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Determinants of worker reproduction in queenless colonies of the ant *Temnothorax crassispinus* (KARAVAIEV, 1926) (Hymenoptera: Formicidae)

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Abstract

Reproductive division of labor between queens and workers in insect societies often relies on a complex system of self-restraint and mutual policing. After queen loss, workers of many social insects quickly begin to produce their own sons from unfertilized eggs. In the ant *Temnothorax crassispinus* (KARAVAIEV, 1926), reproductive division of labor among workers in queenless colonies is maintained through the establishment of social hierarchies in which only top-ranking workers start to reproduce. Here, we investigate, which factors determine whether a worker becomes dominant or not and how many workers per colony lay eggs. Dissection of more than 3300 individuals from 44 colonies showed that workers with above-average mesosoma length and / or a higher number of ovarioles per ovary ("intercastes") tended to have better developed ovaries than other workers. The number of egg layers increased slightly with colony size, and up to seven workers per colony had elongated ovarioles with large, maturing oocytes.

Key words: Kin conflict, worker reproduction, hierarchy length, ovariole number.

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Introduction

The ecological success of social insects relies on their efficient division of labor. Queens monopolize reproduction more or less completely, while the workers, though often capable of laying eggs, refrain from doing so. Instead, they nurse the brood, defend the nest, and forage for food (BOURKE & FRANKS 1995). This regulation of reproduction is often maintained by self-restraint and mutual policing (HEINZE 2004, WENSELEERS & RATNIEKS 2006).

Once the queen has died, workers of most species will quickly begin to produce haploid males from unfertilized eggs (e.g., BOURKE 1988). Queen loss does not lead to complete chaos. Instead, in particular in smaller colonies division of labor is maintained by the formation of rank orders among workers. Only several high-ranking workers pursue their reproductive interests, while medium- and low-ranking workers continue to engage in non-reproductive tasks (e.g., HEINZE & al. 1997). The number of egg layers per colony likely reflects a compromise between the inclusive fitness interests of individual workers, which depend on the relatedness among workers and the costs and benefits associated with worker reproduction (e.g., WENSELEERS & al. 2004, MOLET & al. 2005).

Comparatively little is known about which and how many workers begin to reproduce. There is some evidence that body size and the structure of a worker's ovary, in particular the number of its ovarioles, play important roles in determining who becomes reproductive and who does not: Larger workers or workers with an above-average ovariole number appear to be more likely to lay eggs than others (DARTIGUES & PASSERA 1979, HEINZE & al. 1997, GOBIN & ITO 2003, SMITH & al. 2007, CLÉMENCET & al. 2008).

Concerning how many individuals reproduce per colony, several models predict that the number of egg layers in queenless colonies increases with group size. For example, an evolutionary stable strategy model suggests that 25% of the workers begin laying eggs after queen loss, provided they are all full sisters, the costs associated with egg laying increase linearly with the number of egg layers, and workers do not aggressively prevent each other from reproducing (WENSELEERS & al. 2004). Much fewer individuals will succeed in egg laying when workers form linear dominance hierarchies and when policing occurs. In such a case, the number of hopeful reproductives that engage in dominance interactions is expected to increase with colony size and to level off at larger colony sizes (MOLET & al. 2005). Observations of the skew in worker aggression support this latter prediction for two ponerine ants with totipotent workers (MOLET & al. 2005) and the myrmicine *Temnothorax nylanderi* (FÖRSTER, 1850), in which workers can only lay unfertilized eggs (HEINZE 2008). However, as aggressiveness is not always a reliable predictor of reproductive status (e.g., TRUNZER & al. 1999, HEINZE 2004), additional data are needed to corroborate these observations.

