

Substantial direct fitness gains of workers in a highly eusocial ant

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Abstract

Hamilton's theory of inclusive fitness suggests that helpers in animal societies gain fitness indirectly by increasing the reproductive performance of a related beneficiary. Helpers in cooperatively breeding birds, mammals and primitively eusocial wasps may additionally obtain direct fitness through inheriting the nest or mating partner of the former reproductive. Here, we show that also workers of a highly eusocial ant may achieve considerable direct fitness by producing males in both queenless and queenright colonies. We investigated the reproductive success of workers of the ant *Temnothorax crassispinus* in nature and the laboratory by dissecting workers and determining the origin of males by microsatellite analysis. We show that workers are capable of activating their ovaries and successfully producing their sons independently of the presence of a queen. Genotypes revealed that at least one fifth of the males in natural queenright colonies were not offspring of the queen. Most worker-produced males could be assigned to workers that were unrelated to the queen, suggesting egg-laying by drifting workers.

KEYWORDS

altruism, ants, direct fitness, male production, *Temnothorax*, worker reproduction

1 | INTRODUCTION

Why some individuals help others even at a cost to themselves has long puzzled biologists. Hamilton's theory of inclusive fitness (Hamilton, 1964) provides a fundamental solution to this problem. It states that altruistic behaviour can be stable in evolution, if more copies of the helper's genes can be transmitted to the next generation indirectly via the offspring of the recipient of the help through the helper's altruistic activities (indirect fitness) than through its own direct reproduction (direct fitness).

In cooperatively breeding birds and mammals, helpers in addition to indirect fitness often obtain substantial direct fitness. For example, helpers may produce own offspring after inheriting the mating partner or territory of the former beneficiary of the help, or they may gain experience in brood care, which later benefits their own offspring (Clutton-Brock, 2009; Dickinson & Hatchwell, 2004;

Russell, 2004). Similarly, direct fitness appears to play a considerable role in 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. In addition, they may take over the nest after the death of the latter (Field & Cant, 2007; West-Eberhard, 1978). Direct fitness may occasionally be the only component underlying helping behaviour, for example, when helpers and the recipient of the help are unrelated (Leadbeater, Carruthers, Green, Rosser, & Field, 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 & Schneider, 2007).

In contrast, the chances to obtain direct fitness have traditionally been thought to be much less pronounced for workers of highly eusocial ants and bees (e.g., Hammond & Keller, 2004; Wenseleers, Helanterä, Hart, & Ratnieks, 2004). Workers of most ants and bees

have retained simplified, functioning ovaries and in principle are capable of laying unfertilized, male-destined eggs. Though the hatchability of worker-laid eggs may be reduced (Khila & Abouheif, 2008; Pirk, Neumann, Hepburn, Moritz, & Tautz, 2004), many studies show that workers can produce sons at least in queenless colonies (Bourke, 1988a; Cao, 2013; Choe, 1988; Debout, Schatz, Elias, & Mickey, 2007; Partridge, Partridge, & Franks, 1997). In addition, workers of many species compete aggressively for egg-laying rights (Bourke, 1988a; Cole, 1981; Heinze, 2010; Stroeymeyt, Brunner, & Heinze, 2007; Zanette et al., 2012) and socially dominant workers refrain from costly and dangerous tasks and instead appear to “save their resources” for future reproduction (Barth, Kellner, & Heinze, 2010; Bourke, 1988b; Charbonneau & Dornhaus, 2015). Finally, drifting bee workers appear to selfishly dump their eggs into the queenless or occasionally also queenright nests of unrelated colonies (Alves et al., 2009; Beekman & Oldroyd, 2008; Lopez-Vaamonde, Koning, Brown, Jordan, & Bourke, 2004).

