A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee

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Division of labor among workers is common in insect societies and is thought to be important in their ecological success. In most species, division of labor is based on age (temporal castes), but workers in some ants and termites show morphological specialization for particular tasks (physical castes). Large-headed soldier ants and termites are well-known examples of this specialization. However, until now there has been no equivalent example of physical worker subcastes in social bees or wasps. Here we provide evidence for a physical soldier subcaste in a bee. In the neotropical stingless bee *Tetragonisca angustula*, nest defense is performed by two groups of guards, one hovering near the nest entrance and the other standing on the wax entrance tube. We show that both types of guards are 30% heavier than foragers and of different shape; foragers have relatively larger heads, whereas guards have larger legs. Low variation within each subcaste results in negligible size overlap between guards and foragers, further indicating that they are distinct physical castes. In addition, workers that remove garbage from the nest are of intermediate size, suggesting that they might represent another unrecognized caste. Guards or soldiers are reared in low but sufficient numbers (1–2% of emerging workers), considering that <1% usually perform this task. When challenged by the obligate robber bee *Lestrimelitta limao*, an important natural enemy, larger workers were able to fight for longer before being defeated by the much larger robber. This discovery opens up opportunities for the comparative study of physical castes in social insects, including the question of why soldiers appear to be so much rarer in bees than in ants or termites.

Meliponini | caste evolution

Division of labor among workers is nearly universal in eusocial insects (1–5). In most species, this division is based on temporal subcastes, in which workers specialize in different tasks at different ages ("age polyethism") (2, 4, 5). Physical worker subcastes (i.e., groups of workers with morphological adaptations for particular tasks) are less common (4–6). Only ~15% of all ant genera contain species with physical worker subcastes, and no species of bee or wasp is known to have a division of labor based on physical subcastes (4–6). Where physical worker subcastes occur, they appear to benefit the colony by increasing the efficiency of key tasks such as defense, but have the disadvantage of preventing a colony from rapidly adjusting caste ratios if necessary (4–6). In addition, extreme subcastes, such as large-bodied soldiers, are more costly to rear and have a limited task repertoire (4). It also has been argued that individual-level selection might prevent the evolution of worker polymorphism if workers of different subcastes have varying opportunities to reproduce (5, 7). Given the number of ant and termite species with physical worker subcastes, the lack of evidence for physical worker subcastes in bees and wasps is an intriguing and ongoing puzzle in the study of caste evolution (5).

With several hundred species, stingless bees (Meliponini) are the largest group of highly eusocial bees (8). Their colonies are perennial, typically have a single queen, and contain *ca.* 100–100,000 workers, depending on species (8, 9). It is usually assumed that the division of labor in stingless bees is similar to that

seen in honey bees, with workers of uniform size performing a sequence of tasks as they age (10–13).

Jataí (the Brazilian common name for *Tetragonisca angustula*) are unique among eusocial bees in having a sophisticated system of defense involving two complementary groups of guards (14–16): hovering guards, which station themselves in the air near the nest-entrance tube, and guards that stand inside and around the tip of the wax entrance tube (14) (Fig. 1*A*). In addition, given the short average lifespan of workers (*ca.* 20 d) (17), individual Jataí guards often perform this task for a long duration, 5 d or more (15), compared with approximately 1 d in the honey bee (18). The lengthy careers of Jataí guards are more characteristic of a species with physically specialized guards or soldiers, as in ants or termites, and while carrying out previous studies on Jataí, we noted that guards appeared to be bigger than foragers. This led to the hypothesis that the Jataí worker force includes a distinct physical subcaste of larger-bodied guards.

To test this hypothesis, we analyzed the size and shape of guards, foragers, and waste-removing workers from colonies at two locations 50 km apart in the state of São Paulo, Brazil, where Jataí is a common native species. Because guards aggressively defend the entrance against intruders, we also tested whether worker size is related to fighting performance. To do this, we staged fights between individual *T. angustula* soldiers and workers of *Lestrimelitta limao*, a common obligate robber bee sympatric to Jataí. Jataí is one of the main targets of *L. limao* raids, and attacks frequently result in colony death in the study area (19).