The present study investigates, which and how many workers become dominant in the ant *Temnothorax crassi-*

spinus (KARAVAIEV, 1926), one of the most common ants in deciduous forests of Eastern Central Europe. The life history of *T. crassispinus* resembles that of its well-studied western sibling *Temnothorax nylanderi,* i.e., colonies usually have a single, singly-mated queen (monogyny and monandry, TICHÁ & ŠTYS 2002, STRÄTZ & HEINZE 2004; M. El-Shehaby & J. Heinze, unpubl.). Like *T. nylanderi* (see PLATEAUX 1970, HEINZE 1996a, HEINZE & al. 1997), *T. crassispinus* is characterized by considerable variation in worker body size and ovariole number. Workers with more than the standard two ovarioles often also have a more complex mesosoma structure and better developed ocelli than regular workers. They have been referred to as "intercastes" (PLATEAUX 1970, HEINZE 1998).

Workers in queenless colonies form dominance hierarchies by aggressive interactions similar to those observed in other species of this genus (COLE 1981, HEINZE & al. 1997, STROEYMEYT & al. 2007). We hypothesize that reproductive status of workers in queenless colonies is positively associated with body size and ovariole number and that the number of reproductive workers increases with colony size as predicted by MOLET & al. (2005).

Methods

In May 2010, we collected 44 complete colonies of *Temnothorax crassispinus* from their nests in rotting twigs in a pine-beech-oak forest near Oberisling, Regensburg (48° 58.5' N, 12° 8' E). Colonies contained between 18 and 256 workers (mean 97.3 ± 49.8 SD) and a single queen each. All colonies were transferred into plastic boxes with a plaster floor as described previously (e.g., STROEYMEYT & al. 2007) and kept under near-natural summer conditions at 14h 27°C / 10h 20°C in incubators. The ants were fed with diluted honey and pieces of cockroaches every three days.

To elicit the formation of dominance hierarchies and worker reproduction, we removed the queen from each of 22 randomly chosen colonies, while the remaining 22 colonies served as a control. Experimental and control colonies did not differ significantly in worker number (two-tailed t-test: $t = 0.416$, $df = 42$, $P = 0.679$). Behavior in all 44 colonies was observed by ad libitum sampling (MARTIN & BATESON 1986) during eight observation sessions of 30 min each on days one, four, seven, ten, 13, 16, 19, and 22 after queen removal (i.e., for four hours per colony, total observation time 176 hours). Note that the observations could not be done blindly, as the presence of the queen is very conspicuous in small colonies such as those of *Temnothorax.*

Previous studies with individually marked workers had already shown that removal results in the formation of dominance hierarchies among workers in this and in related species (COLE 1981, HEINZE & al. 1997, STROEYMEYT & al. 2007, WALTER & al. 2011; J. Heinze, unpubl.). In these hierarchies, only a few high ranking individuals develop their ovaries. As it was not aim of this study to describe hierarchy establishment, individuals were not individually marked and individual ranks were not determined. We focused on previously described antagonistic interactions (e.g., HEINZE & al. 1997), in particular violent antennation, biting, "threatening" with opened mandibles, and pulling on antennae or legs, but in addition also noted the occurrence of trophallaxis, i.e., exchange of fluid food. We did not take the duration of individual interactions into account.

Thereafter, all individuals were killed by freezing and their ovaries were dissected under a binocular microscope as described by BUSCHINGER & ALLOWAY (1978). We counted the number of ovarioles and developing eggs and, after arranging the ovarioles in a straight line, measured maximum ovariole length (from the terminal filament to the junction of the two ovarioles). In addition we measured maximum egg length, and, as a proxy of body size, mesosoma length at 50× magnification. Several individuals died during the observation phase and were therefore removed from the analysis. In total, 1818 of the 2066 workers from control colonies and 1947 workers of the 2215 workers from experimental colonies were dissected and measured. Ovaries were not found during dissection in 238 and 168 workers, respectively. Sample size therefore is 1580 for control colonies and 1779 for experimental colonies except where indicated.

Determining the association between colony size and the fraction or number of reproductive workers is not trivial. Ovariole length and the size of maturing eggs are not absolute predictors of reproduction, as the ovaries of workers, who have laid an egg shortly before dissection, may appear less developed than those of workers whose ovaries contain several immature eggs that later will be resorbed or degenerate. Here, we consider ovarioles with a length of ≥ 0.3 mm (appr. ⅔ of max. ovarioles length) as "elongated", shorter ovarioles are considered as "not elongated". Additionally, we measured the length of the largest egg in the ovarioles of each worker. Eggs with more than ⅔ of the maximal egg length in worker ovaries are referred to as "mature eggs". The number of workers with elongated ovarioles or mature eggs was correlated with colony size.

