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Local differences in parasitism and competition shape defensive investment in a polymorphic eusocial bee

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Abstract. Many colonial animals rely for their defense on a soldier caste. Adaptive colony demography theory predicts that colonies should flexibly adjust the investment in different worker castes depending on the colony needs. For example, colonies should invest more in defensive workers (e.g., soldiers) in dangerous environments. However, evidence for this prediction has been mixed. We combined descriptive and experimental approaches to examine whether defensive investment and worker size are adjusted to local ecology in the only known bee with polymorphic workers, *Tetragonisca angustula*. Colonies of this species are defended by a morphologically specialized soldier caste. Our study included three populations that differed in the density of food competition and the occurrence of a parasitic robber bee. We found that colonies coexisting with robber bees had on average 43% more soldiers defending the nest entrance, while colonies facing stronger foraging competition had soldiers that were ~6–7% smaller. We then experimentally relocated colonies to areas with different levels of competition. When released from intense food competition, body sizes of guards and foragers increased. After introducing chemical robber bee cues at nest entrances, we found both a short-term and a long-term up-regulation of the number of soldiers defending the colony. Active soldier numbers remained high after the experiment for a duration equivalent to 2–3 worker life spans. How information about past parasite threat is stored in the colony is currently unknown. In summary, *T. angustula* adjusts both the number and the body size of active soldiers to local ecological conditions. Competitor density also affects forager (or minor) size, an important colony trait with potential community ecological consequences. Our study supports adaptive colony demography theory in a eusocial bee and highlights the importance of colony threats and competition as selective forces shaping colony phenotype.

Key words: bees; colony demography; ergonomic efficiency; phenotypic plasticity; resilience; size polymorphism; social insects; worker size.

Introduction

In nature, resource limitations lead to trade-offs (Stearns 1989). The urgency for defense against parasites, competitors, and predators is highlighted by observations that both plants and animals reduce energy allocation to important life history traits such as growth and reproduction in favor of stronger defensive mechanisms (e.g., Harvell 1992, Yoshida et al. 2004). Phenotypic plasticity of defensive physiology and behavior allows organisms to pay the costs for enhanced protection only when they are outweighed by increased survival chances (e.g., Pettersson and Brönmark 1997, Storm and Lima 2010, Segers and Taborsky 2012).

In colonial animals, defense is often the task of a morphological type, a soldier caste (reviewed in Tian and Zhou 2014). These soldier castes enable superior performance of defensive tasks and increase the probability of colony survival (e.g., Giron et al. 2007, Powell 2009, Grüter et al. 2012, Kamiya and Poulin 2013). On the other hand, soldiers come at a cost to the workforce and may reduce investment in reproduction (e.g., Stern and Foster 1996, Harvey et al. 2000, Kamiya and Poulin 2013, Chouvenc et al. 2015). Thus, a colony has to trade-off the costs of an adequate defense against growth and reproduction. Furthermore, in social insects, soldiers (also called "majors" in ants) are generally larger than members of other worker castes

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("minors") and therefore require more resources to rear (e.g., Hölldobler and Wilson 2009, Segers et al. 2015). The relative proportions of majors and minors in the workforce and their body sizes are an important part of the demography of a colony (Oster and Wilson 1978). To maximize colony-level ergonomic efficiency, a colony is predicted to adjust its demographic traits in response to predation, resource availability, and competition (Oster and Wilson 1978).

Yet, the evidence for adaptive colony demography has been mixed and in various social insect taxa important ecological variables do not seem to affect worker caste ratios (reviewed in Schmid-Hempel 1992, Duarte et al. 2011). It has been suggested that for many social Hymenopterans, environmental changes will come too quickly for colonies to be able to respond by increasing soldier production (e.g., Walker and Stamps 1986, Kaspari and Byrne 1995). Thus, behavioral flexibility of workers (e.g., foragers taking over defensive work) might be important to respond to sudden changes in predation or competition (Wilson 1983, Mertl and Traniello 2009). Alternatively, by overproducing soldiers (e.g., Kaspari and Byrne 1995) a colony could build up a reserve, which can then be mobilized in response to sudden threats to the colony (Kolmes 1986).

In agreement with adaptive colony demography theory, worker size has been observed to vary with environmental conditions (Davidson 1978, Bershers and Traniello 1994, Kamiya et al. 2013, Wills et al. 2014). For example, by studying caste ratios and worker size of the ant *Pheidole morrisi*, Yang et al. (2004) showed that these two colony demographic traits vary between populations in a correlated way: the population with a high proportion of majors had smaller body sizes for both the minor and major caste. The authors suggest that this is the result of a reduction in the threshold size required for larvae to develop into soldiers. Thus if the worker size distribution remains the same but the threshold size is shifted downward, the proportion of soldiers in the colony will increase while the mean size of the major caste will decrease (Fig. 2 in Yang et al. 2004). This threshold model elegantly explains how developmental mechanisms and ecology could be connected in social insects. The study of Yang et al. (2004) highlights the need to measure both worker size and worker number when studying how colony demography reflects environmental parameters.

