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ORIGINAL PAPER

Workers select mates for queens: a possible mechanism of gene flow restriction between supercolonies of the invasive Argentine ant

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Abstract Some invasive ants form large networks of mutually non-aggressive nests, i.e., supercolonies. The Argentine ant *Linepithema humile* forms much larger supercolonies in introduced ranges than in its native range. In both cases, it has been shown that little gene flow occurs between supercolonies of this species, though the mechanism of gene flow restriction is unknown. In this species, queens do not undertake nuptial flight, and males have to travel to foreign nests and cope with workers before gaining access to alien queens. In this study, we hypothesized that male Argentine ants receive interference from workers of alien supercolonies. To test this hypothesis, we conducted behavioral and chemical experiments using ants from two supercolonies in Japan. Workers attacked males from alien supercolonies but not those from their own supercolonies. The level of aggression against alien males was similar to that against alien workers. The frequency of severe aggression against alien males increased as the number of recipient workers increased. Cuticular hydrocarbon profiles, which serve as cues for nestmate recognition, of workers and males from the same supercolony were very similar. Workers are likely to distinguish alien males from males of their own supercolony using the profiles. It is predicted that males are subject to considerable aggression from workers when they intrude into the nests of alien supercolonies. This may be a mechanism underlying the restricted gene flow between supercolonies of Argentine ants. The Argentine ant may possess a distinctive reproductive system, where workers participate in selecting mates for their queens. We argue that the aggression of workers against alien males is a novel form of reproductive interference.

Keywords Aggression · Biological invasions · Gene flow · *Linepithema humile* · Reproductive interference · Supercolony

Introduction

Among invasive alien species, ants are recognized as one of the most harmful groups of animals. They disrupt the ecosystems of invaded habitats by displacing native ants, and adversely affect other animals and plants (Holway et al. 2002). Some species of invasive ants form large networks of mutually non-aggressive nests, i.e., supercolonies, and individual ants can move freely among the interconnected nests. In most native ants whose colonies are composed of one or a few nests, intraspecific territorial aggression among neighboring colonies is common, and this imposes a high cost on reproduction. In contrast, invasive ants that form supercolonies can invest more in brood production because they are released from the cost

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of intraspecific territorial aggression, hence resulting in a huge number of workers per colony (Holway et al. 1998). Invasive ants overwhelm native ants, and often completely displace them owing to their high reproductive ability (Holway 1999).

The Argentine ant *Linepithema humile* (Hymenoptera: Formicidae), one of the most notorious invasive ants, is native to South America but has been introduced into many regions around the world through commercial activities (Suarez et al. 2001; Wild 2007; Wetterer et al. 2009). In their native range, Argentine ants are segregated into multiple, relatively small supercolonies that are mutually aggressive: each supercolony extends from tens to hundreds of meters in diameter (Heller 2004; Pedersen et al. 2006; Vogel et al. 2009). Similar situations can be also found in introduced ranges (Buczkowski et al. 2004; Sunamura et al. 2007). However, the Argentine ants in an introduced range typically form colossal supercolonies that can extend over thousands of kilometers (Tsutsui et al. 2000; Giraud et al. 2002; Corin et al. 2007; Suhr et al. 2009; Helanterä et al. 2009; Sunamura et al. 2009a, b; Vogel et al. 2010; van Wilgenburg et al. 2010a): one or a few introduced native supercolonies are considered to have expanded to that extent with their supercolony identity maintained. The reduced intraspecific aggression in introduced ranges may have provided Argentine ants with greater reproductive success than in their native range (Suarez et al. 1999; Tsutsui et al. 2000).

Argentine ants, like many other invasive ants, do not undertake nuptial flight (Vega and Rust 2001), but new queens and males emerge once a year, in late spring (Markin 1970; Vargo and Passera 1991). New queens mate in their natal nests (Keller and Passera 1992) and establish new nests by budding (Holway et al. 2002). Meanwhile, male Argentine ants can fly and move to distant nests if they do not mate in their natal nests (Markin 1970; Passera and Keller 1990, 1994). In fact, gene flow within a supercolony, presumably via the movement of males, is indicated in nature (Ingram and Gordon 2003).

