Split sex-ratio and variability in aggressive behavior in the ant *Cataglyphis niger*

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Abstract

Several ant species display split sex-ratio distributions, in which colonies specialize in the production of either male or female sexuals. Here, we report split-sex ratio distributions in the desert ant *Cataglyphis niger*. We also show that male- and female-producing colonies display elevated aggression toward conspecifics, compared to colonies without sexuals, and that encounters between workers from female- and male-producing colonies tend to be the most aggressive. Moreover, elevated aggression of workers from sexual-producing colonies overrides the previously reported 'nasty-neighbor' effect and persists even toward ants from distant nests. These results suggest that the need to protect the substantial energetic investment in sexuals leads to elevated aggression levels and alters aggressive behavior patterns.

Introduction

Bimodal sex-ratio distribution

Trivers and Hare's seminal theoretical work on haplodiploid social insects (ants, bees, and wasps) showed that relatedness asymmetries between colony members can lead to different predicted sex ratios when queens or workers control reproduction (Trivers and Hare 1976). In colonies with singly-mated queens, queens and workers should favor 1:1 and 3:1 ratios of

gynes to drones, respectively. Studies from singly-mated species revealed frequent female-biased sex ratios indicating that workers often prevail (Trivers and Hare 1976, Herbers 1984, Nonacs 1986). Conversely, the queen may thwart worker control by specializing in producing either gynes or drones, so that workers are unable to manipulate the sex ratio by selectively eliminating males. This phenomenon is known as split-sex ratio (Grafen 1986, Boomsma and Grafen 1990, Boomsma 1991, Boomsma and Grafen 1991, Chan and Bourke 1994, Pamilo and Seppä 1994, Sundström 1994, Ratnieks and Boomsma 1997, Queller and Strassmann 1998, Chapuisat and Keller 1999, Mehdiabadi et al. 2003, Bourke 2005)

When queens are multiply mated, colonies are expected to vary in relatedness asymmetries depending on the number of mates. Boomsma and Grafen (1990, 1991) argued that the variation in relatedness asymmetries among colonies promotes the evolution of split sex ratios (Boomsma and Grafen 1990, 1991). Furthermore, according to these studies, colonies composed of highly related females are predicted to produce female sexuals, while colonies with lower relatedness will produce males. Here, we report split sex-ratios in colonies founded by multiply-mated queens of *C. niger*, and show that male and female producing colonies differ in their level of aggression.

Aggression in conspecific encounters

In the *C. niger*, as in other social insect species, workers discriminate between nestmates and non-nestmates according to their cuticular hydrocarbons profiles and non-nestmates interactions are typically highly agonistic (Soroker et al. 1994, Soroker et al. 1995b, Soroker et al. 1995a, Lahav et al. 1999). Aggressive behavior between non-nestmates depends on genetic relatedness (Stuart and Herbers 2000, van Zweden and d'Ettorre 2010), environmental cues like nest material, climate, and queen odors (Jutsum et al. 1979, Carlin and Hölldobler 1986, Crosland 1989, Chen and Nonacs 2000), diet (Silverman and Liang 2001, Buczkowski et al. 2005, Grover et al. 2007), worker caste, task, and age (Nowbahari and Lenoir 1989, Wagner et

al. 1998), past experience (Newey et al. 2010a, Van Wilgenburg et al. 2010, Esponda and Gordon 2015), etc. Studies in different species reported variable effects of colony size on aggression toward non-nestmates, including both positive and negative correlation (Crosland 1990, Stuart 1991). These findings suggest that various factors such as life history and reproductive strategy determine whether increased aggression is beneficial for young and small colonies vs. old and large colonies.

