

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

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Abstract

Workers of many species of social Hymenoptera have functional ovaries and are capable of laying haploid, unfertilized eggs, at least in the absence of a queen. Except for honeybees, it remains largely unknown whether worker-produced males have the same quality as queen-produced males and whether workers benefit in direct fitness by producing their sons. Previous studies in the monogynous ant *Temnothorax crassispinus* revealed that a high proportion of males in natural and laboratory colonies are worker offspring. Here, we compare longevity, body size, sperm length and sperm viability between queen- and worker-produced males. We either split queenright colonies into queenright and queenless halves or removed the queen from a fraction of the queenright colonies and then examined the newly produced males. Male quality traits varied considerably among colonies but differed only slightly between queen- and worker-produced males. Worker-produced males outnumbered queen-produced males and also had a longer lifespan, but under certain rearing conditions sperm from queen-produced males had a higher viability.

KEYWORDS

ants, male quality, sperm quality, *Temnothorax*, worker reproduction

1 | INTRODUCTION

The ecological success of social Hymenoptera (ants, bees and wasps) strongly depends on their efficient division of reproductive labour between egg-laying queens and nonlaying workers. The evolutionary stability of worker altruism is explained by Hamilton's theory of inclusive fitness, according to which helpers can gain indirect fitness by boosting the reproductive output of a related beneficiary of their support instead of or in addition to obtaining direct fitness from their own offspring (Hamilton, 1964). While previous research in social insects has focused on the indirect fitness component of workers, recent studies in paper wasps and, outside the Hymenoptera, in wood-dwelling termites show that helpers may gain considerable direct fitness by

sneaking their own eggs onto the egg pile or later inheriting the nest (Korb & Thorne, 2017; Leadbeater, Carruthers, Green, Rosser, & Field, 2011). Understanding the contribution of both fitness might help to better understand the evolution of social life histories and to separate different 'levels' of social complexity (Korb & Heinze, 2016).

In highly eusocial Hymenoptera, workers generally do not mate, but in most species, they are capable of producing haploid male offspring from unfertilized eggs. Because egg-laying workers engage less in foraging, nest defence and maintenance, worker reproduction is costly to the colony (Bourke, 1988a; Dampney, Barron, & Oldroyd, 2004; Gobin, Heinze, Strätz, & Roces, 2003; Tsuji, Kikuta, & Kikuchi, 2012). Worker reproduction is therefore typically prevented by queen pheromones or punished by aggression or eating

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of worker-laid eggs (Ratnieks, 1988). Consequently, workers rarely lay eggs in the presence of the queen but they often do so in queenless colonies (Bourke, 1988b; Choe, 1988; Wenseleers & Ratnieks, 2006). How strongly this translates into direct fitness of workers does not only depend on the frequency of worker egg laying but also the hatchability of their eggs and the reproductive performance of their sons relative to the males produced by the queen. The viability and hatchability of worker-laid eggs appear to be impaired in a number of species (Khila & Abouheif, 2008; Pirk, Neumann, Hepburn, Moritz, & Tautz, 2004), and worker-produced males of honeybees are smaller and less heavy than queen-produced males (Berg, Koeniger, Koeniger, & Fuchs, 1997; Gençer & Firatli, 2005; Utaipanon, Holmes, Chapman, & Oldroyd, 2019). However, in leaf-cutter ants, male body weight rather depends on colony conditions than on origin (Dijkstra & Boomsma, 2007), but little is known about differences in other physiological traits, for example sperm quality.

Ants of the genus *Temnothorax* readily lay unfertilized eggs and rear males in the absence of the queen and occasionally also in queenright conditions (Cole, 1981; El-Shehaby, Abd-el Reheem, & Heinze, 2012; Heinze, Puchinger, & Hölldobler, 1997). Here, we examine whether queen- and worker-produced males of the monogynous ant *Temnothorax crassispinus* (Karavaiev 1926) differ in physiological traits. We either split natural queenright colonies into queenright and queenless parts or removed the queen from recently collected colonies. Colonies were allowed to reproduce until the eclosion of the first males. We compared lifespan, body size, sperm length and sperm viability between worker- and queen-produced males. Variation in sperm size and viability within and between males is strongly affected by selection pressure (Fitzpatrick & Baer, 2011; Gomendio & Roldan, 1991; Hunter and Birkhead, 2002; Koffler, Meneses, Kleinert, & Jaffé, 2016), and sperm morphology might be crucial to sustain the queen with a lifelong sperm supply and to enable a sufficient colony growth.