Recent population genetic studies have demonstrated that gene flow between supercolonies is completely or almost completely absent in two invasive ant species, the Argentine ant (Jaquiéry et al. 2005; Thomas et al. 2006; Pedersen et al. 2006) and yellow crazy ant *Anoplolepis gracilipes* (Thomas et al. 2010). In the Argentine ant, an absence of gene flow between supercolonies was found in both native and introduced populations (Jaquiéry et al. 2005; Thomas et al. 2006; Pedersen et al. 2006; Vogel et al. 2009); however, few studies have addressed the mechanism underlying the restricted gene flow in invasive ants. As mentioned above, Argentine ant queens mate within their natal nests; therefore, in this species, any gene flow between supercolonies would be brought about by the movement of males. Indeed, gene flow between colonies has been shown to be mediated exclusively by males in many other ant species whose queens do not undergo nuptial flight (Ross et al. 1999; Rüppell et al. 2003; Seppä et al. 2004, 2006; Berghoff et al. 2008; Holzer et al. 2009).

In the present study, we hypothesized that male Argentine ants are attacked by workers of alien supercolonies, and the mating success of the attacked males is reduced. If this is the case, it will bring about a restriction of male-mediated gene flow between supercolonies. In ant species whose males have to enter foreign nests to access mates, workers can choose the males who will mate with their queens, as Hölldobler and Wilson (1990) pointed out.

Many ant species, including those that form supercolonies, have been demonstrated or suggested to use cuticular hydrocarbon profiles (i.e., compounds and their relative proportions) as cues for nestmate recognition (Martin and Drijfhout 2009). In Argentine ants, workers from different supercolonies possess different cuticular hydrocarbon profiles and exhibit mutual aggression (Liang and Silverman 2000; Suarez et al. 2002; Greene and Gordon 2007; Torres et al. 2007; Brandt et al. 2009; van Wilgenburg et al. 2010b). Recent studies have shown that qualitative and quantitative differences in cuticular hydrocarbons between workers and queens are used in the recognition of queens by workers in two ant species, the bull ant *Myrmecia gulosa* (Dietemann et al. 2003) and Argentine ant (Vásquez et al. 2008; Vásquez and Silverman 2008a; Cournault and de Biseau 2009); however, whether workers and males from single colonies of ants possess similar hydrocarbon profiles has been rarely studied (e.g., Hojo et al. 2009). If male Argentine ants possess a cuticular hydrocarbon profile similar to that of workers in the same supercolony, the workers can use the profile to recognize and attack males from alien supercolonies.

In the present study, two supercolonies of Argentine ants from an introduced range in Japan were used. First, 10-min behavioral assays were performed to examine whether male intruders are attacked by workers of alien supercolonies. Second, 60-min behavioral assays were performed to investigate the effect of the number of workers on the mortality of intruders. Third, chemical analyses were performed to examine if cuticular hydrocarbons are involved in the induction of aggression against alien males. We show that, under laboratory conditions, worker Argentine ants attack alien males as well as alien workers and that the cuticular hydrocarbon profile of males is very similar to that of workers in the same supercolony. We discuss the potential contribution of this phenomenon to the restricted gene flow between supercolonies of the Argentine ant and argue that the aggression of workers against alien males may be relevant to reproductive interference between biological species.

Materials and methods

Collection and maintenance of ants

Argentine ants were collected on Maya Wharf, Kobe City, Japan (34°41′ N and 135°13′ E). This wharf is part of Kobe Port and harbors two supercolonies ("Japanese main" and "Kobe C") of the Argentine ant (Sunamura et al. 2007). It is approximately 3 km from Port Island, where two other supercolonies ("Kobe A" and "Kobe B") occur. Collection was conducted during 6–9 June 2009, when virgin queens and males were found. Based on the micro-geographical distribution of the supercolonies at Maya Wharf shown in a previous study (Sunamura et al. 2007), three nests each of the Japanese main and Kobe C supercolonies were sampled. Their supercolony identities were confirmed later by behavioral assays in the laboratory. The nests sampled were named M1–M3 for Japanese main and C1–C3 for Kobe C. For each nest, several hundred workers, dozens of males, some broods, and at least one queen were collected, along with some nest materials. Alate queens were not always included. The sampled nests in single supercolonies were >100 m from each other, and the sampled nests belonging to different supercolonies were >30 m from each other.