Results

Size Differences Between Different Types of Workers. We measured 11 morphometric traits and body weight of workers of 12 different colonies. Body weight (wet weight) varied significantly among workers performing the three tasks. The mean weight of hovering and standing guards did not differ significantly [mean, 4.99 ± 0.45 mg vs. 4.92 ± 0.50 mg, respectively; linear mixed-effects (LME) model: $t = 0.79$, $P = 0.43$], but both types of guards were ~30% heavier than foragers, which had a mean weight of 3.83 ± 0.34 mg (standing guards vs. foragers: $t = 12.7$, $P < 0.0001$*; hovering guards vs. foragers: $t = 13.5$, $P < 0.0001$*; *significant after sequential Bonferroni correction) (Fig. 1*B*). At a mean weight of 4.28 ± 0.46 mg, waste bees were intermediate (foragers vs. waste bees: $t = 5.36$, $P < 0.0001$*; waste bees vs. standing guards: $t = 7.4$, $P < 0.0001$*; waste bees vs. hovering
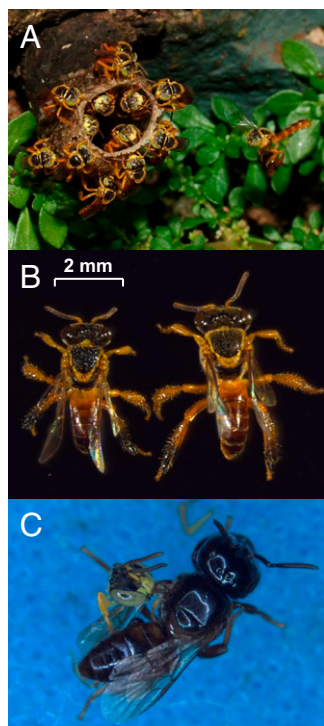
Fig. 1. (*A*) Defending the entrance of a natural nest in a wall cavity; standing guards at the tip of the wax entrance tube and a hovering guard facing the flight corridor leading to the entrance. (*B*) A *T. angustula* forager worker (*Left*) and guard worker (*Right*). The thorax of foragers is usually covered with a thin layer of resin (as shown), the function of which is unknown. Guards have resin on their legs, but not on their thorax. Guards are 30% heavier than foragers. (*C*) The head of a *T. angustula* guard clamped to the wing of a *L. limao* worker at the end of a fight. The *L. limao* worker has decapitated the guard but is unable to remove its head and thus is unable to fly.

Fig. 2. (*A*) Weight distributions of foragers, guards, and waste-removing bees. Values are centered for each colony (colony mean and total mean = 0) to correct for overall colony differences. (*B*) PCA based on morphometric measurements of workers of 12 colonies, showing the effect of worker subcaste. (*C*) Distribution of head width of worker bees that have just emerged from their cells. The arrows indicate the average head width of foragers, waste-removing bees, and guards of the same five colonies.

guards: $t = 8.14$, $P < 0.0001^*$) (Fig. 2*A*). Testing of the other 11 morphometric traits separately led to the same conclusions (Table S1). There were no significant worker weight differences between the colonies from the two sampling locations (z-value = 1.94; $P = 0.08$). Weight variation within foragers or guards was relatively small, with very little overlap between these two types (Fig. 2*A*).

Principal components analysis (PCA) with all 11 morphometric traits (excluding weight) confirmed that workers of different subcastes are of different sizes (Fig. 2*B*). Table S2 shows how much the different traits contribute to the principal components (PCs). The contribution of the different traits to PC 1 is relatively similar (ranging from 0.26 to 33); that is, guards are characterized by larger values for all measured traits. In contrast, PC 2 is strongly affected by head measurements (larger for foragers) and leg measurements (larger for guards). We performed multivariate ANOVA with subcaste and sampling location as explanatory variables and PCs 1–6 (which together explain 95% of all variation in the PCA) as response variables. Subcaste again had a highly significant effect ($F_{2,548} = 21.9$; $P < 0.001$), whereas there was no difference between the sampling locations ($F_{1,273} = 0.06$; $P = 0.99$). We then tested the univariate factors separately and found that subcaste significantly affected PCs 1 and 2 (PC 1: $F = 207.7$, $P < 0.001$; PC 2: $F = 4.24$, $P = 0.015$; PCs 3–6: $F < 1.0$, $P > 0.3$).