The cost of sexuals

The production of sexuals in ants is highly energy-consuming. Gynes and drones are typically larger in size than the average worker. Unlike workers, both gynes and drones need to develop flight muscles, and store sufficient energy reserves for flight. Gynes also accumulate fat and protein in order to decrease, or altogether avoid, foraging during colony founding (Keller and Passera 1988, 1989). Such an energetic investment by the colony is likely to be protected and may result, we hypothesized, in elevated worker aggression between the emergence of sexuals and their departure for mating. This hypothesis is supported by evidence indicating seasonal variation in aggression levels between workers (Ichinose 1991, D'Ettorre et al. 2004, Brandt et al. 2005, Katzerke et al. 2006, Thurin and Aron 2008) which is determined by both intrinsic (e.g. worker density, relatedness within the nest) and extrinsic factors (e.g. temperature, availability and quality of food) (Thurin and Aron 2008). Additional evidence also indicated that some ant species have periodic variation in cuticular hydrocarbon profiles (Vander Meer et al. 1989, Nielsen et al. 1999, Liu et al. 2001), which may alter the response toward non-nestmates. We hypothesized that the reproductive phase of the colony is an important intrinsic factor affecting worker response threshold to various types of threats and that the presence of sexuals in the colony affects intercolonial acceptance threshold in *C. niger*.

The distance between neighboring colonies

Studies suggest that ants can discern whether an ant they encounter comes from a distant nest, or a close one, and that it affects the displayed level of aggression. In *C. niger*, workers tend to be more aggressive toward conspecifics from close nests and exhibit lesser efforts to engage in agonistic behavior when they encounter ants from distant nests, a phenomenon known as the 'nasty neighbor' effect (Saar et al. 2014). This phenomenon is also known in other *Cataglyphis* species (Knaden and Wehner 2003), as well as in other ant genera (Sanada-Morimura et al. 2003, Boulay et al. 2007, Thomas et al. 2007, Wilgenburg 2007, Newey et al. 2010b, Roux et al. 2013). It should be mentioned that, in some species, the opposite effect was also reported, a phenomenon known as the 'dear neighbor' effect (Langen et al. 2000). However, these studies did not investigate the 'nasty/dear neighbor' effects in the context of the reproductive phase of the colony and the presence of sexuals. Another objective of this study, therefore, was to determine whether the 'nasty neighbor' effect persists, or is amplified, during the sexual phase of *C. niger* colonies, when there is a greater resource investment at risk.

Methods

Sampling

Ants from colonies of *C. niger* were sampled in April 2016 from Betzet beach on the northern Israeli coastline (from N33.05162, E35.10245 to N33.07868, E35.10705). This population was previously described as *C. drusus* (Eyer et al. 2017), but our recent species delimitation study shows that it is the same species as *C. niger* (Reiner-Brodetzki et al., in prep). Colonies of this population are monogyne (headed by a single queen), polyandrous (queens are multiply mated) and monodomous (single nest per colony) (Reiner-Brodetzki et al., in prep). Spring is the reproductive season of *C. niger*, and sexuals, i.e., gynes (winged, unmated queens) and males

can be found in nests in early spring. Fifty *C. niger* nests along a transect of 3.1km were dug (one nest every 80m on average) and 30 workers were collected from each nest. Workers were collected from both the entrance of the nests and from inside the nests for a heterogeneous sample. Samples from each nest were kept in a 30x20x10cm box and given water and honey-water. All behavioral assays were conducted within a week after collection. In the 50 colonies sampled, we found gynes in 14 colonies and drones in 7 colonies. None of the colonies had both gynes and males (Fig. 1). Any sexuals found were collected and frozen on the same evening of collection so that workers were left in the boxes without sexuals.