After the samples had been brought to the laboratory, ants used for chemical analyses were killed by freezing and maintained at −20°C. For use in behavioral assays, the remaining ants were maintained in the laboratory in 12× 18×5.5 cm plastic boxes with nest material. The ants were fed sugar water ad libitum, and boiled egg was provided every 3 days. All of the behavioral assays were completed within 14 days after collection.

Behavioral assays

Short-term aggression tests

We examined whether worker ants attack males from an alien supercolony of the same species. Five workers from a single nest were placed in a plastic dish (1.6 cm in diameter) with a Fluon®-coated wall. A worker or a male from another nest (belonging to either the same or a different supercolony) was then introduced into the dish, and their interaction was video-recorded for 10 min. This one-on-five assay simulates a situation in which an ant intrudes into a conspecific nest or trail. By playing back the video, interactions between the native workers and the intruder were scored as follows: 0=disregard (no interest), 1=antennation (repeatedly tapping with antennae), 2= avoidance (moving away), 3=aggression (menacing, biting, or pulling), and 4=fighting (prolonged aggression). The highest score within each minute was recorded for 10 min, and the average was used as an aggression index.

Aggression tests were conducted for the following cases: (1) interactions between five workers and a worker from a different supercolony,(2) interactions between five workers and a worker from a different nest of the same supercolony, (3) interactions between five workers and a male from a different supercolony, and (4) interactions between five workers and a male from a different nest of the same supercolony. All possible pairs of the six nests sampled were examined: nine pairs (3×3) of nests for cases 1 and 3, and six pairs (2×3) of nests for cases 2 and 4. For each nest pair, nests were reciprocally used as the recipient and intruder. Five replicate trials were performed for each combination. Individual ants were replaced for every trial. In 320 trials, the introduction of an individual into the experimental dish failed two times. These two trials were excluded from subsequent analyses.

A Wald test in a generalized linear mixed model (GLMM; Bates and Sarkar 2006) was performed using R software (R Development Core Team 2007). The response variable was the aggression index. The intruder's caste (worker or male), recipient nest (one of the six nests), and supercolony relationship (intra-supercolony trial or inter-supercolony trial) were used as the fixed factors. The intruder's nest (one of the six nests) was entered as a random factor. The model was constructed with Poisson error distribution, Laplace approximation, and a logarithmic link.

Long-term aggression tests

To investigate the mortality of male intruders, aggression tests were performed for a longer period (60 min). Males from nests M3 (Japanese main) and C2 (Kobe C), where a sufficient number of males could be collected, were used in this experiment. Single M3 males were tested against 1, 5, and 20 workers of C2 or M2, and single C2 males were tested against 1, 5, and 20 workers of M3 or C1. Five replicate trials were performed. Ant behavior was video-recorded for 60 min, and later scored for the initial 10 min by the same method as described above. For the remaining 50 min, mortality of the intruders was checked every 10 min. In two of 60 trials, males escaped from the plastic dish by flying away during the experiment. These cases were excluded from subsequent analyses.

To test if the level of aggression differed with the number of recipients, Steel–Dwass tests were performed for intra- and inter-supercolony trials separately, using the highest aggression score in each minute as the variable.

Chemical analyses

Five workers or males from single nests were immersed in 0.5 ml of hexane for 10 min. The extract was loaded onto a column of silica gel (500 mg Wakogel C-200; Wako Pure Chemical Industries, Osaka, Japan) prepared in a glass pipette. The hydrocarbon fraction was eluted with 5 ml of hexane and concentrated to 5 μl under a stream of nitrogen gas, and a 2-μl aliquot was analyzed. Hydrocarbons were identified and quantified with a QP5050A gas chromatograph–mass spectrometer (GC/MS; Shimadzu) system equipped with a DB-1 column (30 m×0.25 mm, 0.25 μm film thickness; J&W Scientific) operated in the electron impact mode. The oven temperature was maintained at 50°C for 2 min, raised by 17°C/min to 220°C, raised by 5°C/min to 300°C, and maintained at 300°C for 32 min. Helium was used as the carrier gas (flow rate, 1.2 ml/min), and hydrocarbons were identified based on mass fragment patterns.

For each of the six nests sampled by caste (worker or male), extraction and GC/MS were performed three or four times. To compare the cuticular hydrocarbon profiles among nests and castes, a principal component analysis of the relative proportions of the hydrocarbons was performed using R software.