Our study shows that workers produce highly viable males. Queen- and worker-produced males were of similar quality. Sperm traits differed strongly among colonies irrespective of queen presence. Worker-produced males lived longer than males from queenright colonies but under natural climatic conditions had less viable sperm.

2 | MATERIAL AND METHODS

Temnothorax crassispinus is a small (approximately 2 to 3 mm) monogynous, monandrous ant, whose colonies consist of up to 300 workers (Strätz & Heinze, 2004). Like in other *Temnothorax* ants, workers are capable of activating their ovaries and producing haploid offspring (El-Shehaby et al., 2012; Pusch, Heinze, & Foitzik, 2006). We compared male traits between experimental queenright (QR) and queenless (QL) colonies in two different experiments. Not all traits could be studied in both experiments.

Experiment 1 was conducted to determine male quality in colonies that were kept as naturally as possible. We used 19 queenright colonies that had been collected in March 2016 near Regensburg, Germany. Potential nest sites (decaying twigs etc.) were carefully

opened and, if they contained *T. crassispinus*, placed into sealable plastic bags, brought to the laboratory and only there removed from their natural nest to avoid the loss of individuals in the field. After counting all adults and brood, we transferred the colonies into artificial nest boxes (10 × 10 × 3 cm³). In ten colonies, we removed the queens before the appearance of the first eggs (four to five weeks after collection) 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 with honey and cockroaches or *Drosophila* twice per week in summer and fall and once every two weeks in winter. To obtain adult males of standardized age, in July 2017 we separated 10 workers and 20 male pupae of comparable developmental stage from nine queenright and ten queenless colonies from the rest of their colonies and checked them daily until the first males had hatched. The experiment started when more than three males had eclosed on the same day and in the same colony fragment. All additional males were removed to avoid an influence of male age differences in the comparisons. In total, 68 males from queenright (mean ± SD: 80.0 ± 21.7 workers) and 69 males from queenless colonies (mean ± SD: 61.6 ± 23.9 workers) were used for the analysis of male longevity (32 QR, 35 QL), male motility (23 QR, 18 QL) and sperm length and viability (13 QR, 16 QL).

In experiment 2, we controlled for any climatic or colony-specific effects to compare male traits primarily by origin. For experiment 2, we split eight colonies collected in September 2017 into standardized queenright and queenless parts, each with 25 workers, 25 larvae and a queen in queenright parts. 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-hr 26°C/12-hr 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 until they reached summer conditions at the end of June 2018. The seasonal cycles were repeated until the first males had eclosed in July 2019, as larval development seemed to be delayed and only few larvae developed into workers after the first hibernation. Sperm viability and length were analysed in 5-day-old males as described below. In total, we analysed sperm cells of 30 males from six queenless colonies (mean ± SD: 30.4 ± 25.3 workers) and 17 males from six queenright colony fragments (mean ± SD: 34.2 ± 28.8 workers) (two queenless and four queenright colony parts did not produce male offspring and had to be excluded from male analysis).

2.1 | Colony productivity, developmental time, male longevity, body size and mobility

In experiment 1, we analysed the lifespan of 32 adult males from five queenright colonies and 35 males from six queenless colonies. Colonies were checked daily until the last male had died. Missing males were excluded from the analysis. Dead males were stored

at -20°C for subsequent measurements of thorax width using a Keyence VHX-500FD digital microscope.