Inter- and intraspecific competition (Davidson 1978, Passera et al. 1996, Harvey et al. 2000, Lloyd and Poulin 2014) and predation (Shingleton and Foster 2000) are thought to be important factors shaping colony demography and drivers of soldier caste evolution in general (e.g., Thorne et al. 2003). For example, Passera et al. (1996) found that *P. pallidula* colonies increase the number of soldiers if they perceive conspecific competitors in a laboratory setting. However, how local ecology shapes colony demography remains poorly understood because there is a lack of field studies that relocate colonies to different environments, presumably because most species cannot easily be moved in nature without the risk that colonies migrate and disappear. An alternative method to examine how the environment affects colony resilience and demography is to manipulate ecological factors around the nest (Passera et al. 1996, Shingleton and Foster 2000, McGlynn and Owen 2002).

Here we combine descriptive and experimental approaches to investigate differences in colony demographic traits between populations of the only known bee with a morphologically distinct soldier caste, the stingless bee *Tetragonisca angustula* (Grüter et al. 2012). Bee colonies in hive boxes are well-suited for relocation experiments as they can be moved to different environments without colonies absconding. *T. angustula* is common in the Neotropics and competition with other stingless bees for food sources can be intense (Hrncir and Maia-Silva 2013). Additionally, *T. angustula* is one of the main targets of the parasitic robber bee *Lestrimelitta limao* (Sakagami et al. 1993) and it has been hypothesized that the specialized soldiers in *T. angustula* are the result of an evolutionary arms race with *L. limao* (Grüter et al. 2012). For three *T. angustula* populations, which differ in competitor density (number of stingless bee colonies) and parasitism (by the robber bee *L. limao*), we compared two colony demographic parameters and their relationship with colony size: (1) mean sizes of soldiers and foragers (minors) and (2) the number of active soldiers. Subsequently, we examined if colony demographic parameters change when competitor density was manipulated through colony relocation and colonies were deliberately exposed to robber bee cues. We predicted that competitor density and parasite prevalence affect defensive investment measured as the number and size of nest entrance guards.

METHODS

Study species

Tetragonisca angustula is a highly eusocial stingless bee with a soldier caste: bees guarding the entrance are on average 30% heavier and of different shape than foragers (minors; Grüter et al. 2012). It nests in cavities in trees, walls and buildings throughout the Neotropics. Colonies contain up to 10 000 workers and are defended by one group of soldiers hovering in front of the nest entrance tube, and another group of soldiers standing on the wax entrance tube (Grüter et al. 2011, van Zweden et al. 2011). During a *L. limao* raid, *L. limao* workers steal resources (wax, honey, pollen, and larval food) and raids occasionally lead to the death of the attacked colony (Sakagami et al. 1993). The ability of *T. angustula* soldiers to detect and kill *L. limao* intruders depends on their number (van Zweden et al. 2011) and body size (Grüter et al. 2012).

Tetragonisca angustula visits many different flower species and it follows an "insinuator strategy", i.e., the non-aggressive foragers leave flower patches in the presence of more dominant stingless bee species (Hrncir and Maia-Silva 2013). Thus, competing stingless bee colonies are likely to have a considerable effect on foraging activities. Its average foraging distance is unknown but a similar sized stingless bee was estimated to forage at approximately 80–400 m (Inoue et al. 1985).