Results

Behavioral assays

Short-term aggression tests

Aggressive behavior was commonly observed between workers from different supercolonies (Fig. 1a). Aggression was initiated by either the intruder or one of the recipient workers. When an individual was subjected to aggressive behavior, it fought back, ran away, or remained still until the aggression ceased. Aggressive interactions often resulted in injuries. More than one recipient worker often participated in the aggression by pulling the legs and antennae of the intruder and preventing the intruder from moving. In 76 of 89 trials with the alien worker–worker combination, score of 4 was recorded at least once in a single trial. Meanwhile, aggressive behavior was not recorded between non-nestmate workers from the same supercolony (Fig. 1a) or between nestmate workers (aggression index=0.24±0.23 in nest M1 and 0.18±0.08 in nest C2).

Workers frequently attacked males from alien supercolonies (Fig. 1b). In 70 of 90 inter-supercolony male–worker trials, a score of 4 was recorded at least once in a single trial. When males were attacked by workers, they tried to run away or remained still. Males never showed aggressive behavior. In addition, they rarely showed escape behavior unless attacked (only two occasions). More than one recipient worker often participated in the aggression by pulling the legs, antennae, and wings of the intruder and preventing the male from moving. In one trial, the male was killed by workers as its abdomen was severed, and in six trials, the wings of males were severed by workers. In contrast, males were rarely attacked by non-nestmate workers of the same supercolonies (Fig. 1b). In 57 of 60 intra-supercolony male–worker trials, the scores recorded were either 0 or 1. Workers did not show any aggressive behavior against nestmate males (aggression index=0.62±0.27 in nest M3 and 0.52±0.27 in nest C2). Overall, males appeared not to be interested in workers, irrespective of the workers' original nests or supercolonies.

The combination of the attributes of the intruder and recipient workers, i.e., whether they belonged to the same supercolony, was an important factor in determining the level of aggression induced (GLMM; Estimate=1.81, SE=0.15, Z=12.1, P<0.001). The intruder's caste (worker or

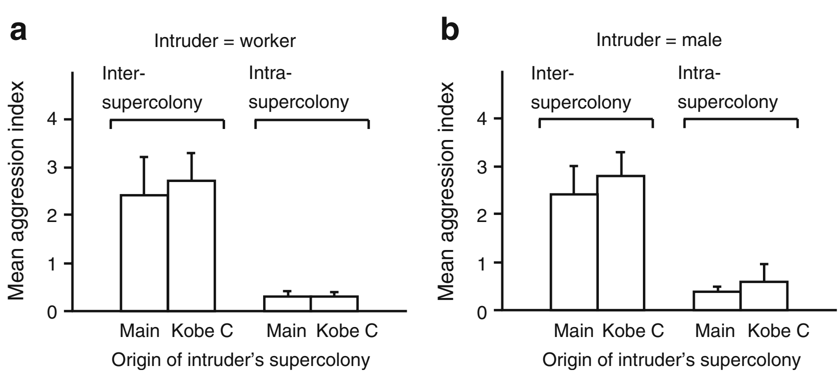

Fig. 1 Results of short-term aggression tests using workers and males of the Argentine ant. One intruder worker (**a**) or male (**b**) was introduced into a plastic dish with five recipient workers. The interaction between the intruder and recipient workers was scored as follows: 0=disregard, 1=antennation, 2=avoidance, 3=aggression, and 4=fighting. The highest score in each minute was averaged across 10 min (aggression index). Aggression indices (mean+SD, N=5) are presented based on the original supercolony of the intruder, and the combination of supercolonies (intra-supercolony or inter-supercolony)

male) or the recipients' nest (and necessarily supercolony) did not affect the level of aggression (Estimate=0.05, SE=0.09, Z=0.53, P=0.60 for intruder's caste; Estimate=−0.03, SE=0.03, Z=−1.30, P=0.19 for recipients' nest).

Long-term aggression tests

The male was killed in two of 29 inter-supercolony trials. In the trial between a M1 male and five C1 workers, the workers killed the male by severing its abdomen within 30–40 min. Similarly, in the trial between a M1 male and 20 C1 workers, the workers killed the male by severing its abdomen within 10–20 min. Injuries (loss of wings) to males were found in three trials. Workers remained aggressive toward alien males throughout the experimental period. The aggression score increased significantly as the number of recipient workers increased (Fig. 2a; P<0.001 for all combinations of comparison by the Steel–Dwass test). In trials with 20 workers, intense aggression (score 4) was observed almost every minute.