All statistical analyses were performed using STATISTICA 6.0. Most data were not normally distributed and we therefore used non-parametric tests throughout, in particular Spearman rank correlations, median tests and Mann-Whitney U-tests. When the same test was performed separately in several colonies, probabilities from these tests were combined using the generalized binomial procedure proposed by DE MEEÛS & al. (2009) and their software MULTITEST V.1.2.

Results

Queen removal resulted in a rapid increase of worker aggression. All types of aggressive behavior (antennation bouts, biting, pulling on legs and antennae, threatening with opened mandibles) were observed significantly more frequently in experimental than in control colonies (Mann-Whitney U-tests, all tests $P < 0.0002$ after correction; Tab. 1). In contrast, the frequency of sociopositive exchange of fluid food (trophallaxis) was significantly lower in experimental colonies ($P < 0.0002$, Tab. 1). In the experimental colonies, the total frequency of aggressive acts among workers increased with colony size (Spearman rank correlation, $n = 22$, $r_S = 0.656$, $P = 0.0009$), i.e., there was more aggression in larger colonies. The frequency of aggressive acts per minute and individual did not vary with colony size ($r_S = -0.194$, $P = 0.388$). In control colonies, neither total aggression ($r_S = 0.214$, $P = 0.339$) nor total aggression per individual correlated with colony size ($r_S = 0.223$, $P = 0.318$), probably because of the low level of aggression.

Workers from queenless colonies had significantly longer ovarioles than workers from queenright colonies, both

Tab. 1: Frequency of interactions per individual and minute (median, quartiles) in 22 colonies of *Temnothorax crassispinus* with queen (control colonies) and 22 queenless colonies (experimental colonies). All differences between frequencies in control and experimental colonies are significant at a P-level of 0.0002 after Bonferroni correction for multiple tests.

Behavior	**Control colonies**			**Experimental colonies**			**Mann-Whitney U-test**	
	median	25%	75%	median	25%	75%	*U*	*P*
antennation bouts	0.031	0.009	0.050	0.128	0.098	0.171	55	< 0.0001
biting	0.004	0.002	0.027	0.022	0.013	0.027	17	< 0.0001
pulling	0.003	0.001	0.005	0.018	0.010	0.022	15	< 0.0001
threatening	0.000	0.000	0.000	0.001	0.001	0.001	1	< 0.0001
trophallaxis	0.001	0.001	0.002	0.000	0.000	0.001	52	< 0.0001

Tab. 2: Ovariole length and mesosoma length (in mm, median, quartiles) of workers from 22 queenright (control) and 22 queenless (experimental) colonies of the ant *Temnothorax crassispinus*. Ovariole length is generally greater in experimental colonies (Mann-Whitney U-test, U = 870365, P < 0.0001), which also holds by comparing only the medians of 22 control and 22 experimental colonies (U = 40.5, P < 0.0001). Ovariole size tends to increase with mesosoma length in control colonies ($r_S = 0.170$, $P < 0.0001$) but not in experimental colonies ($r_S = 0.038$, $P = 0.109$), and higher ovariole number is weakly associated with longer ovariole length (control: n = 1580, $r_S = 0.125$, $P < 0.001$; experiment: n = 1778, rS = 0.061, P = 0.010). After Bonferroni-correction for four rank tests, all three correlations are still significant.