This all suggests that workers of many advanced eusocial Hymenoptera are also under selection to maximize their direct fitness. Nevertheless, most genetic data document only a small contribution of workers to the male offspring of the colony (e.g., Smith, Overson, Hölldobler, & Gadau, 2012; Visscher, 1989; Walin, Sundström, Seppä, & Rosengren, 1998; Wenseleers et al., 2004; Wenseleers & Ratnieks, 2006). For example, in a meta-analysis, Hammond and Keller (2004) reported that in more than 2/3 of all studied species, less than 10% of the males were worker offspring. With a few exceptions, in which workers produce up to 85% of the males in queenright colonies (Barron, Oldroyd, & Ratnieks, 2001; Foitzik & Herbers, 2001; Herbers & Mouser, 1998; Lee et al., 2017; Tóth, Strassmann, Nogueira-Neto, Imperatriz-Fonseca, & Queller, 2002), worker reproduction appears to be largely prevented by inhibitive queen pheromones, aggression towards egg-laying workers, and egg eating (Brunner & Heinze, 2009; Helanterä & Sundström, 2007a; Oldroyd et al., 2001; Orlova & Amsalem, 2019; Ratnieks, 1988; Stroeymeyt et al., 2007; Walter, Brunner, & Heinze, 2011). However, population-wide estimates of direct fitness of workers from both queenright and queenless colonies are needed to better understand why workers strive for direct fitness.

The occurrence of dominance hierarchies among workers and selfish worker policing (Cole, 1981; Franks & Scovell, 1983; Heinze, 2010; Stroeymeyt et al., 2007; Walter et al., 2011) and the ease of collecting and keeping complete colonies makes *Temnothorax* ants suitable models for such an analysis. Here, we aim to determine how frequently selfish behaviour of workers translates into direct fitness by investigating male production in more than 600 colonies of the small Palearctic ant *Temnothorax crassispinus*. Previous studies have shown that colonies of *T. crassispinus* typically contain only a single, singly-mated queen (monogyny, monandry) and seasonally inhabit multiple nest sites (polydomy; Strätz & Heinze, 2004; Tichá & Štys, 2002). Nevertheless, preliminary data suggested an unexpected genetic heterogeneity of colonies and also revealed the presence of developing oocytes in worker ovaries (El-Shehaby, Abd-el-Reheem, & Heinze, 2012; M. El-Shehaby, E. Schifelbein, unpublished data).

We here quantify the contribution of workers to the males produced in the population and estimate their average direct fitness.

2 | MATERIAL AND METHODS

2.1 | Study species and colony collection

Temnothorax crassispinus is a small (approximately 2–3 mm) ant, whose colonies consist of up to 300 workers (Strätz & Heinze, 2004; Tichá & Štys, 2002). Previous genotyping had shown that despite of monogyny and monandry many colonies consist of a mixture of different matriline and patriline (M. El-Shehaby, E. Schifelbein, unpublished data). This suggested a fluid colony and population structure with frequent queen replacements, seasonal polydomy, fusion of neighbouring colonies, and the adoption of alien workers, similar to what has been described in its allopatric sibling species *Temnothorax nylander* (Foitzik & Heinze, 1998, 2000). We therefore complemented studies on the origin of males in “field 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 (“laboratory colonies”).

In total we collected 682 colonies around Regensburg, Germany. Of these, 436 colonies were collected in calendar weeks 10–15 in spring 2016. To avoid losing individuals in the field during collection we placed the whole nest (e.g., rotting twigs) into sealable plastic bags. In the laboratory, we counted all adults and brood and transferred them from their natural nests into separate artificial nest boxes ($10 \times 10 \times 3 \text{ cm}^3$) with an artificial nest consisting of a Plexiglas® frame sandwiched between two microscope slides. Colonies were kept outside the building under natural temperature and humidity conditions (“laboratory colonies”) for several years, at least until their queen had died. Individuals for genetic analyses were collected around 16 weeks after collection in July 2016, shortly after the emergence of the sexual offspring. As the males eclosing in summer 2016 could have been offspring of individuals no longer present upon collection, we repeated the genetic analysis in July 2017 with males that were definitively offspring of individuals in a different set of isolated laboratory colonies. We chose different colonies, as the removal of workers from the colonies studied in summer 2016 might have affected colony productivity.

Laboratory 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.

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 (“field colonies”). Samples of frozen queens and workers were later dissected in a drop of ddH₂O, using a Leica S8 Apo binocular microscope (80×). We counted the number of developing eggs in their ovaries.

2.2 | Microsatellite analysis

We investigated the microsatellite genotypes (for details on microsatellite markers see below) of females (workers and/or female sexuals), males and, if present, the queen from a randomly chosen subset of the collected colonies (in total 106 colonies, QR: 78 queenright colonies, QL: 28 queenless colonies, for details see below). QL colonies may have been parts of polydomous QR colonies, but for simplicity we here refer to all collected units as "colonies." Our sampling in the field was not designed to determine, which nests belong to which polydomous unit.

