

Original Article

Social conflict in ant larvae: egg cannibalism occurs mainly in males and larvae prefer alien eggs

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In social organisms, cooperation is widespread. However, social groups also provide excellent opportunities for individuals to exploit the cooperative efforts of others, thus creating conflict. Social conflict is particularly common in ant societies but although much is known about conflicts between adult individuals, the selfish interests of developing offspring have been largely neglected. One taxonomically widespread way for offspring to engage in competition is through egg cannibalism. Here, we show that larvae of the ant *Formica aquilonia* compete by eating eggs, which increases their survival. Cannibalism behavior differs strongly between sexes, and males cannibalize 3 times as often as females. This points to sex-dependent benefits from cannibalism, for males possibly through removal of future competitors for mating opportunities. Larvae also preferentially eat eggs of distinct origin and odor profile, suggesting that they can detect and react to chemical cues. Thus, similar to adult ants, larvae possess the power to adjust their behavior to available information. We conclude that exploring the behavior of developing individuals can give new insight into social conflict in ants and social animals in general.

Key words: ant larvae, cannibalism, discrimination, kin selection, social conflict.

INTRODUCTION

In social organisms, offspring fitness is shaped by behavioral interactions with other group members, such as competition for common resources (Mock and Parker 1997). Conflict reaches extreme levels when offspring resort to killing each other, a common phenomenon across a diversity of taxa including insects (Dickison 1992; Fincke 1994; Vijendravarma et al. 2013), spiders (Bilde and Lubin 2001), snails (Baur 1992), and birds (Mock et al. 1990). Egg cannibalism in particular is an effective way for developing individuals to gain resources (Elgar and Crespi 1992) and cannibal offspring typically benefit from improved survival (Roy et al. 2007), higher growth and/or developmental rates (Osawa 1992; Roy et al. 2007; Crossland et al. 2011), increased adult body size (Osawa 2002; Michaud and Grant 2004), and competitor removal (Banks 1956; Wise 2006).

Although cannibals benefit from selfish behavior, cannibalism is not without overall costs (Chapman et al. 1999), especially if broods are composed of related individuals (Pfennig 1997). This is because inclusive fitness costs of cannibalism are higher when

preying on close relatives instead of less related brood. Following inclusive fitness theory (Hamilton 1964), the potential for conflict should thus vary according to within-brood relatedness and reach higher levels when broods are mixed, for example, when females mate multiply or when many females breed together.

Because relatedness is an important determinant of potential conflict (Ratnieks and Reeve 1992), the power to assess genetic diversity is likely to be essential in shaping the outcome of intrabrood conflict (Beekman et al. 2003). Specifically, selective behavior to spare kin in mixed brood is only possible if individuals are able to recognize kin. In insects, information on kinship can be encoded in complex chemical cues (Fletcher and Michener 1987; Howard and Blomquist 2005) and developing individuals have been shown to use this information to preferentially choose nonrelatives over kin when cannibalizing (Dobler and Kölliker 2009).

Ants are important models in the study of social conflict (Bourke and Franks 1995), but the selfish interests of larvae have been widely neglected. However, the ecological and social environment of ant societies provides many opportunities for competition between larvae. First, ant brood is reared in batches of eggs and larvae, providing larvae with easy access to highly nutritional eggs.

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Second, ants can spend more than half their lives as larvae, making larval development an important phase of life history (Hölldobler and Wilson 1990). Third, female caste fate, that is, developing into a reproductive queen or nonreproductive worker, is often determined by larval nutrition (Wheeler 1986, 1994). In addition, ant colonies vary in their kin structure (Sundström et al. 2005) and thus in the predicted intensity of competition among larvae. Finally, if ant larvae use chemical information to discriminate among conspecifics in a similar way as adults (Van Zweden and d’Ettorre), they may also possess the power to adjust their behavior to different levels of kinship.

In this study, we investigated brood conflict in the form of larval egg cannibalism in the ant *Formica aquilonia*, a mound building species that forms large networks of interconnected nests called supercolonies (Rosengren and Pamilo 1983). Nests have hundreds of reproductive queens and relatedness among nestmates approaches zero (Pamilo 1982; Pamilo et al. 2005), creating potential for extreme conflict among larvae. We measured levels of cannibalism on different levels of experimentally manipulated brood kinship and in larvae of different sexes. To test if eggs carry recognition cues, we analyzed chemical profiles of eggs produced by queens of distinct supercolonies. Finally, we estimated the effect of cannibalism on the survival of larvae.