Control colonies		**Ovariole length [mm]**			**Mesosoma length [mm]**		
no. of ovarioles	N	median	25%	75%	median	25%	75%
2	1500	0.14	0.12	0.16	0.82	0.80	0.86
3	43	0.17	0.15	0.19	0.86	0.80	0.90
4	27	0.15	0.14	0.19	0.86	0.84	0.92
5	1	0.27			0.90		
6	5	0.19	0.13	0.22	0.90	0.88	0.96
7	–						
8	4	0.13	0.12	0.15	0.92	0.83	0.10
9	–						
Experimental colonies		**Ovariole length [mm]**			**Mesosoma length [mm]**		
no. of ovarioles	N	median	25%	75%	median	25%	75%
2	1741	0.18	0.14	0.22	0.85	0.80	0.90
3	15	0.20	0.19	0.24	0.86	0.82	0.92
4	19	0.21	0.16	0.24	0.85	0.80	0.97
5	–						
6	1	0.19			0.92		
7	–						
8	1	0.10			0.88		
9	1	0.18			0.85		

when considering the whole sample or comparing only the medians per colony (Tab. 2). The ovaries of most workers consisted of two ovarioles ("normal workers"). However, 80 of 1580 workers (5.1%) from 18 control colonies and 37 of 1779 workers (2.1%) from 12 experimental colonies had more than two, up to nine ovarioles ("intercastes"). Over all colonies, the absolute number of intercastes ($r_S = 0.621$, $P < 0.0001$) and their proportion in the colony increased with colony size ($r_S = 0.360$, $P = 0.016$). Across the whole sample, both ovariole number and ovariole length were weakly, but significantly associated with mesosoma length (Tab. 2). The ovarioles of intercastes were signifi-

Tab. 3: Spearman rank correlations between colony size and three indicators of fecundity in workers from 22 queenright (control) and 22 queenless (experimental) colonies of the ant *Temnothorax crassispinus.*

		Control colonies		**Experimental colonies**	
		r_S	P	r_S	P
immature eggs (½ - ⅔ max. length)	number of workers	0.747	< 0.0001	0.738	< 0.0001
	% of workers	0.198	0.378	-0.318	0.149
mature eggs (≥ ⅔ max. length)	number of workers	0.496	0.019	0.398	0.066
	% of workers	0.221	0.324	-0.232	0.300
elongated ovarioles (≥ 0.3 mm)	number of workers	0.233	0.296	0.399	0.066
	% of workers	0.218	0.329	-0.126	0.576

cantly more elongated than the ovarioles of normal workers (median test, control: $n_{2ov} = 1500$, $n_{>2ov} = 80$, $\chi^2 = 19.433$, $p = 0.0002$; experiment: $n_{2ov} = 1741$, $n_{>2ov} = 37$, $\chi^2 = 14.149$, $p = 0.0008$).

Because of the strong variation among colonies concerning ovariole number, mesosoma length, and ovariole length (Kruskal-Wallis tests, all $P < 0.001$) we compared mesosoma length and ovariole length between normal workers and intercastes within each colony with more than three intercastes (6 control, 4 experimental colonies). Summed over these ten colonies, intercastes had a significantly longer mesosoma than normal workers (combined probability from ten Mann-Whitney U-tests, generalized binomial procedure, $P' < 0.001$). More importantly, intercastes also had more elongated ovarioles ($P' < 0.002$). Analyzing workers from control and experimental colonies separately gave similar results (ovariole length: control $P' < 0.01$, experiment $P' < 0.05$; mesosoma length control $P' < 0.01$, experiment $P' < 0.05$; Fig. 1). Within single colonies, ovariole length and mesosoma length were significantly correlated regardless of ovariole number (median and quartiles of 22 Spearman's r_S, control: 0.356 [0.083, 0.204], $P' < 0.002$; experiment: 0.400, [0.102, 0.290], $P' < 0.0001$), i.e., on average, workers with a longer mesosoma had better developed ovaries.

The ovaries of a substantial fraction of workers from both control and experimental colonies contained immature oocytes with lengths between ½ and ⅔ of the maximum egg length (control, median [quartiles]: 19.4 [12.1, 26.6] %, experiment: 45.3 [34.3, 64.8] %). However, in control colonies only few workers met the above-defined cut-off points defining them as "reproductive" (i.e., "elongated ovarioles": 0.00 [0.00, 0.96] %; "mature eggs": 1.67 [0.00, 3.39%]). In experimental colonies, these percentages were significantly larger ("elongated ovarioles": 2.08 [0.68, 5.88] %, $U = 104$, $P = 0.0012$; "mature eggs": 8.77 [5.93, 16.67] %, $U = 47$, $P < 0.0001$).