To determine male mobility, we videotaped the motion of 23 males from six queenright colonies and 18 males from four queenless colonies from experiment 1 for 10 min each. The motility of males was videotaped 12 days after eclosion in a circular arena (diameter 1.1 cm). The recorded videos were analysed by EthoVision XT 10 software (Noldus, Wageningen).

In experiment 2, we analysed the developmental time of male larvae from hibernation to the eclosion of the first adults in six queenless and four queenright colonies (two queenright colonies did not produce males) and counted the number of produced males.

2.2 | Sperm viability and length

We dissected the seminal vesicles of 13 males from five queenright colonies and 16 males from four queenless colonies from experiment 1 and 17 males from six queenright and 30 males from six queenless fragments from experiment 2 four to five days after emergence. We opened the seminal vesicles and accessory glands in Beadle solution (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM CaCl_2 , Ephrussi & Beadle, 1936) and mixed the fluid with 10 μl SYBR Green (Thermo Fisher). After 10 min, we added 2 μl of propidium iodide and incubated the sample for 7 min before analysing the preparations under a fluorescence microscope (Zeiss, magnification 40x). Dead sperm cells fluoresced red and 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

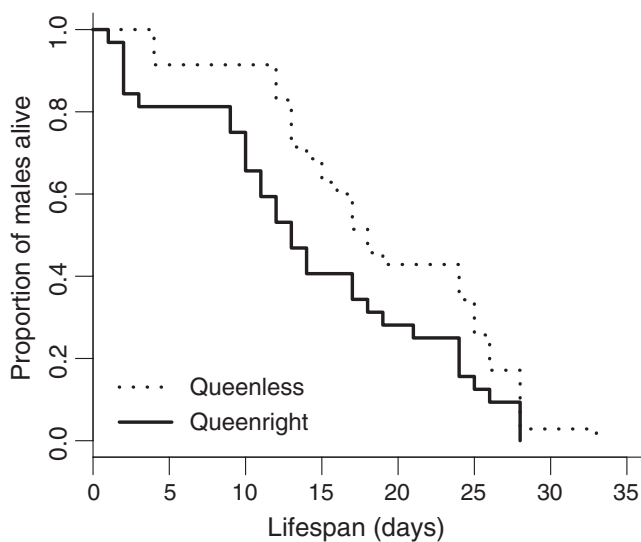


FIGURE 1 Lifespan of *T. crassispinus* males produced in queenless and queenright colonies. Males from queenless colonies appeared to live significantly longer than males from queenright colonies (Kaplan–Meier survival analysis: $\chi^2_1 = 4.3$, $p = .04$), but this effect disappeared when we controlled for colony (linear mixed model: queen presence: $t_9 = -0.926$, $p = .379$, colony included as random factor)

using the software Digivision Pro 20.10.100. To estimate the general measuring error, one sperm was measured ten times in a row.

2.3 | Data analysis

Data were analysed using R 3.5.1 (R Development Core Team, 2008). Nonparametric data (Shapiro–Wilk test, $p < .05$) were analysed using Mann–Whitney U test, Kruskal–Wallis test and generalized linear models (lme4 package, Bates, Mächler, Bolker, & Walker, 2015). Parametric data (Shapiro–Wilk test, $p > .05$) were analysed using linear mixed models with colony ID as random factor ('nlme' package, Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2018). Pairwise Mann–Whitney U test were corrected for a false discovery rate ('fdr', Benjamini & Hochberg, 1995). The coefficient of variation was calculated as $cv = \sigma/\mu$ (σ : standard deviation, μ : mean). Nonparametric data are given as median, 1st quartile (Q1) and 3rd quartile (Q3), parametric data as mean and standard deviation (mean \pm sd).

3 | RESULTS

3.1 | Colony productivity, developmental time, male longevity, body size and mobility

Queenless colony fragments in experiment 2 produced significantly more males (number of males produced, median, Q1, Q3: 32, 16, 52) than queenright fragments of the same source colony (2, 0.8, 4; linear mixed model, queen presence: $t_{37} = -3.15$, $p = .003$; day hatched: $t_{37} = 0.653$, $p = .518$; colony included as random factor). Queenright fragments appeared to focus instead on the production of new workers (worker pupae: 57, 35, 79; only two colonies produced one female sexual each; no worker pupae in queenless colonies). 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 (days from end of hibernation to hatching, median, Q1, Q3; 57, 55, 59) and queenright colonies (61, 56, 63; linear mixed model, queen presence: $t_{37} = 0.21$, $p = .832$; number of males hatched: $t_{37} = 0.63$, $p = .536$; colony included as random factor).