METHODS

Study colonies and collections

Queens and workers were collected from 2 supercolonies (LA: $n = 13$ nests, 59.95000793°N/23.17383804°W; MY: $n = 8$ nests, 59.9859419°N/23.22908954°W) around Tvärminne Zoological Station in southwestern Finland in April 2010 and 2011. The nest fragments were transferred to plastic nest-boxes (40 × 30 × 20 cm) coated with Fluon to prevent ants from escaping and peat and moss provided as nest material. Boxes were kept in a greenhouse under seminatural light and temperature conditions and watered and fed daily with Bhatkar diet (Bhatkar and Whitcomb 1970).

Cannibalism experiments

We presented larvae with eggs of different origins to measure the average level of cannibalism, the effect of egg diversity on cannibalism, and the effect of cannibalism on larvae survival. Subsequent sexing of a fraction of larvae used in experiments allowed testing for differences in cannibalism between sexes.

Bioassays

Larvae and eggs were obtained by transferring queens from different nest fragments to the laboratory and isolating them in individual Petri dishes that were kept in the dark at room temperature. Eggs were removed once or twice daily from the queen dishes and cleaned with strips of paper towel to minimize fungus growth. Eggs were then moved to clean Petri dishes to track their maternal origin and age. Strips of sponge cloth were placed around the eggs and moistened daily to avoid desiccation. Eggs were left to hatch in the dark at room temperature. After 9–11 days, most eggs had hatched (mean hatching success: 75%). Within a few hours after hatching, each larva was moved onto a separate dish and placed ventrally on top of a pile of 5 fresh eggs (1–3 days age), which were obtained in the same way as larvae. Each larva was presented with a batch of 5 eggs representing 4 levels of kinship—1) *sibling*: eggs from the same mother queen ($n = 91$ larvae), 2) *nestmate*: eggs from nestmate

queens ($n = 201$), 3) *colony-mate*: eggs from queens of a different nest within the same supercolony ($n = 87$), and 4) *alien*: eggs from queens of a different supercolony ($n = 80$). For each of these treatments, the number of eggs each larva had consumed was recorded once a day for 2 consecutive days. When a larva was observed actively feeding on an egg, this was also scored as cannibalized.

In the 2 treatments with the highest cannibalism levels (colony-mate and alien), we measured the effect of cannibalism on larvae survival by continuing assays for an additional 4 days and recording survival daily. Larvae were classified as dead when gentle probing with forceps did not result in any response (i.e., moving, opening of mandibles).

Statistical analysis of bioassays

Frequency of cannibalism incidents and the total number of eggs eaten in each treatment were analyzed by fitting a mixed logistic regression model (generalized linear mixed model, GLMM) with presence of cannibalism or number of eaten eggs as response variable, treatment as fixed explanatory variable, and colony as a random factor (function `glmer`, package `lme4` in R version 2.11.1; Bates et al. 2012). Differences between treatments were tested using a priori treatment contrasts (Crawley 2007). The effect of cannibalism on survival was estimated by comparing survival (days) of cannibalistic and noncannibalistic larvae with a Wilcoxon–Mann–Whitney test.

Sex bias in cannibalism

Sex is easily determined in *Formica* ants because males develop from unfertilized, haploid eggs and females from fertilized, diploid eggs. For sexing, a random sample of larvae from sibling (53 of 91 larvae) and nestmate treatments (141 of 201 larvae) was genotyped at 8 polymorphic microsatellite loci designed for *Formica* species and tested for successful cross-amplification in *F. aquilonia*: FE13, FE19, FE21, and FE42 (Gyllenstrand et al. 2002); FL20 and FL21 (Chapuisat 1996); FY4 and FY7 (Hasegawa and Imai 2004). For DNA extraction, entire larvae were placed in individual wells together with a 2.5:100 μ L Proteinase K–Chelex solution and left to incubate overnight at 56 °C. Polymerase chain reactions (PCRs) were run in 10 μ L reactions using 5- μ L QIAGEN Type-It microsatellite multiplex buffer, 3 μ L deionized water, 1 μ L optimized primer mix, and 1 μ L DNA. PCR protocols were run according to QIAGEN recommendations, products analyzed in 1:200 dilutions in a 3730 ABI sequencer, and microsatellite peaks scored using Genemapper software version 4.1. Larvae that were heterozygous at 2 or more loci were classified as females, whereas individuals that were homozygous at all successfully amplified loci were classified as males. Based on allele frequencies from all genotypes, the probability of wrongly assigning a diploid individual to the male category was below 5% when at least 6 loci were used.