In control colonies, the number of workers with elongated ovarioles was not correlated with colony size, while the number of workers with immature or mature eggs increased with colony size (Tab. 3). In experimental colonies, the number of workers with either indicator of fecundity appeared to increase with colony size at marginal significance (Tab. 3). Colony size was neither correlated with the percentage of workers with elongated ovarioles nor that of workers with mature eggs (Tab. 3).

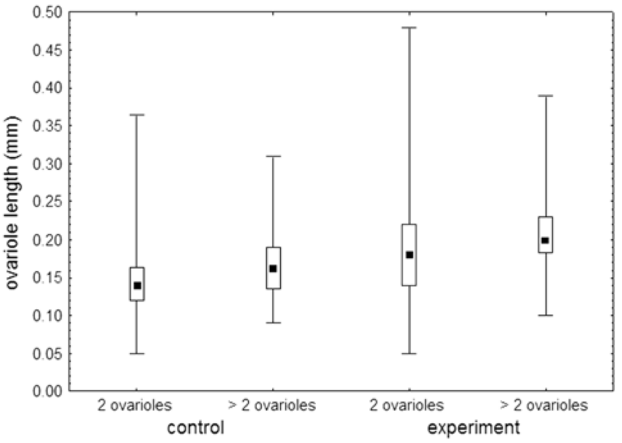

Fig. 1: Ovariole length (median, quartiles and range) in the ovaries of workers of the ant *Temnothorax crassispinus* with two or more than two ovarioles. Workers with more than two ovarioles ("intercastes") have significantly more strongly elongated ovarioles than normal workers in both control and experimental colonies (median test, control: $n_{2ov} = 1500$, $n_{>2ov} = 80$, $\chi^2 = 19.433$, $p = 0.0002$; experiment: $n_{2ov} = 1741$, $n_{>2ov} = 37$, $\chi^2 = 14.149$, $p = 0.0008$).

Discussion

Our study revealed considerable variation in the ovarian status of workers among colonies of the ant *Temnothorax crassispinus* both in queenright and queenless colonies. As expected from previous studies on the maternity of males in other species of this genus (e.g., HEINZE & al. 1997, HAMMOND & KELLER 2004), the ovaries of workers were undeveloped in most of the queenright control colonies, suggesting that workers only rarely if at all contribute to the male offspring of the colony. However, in particular in one colony, several workers had elongated ovarioles. This may indicate that in some colonies, the presence of a queen does not completely prevent worker reproduction. As queens were not dissected in our study, we cannot exclude that in these cases queens were of low fecundity.

Fighting among workers was rare in queenright colonies, but queen removal quickly led to a strong increase in the frequencies of violent antennation, biting, or pulling and in a decrease of the frequency of trophallaxis. While the total number of aggressive acts increased with colony size, the attack rate per individual remained unchanged. A similar result has been reported for foundress associations of paper wasps, *Polistes dominula* (CHRIST, 1791) (see CANT & al. 2006), and queenless worker groups of the bumble bee *Bombus terrestris* (LINNAEUS, 1758) (see AMSALEM & HEFETZ 2011).