Study populations

We studied three populations of *T. angustula*. The Fazenda population is located on a farm near São Simão, São Paulo State, Brazil, approximately 60 km from the other two populations on the campus of the University of São Paulo in Ribeirão Preto. The populations on the university grounds are adjacent to each other (Fig. S1A). The two university populations differ in local ecology: the colonies in the stingless bee yard (population Meliponary) live in a high-density environment (38.25 ± 3.05 [mean ± SE] colonies within a radius of 5 m) of inter- and intraspecific stingless bee colonies (~10 different species). In contrast, the colonies on the campus (population Campus) experience a much lower colony density (0.50 ± 0.20 colonies within a horizontal radius of 5 m). Natural densities vary greatly, but densities of more than 30 colonies in individual trees have been reported (Roubik 1989). The risk of being attacked by the robber bee *L. limao* differs between the three populations. On the Fazenda, *L. limao* colonies have been removed since 1984 to protect colonies from robbing (Nogueira-Neto 1997). Attacks of *L. limao* are, therefore, very rare (Nogueira-Neto 1997; P. Nogueira-Neto, *personal communication*) and we have not observed a raid during several months of fieldwork (F. Segars, C. Grüter, *personal observation*). The Meliponary and Campus populations suffer frequent *L. limao* attacks, with at least four known *L. limao* nests within 1.5 km distance from our *T. angustula* study colonies (Fig. S1A). Although the Fazenda population is in a bee yard, the density is much lower (2.95 ± 0.47 colonies within a horizontal radius of 5 m) than in the Meliponary. The colonies of both the Meliponary and the Fazenda population were kept in wooden hive boxes. These boxes were of variable sizes. The wild Campus colonies lived in cavities of various sizes and types (walls or trees). Preliminary observations indicated that local ecology is a much stronger determinant of colony demography than nesting cavity. The foraging range of some of the Campus and Meliponary colonies were likely overlapping (Fig. S1A).

Population differences in colony demography

We used colony traffic as a noninvasive method to estimate relative colony size (Grüter et al. 2011). We counted the number of bees entering the nest during 30 s three times a day (11:00–12:00, 14:00–15:00, 16:00–17:00) on a day with good foraging conditions. For data analysis, the three measurements were averaged for each colony. At the same time, we counted the number of soldiers guarding the nest entrance. Guarding behavior is highly characteristic (e.g., wing fanning and hovering; Grüter et al. 2011, van Zweden et al. 2011). The number of soldiers actively guarding the colony entrance does not equal the total number of soldiers in a colony, because young bees (2 weeks or younger) perform other behaviors, such as brood care or cell building (Hammel et al. 2016). However, we consider a larger number of soldiers at the entrance a larger investment in defense because guarding is energetically costly (hovering and wing fanning) and these soldiers are unable to perform other tasks such as foraging. Moreover, predation, for example by jumping spiders (Penney and Gabriel 2009), makes guarding a dangerous task.

We studied 19 colonies from the Fazenda, 17 colonies from the Campus, and 15 colonies from the Meliponary. We included the worker mass data from four additional Meliponary colonies studied in Grüter et al. 2012 (thus, $n = 19$ colonies for worker size). We captured 5–10 returning pollen foragers and 5–12 soldiers at the entrance from each colony and weighed each bee to the nearest 0.1 mg using a Sartorius TE64 (Göttingen, Niedersachsen, Germany) analytical balance after immobilizing the bees through chilling. Wet mass in *T. angustula* is highly correlated with other morphological measurements such as head width ($r = 0.89$, $n = 293$; C. Grüter, *unpublished data*) and represents the energetic investment in a worker.

Colony relocation

To test experimentally if colony density affects colony demography we moved 12 colonies from a high- to a low-density environment (Fig. A1B; high to low treatment). Three of these colonies died before the end of our relocation experiment, with two of them showing clear signs of *L. limao* attacks. We also moved six colonies from this low-density location to the high-density location (low to high treatment). Two of those colonies died of unknown causes before the experiment ended. Additionally we used 12 wild colonies on the university campus (Fig. A1B) as an environmental control (stationary). These stationary colonies experience low-density conditions. All colonies used for this experiment (both relocated and stationary ones) were within approximately 500 m of each other and, therefore, experienced similar foraging and climatic conditions (Fig. A1B).

Before relocation, we caught six pollen foragers and six soldiers from each colony and weighed them as described in "Population differences in colony demography". Colony traffic data and counts of soldiers defending the entrance were collected as previously described. Colonies were relocated by closing the nest entrance at nightfall after all foragers returned and carefully carrying them for about 200 m to their new location. The next morning, we opened the nest entrances and left the colonies untouched for 5 months (from June 2013 to November 2013). After this period, we again collected soldiers and foragers for weighing and repeated the traffic and soldier counts. The deceased colonies were excluded from the data analysis (inclusion did not change the effects we found).

L. limao exposure

For this experiment, we used 22 wild colonies on the university campus. Eleven colonies were exposed to *L. limao* cues, while the remaining 11 colonies were exposed to *Tetragona clavipes* cues. *Tetragona clavipes* is a common sympatric stingless bee that has a similar size compared to *L. limao*, but is not known to attack *T. angustula* colonies. *Lestrimelitta limao* volatiles secreted by glands in the head signal the robber's presence to nest workers (Blum et al. 1970, Wittmann et al. 1990). *Lestrimelitta limao* and *T. clavipes* workers were captured at the nest entrances and freeze killed on the day of introduction. We used one crushed head of either a *L. limao* or *T. clavipes* worker per colony, which we gently dropped in the entrances of the colonies from where it usually rolled deeper into the tube. This procedure was performed two times a week for a 5-week period (week 1–5).