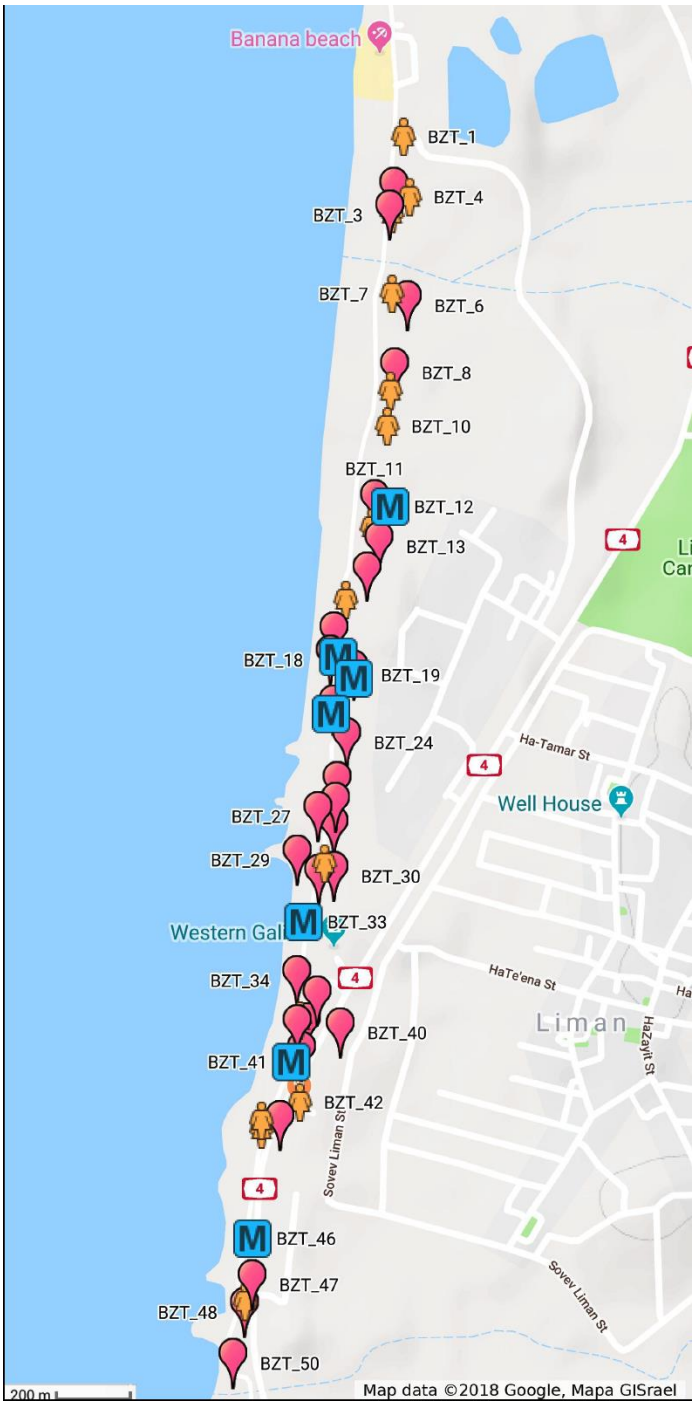

Fig. 1: Map of 50 sampled colonies along a transect in Betzet beach (from N33.05162, E35.10245 to N33.07868, E35.10705). Blue squared M indicate colonies where drones were found. 'Yellow ladies' indicate colonies were gynes were found.

Behavioral assays

All test encounters were dyadic (two workers per encounter) and, in each, workers from either female (F), male (M), or non-sexual (N) colonies were introduced to each other and the level of aggression between them was scored (i.e., six types of encounters all together: FF, MF, MM, NM, NF, NN). Five encounters from each pair of selected colonies were introduced and scored. All encounters were conducted in a neutral arena (9 cm diameter, lined with a filter paper that was changed after each test). Before each test, workers were acclimatized to the arena for 2 min by placing them on the arena surface covered in separate glass tubes. The test began by removing the tubes, and aggressive behavior was recorded for 2 min. Aggression was scored according to the commonly practiced method (Errard and Hefetz 1997) using the following behavioral acts: 1 point - antennation; 2- mandibular threat; 3- short biting with jumping; 4- biting; 5- spraying of formic acid. The aggression index was calculated as follows:

$$\frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} A_i * t_i}{T}$$

where A_i and t_i are the aggression score and duration of each act respectively, and T is the total interaction time (120 sec). Aggression scores were not normally distributed for some types of encounters (Shapiro-Wilk test *p*-values: FF- 0.064, MF- 0.125, MM- 0.005, NF- 0.01, NM- 0.001, NN- 0.042), and thus encounter types were compared using the non-parametric Kruskal-Wallis test.