Allometry. Ants and termites with a soldier caste frequently demonstrate allometry, typically with soldiers having disproportionately larger heads to house the jaw muscles (20). To determine whether *T. angustula* also shows allometry, we examined the relationships between the $\log_{10}$-transformed morphometric
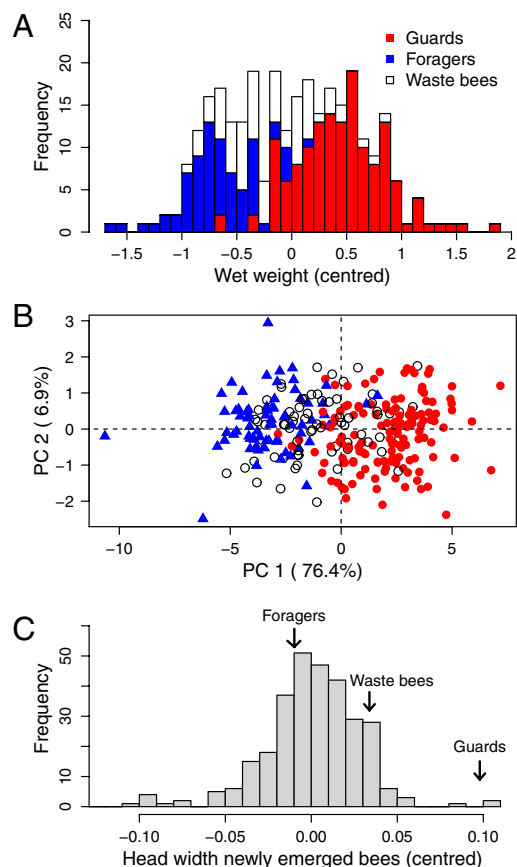
measurements plotted against the $\log_{10}$-transformed cube root of wet weight (Table S3) and plotted against the $\log_{10}$-transformed wing length (Table S4). Allometry occurs if the slope of the relationship differs significantly from 1, that is, if the 95% confidence interval of the slope does not overlap with 1 (4, 21). Positive allometry occurs if the slope is >1, as is usually the case in soldier ants and termites with disproportionately large heads. Six of 11 measurements showed allometry when plotted against weight (Table S3), and 8 of 10 measurements showed allometry when plotted against wing length (Table S4 and Fig. 3 *A*–*C*).

Worker Size Distribution. Because we selected workers based on task, we had no information regarding the overall size distribution of the workers in a colony. To determine this distribution, we measured 300 workers (60 from each of five colonies) emerging from the hexagonal brood cells, which are arranged in horizontal combs (Fig. S1). Most of the bees were of forager size, with an additional peak corresponding to waste-removing bees (Fig. 2*C*). Guard-sized workers were rare, with only 1% of bees of the same size as or larger than the average guard.

Fighting Performance. *L. limao* workers are three times heavier than *T. angustula* workers (13.30 ± 1.29 mg; $n = 80$) and have strong mandibles. They eventually won all of the fights, usually by killing the *T. angustula* soldier, often decapitating it (Fig. 1*C*). Defense was most effective if the Jataí contestant clamped its
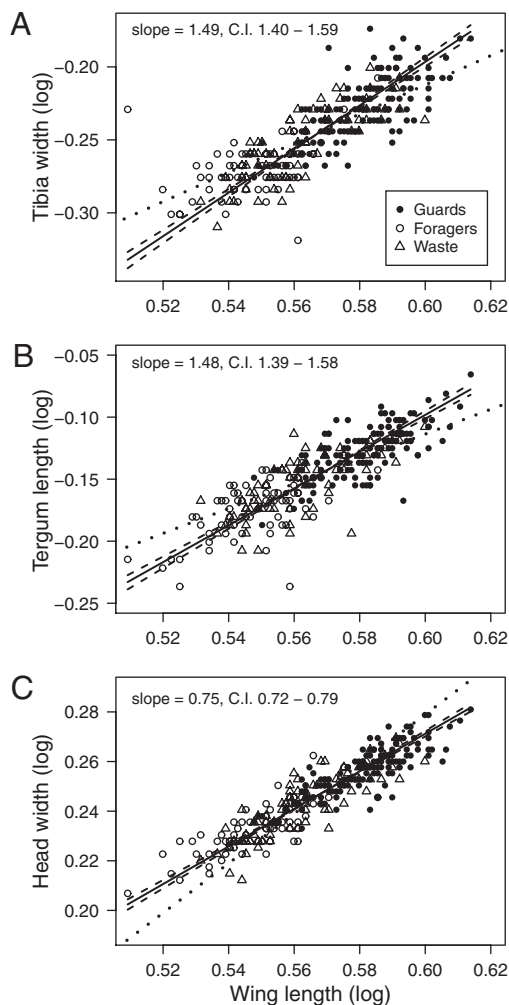
Fig. 3. Examples of allometry of morphometric traits. Open circles represent foragers, solid circles represent guards, and open triangles represent waste-removing bees. The x-axis shows the $\log_{10}$-transformed wing length, and the y-axis shows another $\log_{10}$-transformed morphometric measurement: tibia width (*A*), tergum length (*B*), or head width (*C*). The solid line represents the curve estimated with reduced major axis (model II) regression, and the dashed lines show the 95% confidence intervals of the regression line. These do not overlap with the dotted isometry line (slope = 1).

