathogen load and 19 produced spores of other, unidentified pathogens. One of 33 (3%, one QR colony) of the young control workers that had died during the experiment was infected with *M. brunneum*. Of the 91 dead, untreated old workers, 11 (12%, two QL and 5 QR colonies) produced *M. brunneum* spores, 70 (77%) produced spores of unidentified pathogens, and 10 (11%, 4 QL and 4 QR colonies) did not show any pathogenic growth. Old workers infected with *M. brunneum* were excluded from survival analysis as these resulted from an uncontrolled infection by nestmates. Whereas across the different treatment groups untreated old workers did not differ in lifespan ($\chi^2 = 8.7$, df = 4, p = 0.068, figure 6.1), young spore-exposed workers showed a strongly decreased survival compared to young workers treated with the control solution ($\chi^2 = 238$, df = 4, $p < 0.0001$, for details see table S6.1). In addition to the treatment effect we also observed that colonies were differently sensitive to pathogen exposure (treatment: $\chi^2 = 47.56$, df = 1.0, $p < 0.0001$, colony: $\chi^2 = 27.02$, df = 8.8, p = 0.0012). The percentage of infected workers still alive 10 days after exposure varied significantly among colonies ($\chi^2 = 41.8$, df = 9, $p < 0.0001$, for details see table S6.2). There was no colony effect on survival in young untreated control workers ($\chi^2 = 6.7$, df = 8, p= 0.57). The presence of an uninfected queen did not have any effect on worker lifespan (QRWInf vs. QLInf: p = 0.740, see table S6.1). The survival rate of spore-exposed queens was also strongly reduced ($\chi^2 = 8.6$, df = 2, p = 0.013; survival (days): 5, 6, 6, 7, 10, 10, >74, >74).

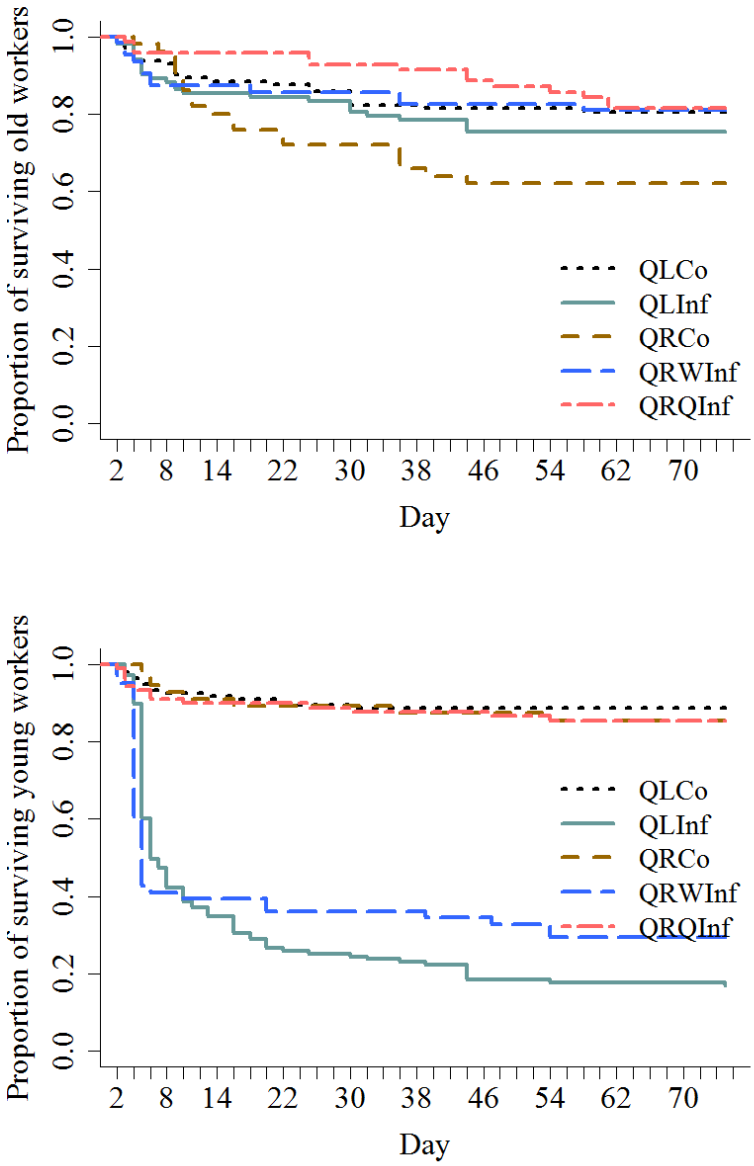

Figure 6.1: Survival of workers of the ant *Temnothorax crassispinus* was significantly decreased in young workers suffering an infection of the entomopathogenic fungus *Metarhizium brunneum* (QLInf, QRWInf) compared to young workers treated with a control solution (QLCo, QRCo, QRQInf) in queenless (QL) and queenright (QR) colonies. Old, untreated control workers did not show a reduction in lifespan between treatment groups. The experiment was terminated 75 days after the treatment and the lifespans of surviving workers were included as censored data. A colony-wise comparison also shows significant differences in survival rates between infected and uninfected individuals (see main text).

Behavior of dying workers and queens

Metarhizium-treated individuals were not prevented from entering the nest or from approaching healthy individuals or brood, e.g., infected and uninfected nestmates did not separate. One infected worker was observed carrying an egg. During the first ten days after treatment the proportion of workers observed outside the nest was significantly higher in colonies in which workers were infected (old workers, control colonies: median 0.017; Q1 0.013 Q3 0.022; old workers, infected colonies: median 0.060; Q1 0.041; Q3 0.079; Mann-Whitney U-test, $U = 71$, $p = 0.0113$, young workers, control colonies: median 0.008; Q1 0.000; Q3 0.011; young workers, infected colonies: median 0.034; Q1 0.021; Q3 0.042; $U = 66$, $p = 0.026$, figure S6.1). Additionally, in colonies with infected workers the number of dead young workers was significantly correlated with the number of young workers observed outside the nest on the previous day (Spearman rank tests, $n = 11$, $0.098 < rS < 1$, mean 0.590, $0.0001 < p < 0.802$, Fisher's combined probability, $df = 22$, $\chi^2 = 106.110$, $p < 0.0001$) but not so in old workers ($n = 9$, $-0.188 < r_S < 0.546$, mean 0.183, $0.129 < p < 0.889$, Fisher's combined probability, $df = 18$, $\chi^2 = 14.183$, $p = 0.717$). Both observations suggest that dying workers leave the nest, as described previously in a related species (Heinze and Walter, 2010). Furthermore, we directly observed 38 *M. brunneum*-infected workers dying outside – they were easily identified by their cramped body posture and stiff locomotion when touched with forceps. In four cases in three different *Metarhizium*-treated colonies we observed untreated, old workers outside the nest carrying the corpse of a young worker. Such behavior was never observed in control colonies.

We could not systematically test the reproductive state of deceased workers because their ovaries were rapidly destroyed by intense hyphal growth. Nevertheless, dissections revealed that of all the examined workers that had died outside the nest, 27% had had eggs in development (for details see below). Furthermore, direct observations of egg laying by four workers that died after spore exposure and the presence of an egg laid in isolation by one of 18 infected workers, which later all died, suggests that several of the workers that had died outside had been reproductive. In conclusion, both non-reproductive and reproductive workers left the nest to die outside.

In contrast, queens were never observed alive outside the nest chamber and while all 158 lethally infected workers, including the egg layers, left the nest before death, the six moribund queens died in the nest (Fisher's exact test, $p < 0.0001$). Three of six

infected dead queens, but none of the infected dead workers, showed mutilations of legs and/or antennae. One infected queen was observed lying motionlessly in a stiff posture inside the nest and one day later was found dead outside the nest. The corpse of another infected queen was carried outside the nest by an untreated old worker. One queen, treated with Triton X, was found decapitated outside the nest four days after treatment. Ovary dissection revealed that the ovaries of the latter queen were undeveloped and that its spermatheca was empty, indicating that it had not been reproductive. This colony was excluded from further analyses.

Effect of pathogen-exposure on worker and queen fecundity

Egg numbers produced before and after treatment were analyzed separately for queenless and queenright colonies, as infected queens died very rapidly while egg production in queenless colonies could be monitored over much longer periods. Workers typically do not become reproductive in the presence of the queen and both treatment and queen presence had a strong effect on eggs present three days before and three days after the treatment (PerMANOVA; treatment: $F = 2.8$, $df = 2$, $p = 0.038$; time: $F = 7.6$, $df = 1$, $p = 0.0024$; queen presence: $F = 10.2$, $df = 1$, $p = 0.0007$).

In queenless colonies, the reproductive rates did not differ between control and infection colonies during seven days before the treatment (Mann-Whitney U-test: $U = 43$, $p = 0.903$), but differed at marginal significance thereafter ($U = 73$, $p = 0.052$). Whereas the weekly egg laying rate did not change in the control group (Wilcoxon signed rank test: $V = 33$, $p = 0.250$), it decreased after infection ($V = 55$, corrected p-value $= 0.0039$, figure 6.2). The number of produced eggs was significantly affected by infection independent of the worker number inside the nest (PerMANOVA; treatment: $df = 1$, $F = 3.6$, $p = 0.049$; number of young workers: $df = 1$, $F = 0.8$, $p = 0.477$; total number of workers: $df = 1$, $F = 0.35$ $p = 0.745$; treatment * number of young workers: $df = 1$, $F = 2.5$, $p = 0.083$).

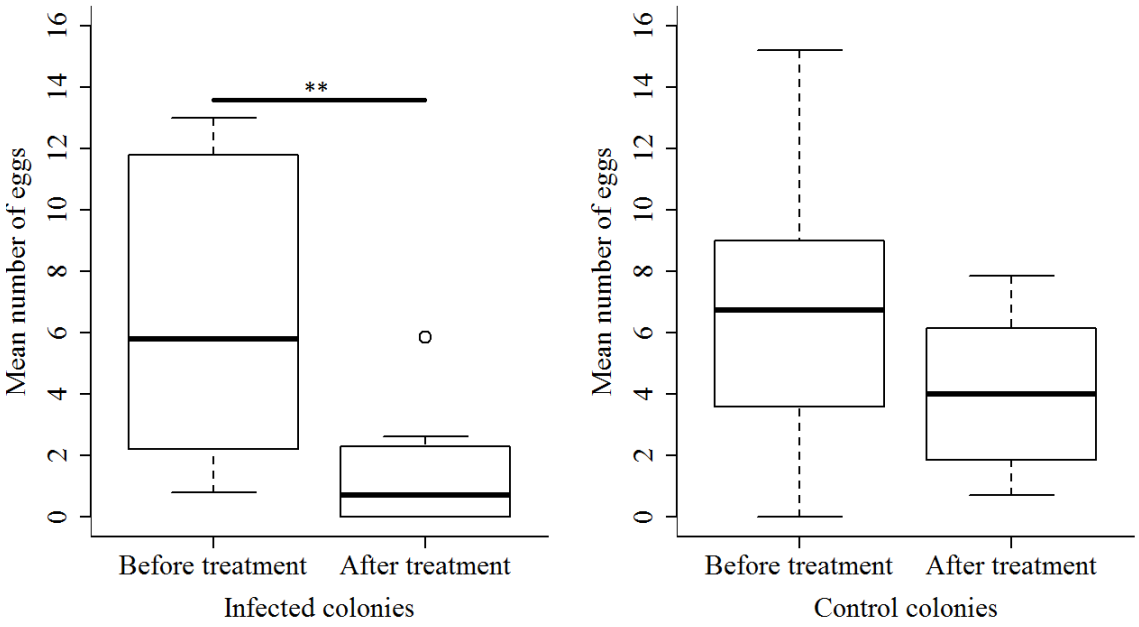

Figure 6.2: Mean number of eggs produced in queenless colonies of *Temnothorax crassispinus* during seven days before and after the treatment. Workers infected with *Metarhizium brunneum* (left) laid significantly fewer eggs than workers of the control group (right) after, but not before the treatment. Boxplots show medians, 25 and 75 quartiles, and 95% percentiles (** $p < 0.01$ corrected for a false discovery rate: "fdr").