Like in other species of *Temnothorax* (see COLE 1981, HEINZE & al. 1997, STROEYMEYT & al. 2007, WALTER & al. 2011), aggression among *T. crassispinus* workers results in dominance hierarchies, in which only a few top-ranking individuals start to lay eggs (J. Heinze, unpubl.). Hierarchy rank was not determined in our study, but both the number of ovarioles per ovary and mesosoma length affected the workers' probability of reproducing after queen removal and therefore might be important in hierarchy formation. Several workers had more than two, up to nine ovarioles. This resembles the situation in *Temnothorax nylanderi*, where a minority of individuals combine traits of the "gynomorphic" queens and the regular, "ergatomorphic" workers (for nomenclature see BUSCHINGER & WINTER 1976, HEINZE 1998), i.e., they have traces of ocelli or thoracic sutures. Such "intercastes" are often larger and have more and better developed ovarioles than regular workers (PLATEAUX 1970, HEINZE & al. 1997). Similarly, the observed intercastes of *T. crassispinus* tended to have more strongly elongated ovaries than their nestmates, i.e., they were more likely to take over reproduction after queen removal. Having more ovarioles might allow workers to mature more oocytes at the same time. The observed association between ovariole number, social status and the likelihood of reproducing after queen loss therefore reminds of the subfertility hypothesis (CRAIG 1983). It posits that division of reproductive labor among totipotent females is based on innate differences in fecundity (e.g., CRAIG 1983, but see SMITH & al. 2009). Intercastes occur regularly in larger colonies of the ant *Crematogaster smithi* CREIGHTON, 1950 and related species and here appear to serve a specific function, in that they produce eggs that are fed to the queen and the larvae (HEINZE & al. 1999). In analogy, one might argue that *Temnothorax* intercastes make ideal replacement reproductives after queen loss. However, the rarity of intercastes, particular of those with six or more ovarioles, suggests that they are produced only sporadically under particular conditions, such as environmental or social disturbances during caste differentiation (HEINZE 1998) and do not constitute an evolutionary adaptation to imminent queen loss.

Regardless of ovariole number, mesosoma length appeared to be positively associated with the likelihood of a worker becoming reproductive. This matches observations in other social insects (e.g., VAN DOORN 1989, HEINZE & OBERSTADT 1999, TIBBETTS & al. 2011). At present it remains unknown whether mesosoma length itself determines the outcome of dominance interactions or only indirectly through a correlation with another causal factor. For example, in many species of social insects social status is strongly affected by the age of an individual (e.g., HIGASHI & al. 1994, HEINZE 1996b, TSUJI & TSUJI 2005). Average mesosoma length might vary from year to year with environmental or social conditions. In our study, the age of individual workers was not known. We therefore cannot exclude that the association between mesosoma length and reproductive status results from younger workers being larger due to changed environmental conditions. In *Leptothorax gredleri* MAYR, 1855, individuals of similar age but different social status did not differ in size (HEINZE & OBERSTADT 1999).

Ant workers occasionally develop oocytes, which later are resorbed without ever maturing or which develop into trophic eggs (e.g., DEJEAN & PASSERA 1974). Hence, determining the exact number or percentage of reproductive workers in insect societies is not a simple task. Nevertheless, our data suggest that the number of workers with elongated ovarioles and maturing eggs increases with colony size. This matches previous observations in paper wasps (CANT & ENGLISH 2006), bumble bees (CNAANI & al. 2007), and ants (MOLET & al. 2005). The proportion of reproductive workers did not vary with colony size and averaged around 8.8% in experimental colonies, i.e., less than expected from the model by WENSELEERS & al. (2004). This discrepancy presumably reflects the impact of aggressive interactions among workers on hierarchy length. Furthermore, it is likely that the costs associated with worker reproduction (e.g., COLE 1986) increase more than linearly with the number of reproductives: non-reproductive workers presumably can compensate for deficits in work caused by a few inactive egg layers, but brood care and foraging might break down at larger proportions of reproductive workers.

Interestingly, the ovaries of a much larger proportion of workers contained immature oocytes. This might suggest that a large fraction of individuals had begun to develop their ovaries after queen loss but later were prevented from fully maturing eggs by the more dominant individuals. Maximally six to eight workers had strongly developed ovarioles even in colonies with 100 or 200 workers. This matches previous observations in *Temnothorax nylanderi*, where the number of workers fighting for dominance after queen loss increased with colony size until it reached a level of about six individuals that were responsible for most of the aggression (HEINZE 2008).

Worker hierarchies in *Temnothorax* provide suitable models to better understand the proximate factors underlying the division of reproductive labor among workers in queenless colonies. Future studies should elucidate the ontogenetic origin of inter-individual differences in ovariole number and body size and the role of age and social experience.

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