Results

In a sample of 50 *C. niger* colonies, gynes were found in 14 colonies and drones were found in 7 colonies (Fig. 1). No colonies had both gynes and drones. Interactions between workers from female producing colonies and male producing colonies (MF) were significantly more

aggressive than any of the interactions which involved workers from colonies that did not produce sexuals (Fig. 2; Kruskal-Wallis test $p < 0.0001$ followed by Dunn's multiple pairwise comparisons corrected for multiple testing (Benjamini and Hochberg 1995): $p = 0.001$, 0.003, and 0.001, for the pairwise comparisons of FM with NF, NM and NN, respectively). FF encounters were also marginally significantly more aggressive in comparison to all type of interactions which involved N colonies (NF, NM and NN $p = 0.055$, 0.055, 0.003, respectively).

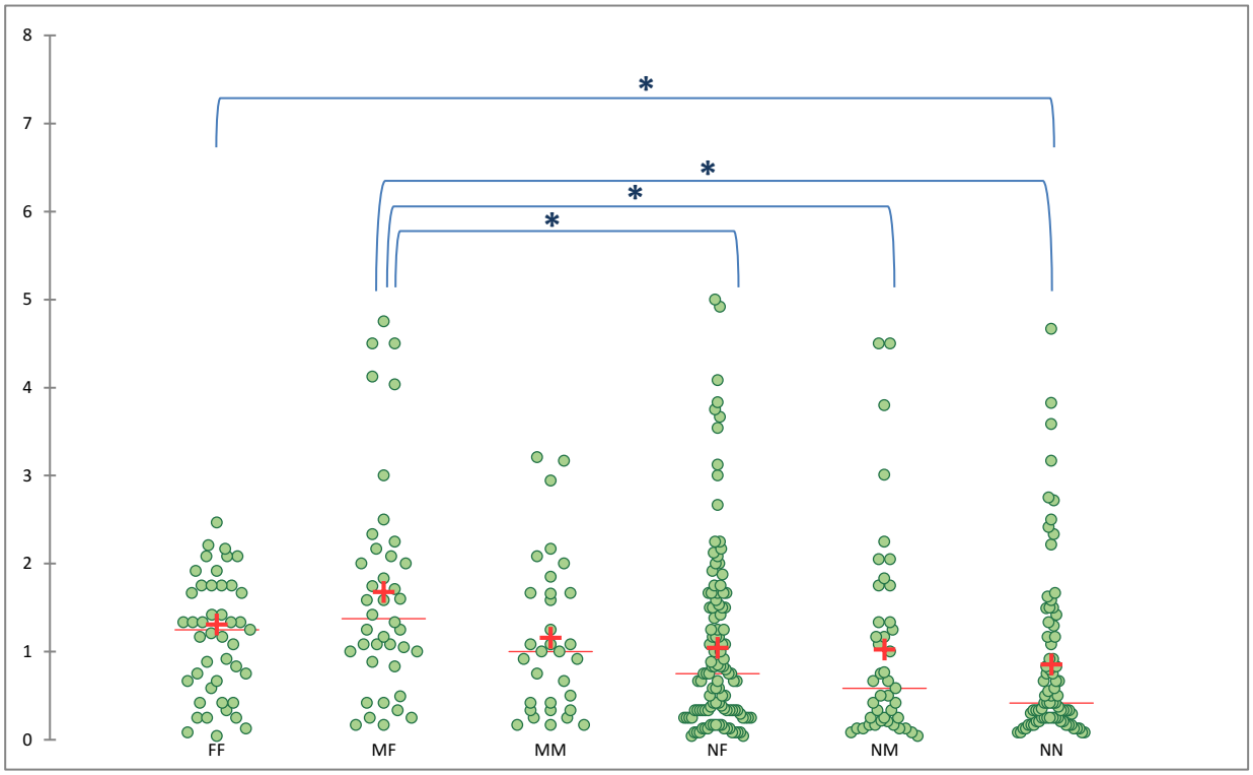

Fig. 2: Distribution of aggression scores for interactions between colonies that produced, or did not produce, sexuals. Aggression scores for interactions between workers from female-producing colonies (F), male-producing colonies (M) and colonies in which no sexuals were found (N). Means are indicated by red + signs and medians are indicated by red horizontal lines. Significant differences are marked with asterisks.