All queenless control colonies had contained eggs before the treatment, but in only six of ten queenless infected colonies workers continued to lay eggs after the treatment (see table 6.1). Furthermore, infected colonies did not only produce fewer eggs after the treatment, but as long as infected workers were present the eggs produced in the colonies frequently disappeared (figure S6.2). The presence of eggs was affected by the number of infected individuals and its interaction with the day of the experiment. The number of infected individuals decreased with time and there was also a colony effect (PerMANOVA; number of infected individuals: $df = 1$, $F = 3.9$, $p = 0.035$; day: $df = 25$, $F = 3.08$, $p = 0.0001$; colony: $df = 9$, $F = 9.14$, $p = 0.0001$; number of infected individuals*day: $df = 25$, $F = 1.8$, $p = 0.004$).

Table 6.1: Number of queenless (QL) and queenright (QR) colonies of the ant *Temnothorax crassispinus* in the different treatment groups containing no eggs during the time before treatment (BT) and after the treatment (AT) (control workers: QLCo, QRCo, QRQInf; infected workers: QLInf, QRWinf; infected queen: QRQInf). In one QRCo colony all workers except one died during the experimental period and this colony was excluded from the 10 weeks after treatment analysis.

Queenless	Before Treatment (without/total)	7 days AT (without/total)	10 weeks AT (without/total)
QLCo	0/9	0/9	0/9
QLInf	0/10	4/10	2/10
Queenright	**Before Treatment (without/total)**	**After Treatment (without/total)**	**10 weeks AT (without/total)**
QRCo	0/5	4/5	1/4
QRWInf	1/5	4/5	2/5
QRQInf	3/8	7/8	2/2 (with queen) 0/6 (queenless)

Queens did not lay any eggs during isolation and egg production in queenright colonies could only be analyzed for the first three days after return to the colony, as the first queen had already died on the third day. The median egg number produced during three days did neither differ among queens before ($\chi^2 = 0.1$, df = 2, p = 0.948) nor after the treatment ($\chi^2 = 0.08$, df = 2, p = 0.959, figure 6.3). Queens appeared to sensitively react to the treatment with an almost complete reproductive shut-down regardless of whether they had been exposed to spores or only Triton-X (PerMANOVA; treatment: df = 1, F = 0.15, p = 0.957; number of young workers: df = 1, F = 0.8, p = 0.395; total number of workers: df = 1, F = 0.03, p = 0.956; treatment * number of young workers: df = 4, F= 0.62, p = 0.556). Although all queenright colonies had contained eggs before the experiment started, no new eggs had appeared in four of 18 colonies even before the treatment. After the treatment, no eggs were laid in seven of eight colonies with an infected queen and four of five colonies each with an uninfected queen and either infected or Triton X treated workers (analyzed per colony until the death of the queen; Fisher's exact test, p = 0.0006). Although queens reacted sensitively to the treatment itself, they also appeared to be capable of adjusting their reproductive rate to the presence of infected workers, as queens of the control group produced more eggs over after treatment than control queens with infected workers (PerMANOVA: treatment: df = 1, F = 7.2, p = 0.0063, day of the experiment: df = 23, F = 1.6, p = 0.03, colony: df = 8, F = 7.4, p = 0.0001, see figure S6.3).

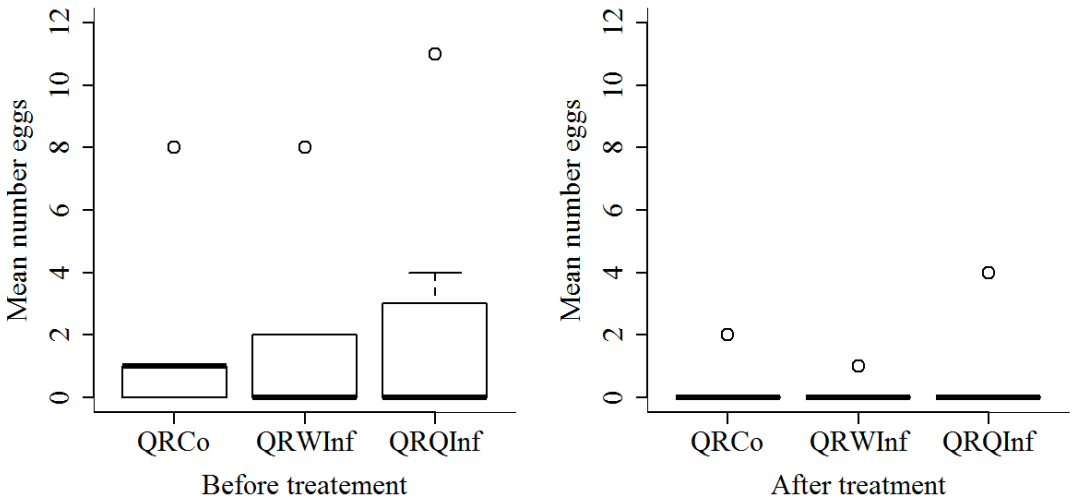

Figure 6.3: Mean number of eggs produced in queenright colonies of *Temnothorax crassispinus* during three days before (left) and after (right) the treatment. Both groups decreased their egg laying rate. The reproductive rate of queens infected with *Metarhizium brunneum* (QRQInf) neither differed from that of control queens (QRCo, QRWInf) before nor after the treatment. The treatment itself seems to result in a reproductive shutdown in queens of all treatment groups. Boxplots show medians, 25 and 75 quartiles, and 95% percentiles.

Interestingly, workers did not start to reproduce in the presence of the queen even when the latter refrained from laying eggs. Six of eight queens died within 10 days after spore contact but workers began to continuously produce eggs in the formerly queenright nests only 37 to 59 days after the queen's death (n = 4, median 40.5). In two additional colonies eggs were sporadically produced but vanished even after 78 days. The initial presence of an infected queen appeared to suppress and delay worker reproduction compared to the removal of a healthy queen (median 17 days, range 6 to 35 days; see also Heinze et al., 1997). At the end of the experiment still no eggs were present in the nests of the two surviving infected queens. Colonies, in which the queen had died, appeared to produce more eggs than control queenright colonies (Mann-Whitney U-test: U = 4, p = 0.053, QRQInf n = 6, median 19, Q1 4.75, Q3 44.5; QRCo n = 5, median = 0, Q1 0, Q3 4; one colony with an uninfected but deceased queen in QRCo and two colonies with a still living, infected queen QRQInf were excluded from the analysis).

The preparation of the ovaries of dead workers showed intense hyphal growth in the gasters of all workers with spore growth after surface sterilization (see figure 6.4). In 27

of 41 infected workers, internal organs, especially the ovaries, were no longer visible and it was impossible to determine their reproductive status. The ovaries of nine workers (64%, four QL and three QR colonies) had at least one egg in development, while the ovaries of five workers appeared to be undeveloped. In contrast, the ovaries of nine uninfected young workers and 20 uninfected old workers were clearly visible and differed in developmental status. One of nine uninfected young workers (11%, one QR colony) and nine of 20 (45%, one worker each in four QR colonies and three QL colonies and two workers in one QR colony) untreated old workers had one or two eggs in development. Traces of previous egg laying (e.g., corpora lutea and/or developing eggs) were found in one infected, dead worker each from two colonies with an uninfected queen.

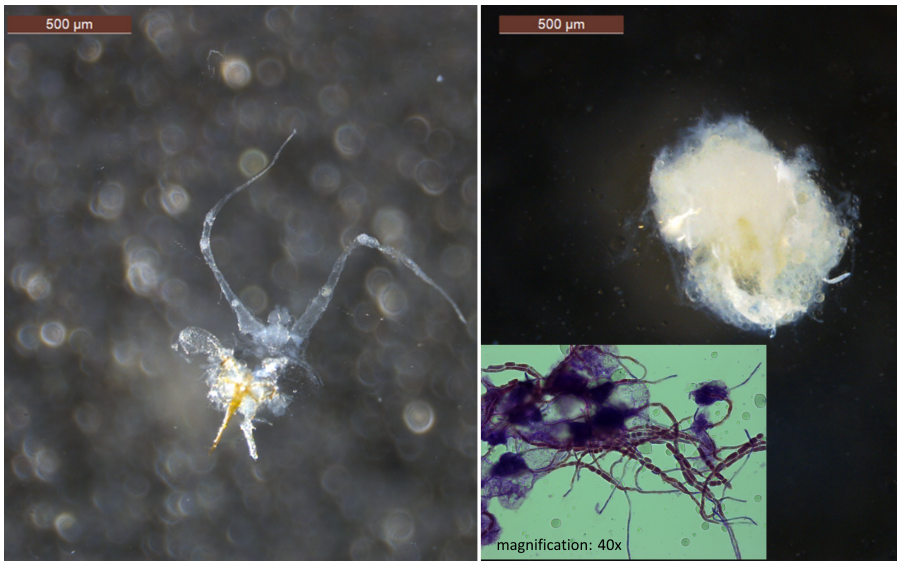

Figure 6.4: Ovaries of control (left, death two days after treatment of colony members) and *Metarhizium*-infected (right, death four days after treatment) *Temnothorax crassispinus* workers. The gaster contents of infected workers are unidentifiable as fungal hyphae have spread throughout the gaster (see microscope picture at the bottom right; magnification 40x), whereas the ovaries of the uninfected workers are clearly visible.

Dissections of the queens revealed hyphal growth throughout the gasters of five of six infected queens. Ovaries and corpora lutea could be detected in only one of the five queens; in the sixth queen, ovaries and corpora lutea were still visible, but no maturing eggs were present (figure S6.4).

6.5 Discussion

The intensive social contact among closely related individuals in densely crowded nests makes insect societies particularly vulnerable to contagious diseases (Hamilton, 1987; Schmid-Hempel, 1998). Social insects were successful in evolution because they evolved a particularly sophisticated multi-layer defense against pathogens and parasites, ranging from cellular to individual and collective responses (Beattie et al., 1986; Bot et al., 2001; Christe et al., 2003; Cremer et al., 2007, 2017; Fefferman et al., 2007; Hughes et al., 2002; Pie et al., 2005; Traniello et al., 2002; Wilson-Rich et al., 2009). Behavioral responses, such as intensive self-grooming, nest cleaning, self-medication (e.g., Bos et al., 2015; Castella et al., 2008; Christe et al., 2003), and self-removal (Heinze and Walter, 2010; Rüppell et al., 2010) have as yet mostly been studied in the worker caste and except for changes in physiology and reproduction (e.g., Alaux et al., 2011; Hassanein, 1951; Overton et al., 2006) little is known about how queens respond to an infection.

We here report that queens and workers of the ant *Temnothorax crassispinus* react differently to an infection with the obligatorily killing pathogen *Metarhizium brunneum*: while moribund workers, regardless of ovarian status, left the nest to die away from it, queens stayed and were carried out of the nest only after their death. Our study shows that the altruistic self-removal is a caste-specific behavior and does not vary with the reproductive status of the workers. Furthermore, the presence of infected nestmates was associated with a strong decrease of egg laying even by uninfected nestmates.

Through altruistic self-removal (Heinze and Walter, 2010; Rüppell et al., 2010) and the withdrawal from social interactions (Bos et al., 2012), infected workers certainly minimize the risk of transmitting a pathogen to brood and adult nestmates. Altruistic self-removal is not caused by a specific manipulation of the pathogen but is induced by the workers to isolate themselves from other colony members to prevent possible risks for nestmates as previously shown for moribund workers by Heinze and Walter (Heinze and Walter, 2010). Horizontal transmission of spores has been documented in several insects (e.g., BaoHui et al., 2010; Hajek et al., 2008; Kaaya and Okech, 1990) and cross-infection may also have been the cause of old, untreated workers dying from an infection with this fungus, in particular as old workers were seen handling corpses. It is therefore easy to see why infected workers leave the nest to die isolated from their nestmates.

Considering the social withdrawal and pathogen control in workers, it is more difficult to understand why infected queens stayed and were removed by workers only after they had died. Even a single-queened colony should benefit from the self-removal or the early expulsion of a fatally infected queen. However, compared to workers most social insect

queens have a longer lifespan and survive better under stressful conditions (Dahlgren et al., 2012; Dussaubat et al., 2016; Rüppell and Kirkman, 2005). They might also have a higher chance to survive an infection than workers by investing more in immune defense (Graeff et al., 2007; Koch et al., 2013; see also below). Queens therefore might remain in the nest as long as there is a chance to recover. Furthermore, Rueppell and colleagues showed that while founding queens of *Temnothorax* are capable of conducting worker tasks and leave the nest to forage, they lose this behavioral plasticity in established colonies and remain in the nest even when workers are removed (Rüppell et al., 2002) whereas reproductive workers may still capable of conducting non-reproductive tasks and leave the nest (e.g., Naeger et al., 2013). Hence, fatally infected queens might simply not have been capable of leaving the nest independently. Workers apparently do not discriminate against infected nestmates before the fungus has begun to produce spores on the cadaver of its host (e.g., Leclerc and Detrain, 2016) and therefore could reduce the contagion risk for the colony only after the queen's death by removing its corpse (López-Riquelme and Fanjul-Moles, 2013; Pull et al., 2013; Renucci et al., 2011).