Statistical analyses of sex bias

Differences between males and females in the number of cannibalism incidents and the number of eaten eggs were tested with a chi-square test for each treatment.

Analysis of chemical and genetic variation

In ants, individual odor profiles are both genetically and environmentally determined (Van Zweden et al. 2010). To test whether *F. aquilonia* larvae have access to information concerning the origin of eggs, we analyzed genotypes of queens and the surface hydrocarbon profiles of their eggs.

Chemical samples

One to 3 queens were isolated from each of 13 nests in supercolony LA ($n = 24$) and 8 nests in supercolony MY ($n = 14$), placed on individual Petri dishes and kept in the dark at room temperature until they had laid 10 eggs. Queens were transferred to individual tubes, killed by freezing, and stored at $-20\text{ }^{\circ}\text{C}$ for genetic analysis (see below). Egg surface chemicals were extracted from a pooled sample of 10 eggs per queen for 10 min in 10 μL pentane (including 5 ng/mL of $n\text{-C}_{20}$ as internal standard). The extract was transferred to a clean vial and 3 μL per sample was injected in an Agilent 6890N gas chromatograph (capillary column: Agilent HP-5MS, 30 m \times 25 μm \times 0.25 μm ; split-splitless injector; carrying helium gas at 1 mL/min) coupled to an Agilent 5375 mass spectrometer (70 eV electron impact ionization). After an initial hold at 70 $^{\circ}\text{C}$ for 1 min, the temperature was raised to 220 $^{\circ}\text{C}$ at 30 $^{\circ}\text{C}/\text{min}$ and then from 200 to 320 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$, where it was held for 5 min. The areas of the 37 peaks common to all egg extracts were integrated and compounds identified on the basis of their mass spectra and retention times (Supplementary Figure S1) compared with the internal standard using Agilent Chemstation software. For analyses by compound group, compounds were separated into linear alkanes ($n = 9$), alkenes ($n = 8$), and methyl-branched compounds ($n = 20$). Prior to analysis, absolute peak areas were normalized according to Aitchison (1986).

Genetic samples

Two legs per queen were removed and genotyped according to the same methods used for larvae sexing. We calculated mean pairwise relatedness between queens (Queller and Goodnight 1989) using GenALEX version 6.5b4 (Peakall and Smouse 2012). To ensure unbiased relatedness estimates, supercolony-specific allele frequencies calculated from worker genotypes (16 workers from 21 nests in each supercolony, data not shown) were used as population allele frequencies.

Statistical analysis of chemical and genetic variation

Matriline origin To test whether egg profiles reflect maternal genotype, we calculated Pearson correlations between pairwise chemical distances (Manhattan distance) of eggs and pairwise genetic distances based on relatedness values of their mother queens. Correlations were calculated for all 37 compounds and each compound group separately. All correlations were tested for significance against a distribution obtained by calculating correlations from 1000 random permutations of the data.

Because discrimination between kin and nonkin eggs is primarily relevant within the nest and the environmental component of chemical profiles can confound analyses, only same-nest pairs of queens and eggs were used in calculating correlations. Also, only queens for whom at least 6 microsatellite loci amplified successfully were included in the analysis. Final analysis was conducted with 8 queen pairs and their corresponding eggs from supercolony LA and 5 queen pairs and their eggs from supercolony MY (total $n = 13$).

Supercolony origin We assessed the availability of cues on a broader level by measuring supercolony-specific variation in all egg profiles ($n = 38$). Principal component analysis (PCA) of all 37 compounds and each separate compound group (alkanes, alkenes, and methyl-branched compounds), followed by linear discriminant analysis (LDA) with leave-one-out cross-validation, were used to measure classification success of eggs according to supercolony of

origin. LDA was based on the principal components that explained at least 90% of the variation in the data set (Supplementary Table S2). In addition, we verified supercolony-specific profile variation using multivariate analysis (MANOVA, function `adonis`, package `vegan`; Oksanen et al. 2012), again for all compounds and each compound group separately.