For the analysis of the effect of the distance between nests (Fig. 3A), all interactions between workers from sexuals-producing colonies were grouped together (i.e., FF+MF+MM = S-S),

interactions between sexuals-producing colonies and colonies where no sexuals were found were grouped together (i.e., NM+NF = S-NS), and a third group consisted of interaction only between workers from colonies where no sexuals were found (NN = NS-NS). Significant negative correlation was found between aggressive behavior and the distance between the nests of the participating parties in encounters between non-sexual colonies (NS-NS; Fig. 3B; Spearman's r=-0.39, p=0.001). This result is in line with previous reports of the 'nasty neighbor' effect in *C. niger* (Saar et al. 2014). Encounters between sexual colonies (S-S) also showed significant negative correlation (Fig. 3C; r=-0.261, p=0.005). However, encounters between sexuals and non-sexuals (S-NS) did not exhibit correlation between aggression and distance (Fig. 3D; r=0.055, p=0.491). In contrast to non-sexual colonies, encounters between sexual colonies persisted in high aggression scores for longer distances, that is, ants remain aggressive even when they encountered ants from nests up to 2km away. Similarly, high aggression was also observed in S-NS interactions at large distances.

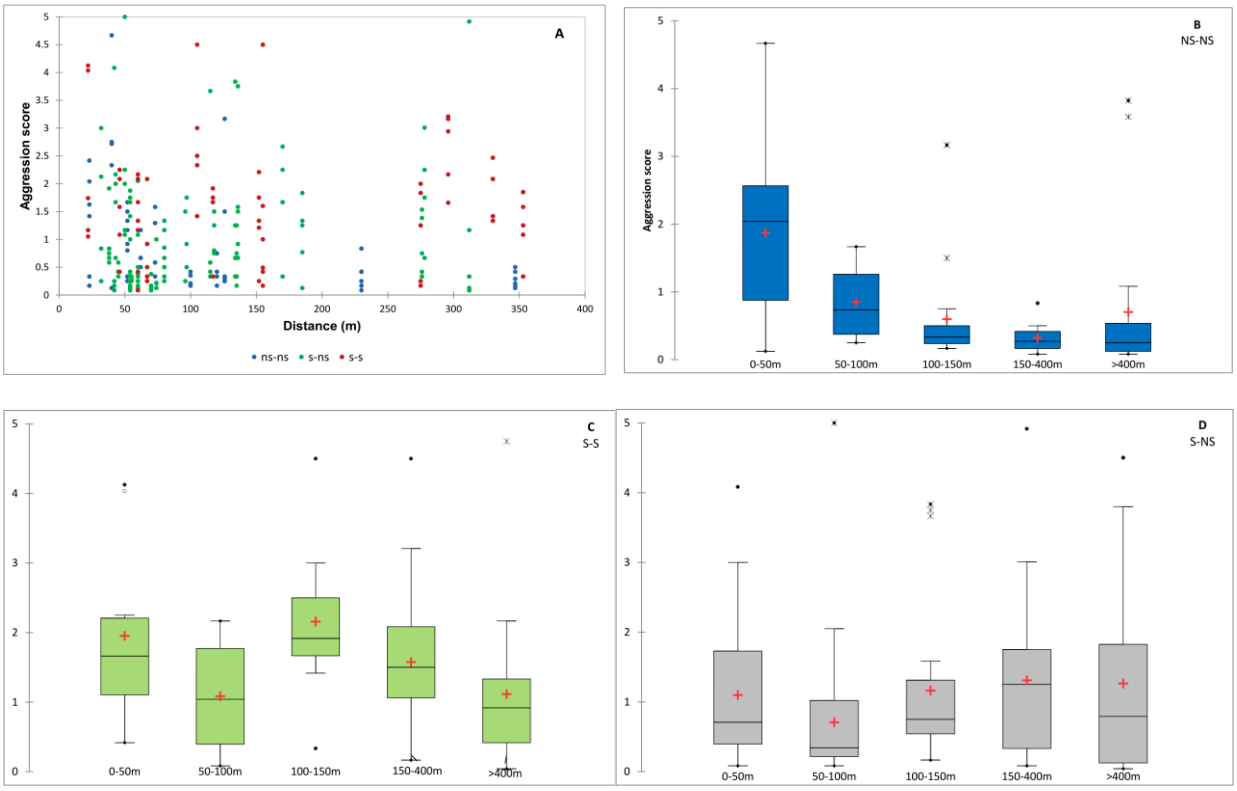

Fig. 3: Aggression scores according to distance in three types of interactions (sexuals and sexuals, non-sexuals and sexuals, non-sexuals and non-sexuals). (A) Scatter plot of observed aggression scores, up to 400m between participating nests. (B) Aggression score by distance intervals in NS-NS interactions (C) Aggression score by distance intervals in S-S interactions. (D) Aggression score by distance intervals in NS-S interactions. Red + marks indicate mean scores.

Discussion

We report a survey of a *C. niger* population during the reproductive season. Our results reveal split-sex ratios in this population. Behavioral assays show that the presence of sexuals in colonies affects both magnitude and patterns of aggressive behavior toward non-nestmates. Workers from female and male producing colonies are expected to protect their energy investment in sexuals, rich with lipid and protein (Keller and Passera 1988, 1989). Our results show that, indeed, the presence of sexuals leads to elevated levels of aggression. Furthermore,