Both control and spore-treated queens refrained from reproduction for several weeks after treatment and even ten weeks later only half of them had recommenced to lay eggs. A reduction of reproductive efforts has previously been observed in honey bee queens infected with the fungus-related pathogen *Nosema apis* (Hassanein, 1951) and *Metarhizium*-infected queens of the ant *Lasius niger* (Pull et al., 2013). In the latter this was suggested to result from increased investment in the immune system, similar to the temporary drop of egg laying rates following an injury in the ant *Cardiocondyla obscurior* (von Wyschetzki et al., 2016). Since *Temnothorax* queens can live for several years (Plateaux, 1986), after a potentially dangerous treatment they might invest more strongly in pathogen defense and the restoration of their body condition than in reproduction. Even the contact with the solvent, handling stress, or the absence of allogrooming and trophallaxis during the isolation phase affected the physiology of queens in a way that they stopped egg laying. In addition, queens appeared to react sensitively to the presence of infected workers by reducing their reproductive efforts.

While a few *Temnothorax* workers quickly begin to lay eggs when the queen is removed from the colony (Brunner and Heinze, 2009; Cole, 1981; El-Shehaby et al., 2012; Heinze et al., 1997), no worker egg laying was observed in the presence of a non-laying, infected queen. This supports the view that the stop of egg laying does not necessarily mean the loss of queen control (see also Röseler and Röseler, 1989 for social dominance of ovariectomized wasp queens). Similarly, only few eggs were laid in queenless colonies after workers had been infected. Freshly laid eggs quickly disappeared, and egg numbers

increased only after most of the infected nestmates had died, probably to prevent the cross-infection of newly produced brood.

Although *Metarhizium* infection strongly decreased the survival of queens and laying workers we could not observe terminal investment (Clutton-Brock, 1984; Duffield et al., 2017), in contrast to what we have previously reported for *Cardiocondyla* ant queens (Giehr et al., 2017). This discrepancy might reflect the different life history of the two species. The single queens of many *Temnothorax* colonies may live for 10 years and longer (Plateaux, 1986), while queens in the multi-queen colonies of *C. obscurior* are short-lived (mean: 26 weeks, Schrempf et al., 2005) and can quickly be replaced by female sexuals, which after eclosing from the brood mate with their brothers in the natal nest (Heinze, 2017). *Temnothorax* queens might therefore preferentially invest in individual and colony immunity as their future reproductive success depends more strongly on their survival than in *Cardiocondyla*. Further studies are needed to investigate how the life span of ant queens is associated with their immune investment.

Conclusion

Our data show that workers and queens of the ant *Temnothorax crassispinus* react differently to infection with the entomopathogenic fungus *Metarhizium brunneum*. Infected queens stayed inside the nest but refrained from reproduction. Workers, independent of their reproductive state, left the nest and died in social isolation. Both, queens and workers reduced reproductive investment after the treatment with *M. brunneum*. Egg numbers increased with the decreasing number of infected individuals in queenless colonies, but workers did not lay eggs in the presence of the queen, even when the queen was sick and did not reproduce. Our study reports for the first time a caste-specific behavior in response to lethal infections in the same species.

List of abbreviations

QR: queenright

QL: queenless

QLCo: queenless control

QLInf: queenless infected

QRCo: queenright control

QRQInf: queenright queen infected

QRWInf: queenright worker infected

Declarations

Ethics approval and consent to participate

Temnothorax crassispinus is an unprotected ant species. All experiments comply with European laws.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the results of this article are given in supplement S1.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JG performed the experiment and analyzed the data; JG and JH designed the study; JG and JH wrote the manuscript and interpreted the data. All authors read and approved the final manuscript.

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7 Sanitary behavior in queenright and queenless ant colonies

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7.1 Highlights

- Most queenless and queenright colony fragments of the ant *Temnothorax crassispinus* form well-defined latrine patches inside the nest.
- Latrine formation inside the nest is independent of queen presence.
- There is no evidence for genetic variation affecting defecation behavior.
- Workers from queenless colonies defecate more frequently outside the nest.
- Latrine formation affects mold growth inside the nest.
- Colonies do not avoid moldy nests, hence microbiota growth seems to be no threat for ant colonies.

7.2 Abstract

Waste disposal is important for maintaining the health of animal societies. Adults and offspring produce large amounts of waste and feces that could contain pathogens or toxins and may need to be stored away from the young or adult individuals. In social insects, the worker caste is responsible for nest maintenance, including sanitary behavior, and waste disposal strategies vary between species. However, individual task allocation is generally affected by queen presence and worker efficiency often decreases in the absence of a queen.

Here we show that most (74%) colonies of the cavity-dwelling ant *Temnothorax crassispinus* construct up to two localized indoor 'latrines', which are used for defecation and only very rarely also as waste dumps. Restriction of defecation to designated areas affects the growth of mold inside the nest. Defecation strategies of colonies are furthermore affected by queen presence, with workers from queenless nests more frequently defecating outside the nest and forming latrines. As colonies do not actively avoid moldy nests, mold seems to not necessarily be a threat to the colony. While solid waste management has been more extensively studied in social insects, this study contributes a rare insight into the organization of non-easily transportable fecal waste.

Keywords
Defecation, Hygienic Behavior, *Temnothorax*, Ants, Sanitary Behavior

7.3 Introduction

The societies of ants, bees, and wasps are generally characterized by division of labor between reproductives and non-reproductive workers. The loss of the reproductive individual(s) typically leads to tremendous changes in colony organization. In particular, in species with small societies, non-reproductives may begin to compete aggressively to obtain direct fitness by producing males from unfertilized eggs (Cole, 1981; Franks and Scovell, 1983; Heinze et al., 1997; Stroeymeyt et al., 2007) or, in a few taxa, even by mating and producing both male and female offspring (Monnin et al., 2002; Peeters and Tsuji, 1993).

Queen loss and the take-over of reproduction by workers affects both individual worker physiology and the structure of the whole society. On an individual level, queen loss affects brain dopamine titers (Cuvillier-Hot and Lenoir, 2006; Harris and Woodring, 1995; Shimoji et al., 2017), learning capability (Evans et al., 2016), and life span (Dixon et al., 2014; Hartmann and Heinze, 2003; Tsuji et al., 1996). On a societal level, queen loss may weaken nestmate discrimination, which in turn facilitates colony usurpation by alien conspecific queens, or by social parasites (Buschinger, 2009; Chapman et al., 2009; Tschinkel, 1996; Van Oystaeyen et al., 2013). Furthermore, queen loss may lower overall activity levels and change collective behavior (Berton et al., 1992; Gobin et al., 2003; Jaycox, 1970; Reeve and Gamboa, 1987; Sumana and Starks, 2004). For example, workers from queenless colonies of the ant *Temnothorax curvispinosus* interact less with the brood and retrieve misplaced larvae more slowly than workers from queenright colonies (Keiser et al., 2018).

More importantly, queenless colonies appear to be less resistant to fungal infections, suggesting that the presence of a queen may affect collective immune responses, and thus the spread of diseases (Keiser et al., 2018). Waste, corpses, and feces may contain infectious agents and therefore are potentially dangerous (Currie et al., 1999; Hart and Ratnieks, 2002; Waddington and Hughes, 2010; Weiss, 2006). Nest hygiene and disease prevention are thus important aspects of the collective behavior of social insects. Many social animals have therefore evolved efficient waste management, including external or internal dumps and latrines (Bernadou et al., 2018; Bot et al., 2001; Czaczkes et al., 2015; Eastwood, 1997; Farji-Brener and Medina, 2000; Hölldobler and Wilson, 1977; Jackson and Hart, 2009; O'Neal and Markin, 1973; Peeters et al., 1994; Waddington and Hughes, 2010).

We found that 27% of *T. crassispinus* colonies are queenless under natural conditions (unpublished results). As queen loss affects worker behavior (Berton et al., 1992; Gobin

et al., 2003; Jaycox, 1970; Reeve and Gamboa, 1987; Sumana and Starks, 2004) and reduces colony resistance to infection in *T. curvispinosus* (Keiser et al., 2018), we asked whether queen loss might affect defecation and sanitary behavior in the related ant *T. crassispinus*. We find that defecation behavior and waste management varies among colonies and also differs between queenright and queenless colony fragments. Our study shows that latrine formation and also queen presence affect microbiota growth inside the colonies, but that ant colonies do not avoid moldy nests. These findings can provide a basis for further studies on sanitary behavior and the co-existence with microbiota in ant colonies.

7.4 Material and methods

Temnothorax crassispinus is a small (2-3 mm), monogynous (single-queened) ant species that lives in small colonies with 20 to 300 workers in acorns or twigs. In summer, many natural nests are queenless (27%, unpublished data), as individual colonies may seasonally inhabit multiple nest sites (seasonal polydomy, Strätz and Heinze, 2004). We collected 29 queenright colonies near Regensburg, Germany, and split each into a queenright and queenless half, resulting in a total of 58 colony fragments. These fragments were then standardized to consist of 25 workers each (with or without queen). We added 25 larvae to all queenright and 10 of 29 queenless colonies, the remaining 19 queenless colonies did not receive larvae, so as to investigate the effects of queen-produced larvae.

Colonies were housed in small three-chambered plastic boxes (10 x 10 x 3 cm^3; one chamber each for nest, food, and water) with a moistened plaster floor under 12 h 26 °C / 12 h 22 °C day / night cycles). The nest was composed of the area surrounded by a plastic frame (554 mm^2) sandwiched between two microscope slides (1.2 x 5 x 0.3 cm^3) with a narrow entrance (0.3 x 1 x 0.3 cm^3). It was covered with an opaque film to ensure that the nest cavity was dark.

Colonies were fed twice per week with fruit flies (*Drosophila melanogaster*) and honey. Honey was colored with commercially available food dyes (red: Allura red AC, E129, 12.5% pure color, 2% aluminum; blue: Brilliant blue FCF, E133, 9.26% pure color, 3.6% aluminum, carrying agent sulfate/chloride, RBV Birkmann GmbH & Co; 4g per liter of solution). Red and blue food coloring was used for queenright and queenless colonies, respectively, for convenience, as in previous experiments the color of food dyes was found to have no influence on defecation behavior (Bernadou et al., 2018; Czaczkes et al., 2015).

Four weeks after colony establishment we took digital photographs of the nest box and nest, which were analyzed blindly. At this time 0 to 4 (median, Q1, Q3: 1, 0, 1) workers had died or vanished per colony. We then noted the presence and number of latrines within each colony. Accumulations of defecation spots were defined as latrines when spots had the color of the food and were concentrated in an area smaller than 1/3 of the nest (see figure 7.1). Randomly distributed spots were not counted as latrines (see photos in supplement S1). Nine queenright and two queenless colonies had to be excluded from the latrine analysis due to excessive mold growth, which prevented an accurate determination of defecation spots. The area covered by latrines and mold, and the distance of the latrines from the nest entrance, and the distance of latrines from brood piles, were measured using ImageJ. Four weeks after the photographs were taken we put 17 related queenless and queenright split-fragments into a new, clean arena (diameter: 14 cm) to allow them to reunite and to observe nest choice relative to queen presence and mold growth. The remaining 12 queenless and queenright fragment were chosen randomly and kept separated for further, independent experiments.

Data were analyzed with R v. 3.2.3 software (R Development Core Team, 2008) with packages "ggplot2" (Wickham, 2009) for the plots and "vegan" (Oksanen et al., 2017) for conducting the PerMANOVA as data were non-parametric in the case of count data or not normally distributed (Shapiro-Wilk normality test: $p < 0.05$). PerMANOVA was used to test the effects of queen presence, larvae presence, latrine presence and outside defecation on the defecation behavior of the colonies. In the results, factors are given in the order they were included in the test. Count data were compared using Chi-square test (χ^2) and pairwise comparisons were conducted using Mann-Whitney U-test ("Wilcoxon rank sum test with continuity correction") followed by a correction for a false discovery rate ("fdr") (Benjamini and Hochberg, 1995). We grouped queenless colonies with and without larvae for analyses, as we did not find any effect of larva presence in queenless colonies. They did not differ in the frequencies of latrine formation (with larvae: 6/19; without larvae: 1/8; $\chi^2 = 1.36$, $p = 0.243$; one colony could not be evaluated due to excessive mold growth), defecation outside (with larvae: 13/19; without larvae: 8/10; $\chi^2 = 0.439$, $p = 0.507$), nor latrine size ($W = 27$, $p = 0.799$), or mold distribution ($W = 99$, $p = 0.865$).