Male survival in experiment 1 was positively correlated with worker number after colony collection (Spearman rank correlation, $r_5 = .457$, $p = .0001$), and males from queenless and queenright colonies did not differ in lifespan (lifespan (days) median, Q1, Q3; queenless: 18, 13, 26; queenright: 13, 10, 19; linear mixed model: queen presence: $t_9 = -0.926$, $p = .379$, colony included as random factor, Figure 1). Males from queenless ($n = 15$, four colonies) and queenright colonies ($n = 9$, four colonies) did not differ in size (thorax width, mean \pm SD, queenless: $540 \pm 42 \mu\text{m}$; queenright: $544 \pm 42 \mu\text{m}$; linear mixed model, queen presence: $t_5 = 0.72$, $p = .502$; worker number: $t_5 = -0.099$, $p = .364$, colony included as random factor). Similarly, the activity patterns did not differ between queenless and queenright males. Males from queenless and queenright colonies covered

the same distance in 10 min (mean \pm SD QL: 65.7 \pm 44.1 cm; QR: 69.1 \pm 71.6 cm) and spent the same time without motion (mean \pm SD QL: 533.9 \pm 45.0 s; QR: 531.9 \pm 70.0 s) (total distance covered: linear mixed model, queen presence: $t_8 = -0.22$, $p = .834$; time spent immobile: linear mixed model, queen presence: $t_8 = 0.25$, $p = .811$, colony included as random factor).

3.2 | Sperm length and viability

Mean sperm length and the variance of sperm length did not differ between experiment and queen presence (linear mixed model, queen presence: $t_{63} = -0.14$, $p = .900$; experiment: $t_{13} = -1.73$, $p = .108$; worker number: $t_{13} = -1.62$, $p = .128$, colony included as random factor, Figure 2). Sperm length was not correlated with thorax width (experiment 1, Spearman rank correlation, $r_5 = .05$, $p = .821$).

The coefficient of variation in sperm length calculated for individual males differed between experiments and was more variable in natural climatic conditions (linear mixed model: queen presence: $t_{57} = 1.35$, $p = .182$; experiment: $t_{14} = -2.45$, $p = .028$; sperm length: $t_{57} = -4.18$, $p = .0001$; see Table 1). The variation in sperm length decreased with increasing sperm length (Spearman's rank correlation; experiment 1: $r_5 = -.51$, $p = .006$; experiment 2: $r_5 = -.44$, $p = .002$), that is, males producing longer sperm also produced sperm of a more homogeneous length.

Across the whole sample, sperm viability was affected by queen presence and sperm length (generalized linear mixed model: queen presence: estimate = 0.21, $z = 5.37$, $p < .0001$; experiment: estimate = 0.29, $z = 0.38$, $p = .708$; worker number: estimate = 0.57, $z = 0.87$, $p = .386$; sperm length: estimate = 0.19, $z = 5.25$, $p < .0001$; colony included as random factor; see Figure 3). Males from queenright colonies reared under natural climatic conditions (experiment 1) had a higher sperm viability than similarly reared males from queenless colonies (Kruskal-Wallis test: $X^2_3 = 14.9$, $p = .002$; pairwise Mann-Whitney U test: $p = .007$, Figure 4). Sperm viability of males from queenright colonies from experiment 1 was also higher compared to that of males from queenright ($p = .002$) and queenless ($p = .002$) colonies from experiment 2. The interaction between the proportion of live sperm and sperm length varied between queenright

and queenless colonies and also between experiments. In queenright colonies from experiment 2, sperm with intermediate length was significantly more viable than small ($p = .024$) or large sperm ($p = .030$) (Kruskal-Wallis test: $X^2_2 = 7.34$, $p = .025$; small sperm: $< Q1$ (36.00 μm), medium sperm: $\geq Q1$ and $< Q3$ (43.40 μm), large sperm: $\geq Q3$, queenless and experiment 1 not significant). Sperm viability was also positively correlated with male thorax width (experiment 1, Spearman's rank correlation: $S = 1,266.7$, $r_5 = .45$, $p = .028$).