Before exposing any colonies to allospecific cues, we counted the number of soldiers at the colony entrances in the early afternoon on three different days (14:00–15:00) and averaged these counts to obtain a reference number for each colony. This reference number was used to calculate the changes in active soldier number in percentages (denoted as relative guard number in *Results*) during and after the weeks in which we exposed the colonies to allospecific cues. On the days of exposure to cues, but before introducing the crushed heads we counted the number of soldiers defending the colony entrance (14:00–15:00). In weeks 1 and 4, we also counted the number of soldiers 30 min after exposure to measure the short-term effects of the exposure. To examine whether there was a long term response of the bees to our treatment we counted active soldiers once again (14:00–15:00) in weeks 11 and 14, respectively, six and nine weeks after we stopped the cue exposure. In week 15, we captured 3–10 guards per colony and measured their head width (cf. Grüter et al. 2012). Between weeks 8 and 11, two colonies (one control and one robber bee colony) suffered a *L. limao* attack and perished. Thus, our control colonies might also have been exposed to *L. limao* cues, though to a much lower level.

A *T. angustula* worker needs approximately 5–6 weeks from egg to eclosion (M. Prato, *personal communication*). After eclosion, a period of behavioral maturation of approximately 2 weeks follows (Hammel et al. 2016). Thus, a new generation of soldiers can be reared in 7–8 weeks. *T. angustula* soldiers guard for an average of 5–6 d (Grüter et al. 2011).

Data analysis

Data was analysed in R 2.9.2 (R Development Core Team 2009) using mainly linear models (LMs) and linear mixed-effect models (LMEs). The LMEs were fitted with the lme function of the nlme package and colony was included as random effect to control for non-independence of data. During LME model selection we first explored the best structure of the random components (comparing random intercept models with random intercept and slope models) using REML (Zuur et al. 2009). Significance of interactions was tested with maximum likelihood (ML) and likelihood ratio tests (LRT). Nonsignificant interactions ($P > 0.05$) were removed from the models and the final LMEs were fitted with REML (Zuur et al. 2009). Model validity was checked by plotting residuals. When differences between the three populations were detected by LMs or LMEs, we compared the populations pairwise with Tukey post hoc tests, using a sequential Bonferroni correction to calculate P values. For the post hoc tests we averaged over interactions and covariates (R package multcomp, Hothorn et al. 2008). In case of significant interactions when we were comparing colony demographics between the three populations, we performed additional models for each population separately.

To test for the an effect of exposure to *L. limao* and *T. clavipes* cues on the number of surveilling soldiers we calculated for each testing day the change in percentages compared to the reference guard number we estimated before the exposure treatment began. Subsequently, we tested for an effect of the cues on relative soldier number with two LMEs (one for during and one for after the exposure treatment) including testing day nested within colony as a random effect. Next to cue type, we also included experimental week as main effect to analyze the responses of the colonies over time. Experimental week was centered to ease interpretation of the models (Zuur et al. 2009).The observations from week 1 and 4 on the short-term effect (within 30 min after exposure to cues) of the treatments on soldier number were analysed with paired t-tests or paired Wilcoxon tests depending on the distribution of the response variable.

RESULTS

Population comparisons

There were population differences in the number of surveilling soldiers at the colony entrance (LM, $F_{2,56} = 21.13$, $P < 0.001$): the Fazenda population, which is exempt from robber bee parasitism, had on average 43% fewer soldiers at the colony entrance than the Campus and Meliponary populations for a given colony traffic (colony size) and mean soldier mass (Tukey post hoc; Fazenda vs. Campus, $t = -6.48$, $P < 0.001$; Fazenda vs. Meliponary, $t = -3.62$, $P = 0.001$; Fig 1A and B). The number of soldiers at the colony entrance did not differ between the Campus and the Meliponary population (Tukey post hoc; Campus vs. Meliponary, $t = 1.76$, $P = 0.08$). For all populations, the number of soldiers at the colony entrance increased with both colony traffic (Fig. 1A) and mean soldier mass (Fig. 1B) (LM, $F_{1,56} = 30.43$, $P < 0.001$ and $F_{1,56} = 5.14$, $P = 0.03$, respectively).