7.5 Results

In almost all colonies (89%, 42/47), the ants defecated inside the nest, and 74% (31/42 colonies) of the colonies with defecation in the nest showed one or two well-defined latrine patches (figure 7.1). In total, 40 latrine patches were formed, mostly along the corners of the nest (37/40 latrines) with distance to the nest entrance (median, Q1, Q3: queenless: 21.15 mm, 9.45 mm, 48.81 mm; queenright: 16.89 mm, 10.92 mm, 29.14 mm; $W = 140$, $p = 0.421$). Queenless and queenright colonies did not differ in the presence ($\chi^2 = 0.260$, $df = 1$, $p = 0.6125$) or number of latrines ($\chi^2 = 0.816$, $df = 2$, $p = 0.665$). Furthermore, comparing the presence of latrines between the queenright and queenless halves of the same colony did not reveal any colony-specific trend (Spearman's rank correlation, $n = 20$, $r_S = -0.132$, $p = 0.578$).

Latrines made up to 26 % (150.67 mm²) of the inner nest (area median, Q1, Q3: queenless: 26.18 mm², 19.01 mm², 41.21 mm²; queenright: 23.37 mm², 18.16 mm², 62.33 mm²; $W = 118$, $p = 0.984$). Only a minority of latrines (queenless: 2/21, 9.5%; queenright: 2/19 latrines, 10.5 %) contained other refuse particles (one piece of *Drosophila* located in one latrine each in two queenright colonies and two queenless colonies) both in queenless and queenright colonies, and none of the latrines contained dead ants, i.e., latrines did not serve as general waste dumps. Only 21 % (14/58) of all colonies stored food items (fresh pieces of *Drosophila*) inside the colony, but never in a centralized place. Rather, food items were widely distributed throughout the nest (indicated by the yellow circles in supplement S2 & S3).

Brood piles were always kept away from the latrines (distance median, Q1, Q3: queenless: 30.93 mm, 26.20 mm, 41.88 mm; queenright: 29.12 mm, 23.74 mm, 34.53 mm) and non-centralized defecation areas. Only in two queenright colonies single larvae were found on defecation spots. In most queenless colonies (21/29) workers also defecated outside the nest, especially under the nest, while external defecation was significantly less common in queenright colonies (11/29; $\chi^2 = 6.97$, $df = 1$, $p = 0.0017$, see figure S7.1). Workers from colonies with well-defined latrines inside the nest defecated more frequently outside the nest (25/31) than workers from colonies with random defecation spots inside the nest (4/11) ($\chi^2 = 7.5$, $p = 0.006$; PerMANOVA: defecation outside the nest: queen presence $F = 1.1$, $df = 1$, $p = 0.22$, presence of larvae $F = 2.8$, $df = 1$, $p = 0.1$, latrine presence inside the nest $F = 8.4$, $df = 1$, $p = 0.0052$). When defecating outside, workers from queenright colonies were more likely to form localized latrines under the nest than workers from queenless colonies (5/21 queenless colonies, 9/11 queenright colonies; $\chi^2 = 9.9$, $df = 1$, $p = 0.002$) (PerMANOVA latrine formation outside the nest:

queen presence F = 14.6, df = 1, p = 0.0005, presence of larvae F = 0.75, df = 1, p = 0.39, latrine presence inside the nest F = 0.55, df = 1, p = 0.63).

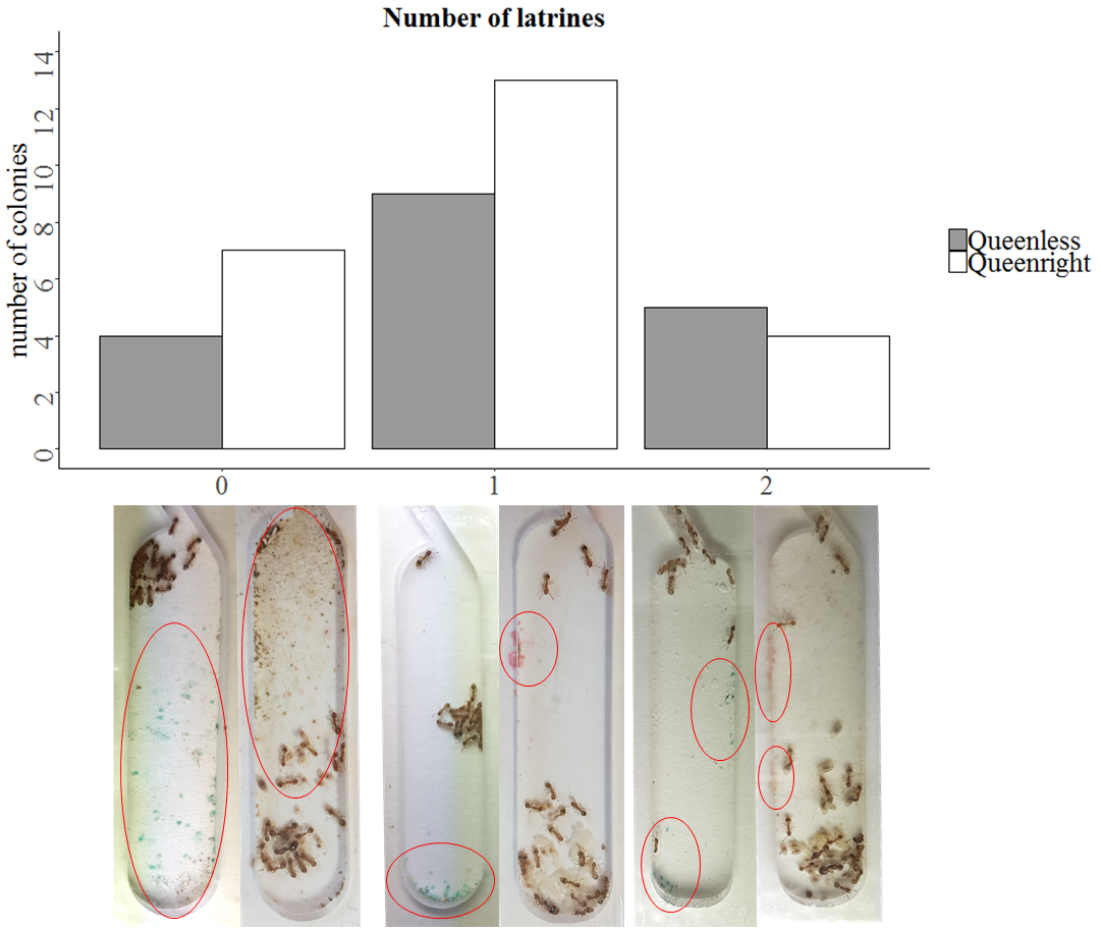

Figure 7.1: Number of colonies without (0) with one (1) or with two (2) locally defined latrines in queenless (black) and queenright (grey) *Temnothorax crassispinus* nests. Defecation and latrine areas are marked with a red circle. In the two leftmost nests, defecation spots are randomly distributed throughout large parts of the nest and not locally concentrated in a latrine area.

Under laboratory conditions, *T. crassispinus* nests were quickly covered by mold (area median, Q1, Q3: 44.37 mm², 0.00 mm², 403.95 mm²) and the degree of microbiota growth inside the nest appeared to be linked to the presence of the queen and the individuals' defecation behavior (PerMANOVA: queen presence: F = 3.71, df = 1, p = 0.041, larvae presence: F = 1.27, df = 1, p = 0.29, latrine presence: F = 13.61, df = 4, p = 0.001, defecation outside the nest: F = 1.37, df = 1, p = 0.27, see figure 7.2). Colonies were less moldy when workers defecated outside (median, Q1, Q3:

0.00 mm², 0.00 mm², 45.75 mm²) than colonies with only inside defecation (median, Q1, Q3: 450.57 mm², 83.95 mm², 563.06 mm²; $W = 704$, $p < 0.0001$). Furthermore, the formation of localized latrines inside the nest (median, Q1, Q3: 14.28 mm², 0.00 mm², 47.21 mm²) appeared to reduce mold growth more than random defecation (median, Q1, Q3: 291.71 mm², 122.41 mm², 473.33 mm²; $W = 300.5$, $p = 0.00014$) and the area covered by latrines and mold growth were positively correlated (Spearman's rank correlation: $r_S = 0.45$, $p = 0.01$).

When we allowed 17 split colonies to reunite four weeks later, eight colony fragments moved in with their nestmates in the more or equally moldy nest, five colonies merged in the less moldy nest and in four cases colonies did not reunite at all. Interestingly, regarding the colonies that moved, six left their clean, mold-free nests to move into nests with considerable microbiota growth. This indicates that the ants do not avoid moldy nests.

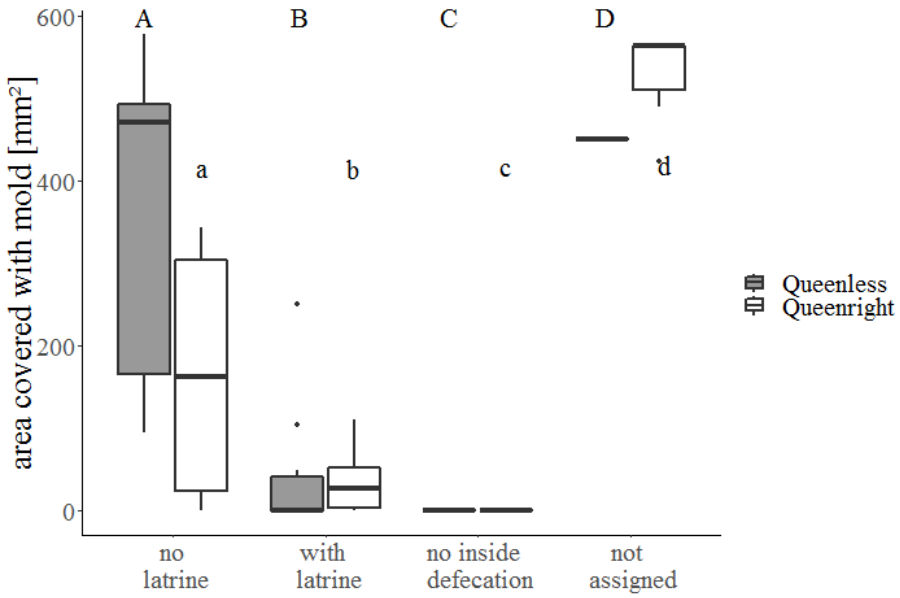

Figure 7.2: Area of the inner nest covered by mold in relation to the individuals' defecation behavior and queen presence. In colonies without latrines ("no latrines") the inner nest is covered up to 100% by mold, but the formation of latrines inside the nest ("with latrines") significantly reduces mold growth. The avoidance of inside defecation ("no inside defecation") can stop mold growth completely. In a few colonies, mold growth was so intense that they could not be assigned ("not assigned") to any of the other groups. Boxplots show median, 25 and 75 quartiles, and 95% percentiles. Post hoc comparisons among groups significantly different at $p = 0.05$, adjusted following Benjamini and Hochberg (1995), are displayed by different letters (lower case for queenright colonies, capitals for queenless colonies). Mold growth does not differ significantly between queenless and queenright groups.

7.6 Discussion

In most (89%) studied colonies of the ant *T. crassispinus*, individuals defecated inside the nest, and in most nests (74%) they defecated only in particular areas. These areas appear to be defecation sites (latrines or toilets c.f. Czaczkes et al., 2015), as their color matched the artificially colored food. In contrast to waste disposal sites or middens (Peeters et al., 1994; Rettenmeyer, 1963; Wilson and Brown, 2005), only two of 40 latrines contained left-over food items or other waste. *T. crassispinus* rarely store large amounts of food in their nests and the random distribution of the few scattered food residues indicated that they do not deposit left-over food items in specific sites. Instead, workers were seen removing decaying food from the nest (unpublished observations).

Many ant species have waste disposal areas, where they put droplets of feces and other waste products (Bernadou et al., 2018; Hart and Ratnieks, 2001; Hölldobler and Wilson, 1978; Weiss, 2006). However, the formation of latrines inside ant nests has so far only been reported for a few formicine ants (e.g. Hölldobler and Wilson, 1977), including *Lasius niger* (Czaczkes et al., 2015), the ponerine ant *Platythyrea punctata* (Bernadou et al., 2018) and *Crematogaster smithi* (S. Cremer & J. Oettler, unpublished). Feces and other excretions can promote pathogen growth and thus pose a threat to insect societies (Copley et al., 2012; Hart and Ratnieks, 2001). Together with other types of pathogen defense (e.g. Cremer et al., 2007), defecation in well-defined areas might minimize infection risk. While foragers may easily defecate outside the nest (Bernadou et al., 2018), the presence of indoor latrines may protect younger workers and the queen from predation and other external hazards (Czaczkes et al., 2015).