To determine the origin of males in natural QR colonies we genotyped females and males from 19 QR laboratory colonies collected in spring 2016 after sexuals had eclosed in July 2016 (7–12 females from 19 colonies, 7–12 males from 18 colonies, microsatellite loci 1–6, see below). In July 2017 we repeated this analysis to determine the origin of males that had definitively been produced in isolated QR laboratory colonies (10–12 females, 10–12 males from 29 QR colonies collected in spring 2016, microsatellite loci 1–6). To increase the information about the presence of males, which were not offspring of the queen, we also genotyped 12 females and 12 males from each of 58 field colonies collected in summer 2016 after sexuals had eclosed (30 QR, 28 QL; to improve resolution we used microsatellite loci 1–7). To investigate whether males with aberrant genotypes were offspring of nest-mates, whose genotypes had been missed among the first 12 studied workers, we genotyped all remaining workers (median, Q1, Q3; QR: 106, 62, 162 workers; QL: 45, 29, 61) from eleven of these field colonies (six QR, five QL) at loci with diagnostic value (loci 3–5).

DNA was extracted using a CTAB method (modified from Sambrook & Russell, 2001). In total, we used seven microsatellite markers: (a) GT1 (Bourke, Green, & Bruford, 1997), (b) GT218 (Hamaguchi, Itô, & Tanaka, 1993), (3) 2MS17 (Suefuji, Trindl, & Heinze, 2011), (4) L5 (Foitzik, Haberl, Gadau, & Heinze, 1997), (5) L18 (Foitzik et al., 1997), (6) Ant3993 (Butler, Siletti, Oxley, & Kronauer, 2014) and (7) Ant11893 (Butler et al., 2014). The 10 µl PCR reaction volume consisted of 5 µl Taq DNA polymerase, 3 µl ddH₂O, 0.5 µl unlabelled reverse primer, 0.5 µl labelled 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 s), 57°C (annealing, 80 s) and 72°C (elongation, 25 s), 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 s, and annealing temperature was decreased to 54°C.

We mixed 0.07–0.2 µl of the PCR product (depending on DNA quantity) with 25 µl formamide and 0.1 µl T486 size standard, and analysed it in an ABI PRISM 310 Genetic Analyser (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). Because PCR failed or had unclear results, genotypic information is not available for all individuals at all loci.

2.3 | Data analysis

For each queenless colony, we reconstructed the 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 (all genotypes are accessible at DOI: <https://doi.org/10.5283/epub.43512>). Workers and males were defined as alien individuals when their alleles did not match the determined maternal or paternal alleles of the colony 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 analysed 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 least six of the seven studied loci.
2. Grandsons of the queen, that is 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 with 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.

In addition, we used GENALEX 6.5 (Peakall & Smouse, 2006, 2012) to estimate pairwise queen-worker, queen-male, and worker-worker relatedness for a subset of 15 laboratory colonies (15 queens, 176 workers, 160 males), for which the genotypes were most complete. Worker-male relatedness could be determined only in six colonies (10–12 workers and males each from six colonies, in total 71 workers and 69 males; data are accessible at DOI: <https://doi.org/10.5283/epub.43512>). The mean values for pairwise relatedness across all colonies and their standard errors were obtained by bootstrapping over the colony means (999 replicates) using the respective module at wessa.net (Wessa, 2020).

Data were analysed using R 3.2.3 software (R Development Core Team, 2008). Values are given as median and 1st (Q1) and 3rd (Q3) quantiles. Data of eggs per worker were not normally distributed and

were analysed with a generalized linear model using a gamma distribution (“LME4” package; Bates, Mächler, Bolker, & Walker, 2015). The residuals were checked using the DHARMA package (Hartig, 2019). We included the mean number of eggs per worker as dependent variable and queen presence, worker number and the proportion of reproductive workers as independent variables (in mentioned order). The colony was included as a random factor.

The explored statistical model was as follows:

$$\text{Eggsperworker} \sim \text{queenpresence} + \text{workernumber} \\ + \text{proportionofreproductiveworkers} + (1|\text{colony}).$$

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 & Hochberg, 1995).