it appears that aggression levels are highest when workers from female producing colonies interact with workers form male producing colonies. This may be a result of differences in CHCs between drones and gynes (Cuvillier-Hot et al. 2001, Smith et al. 2014), which may be incorporated in the CHC profiles of the workers, thus making the identification of a non-nestmate quicker and eliciting a greater aggressive response. Another explanation for increased aggression levels between workers from female and male producing colonies may be differences in intra-colony relatedness. Crozier suggested that individuals with rare labels should be rejected more often than individuals with common labels (Crozier 1986, Crozier 1987, 1988, Elgar and Crozier 1989) and that individuals from high-diversity colonies should be rejected more frequently than individuals from low-diversity colonies. And indeed, there are reports that colonies with greater genetic relatedness tend to be more aggressive toward colonies of lesser genetic relatedness (Tsutsui et al. 2003). Since colonies with greater genetic relatedness may also tend to produce gynes (Boomsma and Grafen 1990, 1991, Sundström 1994, Evans 1995, Bourke 2015) this may explain the elevated levels of aggression we observed in FM interactions.

It is also expected that, in the presence of sexuals, workers would invest greater efforts in protecting their energetic investment in them and exhibit greater aggression response toward intruders in general. In this study, in addition to estimating the magnitude of aggressive responses, we, also, examined whether the presence of sexuals alters the pattern of aggressive behavior. Our results show that, while colonies that did not produce sexuals exhibit a clear ‘nasty neighbour’ effect, as previously reported (Saar et al. 2014), colonies with gynes or males persist in aggressive behavior even when they encounter ants from distant nests. Although S-S interactions do have a significant negative correlation between distance and aggressiveness, aggression levels decrease only at ~500m from the nest. S-NS interaction, on the other hand, do not exhibit any correlation between distance and aggressiveness and ants persist in

aggressive behavior for even longer distances. This result may suggest, again, that risk assessment in the presence of sexuals play a role in deciding to invest in aggressive behavior.

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