Fig. 4. Relationship between *T. angustula* guard size (head width) and the durations of fights with a single robber bee *L. limao* worker. Each circle represents the average of 10 guards from a particular colony (*n* = 8 colonies; 80 fights). Average guard size differed between colonies but was consistent within a colony. Open circles represent measurements of the fighting guards taken after the fights. Heads were often damaged and difficult to position in a way that allowed precise measurements, which likely led to lower values. Thus, we also measured 10 other guards from the same eight colonies (solid circles); these values confirmed the relationship.

head onto a wing or antenna of a robber (Fig. 1*C*). When this happened, the *L. limao* robber bee was usually (70%, *n* = 30 instances) unable to remove the soldier during the 15 min of observation and, as a consequence, remained unable to fly. There was a significant relationship between guard size and fight duration; the larger the guard, the longer the guard persisted in a fight (linear regression: $R^2 = 0.51$, $P = 0.045$; $n = 8$ colonies) (Fig. 4). After fights, Jataí guards often had serious injuries that made precise size measurements of head width difficult. Thus, we also measured 10 healthy guards of each of the eight colonies to get a second measurement of guard size for each colony. This confirmed our result by demonstrating a positive relationship between average guard size in a colony and fight duration ($R^2 = 0.66$; $P = 0.008$).

Discussion

Our results indicate that *T. angustula* guards constitute a physically distinct worker subcaste. Guards are not only bigger than foragers (30% heavier), but also of different shape, with 6 of 11 morphometric traits showing allometry when plotted against
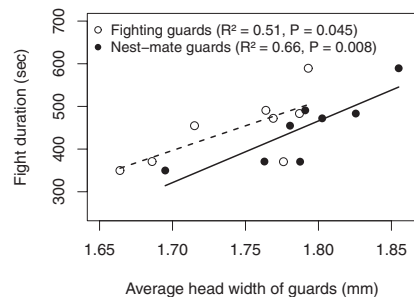
weight and 8 of 10 traits doing so when plotted against wing length. Jataí guards have relatively larger hind legs for their body weight, but a smaller head than foragers. The size variation within each subcaste is relatively small, with negligible overlap between guards and foragers (Fig. 2 *A* and *B*). Unlike the situation in many ants, Jataí guards use their mandibles not for cutting or crushing an enemy, but rather for grabbing and clamping. Thus, they might not need allometrically larger heads to accommodate powerful jaw muscles, as in *Pheidole* or *Atta* ants (4, 20). On the other hand, larger hind legs may help when grappling with an intruder. In contrast, foragers might have relatively larger heads if this is important in the acquisition and processing of sensory information related to foraging. Further research is needed to test specific hypotheses about the function of these allometric differences between guards and foragers.