The three populations differed in the relationship between colony traffic and soldier mass (population × colony traffic; LME, $n = 607$, LRT = 17.28, $P < 0.001$; Fig. S2). When we analyzed the relationship between colony traffic and soldier mass separately for each population, we found that, for the Meliponary population, soldier mass increased with colony traffic (LME, $n = 163$, $t = 3.86$, $P = 0.002$). For the Fazenda and the Campus populations, colony traffic was unrelated to soldier mass (Fazenda, $n = 190$, $t = -1.69$, $P = 0.11$; Campus, $n = 254$, $t = -0.22$, $P = 0.83$; Fig. S2). Overall, the colonies in the Meliponary had on average 6.33% smaller soldiers than the colonies on the Campus (Tukey post hoc, Meliponary vs. Campus, $z = -2.54$, $P = 0.02$) and 6.91% smaller soldiers than colonies on the Fazenda (Fazenda vs. Meliponary, $z = 2.71$, $P = 0.02$). Fazenda and Campus colonies did not differ in mean soldier mass (Fazenda vs. Campus: $z = 0.36$, $P = 0.72$).

In all three populations, forager mass increased with colony traffic (LME, $n = 364$, $t = 2.74$, $P = 0.008$; Fig. S2) and there was a trend for forager mass to differ between the populations (population, LRT = 4.63, $P = 0.10$; population × colony traffic, LRT = 5.62, $P = 0.06$).

Colony traffic affected the difference in mass between foragers and soldiers differently in the three populations (colony traffic × population, LM, $F_{2,54} = 9.05$, $P < 0.001$; Fig. S2): with increasing forager traffic the mass difference became smaller in the Fazenda population (LM, $F_{1,17} = 12.33$, $P = 0.003$), while the opposite was true for the Meliponary population (LM, $F_{1,12} = 6.99$, $P = 0.02$). Forager and soldier difference was not related to colony traffic for the colonies on Campus (LM, $F_{1,25} = 0.54$, $P = 0.49$).

There were also differences between the three populations in how mean forager mass of a colony was related to mean soldier mass (population × mean forager mass, LM, $F_{5,59} = 6.31$, $P < 0.001$; Fig. A3). For the Campus and Meliponary colonies, the size of their foragers was significantly related to the size of their soldiers (LM, Campus, $F_{1,26} = 5.61$, $P = 0.03$; Meliponary, $F_{1,16} = 12.88$, $P = 0.002$), however, forager and soldier size were not linked in the Fazenda population (LM, $F_{1,17} = 0.38$, $P = 0.55$).

Colony relocation

Both the high-to-low and stationary colonies had a higher colony traffic in November compared to June (LME, $n = 41$, $t = -2.69$, $P = 0.01$). There was no statistical difference in colony traffic between the two groups (stationary vs. high-to-low, $t = 0.81$, $P = 0.43$; treatment group × month, LRT = 1.63, $P = 0.20$). There was also no difference in surveilling soldier

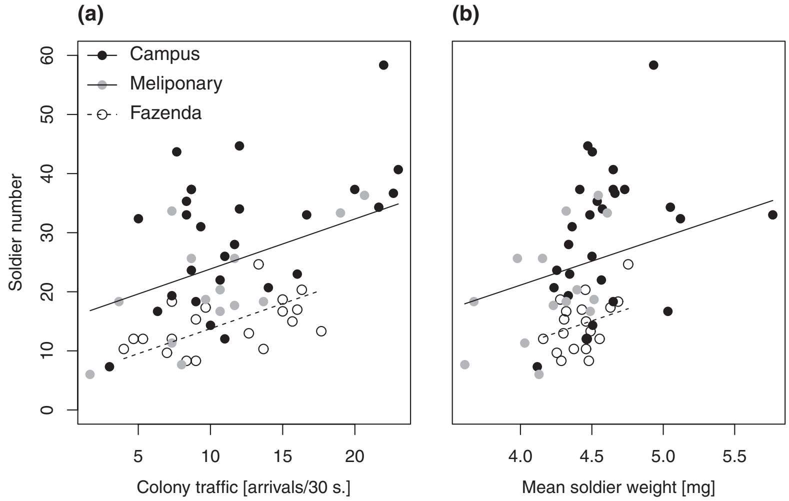

FIG. 1. The relationships between (A) colony traffic and surveilling soldier number and (B) mean soldier mass and soldier number. Each point represents a colony. In both figures, the solid line is the best fit line for both the Campus and the Meliponary population, as there was no significant difference between the two (see *Results* section). The best fit lines were made with the parameter estimates from the linear model. Because the model contains two covariates, mean soldier mass was kept constant in panel (A) and traffic was kept constant in panel (B) (by adding the mean value for all populations).