3 | RESULTS

3.1 | Reproduction in field colonies

About 30% of the collected colonies were queenless (QL; spring 2016: 27%, 116/436; summer 2016: 33%, 80/246 colonies). The percentage of queenless colonies did not differ between the sampling dates ($\chi^2 = 2.69$, $df = 1$, $p = .101$). A minority of the queenright (QR) colonies contained two queens (early spring 2016: 12%, 37/320; summer 2016: 3%, 5/166). Previous dissection results from colonies collected around Regensburg (e.g., Strätz & Heinze, 2004) had shown that unmated female sexuals may occasionally hibernate in their natal nests but later are expelled. This might explain the significant decrease of the percentage of colonies with multiple queens from spring to summer ($\chi^2 = 10.12$, $df = 1$, $p < .002$).

When collected, QL colonies contained on average fewer workers and brood than QR colonies in spring and summer 2016 (Table 1). The proportions of female sexuals, males, and new workers varied

among colonies: half of all field colonies (50%, 123/246) contained both female and male sexual offspring, approximately one fifth of the colonies each had exclusively either male (17%, 43/246) or sexual female offspring (20%, 47/246), and 13% had only new workers (10%, 24/246) or no pupae at all (3%, 9/246). Regarding only those colonies that contained pupae, the differences were independent of queen presence ($\chi^2 = 6.2$, $df = 3$, $p = .1$).

Similarly, the total number of sexual offspring produced per field colony in summer 2016 and the proportion of male offspring did not differ between QL and QR colonies (Table 1). The proportion of males decreased significantly with increasing colony size in QR colonies (Spearman rank correlation: $r_s = -.21$, $p = .016$), but not so in QL colonies ($r_s = -.04$, $p = .726$), that is, larger QR colonies invested preferentially in female sexuals and smaller QR colonies in the cheaper male sexuals. In QL field colonies the number of female sexuals was limited regardless of colony size, presumably due to the lack of fertilized eggs.

The dissection of 36 queens and 769 workers from 64 field colonies (12 workers per colony, 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, that is, 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: $W = 276$, $p = .002$). Half of the workers had active ovaries (median, Q1, Q3; QL: 50%, 29%, 58%; QR: 50%, 25%, 58%, Figure 1a) and neither the proportion of reproductive workers ($W = 507$, $p = .923$) nor the mean number of developing eggs in worker ovaries ($W = 491$, $p = .908$; median, Q1, Q3; QL: 0.92, 0.54, 1.3; QR: 1.0, 0.42, 1.25, Figure 1b) differed between QL and QR colonies. Furthermore, there was no difference in egg numbers ($W = 871$, $p = .563$) or in the proportion of workers with active ovaries ($W = 999$, $p = .628$) between workers related and not related to the queen (see below).

Dissections of all workers from additional 10 queenright and 11 queenless field colonies from summer 2016 corroborated these findings: 258 of 530 workers from QL colonies and 382 of 803 workers

TABLE 1 Worker number and offspring number in natural queenless and queenright *Temnothorax crassispinus* colonies collected in spring and summer 2016

	Number of workers median (Q1, Q3)		Number of brood items median (Q1, Q3)		Number of sexuals median (Q1, Q3)		Number of males/total sexual offspring median (Q1, Q3)	
	QL	QR	QL	QR	QL	QR	QL	QR
Spring	70 (21, 120)	160 (100, 220)	50 (8, 250)	150 (80, 250)				
	$W = 8,806$ $p < .0001$		$W = 6,567$ $p < .0001$					
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)
	$W = 3,632$ $p < .0001$		$W = 3,294$ $p < .0001$		$W = 6,285$ $p = .618$		$W = 4,497$ $p = .815$	

Note: Queenright colonies were larger and contained more brood items independently of season, but the sex ratio did not differ between queenless and queenright colonies.