In *T. crassispinus*, the formation of localized latrines appears to reduce mold growth. Natural colonies of *T. crassispinus* live in relatively ephemeral sites, such rotting twigs, under bark, and in hollow acorns, and mold prevention might increase the stability of the nest material. The resting workers avoided moldy patches inside the nest, and brood items were kept away from them (see also Karlik et al., 2016). Nevertheless, single individuals themselves appeared not to be strongly affected by mold. When we allowed colony fragments to reunite at the end of the experiment, some of them moved into the moldier nest. Behavioral observations revealed that active workers frequently handled the mold, relocating it, removing fragments or using pieces of mold to close the nest entrance (unpublished data). Furthermore, colonies remained in a moldy nest rather than moving into new nest sites. These observations are consistent with further studies, which show that mature ant colonies (*Myrmica rubra*, Leclerc and Detrain, 2016; *Monomorium pharaonis*, Pontieri et al., 2014) or founding queens (*Formica selysi*, Rothenbuhler, 1964) often actively choose pathogen contaminated substrate for nest foundation. These studies conclude, that intentional pathogen contact could lead to immunization (Brütsch et al., 2017; Leclerc and Detrain, 2016; Pontieri et al., 2014).

Colonies differed in sanitary behavior, but why workers in a minority of colonies defecated randomly throughout the nest or only outside remains unclear. Variation in hygienic behavior in honey bees has been suggested to have a genetic basis (Rothenbuhler, 1964), but in our study latrine formation was not significantly correlated between queenless and queenright fragments of the same colony. Instead, differences appeared to be in part associated with presence or absence of the queen: workers from queenless colonies defecated more frequently outside the nest than workers from queenright colonies. We can only speculate as to why queen loss results in a slightly changed defecation

behavior. While workers refrain from laying large numbers of eggs in the presence of the queen, several socially dominant workers oviposit in queenless colonies (El-Shehaby et al., 2012) and the number of brood items is often larger than in queenright colonies of the same size (unpublished data). This means that more food needs to be retrieved, and, as in *Platythyrea punctata* (Bernadou et al., 2018), increased foraging activity might be associated with more outside defecation.

Nevertheless, the occurrence of latrines inside the colony documents sanitary behavior in *Temnothorax* ants and the capability of workers to form well-defined latrine patches. The connection between latrines and mold growth inside the nest might indicate that these products and the consequent growth of microbiota might not be harmful for the colony, and may indeed be beneficial. Indeed, Varoudis et al. (2018) showed that frass and plant tissue play an important role in structuring natural nest sites of this species.

7.7 Conclusion

Our study reveals that sanitary behavior, as in our case latrine formation, is not only of importance in highly structured social systems as honey bees (Rothenbuhler, 1964) or *Lasius niger* ant colonies (Czaczkes et al., 2015), but can also be found in less complex societies, such as the small *Temnothorax* colonies. Localized latrines seem to improve nest cleanliness and reduce mold growth in *Temnothorax* ants. In contrast to solid waste management, studies on defecation behavior in social insects are rare, and further studies are needed to understand the function of latrines, their localization, and their role in microbiota growth.

Declarations

Supplementary data

The pictures of the ant colonies are given as supplemental material S2 and S3 in supplement S1. Raw data are also given in supplement S1.

Declaration of interest

The authors declare that they have no competing interests.

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Authors' contributions

JG performed the experiment, designed the study and analyzed the data; JG, TJC and JH wrote the manuscript and interpreted the data. All authors read and approved the final manuscript.

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8 General discussion

Traditionally, direct fitness for ant workers is supposed to be low in the presence of a queen, due to the reproductive and non-reproductive division of labor (Hölldobler and Wilson, 1990; Robinson, 1992). However, in many ant species, workers retained functional ovaries and are capable of laying haploid eggs (Bourke, 1988*b*; Cole, 1986; El-Shehaby et al., 2012; Heinze et al., 1997). So far it is assumed, that worker reproduction is suppressed under queenright conditions and occurs only after the queen's death or in the queen's absence in queenless colonies (Alaux et al., 2004; Bourke, 1988*b*; Holmes et al., 2013; Hoover et al., 2003; Keller and Nonacs, 1993). However, most ant queens outlive their workers (Keller and Genoud, 1997; Keller, 1998) and a future reproductive success is assumed to be negligible for the majority of workers. Accordingly, workers increase their indirect fitness by rearing queen-produced offspring and mainly benefit from inclusive fitness (Hamilton, 1964*a*,*b*).

Doubts are arising whether Hamilton's theory of inclusive fitness (Hamilton, 1964*a*,*b*) is sufficiently explaining altruistic helping behavior in nature (Nowak et al., 2010). One central point of this theory is the relatedness between the actor and the recipient of the help. Although workers are highly related to their sisters under monogynous, monandrous conditions (Queller and Strassmann, 1998), the average relatedness among all siblings in a colony is still comparable to other monogamous, non-eusocial species and thus, indirect fitness might not be enough for a lifetime resignation from reproduction in social insects. Indeed, although relatedness built the foundation for the evolution of altruism, it is probably maintained by decentralized punishments among workers (Wenseleers and Ratnieks, 2006). However, it remains unclear whether social insect workers are nevertheless capable to overcome reproductive suppression and to increase their direct fitness by independent reproduction.

The aim of this thesis was to estimate the importance of direct fitness for workers of a highly social ant species. Our study reveals, that independent reproduction can contribute significantly to the direct fitness of ant workers and cannot be neglected in the context of social evolution. Worker reproduction was not only restricted to queenless colonies but both, queenright and queenless colonies, seem to provide an environment that facilitates successful reproduction of workers in nature. About 30% of the analyzed males were not produced by the estimated queen, but were derived from workers, as supported by the high proportion of workers with activated ovaries. Interestingly, worker reproductive success was rather low in the maternal colony but seems to be increased when workers are not related to the colony. However, it needs to be mentioned, that our evaluation of worker-produced males is based on a conservative estimate. Half of the worker-produced males will inherit one of the queen's alleles and we are not able to

assign these males correctly if they were produced by workers in this case. Additionally, potential null alleles (Dakin and Avise, 2004) and a high percentage of homozygous individuals in some colonies aggravate a successful assignment in these cases.

Interestingly, parts of the genotyped males were even completely unrelated to other female nestmates, indicating that workers did not reproduce exclusively in their residential colony but might also dump eggs in alien colonies (Nanork et al., 2007; O'Connor et al., 2013; Tallamy, 2005). If unrelated males were produced by females of a previous, extinct lineage we would expect to find a female relative, since *T. crassispinus* workers can live for several years (Sendova-Franks and Franks, 1995, and see chapter 3), although we cannot completely exclude this possibility. Generally, reproduction of migrating females is not unlikely, as nestmate recognition is limited in *Temnothorax* ants and unrelated workers are often not prevented from entering the nest (Foitzik et al., 2007). Furthermore, migrations between nests might facilitate brood raids among colonies as observed in related social parasites (Buschinger et al., 1980) and could also explain unrelated haploid and diploid brood found inside queenless and/or queenright colonies. The genetic heterogeneity might therefore be a byproduct io instable colony structures caused by frequent colony fusions and fissions, associated with seasonal polydomy or colony usurpation (Foitzik and Heinze, 1998, 2000; Foitzik et al., 2007; Strätz and Heinze, 2004).

Although worker policing has been described frequently (Brunner and Heinze, 2009; Helanterä and Sundström, 2007*a*; Oldroyd et al., 2001; Ratnieks, 1988*b*; Stroeymeyt et al., 2007), it seems not sufficient to entirely prevent a maturation of eggs produced by workers related to the queen. Productivity of queen-related workers strongly increased in queenright colonies under laboratory conditions, despite a constant proportion of unrelated workers within the colony (see chapter 3). We do not expect that this is caused by a decreased fecundity of the queen, as its fecundity does not decrease with increasing age (Heinze and Schrempf, 2012; Keller, 1998; Schrempf et al., 2017) and the observed sex ratios do not indicate a lack of sperm supply in queens. However, genetic and observational data revealed a rather short lifespan in queens, either naturally or caused by premature matricide, which significantly contributed to the direct fitness of workers and the inclusive fitness of the entire colony.

In more than one third of the queenright colonies, queens were expelled from the nest or killed by workers several weeks after colony collection. Matricide has so far only been observed, in polygynous (several reproductive queens) or annual species and is supposed to be costly in a monogynous species that cannot replace the only queen (Balas, 2005; Bourke, 1994; Keller et al., 1989; Loope, 2016). In our study, matricide was

observed in large and matured colonies with sufficient work force and survival probability to raise their independently produced offspring. Indeed, orphaned colonies were highly productive and persisted for several years and thus, might considerably contribute to male production in natural populations.

In still growing colonies, seasonal polydomy might additionally facilitate an escape from queen control. Although ovary development was not impaired by queen presence, the seasonal separation of queenless colony fragments from the queen, might facilitate the maturation of worker-produced eggs and reduce reproductive costs in nature. Indeed, we could show that at the end of the reproductive period, worker-produced larvae could be introduced successfully into queenright colonies, where they hibernate until male eclosion the following year (see chapter 5).

The direct fitness of workers is mainly determined by the reproductive success of their sons and we could not find striking effects on male fitness traits by origin (see chapter 4). In contrast to honey bees, where male traits are affected by the type of brood cell they are raised in (Berg, 1991; Berg et al., 1997; Gençer and Kahya, 2011; Gençer and Çetin Firatli, 2005), fitness traits in ant males seemed to be rather genetically determined. Although worker-produced males outnumbered and outlived queen produced males, sperm length and viability differed generally strongly between colonies, the latter especially in the presence of a queen. The observed sperm length variation within and among males might result from developmental deviations. Sperm cells are clonal in haploid males, and heterogeneous sperm sizes indicate a rather low selection pressure on sperm length, as assumed for monandrous species due to absent mate competition (Baer et al., 2003; Fitzpatrick and Baer, 2011; Fitzpatrick and Lüpold, 2014; Hunter and Birkhead, 2002; Kleven et al., 2008; Simmons and Fitzpatrick, 2012; Wigby and Chapman, 2004).

Alternatively, heterogeneous sperm might be a side effect caused by the observed interaction between sperm length and viability. Ant queens can live up to several decades (Keller and Genoud, 1997; Keller, 1998; Plateaux, 1986) and strongly depend on the viability of the stored sperm to successfully fertilize eggs. The importance of sperm durability could result in a reinforced selection pressure on sperm viability and may cause a trade-off between both traits. Indeed, sperm viability was generally rather high for a singly mated species (see multiply mated honey bees Collins and Donoghue, 1999), and unmanipulated, queenright colonies kept under natural conditions produced males with the most viable sperm. Additionally, studies in honey bees revealed a negative effect of high temperatures on sperm cells (Stürup et al., 2013) and the artificial climate in the laboratory might have had harmful effects on sperm viability in queenright colonies

(series 2), although this cannot explain the similar sperm quality in queenless colonies of both series. Furthermore, we cannot exclude that parts of the males from queenright colonies in series 1 and/or series 2 were produced by workers, as worker-reproduction increases in laboratory (see chapter 2).

Unfortunately, we could not analyze the mating success of queen- and worker-produced males during mating flights in our studies. The mating period in *T. crassispinus* is limited and generally requires certain conditions (Plateaux, 1986), which we could not sufficiently reconstruct to induce mating attempts under standardized conditions. Furthermore, males are generally sensitive and marking of queen- and worker-produced males by tarsae clipping or coloration caused high losses during preliminary studies and surviving males did not try to mate afterwards. Finally, mating among several males and females succeeded only once, under not standardized conditions. Preliminary observations indicate non-random mating (J. Giehr, personal commment), presumably by female choice, and agree with previous studies in other *Leptothorax* ants (Oberstadt and Heinze, 2003). Nevertheless, the high abundance of worker-produced males in queenless, but also queenright colonies, together with an apparently high sperm quality might be contributing substantially to worker direct fitness in nature. However, whether mating success is associated with other, non-tested traits has to be examined in further studies.