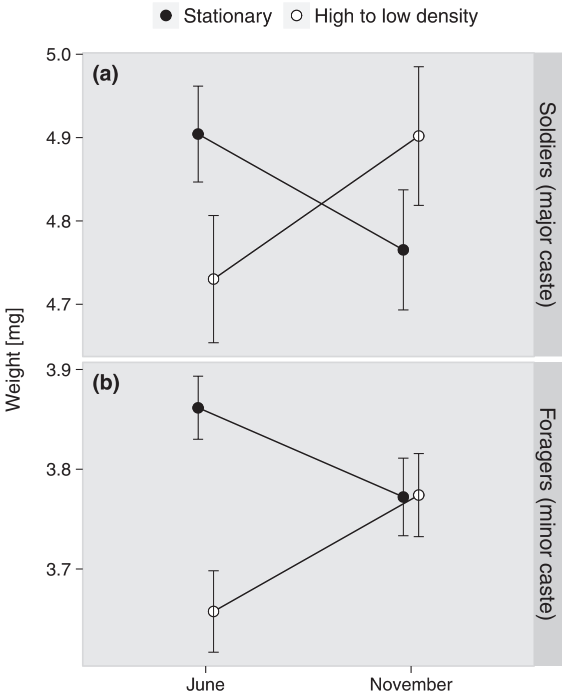

FIG. 2. The effect of relocation from a high- to low-density environment on the size of soldiers and foragers. The points and error bars represent the mean ± SE mass for the treatment groups.

number between the stationary and high-to-low colonies (LME, $n = 41$, $t = -0.38$, $P = 0.71$) and soldier number did not change over time for both groups (June vs. November, $t = 0.26$, $P = 0.80$; density group × month, LRT = 0.10, $P = 0.76$).

When analyzing worker size, we found a significant interaction between treatment group (high-to-low and stationary) and month (June and November; LME, $n = 503$, $t = -3.74$, $P < 0.001$). More specifically, before relocation, foragers and soldiers of the colonies at the high-density site were significantly smaller than foragers and soldiers of the stationary (low-density) colonies (LME, $n = 251$, $t = -2.54$, $P = 0.02$; Fig. 2), while at the end of the relocation experiment, workers of the stationary and the high-to-low colonies no longer differed in mass (LME, $n = 252$, $t = 0.005$, $P = 0.99$, Fig. 2). The changes in mass depending on month and treatment were similar for both soldiers and foragers (i.e., all interactions with worker type were nonsignificant).

The colonies that were moved from a low-density site to the high-density site were not included in the model because of the small sample size. However, when analyzing this group separately, we found that soldiers became significantly smaller in the high-density environment (before, 5.14 ± 0.09 mg [mean ± SE]; after 4.72 ± 0.10 mg; LME, $n = 47$, $t = 3.19$, $P = 0.003$), while foragers did not change significantly in mass (before, 3.68 ± 0.04 mg, after, 3.73 ± 0.06 mg; $n = 42$, $t = -1.42$, $P = 0.17$).

L. limao exposure

Compared to the control colonies, the colonies treated with robber bee cues showed a significant increase in the number of surveilling soldiers (relative to the pretreatment estimate) already after the first exposure (LME, $n = 198$, robber bee vs. non-robber bee cue, $t = 3.79$, $P = 0.001$; Fig. 3). The difference between the two treatments increased over the duration of our exposure treatment (cue type × experimental week, LRT = 13.92, $P < 0.001$; experimental week, $t = -1.39$, $P = 0.17$). After we stopped the treatment (Fig. 3;

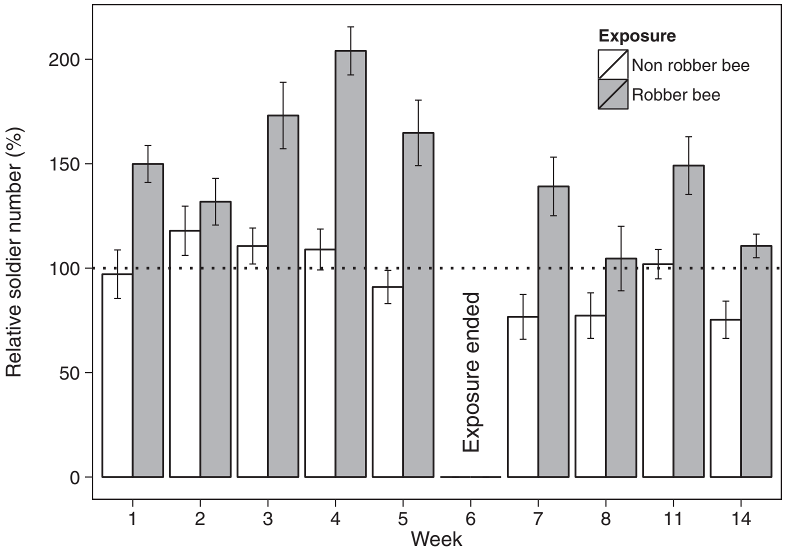

FIG. 3. The bar plots show for both treatment groups the mean (±SE) percentage of change in surveilling soldier number relative to a colony-specific estimate, which is based on counts done before the start of the exposure treatment (the dotted line indicates the reference level). The relative soldier counts shown for week 1 were recorded after the colonies were exposed to allospecific cues for the first time earlier in the week. The statistical tests were done between the treatment groups (see *Results* section).