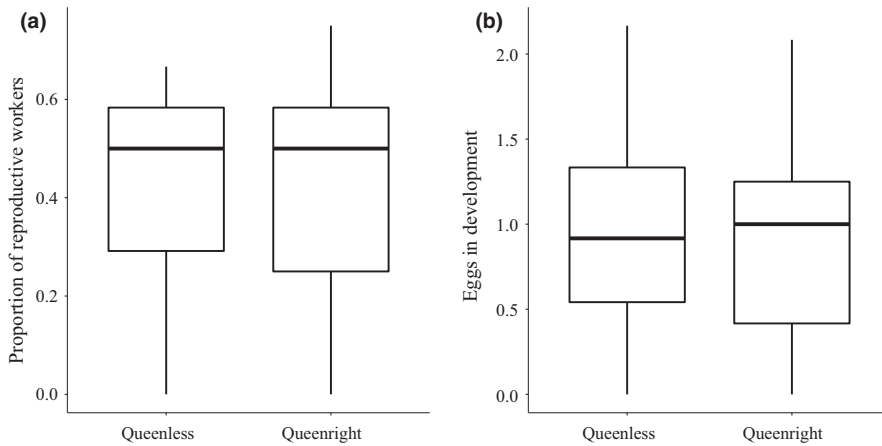


FIGURE 1 Number of reproductive workers (a) and number of eggs that are in development (b) in workers from queenless and queenright *Temnothorax crassispinus* colonies. Workers were equally capable of activating their ovaries in queenless and queenright and workers from both groups had on average one egg in development. Boxplots show median, 25 and 75 quartiles and 95% percentiles

from QR colonies had at least one egg in development ($\chi^2 = 0.16$, $p = .692$; see also El-Shehaby et al., 2012). Individual worker fecundity (mean number of eggs per worker) increased with the proportion of reproductive workers independently of queen presence or worker number (generalized linear model: queen: estimate = -0.07 , $t = 13.72$, $p = .644$; worker number: estimate = -0.04 , $t = -0.55$, $p = .581$, proportion reproductive workers: estimate = -0.3 , $t = -4.30$, $p < .0001$).

3.2 | Genetic colony composition and maternity of males in field 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 QR field colonies (27%) from summer 2016, the genotypes of the analysed queens were not compatible with those of the present workers. 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 closely 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: 0.1%, 0%, 17%). 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 12.5%, 0%, 25%).

In accordance with the dissection results, which suggested considerable worker reproduction, microsatellite analyses revealed that more than half of the QR colonies collected in spring (67%, 12/18) and summer 2016 (57%, 17/30, $\chi^2 = 0.47$, $p = .493$) 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: $W = 699$, $p = .03$; absolute numbers: spring 2016: 35/206 males; summer 2016: 112/360, $\chi^2 = 13.6$, $df = 1$, $p = .0002$). Only very few males shared the parental allele at each locus with

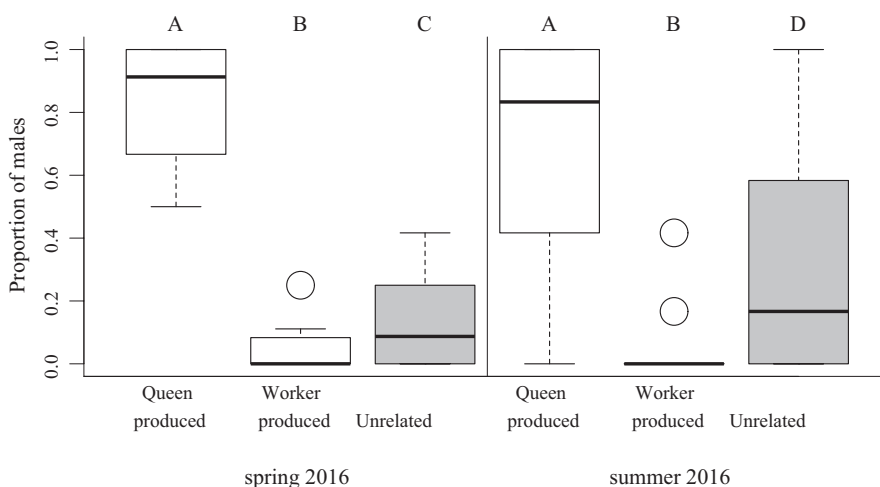


FIGURE 2 Proportion of males, which were produced by the queen, by workers related to the queen or by unrelated workers in queenright *Temnothorax crassispinus* colonies collected in spring (left) and summer 2016 (right). Boxplots show median, 25 and 75 quartile and 95% percentiles and outliers. Pairwise comparisons (Mann–Whitney U -test) among seasons and groups significantly different at $p < .05$ (adjusted following Benjamini & Hochberg, 1995) are displayed by different letters

workers related to the present queen and thus 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). 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). Similarly, 89% (25/28) of the QL field 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.