RESULTS

Cannibalism experiments

Bioassays

Cannibalism levels rose gradually as diversity of eggs increased from sibling to alien treatments. In the sibling treatment, 10% of larvae (9/91) engaged in at least one act of cannibalism, whereas in the nestmate treatment, 13% (27/201) engaged in cannibalism. In total, larvae ate 2% and 3% of offered eggs in sibling and nestmate treatments, respectively. In the colonymate treatment, 21% of larvae (18/87) were cannibals and consumed a mean of 4% of eggs. In the alien egg treatment, 24% (19/80) of larvae engaged in cannibalism and ate 5% of eggs. Both number of cannibalistic incidents and the number of eaten eggs were significantly lower in the sibling treatment compared with the alien treatment (response: incidents, degrees of freedom [df] = 3, $P = 0.039$; response: number of eaten eggs, df = 3, $P = 0.025$; Figure 1 and Table 1). There were no significant differences in the number of cannibalistic incidents or eaten eggs among the other treatments.

Cannibalism levels were highest in colonymate and alien treatments; therefore only these 2 treatments were continued for an additional 4 days in order to measure larvae survival. Because cannibalism levels did not differ between the two, we pooled data from both treatments for survival analysis. Overall, cannibals survived significantly longer than noncannibals (mean in days \pm standard error for cannibals: 2.94 ± 0.12 and noncannibals: 2.45 ± 0.07 ; Wilcoxon–Mann–Whitney test, $n = 167$, $W = 1642$, $P = 0.002$).

Sex bias in cannibalism

Of the 194 genotyped larvae, 88 were female and 106 were male (sibling treatment: 21 females and 32 males; nestmate treatment:

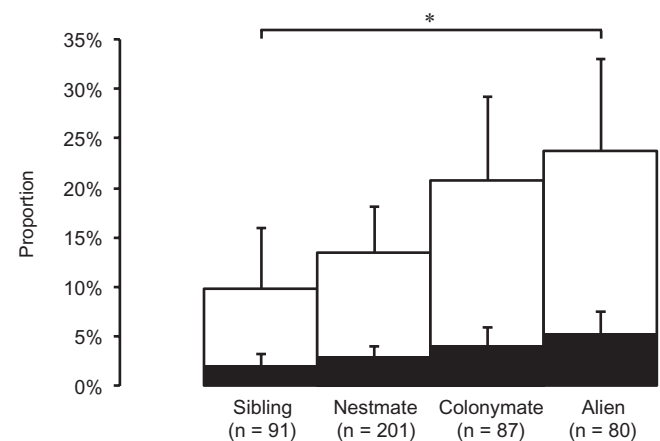


Figure 1

Mean proportion of cannibalism incidents (white) and number of eaten eggs (black) across treatments. Bars indicate 95% confidence intervals. Differences were tested for each response separately with a GLMM for binomially distributed data using treatment as fixed and colony as random variables. * $P < 0.05$.

Table 1
GLMM analysis of incidents of cannibalism (a) and number of eaten eggs (b)

Fixed effects					Random effect, colony ($n = 18$)	
Treatment	Estimate	Standard error	z-Value	P	Variance	Standard deviation
(a)						
Intercept (sibling)	-2.248	0.373	-6.025	<0.001		
Nestmate	0.364	0.429	0.849	0.396	0.134	0.366
Colonymate	0.807	0.482	1.673	0.094		
Alien	0.987	0.480	2.054	0.040		
(b)						
Intercept (sibling)	-3.932	0.352	-11.169	<0.001		
Nestmate	0.441	0.399	1.104	0.269	0.084	0.289
Colonymate	0.734	0.444	1.651	0.099		
Alien	0.973	0.435	2.237	0.025		

67 females and 74 males). In the sibling treatment, male larvae engaged in cannibalism significantly more often than female larvae (proportion of cannibalism incidents in females: 0% [0/21 larvae] and males: 21.8% [7/32 larvae]; Pearson's chi-square test: $\chi^2 = 4.27$, $df = 1$, $P = 0.038$). The pattern was similar in the nestmate treatment with 5.9% (4/67 larvae) cannibalism in females and 20.2% (15/74 larvae) in males (Pearson's chi-square test: $\chi^2 = 4.75$, $df = 1$, $P = 0.013$; Figure 2). Males also ate significantly more eggs than females in both treatments (Pearson's chi-square test; sibling—females: 0%, males: 4.4%; $\chi^2 = 4.72$, $df = 1$, $P = 0.029$; nestmate—females: 1.5%, males: 4.6%; $\chi^2 = 5.59$, $df = 1$, $P = 0.018$).