4 | DISCUSSION

The degree of worker altruism differs considerably among insect societies, from no help in the drywood termite *Cryptotermes secundus* (Korb, 2017) to extreme self-sacrifice in stinging honeybees and exploding *Camponotus* workers (Shorter & Rueppell, 2012) and workers that completely lack ovaries in a number of ant genera (e.g. Downing, Cornwallis, & Griffin, 2017). A better understanding of this variation in social complexity requires an estimate of how strongly direct fitness gains contribute to worker inclusive fitness. Workers of many social Hymenoptera can in principle increase their direct fitness by producing males from unfertilized eggs, at least in the absence of a reproductive queen (Bourke, 1988; Choe, 1988; Cole, 1986; El-Shehaby et al., 2012; Heinze et al., 1997; Holmes, Oldroyd, Duncan, Allsopp, & Beekman, 2013; O'Connor, Park, & Goulson, 2013; Visscher, 1989). However, whether worker-produced males have the same quality as queen-produced males and whether workers thus gain considerable direct fitness remains largely unknown in ants (Berg et al., 1997; Dijkstra & Boomsma, 2007; Gençer & Firatli, 2005; Utaipanon et al., 2019). Here, we document that queen- and worker-produced males are of comparable quality in *Temnothorax crassispinus* ants and that variation among males is more frequently determined by colony 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 in the same season. Queen- and worker-produced males did neither differ in motility nor in body size, as previously reported for leafcutter ants (Dijkstra & Boomsma, 2007, but see honey bees Berg et al., 1997; Gençer & Firatli, 2005).

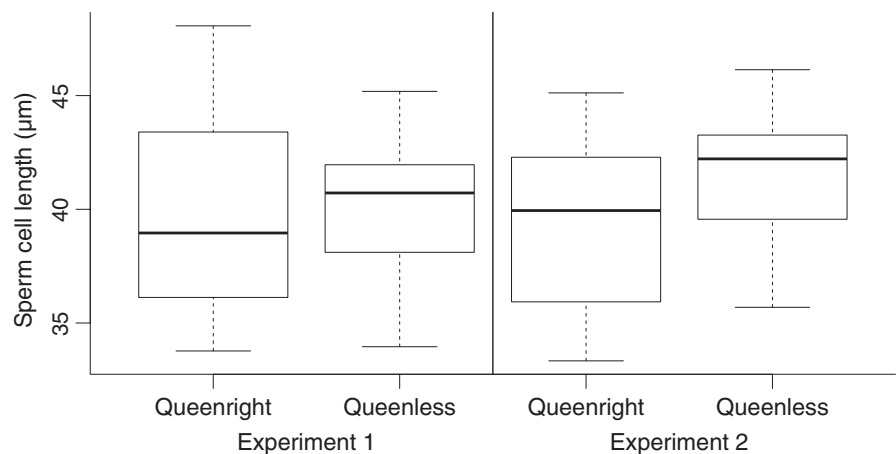


FIGURE 2 Sperm length (μm) of males from queenright and queenless colonies of the ant *Temnothorax crassispinus*. Length did not differ with queen presence and experiment (see results for details). Boxplots show medians, 25 and 75 quartiles, and 95% percentiles

TABLE 1 Sperm cell length, coefficient of variation and proportion of live sperm in males of the ant *Temnothorax crassispinus* produced in queenright and queenless colonies from experiment 1 and experiment 2 (for details see results)