In queenright field colonies, the number of alien males per present alien worker (2:1) was significantly higher (Kruskal–Wallis test: $X^2 = 80.06$, $df = 2$, $p < .0001$) than the number of males produced by the queen (0.86:1, $p = .022$) or per worker related to the queen (0.06:1; $p < .0001$; males produced by queen-related workers vs. queen-produced males $p < .0001$).

As genotyping only 12 workers per colony might have missed potential mothers of alien males we genotyped all the workers from eleven field colonies. Of those males whose genotypes had not matched the majority of the 12 previously genotyped workers, 23 from eight colonies could be assigned to workers produced by the queen, and 20 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: 23%, 21%, 29%). 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%) analysed males (median, Q1, Q3 per colony: 3, 2, 8) did not match any worker genotype on at least one of three analysed 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.

The relatedness among nestmate workers in 15 colonies ranged from 0.57 to 0.86 (mean $0.685 \pm SE 0.011$) and was significantly lower than the value 0.75 expected for full sisters (one sample t -test, $t = 6.023$, $p < .001$). Reflecting the large variation in the number of alien individuals in the colonies, queen-worker and queen-male relatedness in a sample of 15 colonies ranged from -0.09 to 0.70 (mean 0.351 ± 0.065) and from -0.029 to 0.543 (0.283 ± 0.050), respectively. Within colonies, the relatedness of workers to males

ranged from 0 to 0.5 (average across six colonies 0.223, range 0.121–0.390).

3.3 | Maternity of males in laboratory colonies

Compared to field 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, $X^2 = 5.76$, $df = 1$, $p = .016$), and about half of the males (median, Q1, Q3; 50%, 17%, 73% per colony) were not sons of the present queen (Figure 3). Whereas in field 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%, 65%; compared to a median of 0% in field colonies, $W = 354$, $p < .0001$, Figure 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 field colonies (median, Q1, Q3; 0%, 0%, 17% compared to a median of 8% in field colonies; $W = 330$, $p = .178$). 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%; $W = 138$, $p = .645$).

The increased worker reproduction in laboratory colonies caused a shift in the proportion of males among all produced sexuals (Kruskal–Wallis test: $X^2 = 32.1$, $df = 2$, $p < .0001$). 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%, $p = .92$), QR laboratory colonies produced

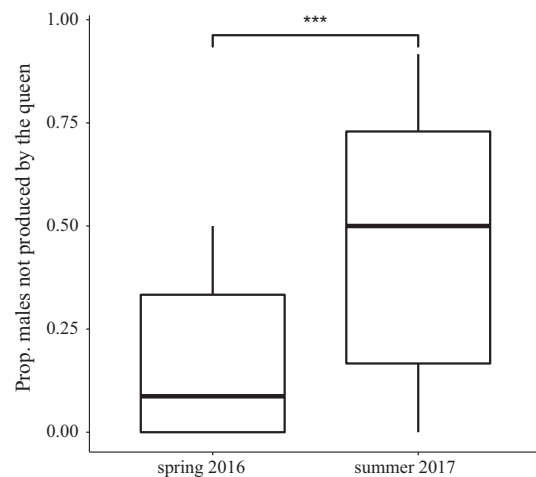


FIGURE 3 Proportion of males, which were not produced by the resident queen, eclosing in 2016 and 2017 in queenright *Temnothorax crassispinus* colonies. The proportion of males produced by other females than the queen increased significantly after one year in semi-natural conditions (median, Q1, Q3; summer 2017:50%, 17%, 75%; spring 2016: 9%, 0%, 33%; $W = 405$, $p = .0004$). Boxplots show median, 25 and 75 quartile and 95th percentiles (** $p < .001$)