In intra-supercolony trials, males were not subjected to any aggressive behavior (Fig. 2b). The aggression scores did not change significantly as the number of workers increased (P>0.05 by the Steel–Dwass test). No males died in these trials.

Chemical analyses

Overall, 54 hydrocarbons with a skeletal carbon chain length of 14 to 37 (C14–C37) were identified (Online resource 1). Twenty-eight hydrocarbons of C14–C30 consisted of straight and monomethylalkanes, and three alkenes. Twenty-six hydrocarbons of C31–C37 consisted of straight, monomethyl, dimethyl, and trimethylalkanes. All hydrocarbons except one (triMeC35: peak 47 in Online resource 1) were shared among the samples, irrespective of the supercolony and caste. triMeC35 was abundant in the samples from the Kobe C supercolony but was undetectable in the samples from the Japanese main supercolony.

In a principal component analysis of the amount of common 53 hydrocarbons, the first, second, and third principal components explained 30.1%, 25.4%, and 9.5% of the total variance, respectively. When plotted on the plane of the first and second principal components, samples were not necessarily clustered by nests. The first principal component separated the samples into two groups, which agreed with the supercolonies (Fig. 3). Hydrocarbons that highly contributed to the first principal component (correlation coefficient >0.6 or <−0.6) were monomethyl, dimethyl, and trimethylalkanes of C31−C37 (17 peaks: Online resource 1). Some were more abundant in the Japanese main supercolony, and others were more abundant in the Kobe C supercolony. The second principal component separated workers from males (Fig. 3). Hydrocarbons that highly contributed to the second principal component (correlation coefficient >0.6 or <−0.6) were straight and monomethylalkanes of C14−C30 (12 peaks), and dimethylalkanes of C31–C37 (four peaks) (Online resource 1). All of the C14–C30 compounds were more abundant in workers. The difference in the relative abundance of dimethylalkanes between castes was not clear. One of the dimethylalkanes was relatively abundant in males, but the other three were abundant in the males of Kobe C only. In short, no hydrocarbon was notably more abundant in males than in workers.

Discussion

In the present study, we obtained evidence for our working hypothesis that Argentine ants possess a reproductive system in which the workers participate in selecting the mates for their queens. Argentine ant workers distinguished between males of alien supercolonies and those of their own, and attacked the alien males (Fig. 1). The aggression level against alien males was similar to that against alien workers. The frequency of severe aggression against alien males increased as the number of recipient workers increased due to the increase in encounters between males and workers (Fig. 2). A similar dependence on density was found in aggression tests between workers of Argentine ants conducted by Roulston et al. (2003). Recently,

Fig. 2 Relationship between the number of recipient workers (1, 5, or 20) and the level of aggression against a male intruder. Aggression tests were performed as described in the legend of Fig. 1 but with a different number of recipient workers. **a** Inter-supercolony nest pairs. **b** Intra-supercolony nest pairs

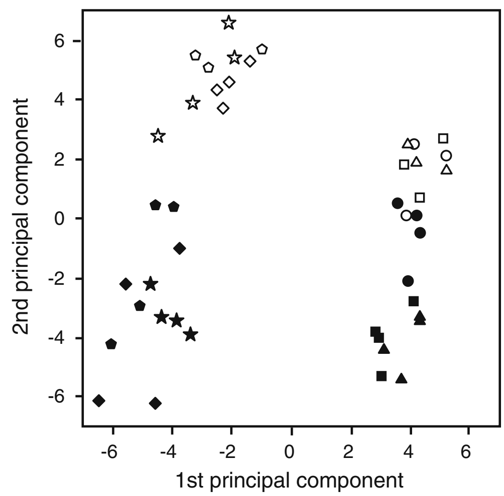

Fig. 3 Results of a principal component analysis (PCA) of the cuticular hydrocarbons of workers and males from two supercolonies of the Argentine ant. The PCA was performed with the relative proportion (peak area) of 53 hydrocarbons detected by GC/MS. triMeC35, present in only one of the two supercolonies, was not included in the analysis. Three or four samples of ants were analyzed per nest and caste. *Symbols* used for nests from the Japanese main supercolony: M1 (*circles*), M2 (*triangles*), and M3 (*squares*). *Symbols* used for nests from the Kobe C supercolony: C1 (*diamonds*), C2 (*pentagons*), and C3 (*stars*). *Closed symbols*: workers; *open symbols*: males

aggression of workers against alien males was also found in the invasive yellow crazy ant (Drescher et al. 2010). To our knowledge, the Argentine ant and the yellow crazy ant are the only social insect species in which male choice by workers has been demonstrated.