	Experiment 1		Experiment 2	
	Queenright	Queenless	Queenright	Queenless
sperm cell length (μm) mean \pm sd	39.8 \pm 3.7	41.7 \pm 3.1	39.9 \pm 4.9	40.7 \pm 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

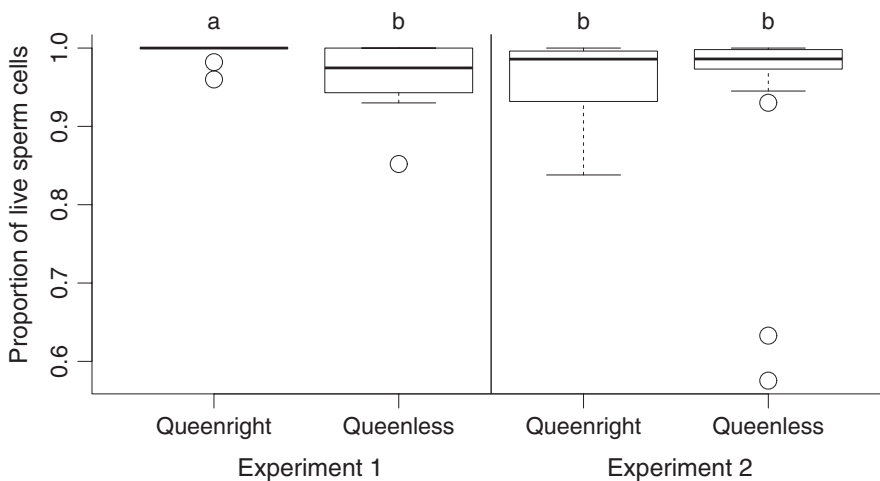


FIGURE 3 Proportion of live sperm cells in queenright and queenless colonies of the ant *Temnothorax crassispinus*. Viability was significantly higher in males from queenright colonies kept under natural climatic conditions (experiment 1). Boxplots show medians, 25 and 75 quartiles, and 95% percentiles and outliers. Pairwise comparisons among groups significantly different at $p < 0.05$, adjusted following Benjamini and Hochberg (1995), are displayed by different letters

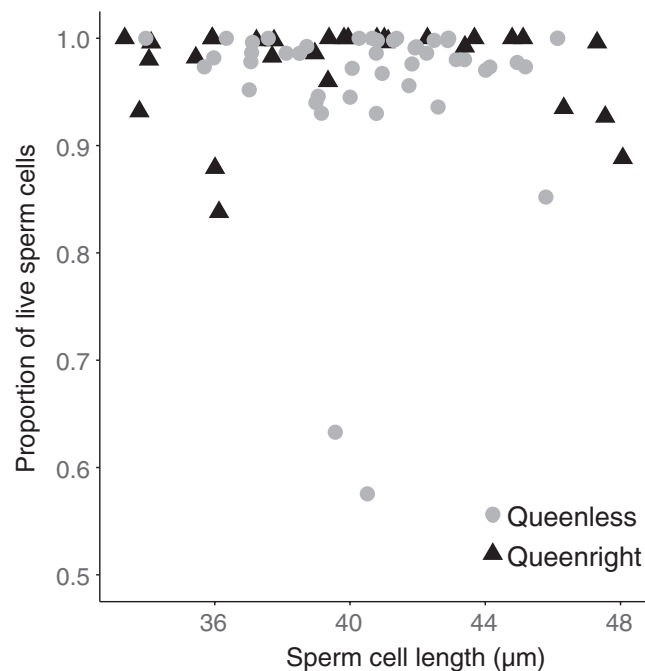


FIGURE 4 Proportion of live sperm cells in relation to sperm cell length in *Temnothorax crassispinus* males depending on queen presence. Sperm cell length strongly affected the proportion of live sperm

Both are important traits for the male's mating success (Abell, Cole, Reyes, & Wiernasz, 1999; Berg, 1991; Berg et al., 1997; Davidson, 1982; Gençer & Firatli, 2005; Gençer & Kahya, 2011; Wiernasz,

Sater, Abell, & Cole, 2001) as they determine a male's flight capability (Stone, Loder, & Blackburn, 1995) and reflect its nutritional status and quality (Fitzpatrick & Lüpold, 2014). The longer lifespan of males from larger colonies might also be based on a better nutritional status. This might increase the mating success also in males from large queenless colonies, as they might benefit from less competition at the end of the reproductive season (see also honey bees Gençer & Kahya, 2011; Page & Erickson, 1988).