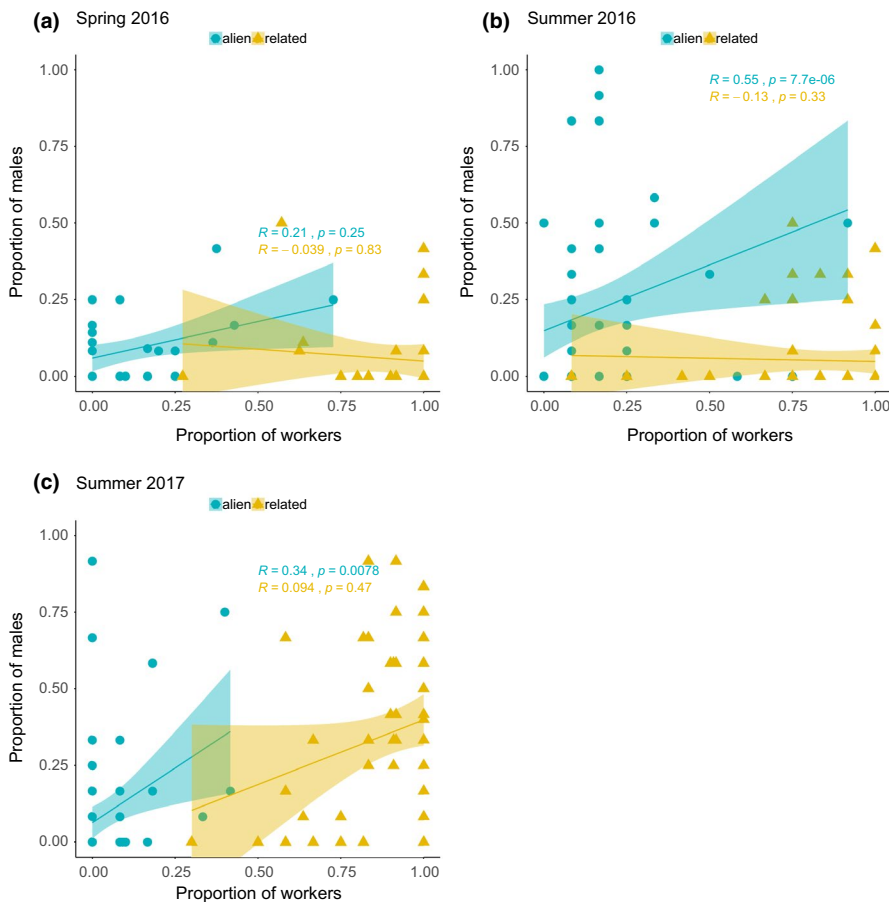


FIGURE 4 Correlation between the proportion of *Temnothorax crassispinus* males and workers that were either related (yellow) or not related (blue, “alien”) with the queen (queenright colonies) or the majority of nestmate workers (queenless colonies). The number of males that were produced by workers, which were related with the queen, increased under laboratory conditions in summer 2017 (see Section 3) [Colour figure can be viewed at wileyonlinelibrary.com]

significantly more males in 2017 (median, Q1, Q3; 88%, 74%, 95%; vs. field colonies 2016: $p < .0001$, vs. laboratory colonies 2016: $p < .0001$).

4 | DISCUSSION

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, that is offspring of the queen's workers, or produced by unrelated individuals. Our study therefore reveals considerable direct fitness benefits for workers of this species, which appears to be even more pronounced in the case of reproducing workers unrelated to the queen.

The genotypes of queens and workers from queenright field colonies suggest a rather instable structure of the studied *T. crassispinus* population, with frequent queen turnovers, colony usurpations, and fusions, similar to what has been observed in its sibling species *T. nylanderii* (Foitzik & Heinze, 1998, 2000; Foitzik, Sturm, Pusch, d’Ettorre, & Heinze, 2007; Strätz & Heinze, 2004). Furthermore, queenless field colonies might have lost their queen or be fragments of queenright colonies (Cao, 2013; Debout et al., 2007; Smith et al., 2012; Strätz & Heinze, 2004). This population fluidity appears to result from high queen mortality (J. Giehr, unpublished), very high nest densities, the fragility of nest sites, and presumably also a

nestmate recognition system strongly based on environmental cues (Foitzik et al., 2007; Heinze, Foitzik, Hippert, & Hölldobler, 1996). In field 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 reunite for hibernation (Giehr, Senninger, Ruhland, & Heinze, 2020).