Only 1% of the emerging Jataí workers were as large as or larger than the average guard (Fig. 2*C*). This matches the situation in ants and termites with physical castes. In ants, soldiers or majors also represent a small minority of the workforce (4, 5), but usually >3% (22–24). In the termite *Cryptotermes domesticus* (24) soldiers compose only 1–2% of the workforce. Given that Jataí colonies contain up to 10,000 workers (25) but have only 20–40 guards standing or hovering at the entrance at any one time (15), the rearing of 1–2% guard-sized workers would fill this labor need, considering that Jataí guards carry out this task for extended periods (15). Wilson (22) argued that the degree of behavioral specialization of the soldier caste is negatively correlated with the number of soldiers in a colony; in support of this argument, the proportion of soldiers was found to be positively correlated with the number of tasks that the soldiers perform in *Pheidole* ants (23). This also fits with data showing that Jataí guards have considerable fidelity to guarding (15).

Our data indicate that waste-removing bees are of intermediate size (Fig. 2 *A* and *B*). This raises the possibility of further morphological specialization in the caste system, with the possibility of minors, majors, and medias, as found in some ants (21). In some primitively eusocial bee species (i.e., Halictidae and *Bombus* spp.), worker size variation also can be linked to division of labor (9, 12). In many bumblebee (*Bombus*) species, larger workers tend to forage and smaller workers tend to work inside the nest (9, 26). Bumblebees do not have distinct physical worker subcastes, however; overall task specialization is weak (27), with many smaller workers foraging and larger workers performing in-nest tasks (28), resulting in a unimodal size distribution (26).

How do Jataí colonies benefit from having larger-sized workers as guards? In our experiments, larger guards were able to fight significantly longer against *L. limao* workers before being defeated (Fig. 4), indicating that larger soldiers are more effective fighters. This could be important in slowing the buildup of an attack by a colony of *L. limao* robber bees, giving the victim colony additional time to mobilize its defenses. Attacks also might be prevented if guards can immobilize the *L. limao* scout bees, which gather information about potential victims before a raid is initiated (29). Some other features of the sophisticated defense system of *T. angustula* are also thought to have evolved in response to attacks by *L. limao* (16, 30–32); for example, hovering guards attack black model bees (the same color as *L. limao*), but not models of other colors. Furthermore, Jataí workers use the characteristic lemon smell of *L. limao* as a kairomone, and respond to it by exiting their nest to defend (16, 30–32). Specialized defensive adaptations to a specific sympatric predator species are known in other bees as well; for example, Asian honey bees (*Apis cerana japonica*) can effectively prevent attacks of sympatric Asian giant hornets (*Vespa mandarinia*) by killing wasp scouts in a highly coordinated fashion (33). In contrast, imported European honey bees (*Apis mellifera*) lack such a response when attacked by the same predator. Like Jataí, Asian honey bees use chemicals produced by the intruder as kairomones (14, 33).

Brood combs in *T. angustula* are composed of a horizontal layer of uniform hexagonal cells (Fig. S1). As in all stingless bees, these cells are mass-provisioned; that is, newly constructed cells are filled with food by the workers and then permanently sealed immediately after the queen lays an egg in each (11). How colonies manage to produce workers of different sizes in the appropriate ratios is an intriguing question. In *Bombus impatiens*, brood cells are of irregular size, and worker size is determined by differential feeding that depends on the location of the larvae in the nest (34). More research is needed to understand the role of cell size, larval location, and food provisioning in the production of workers in *T. angustula*.

Taken together with the results of previous studies showing long-term guarding persistence by individuals of up to 20 d (15) and exceptional ability to detect both conspecific (14, 35) and heterospecific intruders (16), our findings demonstrate that Jataí is unparalleled in social bees in terms of the behavioral and morphological specialization of its guards, and thus that there is no fundamental reason preventing the evolution of physical worker subcastes in bees. However, the flying lifestyle of workers (5), developmental constraints (6), or the rearing of brood in hexagonal cells might set an upper limit of worker size polymorphism that prevents extreme polymorphism, as found in, for example, the leafcutter ants *Atta*. Is Jataí unique among stingless bees in having physical worker subcastes? Evidence of size-related task performance in stingless bees is scant, although there are some intriguing suggestions that this characteristic might not be confined to Jataí (36). A study of the Sumatran stingless bee *Tetragonula minangkabau*, which unfortunately did not measure worker sizes, found that division of labor is based on long-term individual careers rather than on age polyethism as in other stingless bees and the honey bee (37). This suggests that the organization of work in stingless bees is more diverse than assumed and sometimes may differ significantly from that in honey bees. Our discovery of a soldier caste in a social bee represents a remarkable example of a defensive adaptation in social bees. It also serves as a reminder that stingless does not mean defenseless.