The high abundance of worker-produced males in nature arises questions about the different strategies used by workers to escape queen control. Based on the high proportion of queenless colonies in nature (chapter 2), matricide is not unlikely to happen naturally and the large amount of produced males in orphaned colonies can contribute significantly to worker direct fitness the following years. However, killing the only fully fertile queen is nevertheless a rather costly strategy, especially when the colony is still growing. Although workers are capable of activating their ovaries in queenright colonies (chapter 2), previous studies reported a rather high policing rate (Brunner and Heinze, 2009; Stroeymeyt et al., 2007), which might aggravate reproduction in the presence of a queen. Workers might therefore use the absence of the queen in polydomous split fragments to successfully raise their independently produced eggs to larvae and to introduce them into queenright colonies during colony fusion before hibernation (see chapter 5).

Although natural queenless colonies contained larvae of both castes and sexes (see chapter 2), worker reproduction is not inhibited by queen-produced larvae (but see *Novomessor* ants Ebie et al., 2015) and the introduction of worker-produced larvae into queenright colonies before hibernation was not selectively prevented by other workers. Workers seemed to be capable of recognizing their own brood (see also Camargo et al., 2006), but larvae did not vanish selectively after colony reunification and all larvae were

equally cared. Reunited colonies produced significantly more males than queenright colonies that did not fuse with related queenless colonies. This indicates, that worker-produced larvae contributed significantly to male production and reproduction in split fragments might be a successful strategy for workers to gain direct fitness. However, colony reunification had negative effects on colony growth and worker number, and might cause a trade-off between selfish reproduction and indirect fitness for workers. Based on the observed heterogeneity in natural colonies (chapter 2), this impairment might be outbalanced by an adoption of unrelated individuals in nature.

Nevertheless, selfish behavior appeared to be costly for the colony and many studies examined consequences arising from obvious selfish behaviors like e.g. egg laying, (Bourke, 1988*b*; Dampney et al., 2004; El-Shehaby et al., 2012; Tsuji et al., 2012), alteration of sex ratios (Owen and Plowright, 1982; Queller et al., 1993; Sundström et al., 1996), punishments of other workers (Foster et al., 2002; Helanterä and Sundström, 2007*a*; Kikuta and Tsuji, 1999; Oldroyd et al., 2001; Stroeymeyt et al., 2007) or killing queens (Balas, 2005; Bourke, 1994; Inoue et al., 2015; Keller et al., 1989; Loope, 2015). However, it has been rarely tested to what extend workers behave selfish to increase their future direct fitness. Queens for instance, try to maximize their reproductive success by increasing their fecundity with decreasing body constitution (Giehr et al., 2017) and reproduction until death, even under impending threats to other nest members (Giehr and Heinze, 2018). In contrast, artificially aged workers put the condition of the colony before their own benefits and leave the colony to die in social isolation (Heinze and Walter, 2010; Rüppell et al., 2010), possibly to prevent a spread of diseases. However, this behavior has never been put in the context of future reproductive success in workers. In our study, contaminated workers forwent direct fitness and left the colony independently of prospective fecundity (Giehr and Heinze, 2018, or see chapter 6). Moreover, workers seemed to ascertain the risk of contagion and to resume reproduction only after the removal of contaminated individuals. Interestingly, this indicates that reproduction does not cause similar behavioral restrictions as observed in queens, which seem not to be capable of leaving the colony after the first workers eclosed (Rüppell et al., 2002).

The self-removal of health compromised workers possibly serves to prevent the spread of diseases and to minimize the infection risk for the colony, although social insects are characterized by an efficient collective immune defense (Cremer et al., 2007). However, one major health risk in crowded societies is the accumulation of debris and feces and although most ant species efficiently take care of waste disposal (Bot et al., 2001; Hart and Ratnieks, 2002; Waddington and Hughes, 2010), removal of contaminated material (Hart and Ratnieks, 2001; Pull et al., 2018) or the formation of latrines (Bernadou et al.,

2018; Czaczkes et al., 2015), it is not known to what extend this is based on social organization and/or genetic lineage (see honey bees Lapidge et al., 2002). The prospect of selfish reproduction causes changes in worker behavior (Barth et al., 2010; Bourke, 1988*a*; Cant and Field, 2001; Dampney et al., 2004; Monnin and Ratnieks, 1999), which might additionally affect sanitary behavior and could again increase the overall risk of diseases in queenless colonies. We found, that *T. crassispinus* ants form well-defined latrines inside the nest independent of queen presence, which seemed to control mold growth inside the nest (Giehr et al., 2019). However, microbiota growth appeared to be reduced, despite random defecation in the presence of a queen. Behavioral observations revealed, that mold was often actively removed from the nest by workers and selfish reproduction could possibly affect this behavior in workers from queenless colonies. The outgrowth of surface sterilized, dead workers revealed a high proportion of internal pathogens (Giehr and Heinze, 2018) and thus, reproducing workers might refrain from nest cleaning to avoid contact with pathogens or other microbiota. The uptake of contaminated particles might be more risky for reproductive individuals, as *T. crassispinus* workers are not capable of spraying acid extensively to disinfect particles or large areas as formicine ants (Pull et al., 2018; Tragust et al., 2013). However, further studies are needed to investigate the effects of worker reproduction on further sanitary behavior traits and its effect on colony health.

9 Summary and conclusion

The importance of direct fitness for ant workers has long been neglected but might be an important component in the explanation of altruism and eusociality. *T. crassispinus* workers are capable of gaining considerable direct fitness in natural queenright and queenless colonies and this species might have evolved different strategies to cope thereby arising consequences. Although there seems to be generally a high queen turn-over in nature, workers possibly do not wait until the death of the queen to gain direct fitness, but might benefit from her premature death after the colony is fully grown. The high productivity observed in queenless colonies after the queen's death, might outbalance the indirect fitness costs resulting from absent prospective female production.

However, in nature it might not be absolutely necessary to kill the queen to escape queen control. Workers might avoid reproductive restrictions in queenless fragments of polydomous colonies and can presumably introduce worker-produced larvae successfully into queenright colonies before hibernation. Colony reunification increases the number of eclosing males in queenright colonies and worker-produced males seem not to be restricted in fitness, but might contribute significantly to the direct fitness of workers. Although worker reproduction seems to thrive on the expense of colony growth, genetical analyses indicate, that colonies are able to cope with the costs by adopting new females, which again might benefit from the newly arising reproductive opportunity. The adoption of unrelated females might generally be a strategy to increase the colony size after striking incidents, as e.g. the death of the queen or a major loss of workers and its prevention in the laboratory might be responsible for the strong decrease in worker number.

Although, selfish worker reproduction revealed negative impacts on colony growth and is possibly responsible for the observed matricide, it does not cause an assessment of current and future reproductive success in workers. In contrast to queens, that stay inside the colony and reproduce until death, sick reproductive workers left the colony, presumably to minimize potential risks for their nestmates independent of their fecundity. However, selfishness might result in a reduced nest cleaning under queenless conditions, which again might put the entire colony at the risk of diseases.

In summary, our studies reveal, that around 30% of the natural male population are produced by workers in the monogynous, monandrous ant *Temnothorax crassispinus*. Worker-produced males differ only slightly from queen-produced males in the tested fitness traits and can substantially contribute to the direct fitness of ant workers in nature. Finally, the prospect of direct fitness gains presumably promotes the preservation of organizational structures and reproductive rank orders inside the colonies and thus, contribute considerably to the inclusive fitness of the workers.

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Supplement S1
Raw data (all chapters)

All raw data and additional files referred to in this thesis can be found on the attached compact disc Supplement S1. Data are arranged in a single file per chapter consisting of several sheets and tables. Further supplementary notes and information can be found on the right margin of each table.

Supplement S2
DNA extraction protocol

DNA extraction with CTAB method (modified from Sambrook & Russell 2001)

Material list:

- Thermomixer
- Pipettes for transferring volumes of 2 µl, 40 µl, 150 µl, 300 µl, 350 µl, and 500 µl.
- Enough space in a freezer (-20 °C) to store one Eppendorf cup per sample, one Eppendorf cup rack, and a few Falcon tubes.
- Labelling stickers that fit on Eppendorf cup lids.
- Crushed ice in a container large enough to store all samples in Eppendorf cups.
- TE buffer (pH8)
- 70% EtOH (p.a.)
- 100% EtOH (p.a.)
- Isopropanol
- Chloroform / Isoamyl alcohol 24:1
- Sodium acetate (1/10 Vol., 3 M, pH = 4.8)
- CTAB solution: 0.75 M NaCl, 50 mM Tris/HCl (pH = 8.0), 10 mM EDTA, 1% Hexadecyltrimethylammoniumbromid (add after autoclave); you need 500 µl per sample
- Water bath if thermomixer is not used for warming up.
- Clean pestles for crushing the material.
- Fluid nitrogen
- Centrifuge for 4 °C and room temperature (14000 rounds per minute).

- PCR water (Sigma Aldrich Chemie GmbH) for DNA storage or TE (Tris/EDTA) buffer for long-term storage of DNA (40 µl each for ants, thoraces or larvae, 25 µl each for eggs or legs).

Part I

Preparations:

- Prepare 1% CTAB solution (500 µl per sample) and warm it to 65 °C (either in the water bath or in the Thermomixer, for the latter use large Eppendorf cups).
- Prepare crushed ice for cooling samples in Eppendorf cups.
- Fluid nitrogen
- Proteinase K (MBI Fermentas) (2-10 µl per sample)
- Chloroform / Isoamyl alcohol (24:1)
- Vortexer

Procedure:

If ants are not small, do not use gaster for extraction (cut it off with a scalpel).

Ants that have been stored in alcohol must be equilibrated with 70% EtOH (p.a.) and distillate water.*

Place each (frozen or equilibrated) individual (ant worker, legs, larva or egg) in a 1.5 ml Eppendorf cup (without fluid), put label (colony number, individual ID) on the cup, put cups on ice.

Put fluid nitrogen in each Eppendorf cup, quickly close the lid for a short time (for preventing the sample to "jump out"), and immediately crush animals with clean pestles; then add 500 µl of warmed (65 °C) 1% (or 2%) CTAB solution.

Incubate the mixture for 1 h at 65 °C in the Thermomixer.

Let the mixture cool down to 55 °C (and set the Thermomixer to 55 °C), then add 2 µl-10 µl Proteinase K and incubate in the Thermomixer for 3-4 h at 55 °C or overnight (old animals always overnight).

Part II

Preparations:

- Prepare Chloroform / Isoamyl alcohol 24:1 (500 µl per sample).

- Prepare NaAc (1/10 Vol., 3 M, ph 4.8) (40 µl for each sample).
- Prepare one fresh 1.5 ml Eppendorf cup for each sample (with equal label).
- Put Isopropanol (350 µl for each sample) in the freezer (-20 °C).

Procedure:

Add 500 µl Chloroform / Isoamyl alcohol, vortex, and centrifuge for 5 min at 14000 rpm (rounds per minute) (RT).

After centrifugation put upper phase of each sample in a fresh cup (if individuals are very old or dirty, begin this procedure again from the start). Be cautious not to get the tiniest part of the lower phase mixed with the upper phase!

Add 40 µl NaAc (1/10 Vol., 3 M, ph 4.8) mix and 350 µl Isopropanol (-20 °C), shortly shake the cup, then incubate 30 min at -20 °C or overnight (old animals at least 1 h) in the freezer; if not overnight.

Cool down centrifuge in time.

Part III

Preparations:

- Put 70% and 100% EtOH (p.a.) in the freezer (-20 °C) (300 µl of each per sample).
- Prepare 70% EtOH (p.a.) (150 µl per sample, room temperature (= RT)).
- Prepare PCR water (Sigma Aldrich Chemie GmbH) or TE buffer (pH8) (the latter for long-term storage) for resolving the DNA pellet (40 µl each for ants, thoraces or larvae, 25 µl each for eggs or legs). Alternatively: TE-Buffer!
- Prepare a cooled metal cup rack (by putting it in the freezer at -20 °C) or crushed ice for handling the samples while cooled.
- Cool down the centrifuge in time.

Procedure:

Centrifuge for 30 min at 4 °C and 14000 rpm; discard supernatant carefully with pipet.

Place the cups on ice or in a cooled rack; wash** each pellet with 300 µl 100% EtOH (p.a.) (-20 °C), then centrifuge 10 min at 4 °C and 14000 rpm; discard supernatant carefully with pipet.

Place the cups on ice or in a cooled rack; wash** pellet with 300 µl 70% EtOH (p.a.) (-20 °C), then centrifuge 5 min at 4 °C and 14000 rpm; discard supernatant carefully with pipet.