week 7–14) the previously administered robber bee cues still had a noticeable effect on soldier number (LME, $n = 128$, $t = 2.63$, $P = 0.02$; Fig. 3). Remarkably, for the duration of our observations, the difference in the number of soldiers between colonies of both groups did not decrease (i.e., no interaction between cue type × experimental week, LRT = 1.08, $P = 0.30$; experimental week, $t = -0.23$, $P = 0.82$).

Next to a long-lasting change in the amount of soldiers at the nest entrance the colonies exposed to robber bee cues also showed an instant response to our treatment: 30 minutes after we applied the cues for the second time in week 1, the soldier numbers relative to the pretreatment counts significantly increased from 149.9% up to 402.1% (±153.2 SE; paired Wilcoxon, $n = 22$, $V = 66$, $P < 0.001$). The control colonies exposed to non-robber bee cues did not show this immediate response (from 97.1% ± 11.6% to 103.0% ± 12.5%, paired t test, $n = 22$, $t = -0.67$, $P = 0.52$). When we tested the short-term response in week 4, the colonies no longer showed this instant strong response to the robber bee cues: directly before exposure the relative soldier numbers were 199.8% ± 15.8% while, 30 minutes later after we administered the cues, these numbers went up to 213.2% ± 29.8% (paired t test, $n = 10$, $t = -1.82$, $P = 0.10$). Again the control colonies showed no response (from 117.3% ± 10.5% to 101.7% ± 13.5%, $n = 11$, $t = 1.52$, $P = 0.16$).

At the end of the experiment, there was no size difference between the soldiers of the colonies that were exposed to robber bee cues and the soldiers of the colonies that were exposed to non-robber bee cues (LME, $n = 153$, $t = -0.66$, $P = 0.52$). Colony traffic ($t = 0.02$, $P = 0.98$) and its interaction with treatment (LRT = 0.40, $P = 0.52$) also did not affect soldier size.

Discussion

Our results show that small scale ecological differences between populations are linked to differences in worker size and defensive investment in *T. angustula* colonies. Furthermore, experimental changes in important ecological parameters lead to changes in colony-level phenotypic traits.

Density of food competitors

Colonies in a high-density environment produced smaller soldiers and tended to have smaller foragers but were of similar colony size. It should be noted that this finding is based on a comparison of colonies from three populations. It would be interesting to include more populations in future studies, perhaps also from other South American biotopes. However, our relocation experiment supports the hypothesis that worker size is affected by food competitors in the immediate surroundings: body size of both soldiers and foragers increased for colonies that we relocated from an environment with many con- and heterospecific stingless bee colonies to an environment with a low density of competitors. A control group of stationary colonies under low-density conditions did not increase worker size over the same period, suggesting that the increase in relocated colonies is not due to general environmental changes (e.g., season or food availability). In stingless bees, newly relocated colonies normally experience a reduction in worker population and worker size (Ramalho et al. 1998), while the colonies we moved from high to low density showed the opposite pattern. Although our other experimental group, which was moved from low to high density, was small, the results for this group partly supported our expectation that competitor density affects worker size: the soldier caste decreased in size, while the forager worker caste did not show a significant change. This change in soldier size in the opposite direction compared to high-to-low colonies shows that the change in worker size of high-to-low colonies was not due to the relocation per se. As far as we know, this is the first study to experimentally demonstrate that the density of food competitors affects worker size in bees. In other social insects, there is observational evidence that competition affects worker size (see Davidson 1978, Kamiya et al. 2013).

Worker size has several potentially important effects: larger bees have been shown to forage more efficiently (e.g., Goulson et al. 2002, Spaethe and Weidenmüller 2002), cover a larger foraging area (Greenleaf et al. 2007) and are better fighters (Grüter et al. 2012). On the other hand, a colony can obtain more workers for a given energetic investment if it produces smaller workers (e.g., Yang et al. 2004) and maintaining a larger population of foragers and soldiers could be important for food competition and defense (Bershers and Traniello 1994, van Zweden et al. 2011). Colonies of the ant *P. pallidula* increased soldier production after being exposed to conspecific competitors (Passera et al. 1996), but it is not currently known whether colonies also adjusted the mean body sizes of soldiers and minors. Interestingly, we found that the numbers of soldiers defending the nest entrance and the foraging traffic were unaffected by colony relocation to a high-density environment. Under high food competition, *T. angustula* seems to prioritize having smaller workers in stable numbers over larger workers and a smaller worker population. However, it remains to be tested whether the size reduction we observed is a side effect of a decrease in food availability or an adaptive response.