Analysis of chemical and genetic variation

Matriline origin

Eggs laid by genetically distant queens were not more distinct in their chemical profiles than eggs laid by closely related queens, indicating that egg odors do not contain information about matriline origin (Pearson correlation coefficient, $n = 13$; all compounds: $r^2 = -0.20$, $P = 0.26$; alkanes: $r^2 = -0.25$, $P = 0.21$;

alkenes: $r^2 = 0.02$, $P = 0.55$; methyl-branched compounds: $r^2 = -0.14$, $P = 0.34$).

Supercolony origin

Egg samples (92%) were classified correctly by supercolony origin (PCA + LDA on PCs 1–10; Supplementary Table S2). Multivariate analysis confirmed this result (MANOVA—factor: supercolony, $df = 1$, $F = 2.72$, $R^2 = 0.07$, $P = 0.016$). When compound groups were analyzed separately, classification was similar using only methyl-branched compounds (LDA on PCs 1–8: 84% correct classification; MANOVA—factor: supercolony, $df = 1$, $F = 5.71$, $R^2 = 0.17$, $P = 0.0019$) but not when using linear alkanes (LDA on PCs 1–3: 52% correct; MANOVA—factor: supercolony, $df = 1$, $F = 0.25$, $R^2 = 0.007$, $P = 0.82$) or alkenes (LDA on PCs 1–4: 52% correct; MANOVA—factor: supercolony, $df = 1$, $F = 1.62$, $R^2 = 0.04$, $P = 0.18$).

DISCUSSION

Our experiments show that potential conflict among ant larvae is reflected in their behavior in the form of egg cannibalism. Around 10% of larvae cannibalized sibling eggs, and incidence of cannibalism rose to 24% when larvae were presented eggs from an alien supercolony. This is the first evidence that ant larvae can adjust their behavior to brood composition and available sensory information. Cannibalism increased larvae survival and male larvae engaged in cannibalism 3 times as often as females. These results emphasize the importance of including developing individuals in studies of conflict in ants and social insects in general and offer a new perspective on the role of males in ant societies.

In ants, egg eating can play an important role during early colony life when founding queens lay nonviable, trophic eggs to feed the first generation of larvae (Crespi 1992). In contrast, cannibalism of viable eggs by adults and feeding of eggs to older larvae are thought to occur as a result of resource shortages (Wilson 1971; Sorensen et al. 1983; Heinze et al. 1999) and help increase colony resistance to starvation (Rueppell and Kirkman 2005). Our experiments confirm that eggs are highly nutritious and that their consumption improves larvae survival in the absence of other food sources and thus can provide a means for larvae to regulate their own food intake. In addition, egg eating may allow larvae to grow faster than their nestmates and possibly avoid being eaten themselves, a benefit of cannibalism in other taxa (Polis 1981). Especially female larvae, whose adult caste depends on larval nutrition, would gain from the increased growth and/or developmental rates commonly associated with cannibalism (Fox 1975; Polis 1981; Roy et al. 2007; Crossland et al. 2011). In stingless bees, some

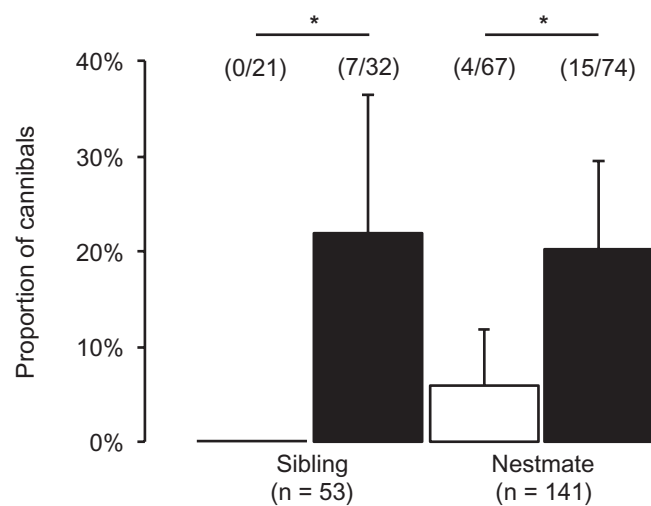


Figure 2

Proportion of cannibalism incidents performed by females (white) and males (black) in sibling and nestmate treatments. Numbers above bars indicate the number of cannibalistic larvae/total number of larvae from each sex. Differences between sexes were analyzed with a chi-square test. * $P < 0.05$.

females selfishly determine their caste, which may be linked to underlying physiological differences between larvae (Wenseleers et al. 2005). Conversely, cannibalism could be a behavioral mechanism of selfish caste determination in female ant larvae, although this remains to be shown (Rüger et al. 2007).