Wash** pellet with 150 µl 70% EtOH (p.a.) (RT), then centrifuge 5 min at RT and 14000 rpm; discard supernatant carefully with pipet.

Dry DNA pellet in open Eppendorf cup for 2 min at 50 °C (in the Thermomixer) or for at least 10 min (better 30 min) at RT (in the flue (Abzug)!).

Resolve the pellet in PCR water or TE buffer (pH8): use 40 µl each for ants, thoraces or larvae, 25 µl each for eggs or legs. Give enough time for dissolving (e.g. 1 h in the fridge).

*Equilibration of ants: Place the material of each sample for 5 min in 250 µl 70% EtOH (p.a.), then replace the EtOH with distilled H_2O and leave it for 5-10 min.

**Wash = replace entire fluid: imagine from the orientation of the Eppendorf cup in the centrifuge where in the cup the pellet must be, set the pipette tip at the opposite side of the pellet on the bottom of the cup, and draw the entire fluid out of the cup.

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Supplement S3
Supplemental material chapter 3

Table S3.1: Relatedness of queens expelled from queenright *Temnothorax crassispinus* colonies in spring 2016. Only 11 out of 27 analyzed queens could be confirmed as the foundress of the colony, four queens were presumably sisters of the present workers and 12 queens were completely unrelated.

Colony	Queen number	Queens estimated as foundress	Queens related to workers	Unrelated queens
A108	2	1		1
A112	1	1		
A68	1			1
B10	1	1		
B12	2			2
B123	1	1		
B126	1	1		
B128	1			1
B129	1			1
B130	1		1	
B131	1		1	
B134	1			1
B140	2	1		1
B143	1	1		
B53	1	1		
B88	1	1		
P123	1			1
P18	3		1	2
P2	1		1	
P42	1	1		
P89	1			1
P94	1	1		

Investigating a disease outbreak

Results (fungi)

Queens from infected colonies (n=21)

Fungus	No. samples	BLAST percentage	Comment
Cladosporium sp.	n=6	99% (n=1); 100% (n=5)	generalist entomopathogen
Paecilomyces hepiali	n=3	100%	insect endoparasite
Yarrowia lipolytica	n=2	100%	alkane assimilating yeast
Lecanicillium lecanii	n=1	100%	generalist entomopathogen
Fusarium sambucinum	n=1	99%	plant parasite; found in moth larvae
Penicillium rubens	n=1	99%	food mold
Purpureocillium lilacinum	n=1	97%	generalist entomopathogen
Mortierella sp.	n=1	92%	non-pathogenic soil fungus
unidentified	n=10		

Workers and brood from infected colonies (n=25)

Fungus	No. samples	BLAST percentage	Comment
Beauveria bassiana	n=15	100%	generalist entomopathogen
Fusarium sp. (petrophilium)	n=4	100%	entomopathogen? Human pathogen
Trichosporon sp.	n=4	100%	found in insect guts
Yarrowia lipolytica	n=3	100%	alkane assimilating yeast
Candida orthopsilosis	n=3	100%	human parasite; low virulence against moths
Lecanicillium lecanii	n=2	99%	generalist entomopathogen
Acremonium sp	n=1	98%	found in insects; entomopathogenic properties
unidentified	n=2		

NB. *Candida orthopsilosis* found in 1 out of 6 'healthy' samples
Beauveria bassiana found in 6 out of 6 'other species' samples *(Leptothorax acervorum* and *T. unifasciatus*)

Figure S3.1: Excerpt from an analysis on possible fungal infections of expelled *Temnothorax crassispinus* queens and dead workers and brood items conducted by Nathalie Stroeymeyt (University of Fribourg, Switzerland). The results indicate that the queens were not expelled as a response to an infection with lethal pathogens, however, some workers and brood items were infested by *Beauveria bassiana* several weeks after the loss of the queen.

Supplement S4
Supplemental material chapter 4

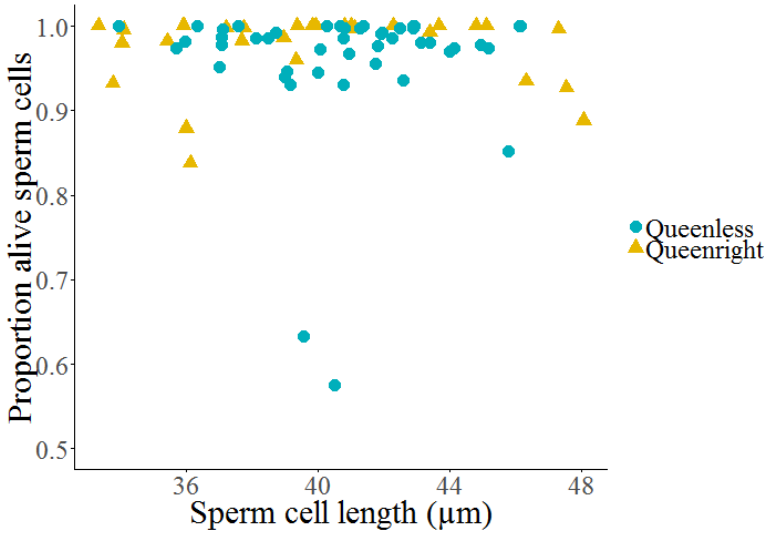

Figure S4.1: Proportion alive sperm cells in relation to sperm cell length in *Temnothorax crassispinus* males depending on queen presence. Sperm cell length strongly affects the proportion of alive sperm, although there is no correlation between both traits (Spearman's rank correlation: S = 73346, p = 0.982, r_S = -0.003).

Supplement S5
Supplemental material chapter 5

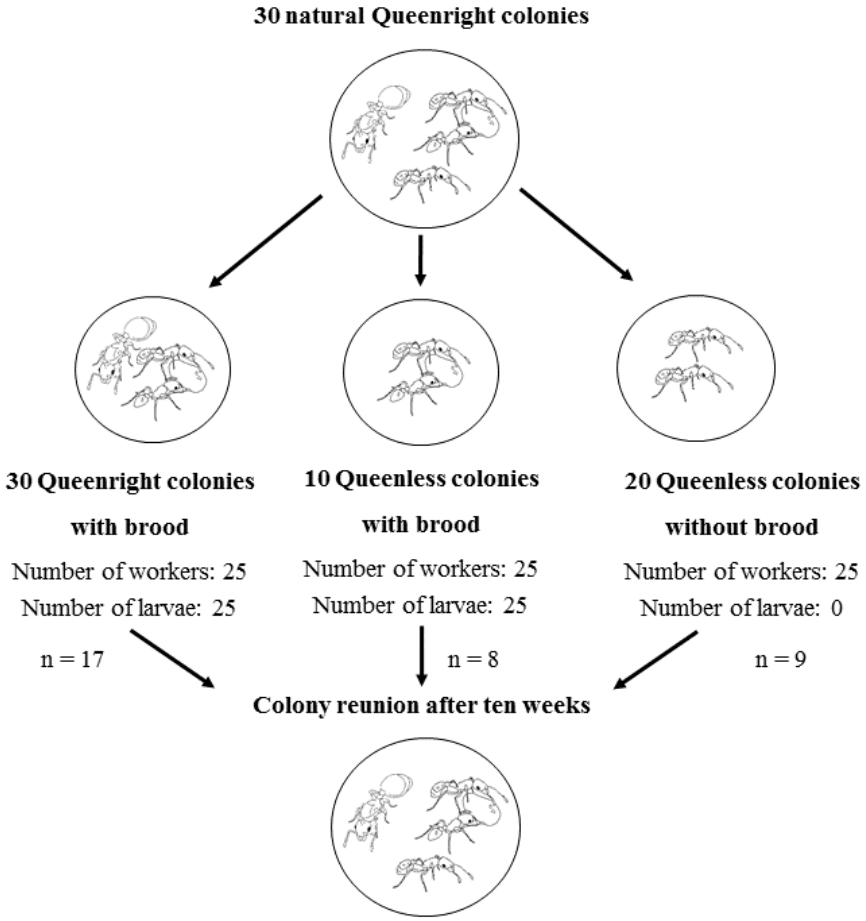

Figure S5.1: Schematic representation of the experimental set up. In total, 30 natural queenright colonies were splitted in 10 queenless colonies with queen-deprived brood and 20 queenless colonies without queen-deprived brood. All colonies were allowed to reproduce independently for five weeks. Colonies were reunited in a newly introduced arena five weeks later, when eggs developed successfully into larvae and showed a visible blue (queenless larvae) and red (queenright larvae) staining.

Supplement S6
Supplemental Material Chapter 6

Table S6.1: Pairwise survival comparison of young *Temnothorax crassispinus* workers in queenless (QL) and queenright (QR) colonies, treated with a control solution (QLCo, QRCo, QRQInf) or infected with *Metarhizium brunneum*, (QLInf, QRWInf). Significant p-values (corrected for a false discovery rate "fdr") are marked in bold.

	QLCo	QLInf	QRCo	QRWInf
QLInf	**p < 0.0001**			
QRCo	p = 0.74	**p < 0.0001**		
QRWInf	**p < 0.0001**	p = 0.74	**p < 0.0001**	
QRQInf	p = 0.74	**p < 0.0001**	p = 0.95	**p < 0.0001**

Table S6.2: Pairwise survival comparison of young *Temnothorax crassispinus* workers of queenless colonies infected with *Metarhizium brunneum*. Significant p-values (corrected for a false discovery rate "fdr") are marked in bold.

Colony	1	2	3	4	5	6	7	8	9
2	p = 0.149								
3	p = 0.714	p = 0.553							
4	p = 0.754	p = 0.177	p = 0.652						
5	**p = 0.011**	p = 0.144	p = 0.0551	p = 0.007					
6	**p = 0.007**	**p = 0.019**	**p = 0.021**	p = 0.005	p = 0.304				
7	p = 0.737	p = 0.442	p = 0.572	p = 0.399	**p = 0.014**	**p = 0.005**			
8	p = 0.252	p = 0.714	p = 0.635	p = 0.384	p = 0.060	**p = 0.012**	p = 0.635		
9	**p = 0.021**	p = 0.373	p = 0.111	p = 0.051	p = 0.653	p = 0.149	p = 0.095	p = 0.188	
0	**p = 0.012**	p = 0.111	**p = 0.049**	p = 0.012	p = 0.694	p = 0.553	**p = 0.013**	p = 0.055	p = 0.553

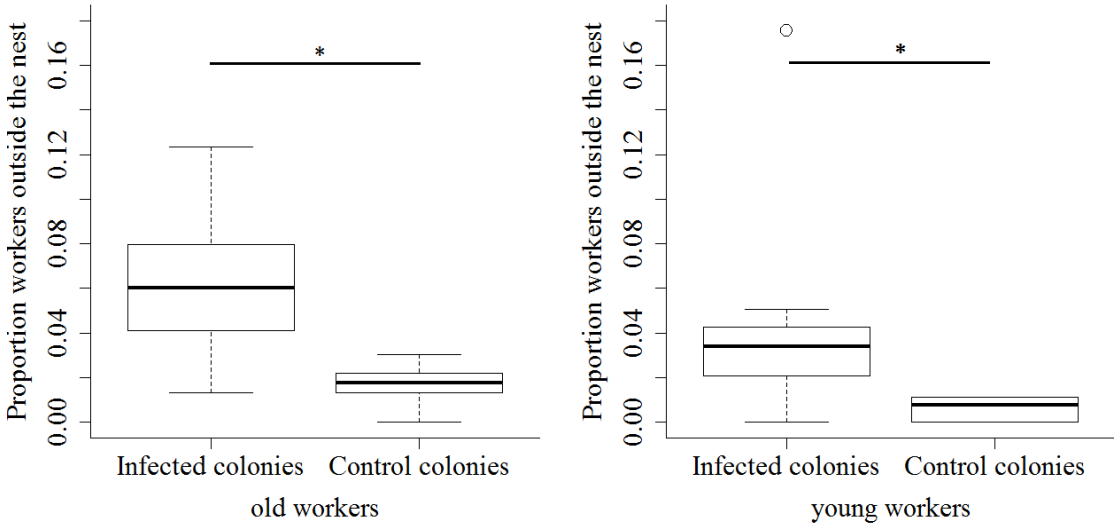

Figure S6.1: Proportion of old (left) and young (right) *Temnothorax crassispinus* workers leaving the nest in colonies with young workers either infected with *Metarhizium brunneum* (infected colonies) or treated with a control solution (control colonies) independent of queen presence. Both young and old workers leave the nest more often when they themselves or nestmates are infected. Boxplots show median, 25 and 75 quartiles and 95% percentile (* $0.05 > p > 0.01$; corrected for a false discovery rate: "fdr").