For all three populations, forager mass was positively related to colony size, which is common for social insects (Oster and Wilson 1978). In contrast, only in the high-density population did soldier body size increase with colony size. One interpretation is that small colonies in the highly competitive environment could not afford large soldiers, while simultaneously keeping the number of active soldiers high. Additionally, in the high-density population the size difference between soldiers and foragers decreased in smaller colonies, while this relationship was absent and opposite in the Campus and Fazenda populations. Generally, soldier size and within-colony size variation have been found to increase with colony size in ants (e.g., Wood and Tschinkel 1981, Huang and Wheeler 2011, but see Tschinkel 1998). We suggest that population differences in the relationships between colony size, worker size and within-colony size variation arise through ecological pressures favoring different balances between worker size and numbers (see also Bershers and Traniello 1994).

Robber bee sympatry

Lestrimelitta limao is a widespread threat of *T. angustula* but robber densities are likely to vary locally. In our study, the populations experiencing attacks from robber bees had a higher number of soldiers at the colony entrance. Thus, colonies existing alongside robber bees seem to permanently up-regulate colony defense. Our observation that colonies that are not exposed to robber bees have a reduced colony defense is in agreement with ergonomic efficiency theory (Oster and Wilson 1978). The difference in soldier number depending on robber bee threat could be an example of local adaptation (Yang et al. 2004) or the result of adaptive phenotypic plasticity (Passera et al. 1996). *Tetragonisca angustula* colonies were able to instantly raise defense (within 30 min), presumably by activating a reserve of soldiers inside the colony. A reserve of soldiers might lead to a resilient social system, which is expected to evolve when the biotic environment contains potentially catastrophic threats (Kolmes 1986). This is the case in our study species, where robber bee raids represent sudden events that last from a few hours to a few days (Sakagami et al. 1993). Mobilizing a reserve is much faster than rearing a new generation of soldiers, which can take several weeks in *T. angustula* (Segers et al. 2015). Keeping soldiers inside the colony as long as they are not needed is likely to benefit colonies by reducing soldier energy consumption and mortality (e.g., predation by jumping spiders; Penney and Gabriel 2009). A repeated exposure over several weeks lead to a prolonged increase in the number of surveilling soldiers outside the nest and we measured an increased number of soldiers nine weeks after the treatments were terminated. These soldiers were either still developing as larvae in sealed cells or not present at all when we exposed the colonies to robber bee cues. Thus, *L. limao* exposure might lead to an increased soldier production on a long-term basis. Alternatively, colonies might have allocated a larger number of reserve soldiers to active guarding. Colonies exposed to robber bee cues did not have smaller soldiers at the end of the experiment making it unlikely that they recruited workers from smaller size classes. How the information about an increased threat from *L. limao* is stored in the colony is currently unknown and represents an interesting topic for future research.

The developmental threshold model proposed for dimorphic *Pheidole* ants (and which might also hold in the ants *Pogonomyrmex badius* and *Solenopsis invicta*, see Tschinkel 1998) explains how soldier ratios can be adjusted to local ecology: colonies achieve an increase in soldier number by reducing the threshold size required for development into soldiers (Yang et al. 2004, McGlynn et al. 2012). As a result, a population with a higher proportion of soldiers per colony has on average smaller soldiers (Yang et al. 2004). We can use our data to test this model in *T. angustula*, if we assume that the number of active guards is proportional to the number of soldiers produced by a colony. We found that the Fazenda population has fewer but larger soldiers than the Meliponary population and fewer soldiers than the Campus population, but is of a similar size. Thus, our data is inconclusive as to whether it supports the developmental threshold model for stingless bees. By hand-rearing workers, future studies can test whether *T. angustula* populations that live sympatrically with robber bees have lower size thresholds at which larvae are destined to become soldiers.

CONCLUSION

Our results confirm the prediction of colony demography theory that worker size and worker size variation should differ between populations in different habitats (Oster and Wilson 1978). We demonstrated experimentally that worker size changes with colony density. However, the decrease in worker size in high-density areas might be a non-adaptive colony response to reduced food availability. Worker size is an important trait of social insect colonies with community ecological consequences (Greenleaf et al. 2007, Mertl and Traniello 2009, Bihn et al. 2010). Additionally, we have shown that colonies increase ergonomic efficiency by adjusting the number of soldiers at the colony entrance to the perceived threat.

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