Methods

We studied eight colonies at Fazenda Aretuzina (21°26.4 S, 047°35 W; São Simão, SP, Brazil) and four colonies at the University of São Paulo (21°9.4 S, 047°51.3 W; Ribeirão Preto, SP, Brazil). The two locations are ~50 km apart. *T. angustula* is a common species, ranging from Veracruz, Mexico to Misiones, Argentina (8). Mature colonies contain ~10,000 bees (25). Study colonies were kept in wooden hives.

We collected four types of workers according to their behavior. Foragers were identified by their pollen loads after returning to the hive. Waste-removing bees were identified when they exited the entrance tube carrying waste material in their mouthparts. Hovering guards were identified by their characteristic hovering behavior near the nest entrance (30). Standing guards were identified by their continuous presence on the entrance tube (14). We captured each bee individually and with care; other methods, such as netting of several bees at the entrance (36), do not allow for a distinction between the different types of bees. We selectively caught only standing guards in a small, arbitrarily chosen area on the lower side of the entrance tube to avoid any unconscious tendency to take larger individuals (i.e., there was never a choice of bees at this location). We captured six bees per type per colony and measured wet weight, head width and height, eye width and length, tibia width and length, thorax width and height, third tergum length, and forewing length. Weight was measured to the nearest 0.1 mg using a Sartorius TE64 analytical balance. Images of body features were obtained using a Leica DFC500 camera attached to a Leica MZ16 stereomicroscope. Measurements were made from the images using Leica IM50 software, version 5.

Size Distribution of Worker Bees Emerging from Brood Cells. Brood combs from five colonies were kept in an incubator maintained at 28.5 °C until 60 young workers per colony had emerged ($n = 300$ bees). Head width was measured following the methodology described above. (The exoskeleton of holometabolous insects, including stingless bees, does not grow after adult emergence).

Fighting Ability of Guards. *L. limao* workers were collected from a wild colony on the University of São Paulo campus and temporarily kept in groups of 30–50 workers in large (15 cm) Petri dishes and fed with honey. For each fight, a single *L. limao* worker was removed from the Petri dish by gently grasping it with soft forceps and then taken to an experimental *T. angustula* colony and held near the entrance tube until either a standing or a hovering guard attacked it. The fighting pair was then put into a medium-sized Petri dish (10-cm diameter) and left to fight for a maximum of 15 min. The few fights that were unfinished after this time were terminated. The fight was considered over when the *L. limao* worker managed to remove the attacking *T. angustula* guard. We repeated this experiment with 10 guards (both hovering and standing) per colony and eight different colonies (80 fights in total).

Statistical Analysis. All statistical analyses were done using R 2.9 (38). We analyzed the size differences in two ways. First, using general linear models and LME models, we tested differences between subcastes for each morphometric trait separately. We used LMEs because these models control for the nonindependence of data from the same colony by including colony as a random effect (39, 40). For model selection, we used the protocol proposed by Zuur et al. (40). All tested interactions between fixed effects were nonsignificant ($P > 0.05$) and thus were removed for the final model. We then used all 11 morphometric traits in a global analysis using standard PCA and multivariate ANOVA to test for differences between subcastes.

To test for allometry, we log$_{10}$-transformed all measurements and plotted this against the log$_{10}$-transformed cube root of each individual's wet weight (Table S3) and the log$_{10}$-transformed wing length (Table S4). Using log-transformed values in the analyses allowed us to determine whether the relationships between variables are isometric or allometric (4, 21); this is because the slope, b, of the regression $\log(y) = \log(a) + b \log(x)$ equals the power term from the geometric relationship $y = ax^b$. The exponent term from the geometric relationship $y = ax^b$ encompasses both allometry ($b \neq 1$) and isometry ($b = 1$). We used reduced major axis regression (model II) to estimate the slopes and 95% confidence intervals (41). To test whether slopes differed from isometry, we determined whether the 95% confidence interval for the slopes overlapped with 1. When we tested datasets multiple times, we adjusted the significance levels using the sequential Bonferroni method (41).

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