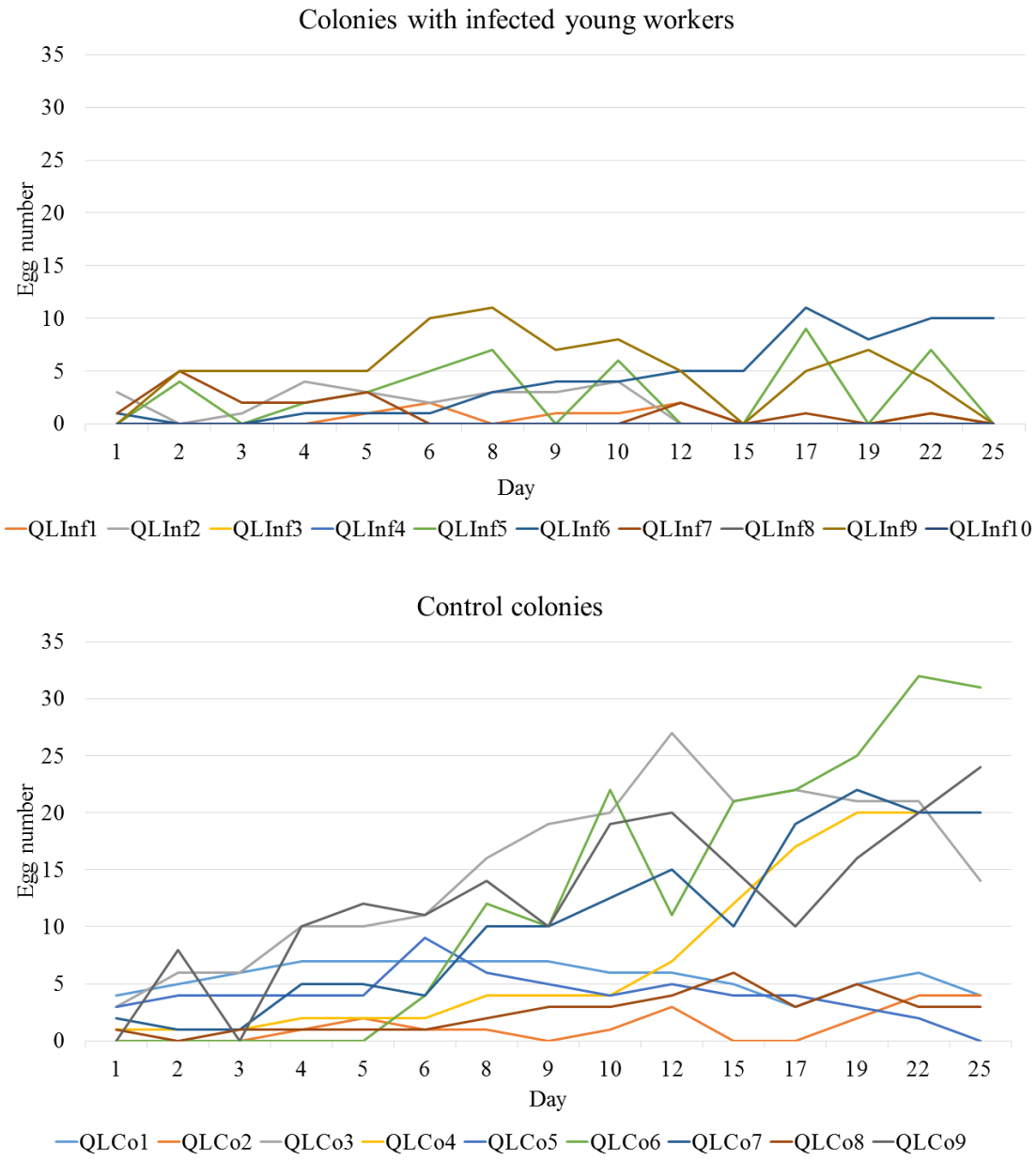

Figure S6.2: Reproductive rate of queenless *Temnothorax crassispinus* colonies during the first 25 days after the treatment. Eggs in infected colonies (top) vanish frequently and the colonies produce less eggs than control colonies (bottom).

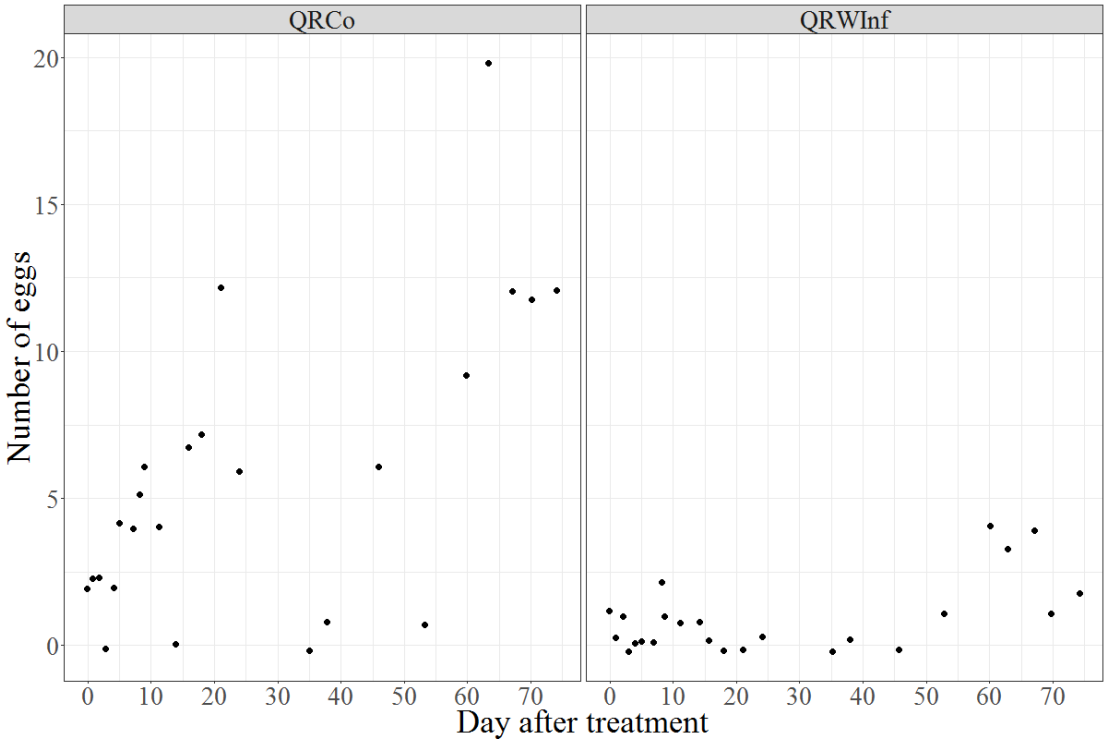

Figure S6.3: Number of eggs produced in queenright *T. crassispinus* control colonies (QRCo, left) or colonies with a control queen and *M. brunneum* infected workers (QRWInf, right). Whereas control queens increase their egg laying rate with time, the small number of eggs produced in colonies with infected workers vanish repeatedly.

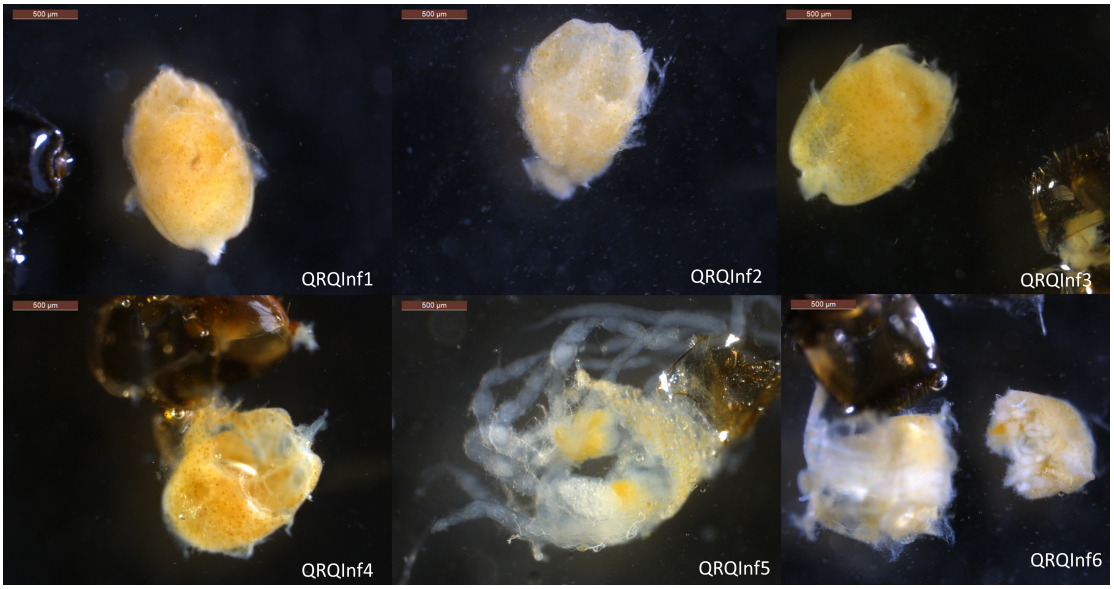

Figure S6.4: Ovaries of *Temnothorax crassispinus* queens infected with *Metarhizium brunneum*. Developmental status of the ovaries cannot be analyzed in five out of six queens as the gaster show excessive spore growth.

Supplement S7
Supplemental Material Chapter 7

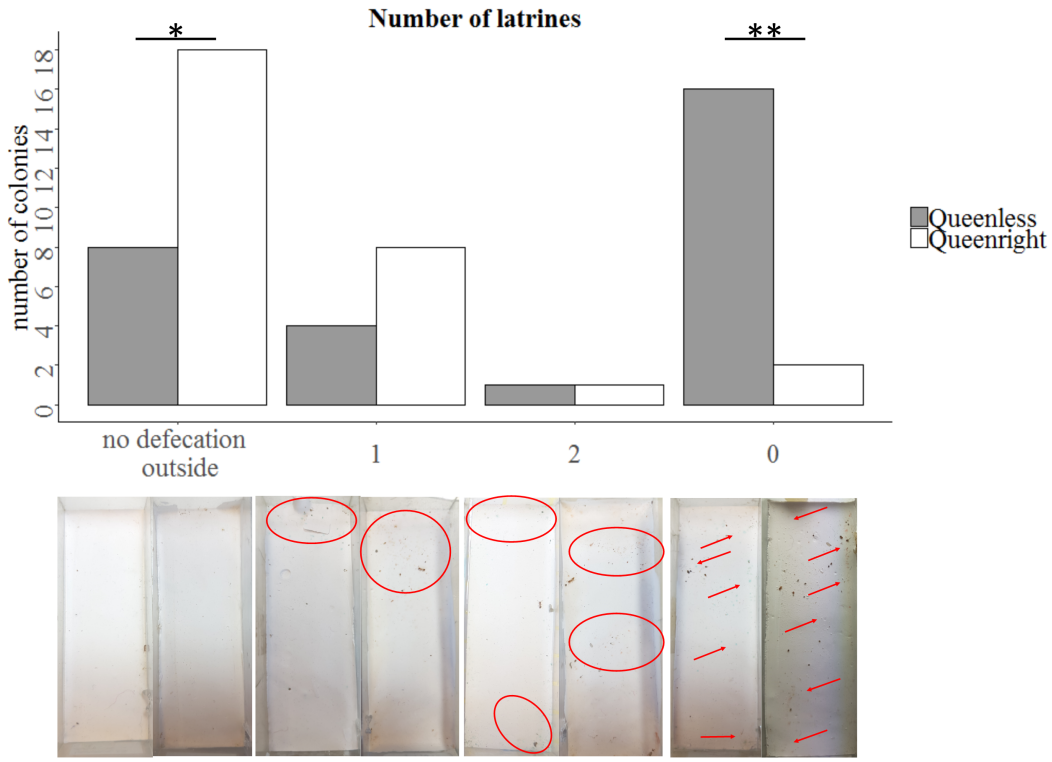

Figure S7.1: Number of queenless (grey) and queenright (white) *Temnothorax crassispinus* colonies with no defecation outside, one (1), two (2) or no (0) localized toilets outside the nest. A high proportion of queenright and a few queenless colonies did not defecate outside at all (* $p < 0.05$, ** $p < 0.01$ corrected for a false discovery rate: "fdr"). Red circles mark the defined toilets and red arrows highlight scattered defecation spots.