The strong male bias in our experiments furthermore raises the question of sex-specific benefits from cannibalism (Osawa 2002). In *F. aquilonia*, male and female reproductives are reared from the first batch of eggs laid in spring (Rosengren et al. 1987; Gösswald 1989). The larvae used in our experiments were obtained from this brood cohort; thus, it does not seem likely that the observed male bias was due to differences in cannibalism between worker-destined and sexual brood. Instead, males may engage in cannibalism more often if the associated gain in nutrition increases sperm quantity or quality (Delisle and Hardy 1997). This could be especially relevant in ants, where spermatogenesis typically occurs only during larval development (Hölldobler and Bartz 1985). In addition, developmental consequences of cannibalism such as increased adult body size (Osawa 2002) may select for higher levels of cannibalism in males. For instance, leg and wing length of adult males have been positively correlated with mating success in the ant *Pogonomyrmex occidentalis* (Wiernasz et al. 1995). Finally, if competition for mating opportunities among males is stronger than in females, removal of competitors through egg cannibalism may be more advantageous to males. This could be important in *F. aquilonia*, where mating often occurs in or near the nest (Fortelius et al. 1993), but further studies of population structure would be needed to confirm this.

Intrabrood relatedness and the ability of individuals to assess kinship are known to mediate the occurrence and intensity of cannibalism in animals (Pfennig 1997). However, in our experiments, levels of cannibalism did not differ significantly between sibling, nestmate, and colony-mate treatments. Together with the lack of evidence for matriline-specific cues in egg odors, this suggests that lack of information constrains discrimination within colonies. To our knowledge, the only other study of discriminative egg cannibalism in ant larvae also failed to find evidence for nestmate recognition (Urbani-Baroni 1991) although chemical cues were not investigated here. In social insects, nest-level costs of individual selfishness are thought to select against the evolution of kin-informative cues (Ratnieks 1991; Keller 1997) and only a few studies have found such cues in ants (Nehring et al. 2011; Helanterä et al. 2013). In line with this, evidence for kin-preferential behavior is limited (Van Zweden et al. 2010, but see Hannonen and Sundström 2003 for an example in *Formica fusca*), even though patterns resembling nepotism may arise through passive processes (for example in *Formica exsecta*, Holzer, Kümmerli, et al. 2006). Our results suggest similar constraints act on kin discrimination in larvae.

On the supercolony level, eggs differed in their chemical profiles. Methyl-branched compounds in particular showed a strong supercolony signal, confirming that substance classes differ in their importance as recognition cues (Van Zweden et al. 2010). In ants, efficient discrimination between colony members and intruders is essential in maintaining the integrity and organization of colonies (Hölldobler and Wilson 1990) and adult workers use colony-specific odors to discriminate nestmates from non-nestmates (Van Zweden and d'Ettoire). In supercolonial ants, exchange of workers, brood, and queens between nests of the same supercolony can lead to blending of odors on a large spatial scale, and our results indicate that supercolony-level cue mixing in the maternal environment plays an important role in determining egg odors in *F. aquilonia*.

Indeed, when larvae were presented with eggs carrying odors distinct from those they typically encounter within their own supercolony, cannibalism levels increased significantly. Thus when differences are extreme, ant larvae react to chemical cues. In nature, larval discrimination of alien eggs may play a role when queens enter foreign nests. Ant supercolonies often originate from a single founding colony and can have strict boundaries (Holzer, Chapuisat, et al. 2006; Helanterä 2009). Still, queens may move across supercolonies and attempt adoption in foreign nests (Holzer et al. 2008). Several species of wood ants including *F. aquilonia* dominate our study area and exchange of queens between supercolonies or even intrusion by heterospecific queens is thus not improbable (Czechowski 1996).

Overall, we found that egg cannibalism in *F. aquilonia* is shaped by the ability of larvae to assess the chemical diversity of eggs and intrinsic differences between sexes regarding its intensity. Notably, sex-specific differences regarding the benefits of egg eating may play a role in mediating larval cannibalism. Ant larvae are thus not the powerless individuals they have been seen as so far. Instead, our study shows that developing offspring and male larvae in particular may be a neglected force in ant societies. Moreover, this work demonstrates that ants, whose kin structure variation and chemical information processing are already well understood, can be fruitful models for studies of intrabrood conflict.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>

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