Cuticular hydrocarbon profiles of workers and males in the same supercolony were very similar. Long-chain hydrocarbons appeared to contribute to the distinction of supercolonies, and minor differences between castes were found mostly in the abundance of short-chain (<C30) hydrocarbons. This is consistent with the results of previous studies that suggested the importance of long-chain hydrocarbons, C33–C37 in particular, for nestmate recognition (Liang et al. 2001; Brandt et al. 2009; van Wilgenburg et al. 2010b). The present results suggest that workers recognize and attack males of alien supercolonies based on profiles of cuticular hydrocarbons. Workers are unlikely to distinguish between alien males and alien workers, and hence attack them both.

It is very likely that male Argentine ants are subjected to considerable aggression by workers when they intrude into the nests of conspecific but foreign supercolonies. Aggression by workers would decrease the possibility of a male reaching a queen, and hinder the mating of a male, and deprive a male of its physical strength. Thus, we argue that the aggression of workers against alien males may be a mechanism restricting gene flow between supercolonies of Argentine ants. Indeed, we witnessed two cases of a male being attacked by workers near the trail and nest entrance during the sampling of ants on the Maya Wharf. Further study is necessary to clarify the frequency of these attacks in nature. Moreover, since queens were not included in the present behavioral assays, it should be investigated whether workers still attack alien males in the presence of their virgin queens.

In the present study, the mortality of males from aggression by workers was low. It remains unclear to what extent the aggression of workers against alien males contributes to the restriction of gene flow between supercolonies of the Argentine ant. The presence of other mechanisms should also be taken into consideration. It should be examined whether queens accept or reject males from alien supercolonies as mates and whether offspring develop normally after successful copulation.

Vásquez and Silverman (2008b) found that some pairs of mutually aggressive Argentine ant colonies eventually fused into single colonies, albeit under laboratory conditions. This finding suggests that males may eventually be accepted by alien workers under certain conditions. However, the fusion of colonies occurred mostly in pairs with low levels of initial aggression and high levels of cuticular hydrocarbon and genetic similarity; some pairs remained mutually aggressive even after 6 months (Vásquez and Silverman 2008b). Further studies are necessary to elucidate the conditions for acceptance of individuals from alien supercolonies in nature.

There might be some evolutionary significance in the aggression of workers against alien males in the Argentine ant. According to the literature, this phenomenon appears uncommon in other ant species whose new queens stay within their natal nests, not undertaking mating flight. In those species, male-mediated gene flow has been reported to be common (Ross et al. 1999; Rüppell et al. 2003; Seppä et al. 2004, 2006; Berghoff et al. 2008; Holzer et al. 2009). For instance, workers of the army ant accept alien males without aggression (Franks and Hölldobler 1987). The aggression of workers against alien males might therefore be a phenomenon characteristic to Argentine ants and some other species.

Another significant aspect of worker–male interaction in Argentine ant supercolonies is its relevance to reproductive interference. Reproductive interference is defined as any kind of interspecific interaction (usually between related species) during the process of mate acquisition that adversely affects the fitness of at least one of the species involved (e.g., wasting time, energy, nutrients, or gametes) and is caused mostly by incomplete species recognition (e.g., misdirected courtship and heterospecific mating attempts; reviewed by Gröning and Hochkirch 2008). Reproductive interference has recently received much attention as a factor governing the geographical distribution of species and community structure (Kishi et al. 2009; Takakura and Fujii 2010). If single supercolonies are regarded as single biological species (Moffett 2010), the phenomenon found in this study agrees with the definition of reproductive interference. Another characteristic of reproductive interference is the presence of a density-dependent effect (Gröning and Hochkirch 2008), which was also found in the Argentine ant (Fig. 2). To date, most known cases of reproductive interference involve interactions between mating males and females, and even when non-mating individuals are involved, they are passively caught up in the phenomena (Gröning and Hochkirch 2008). Therefore, the present case of the Argentine ant can be interpreted as a novel form of reproductive interference, in which non-reproductive individuals (workers) are actively involved by attacking reproductive individuals (males).

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