Sperm length showed considerable variation between experiments. It did not differ between queen- and worker-produced males, but sperm length was more variable in natural climatic conditions, but decreases with increasing sperm length. Sperm traits of social Hymenoptera are shaped by storage constraints of the queen and the intensity of sperm competition, which may lead to opposite selection pressures. On the one hand, the limited capacity of the sperm storage organ of the queen might select against long sperm tails (Baer, Dijkstra, Mueller, Nash, & Boomsma, 2008). Queens mate early in life and thereafter use stored sperm to fertilize their eggs. On the other hand, sperm competition may affect sperm size and also lead to less variable sperm (Baer, Schmid-Hempel, Høeg, & Boomsma, 2003; Gomendio & Roldan, 1991; Parker, 1993; García-González & Simmons, 2007; Immler et al., 2011; Simmons & Fitzpatrick, 2012; Tourmente, Delbarco Trillo, & Roldan, 2015; but see Baer et al., 2008; Aron, Lybaert, Baudoux, Vandervelden, & Fournier, 2016). The degree of sperm competition is presumably rather low in singly mated *T. crassispinus* ants (Strätz & Heinze, 2004), and selection on sperm velocity (Baer

et al., 2003; Gomendio & Roldan, 1991) or the rapid filling of the spermatheca (Boomsma, Baer, & Heinze, 2005) should be negligible. This might explain the relatively large intra-male variation in sperm length compared to polyandrous social Hymenoptera (e.g. Fitzpatrick & Baer, 2011; Woyke, 1983).

As is typical for social Hymenoptera in which sperm is stored by the queen for years or even decades (Baer, 2011; Boomsma, 2009; Boomsma et al., 2005; Zareie, Eubel, Millar, & Baer, 2013), sperm viability was generally high and did not differ between males from related queenless and queenright colonies. Sperm of an intermediate length had the highest viability. A minimum size of sperm cells might be required to sufficiently supply sperm with energy (Fitzpatrick & Lüpold, 2014), but the production of long sperm cells is costly and might cause a trade-off with viability (Birkhead, Pellatt, Brekke, Yeates, & Castillo-Juarez, 2005; Evans, 2011; Fitzpatrick & Lüpold, 2014; Simmons & Moore, 2009), as reported for sperm length and number (LaMunyon & Ward, 1998; Parker, 1982; Pitnick, 1996). The positive association between thorax width and sperm viability might indicate that larger males could more easily bear the costs of sperm production, probably due to better nutritional condition (Fitzpatrick & Lüpold, 2014). Sperm viability was highest in queenright colonies reared under natural climatic conditions (experiment 1), that is, natural temperature cycles might be more beneficial for sperm development and storage than standardized laboratory conditions (experiment 2). The difference in sperm viability between queenless and similarly kept queenright colonies from experiment 1 remains unexplained. Finally, workers are generally highly productive in laboratory and we cannot exclude that some of the males in queenright colonies were produced by workers.

In summary, our study shows that worker-produced males are of similar quality as queen-produced males and did not differ from the latter in body size or motility. Sperm generally had a high viability, which matches the life history of social insects, in which sperm viability determines the lifetime reproductive success of males and females (Boomsma et al., 2005; Zareie et al., 2013). Sperm traits differed little between queen- and worker-produced males. Queen- and worker-produced males had a similar lifespan but worker-produced males outnumbered queen-produced males, which might be important at the end of the reproductive season. In consequence, workers may therefore gain considerable direct fitness by producing own male offspring.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

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

ETHICAL APPROVAL

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

DATA AVAILABILITY STATEMENT

The data sets of the article are available in Supplement S1.

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