Queen turnover 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 (J. Giehr personal observation; Plateaux, 1986; Sendova-Franks & 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 neighbouring nests, as occurs in bees (Beekman & Oldroyd, 2008; O’Connor, Park, & Goulson, 2013; Pearson, Raybould, & Clarke, 1997; Tallamy, 2005). As fertile queens do not leave the nest (e.g., Giehr & Heinze, 2018), it is unlikely that individuals with aberrant genotypes are offspring of “drifting queens.”

Finally, brood raiding, as observed in related social parasites (Buschinger, Ehrhardt, & Winter, 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 those males, which were not offspring of the present queen, 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 field colonies had active ovaries, independently of queen presence and relatedness to the colony (see also El-Shehaby et al., 2012). Second, the origin of males in laboratory colonies, which in our study were kept isolated from other colonies, clearly showed that also workers related to the present queen readily lay eggs.

This reveals a high realized reproductive potential of *T. crassispinus* workers compared to other social insect species (ants: Helanterä & Sundström, 2007b; Smith et al., 2012; Walin et al., 1998; honey bees and wasps: Foster, Ratnieks, Gyllenstrand, & Thorén, 2001; Ratnieks, 1993) including many congeneric species, in which the development of worker-produced eggs is prevented by worker policing or queen pheromones (Helanterä & Sundström, 2007a; Stroeymeyt et al., 2007; Walin et al., 1998; Walter et al., 2011). Previous observations had shown that workers interact aggressively by antennal boxing and biting in both queenless and queenright colonies (El-Shehaby et al., 2012). Hence, it appears that workers compete for egg-laying opportunities and form social and reproductive dominance hierarchies, similar to what has been observed in other species of this genus (Cole, 1981; Franks & Scovell, 1983; Heinze, Puchinger, & Hölldobler, 1997; Walter et al., 2011). The fluid colony structure in the study population, with colony mergers and usurpations leading to unpredictable variation of the relatedness between workers and the brood they care for, might increase worker selfishness. In addition, frequent queenlessness might also select for worker reproduction (Nanork et al., 2007). Balanced sex ratios in field colonies indicate that worker reproduction is not associated with high costs, but the strong increase of male bias under laboratory conditions might indicate negative effects under artificial conditions: egg-laying workers are known to work less and to refrain from costly tasks (e.g., Barth et al., 2010; Bourke, 1988a; Charbonneau & Dornhaus, 2015). This, and in particular reproduction by alien workers, should select for worker policing (Ratnieks, 1988), which, however, appears to be not very efficient in the studied colonies.

Of particular interest is the apparent higher direct fitness of alien workers. In bees, drifted workers engage less in brood care (Pfeiffer & Crailsheim, 1999) and have a higher reproductive potential by escaping queen control and worker policing (Birmingham, Hoover, Winston, & Ydenberg, 2004; Lopez-Vaamonde et al., 2004; Nanork et al., 2007; Nanork, Paar, Chapman, Wongsiri, & Oldroyd, 2005; O'Connor et al., 2013).

Though the Western sibling species *T. nylanderii* shows a similar colony and population structure as *T. crassispinus* (Foitzik & Heinze, 1998, 2000, 2001; Hammond & Keller, 2004), reanalysing data from Foitzik and Heinze (2001), estimated worker-male

contribution in queenright colonies to be less than 10%. Similarly, workers appear to only infrequently succeed in producing males in queenright colonies of other non-parasitic species of this genus (e.g., Brunner & Heinze, 2009; Brunner, Kroiss, Trindl, & Heinze, 2011; Heinze et al., 1997; Stroeymeyt et al., 2007).

In summary, our data show that *T. crassispinus* workers are capable of raising own sons and gaining considerable direct fitness in field colonies. Assuming that approximately 25%–30% of the males in natural populations are offspring of 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 0.1% 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., Bourke, 1988a; Cole, 1981; Franks & Scovell, 1983; Heinze et al., 1997; Stroeymeyt et al., 2007).

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AUTHOR CONTRIBUTIONS

J.G. and J.H. designed the study; J.G., J.W., L.S., K.R. and T.K. performed the experiments; J.G. and J.H. analysed the data; and J.G. and J.H. interpreted the data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

Raw data, individual genotypes and detailed pairwise relatedness analysis are available at <https://epub.uni-regensburg.de/43512/> (DOI: <https://doi.org/10.5283/epub.43512>; Giehr, Wallner, et al., 2020).

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