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TITLE DOCUMENT

**Small workers are more persistent fighters than soldiers in the highly polymorphic
Atta leaf-cutting ants**

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ABSTRACT - Social animals often coordinate behavioural activities for the gathering, maintenance, and protection of resources. In eusocial insects, morphological and/or behavioural heterogeneity among worker caste allows division of labour and, in some instances, the specialisation of individuals in specific tasks. In leaf-cutting ants (genera *Atta*), the division of labour is based on body size differences (alloethism), meaning that differently sized workers will likely perform different tasks. Although alloethism is now well understood during foraging and processing of food resources in *Atta*, the relationship between body size and aggressive response has been poorly investigated, and so far, it is not well understood how different morphological subcastes are integrated into the defensive strategy. Here, we quantified intra- and hetero-specific aggression, in a within-colony social context (inside the nest), aiming to test which worker size class (subcaste) exhibit the most aggressive response against non-nestmates (NNMs). Workers were divided into three distinct subcastes: minors, majors, and soldiers and reared in sub-colonies containing two individuals of each subcaste, as well as brood and the symbiotic fungus. We found a low probability of aggression towards conspecific NNMs, and a high probability towards heterospecific NNMs. Quantification of aggression across subcastes showed that minors directed the most overt physical attacks towards heterospecific NNMs. We discuss that the nest, where minors perform most of their tasks, might constitute a strong social contextual stimulus for them, lowering their response threshold and increasing their aggression towards foreign ants. We also discuss that possible signals (ie, chemical or mechanical) emitted during interactions involving minors stimulate larger workers to engage in fights, a mechanism

that has been previously demonstrated in other ant species. The integration of size-specific behavioural traits might constitute a very efficient defensive mechanism.

Key-words: Division of labour, alloethism, aggression, *Atta*, subcastes, soldier,

Body size is a key trait of animals, constraining how they interact with each other and the physical environment (Kalinkat et al, 2015). For instance, from insects to mammals, resolution of competitive conflicts between members of a group is often solved based on aggressive interactions in which body size plays an important role (Wright et al, 2020). Body size is a good predictor of the outcome of competitive conflicts because larger individuals are generally more aggressive and display a larger number of aggressive acts towards smaller individuals, thus having higher chances of gaining access to the disputed resources (Reques & Tejedo, 1996; Wright et al, 2020; Mikkola et al, 2021). In mountain gorillas (*Gorilla beringei*), larger males are more aggressive than smaller ones and alpha male size is positively correlated with tenure length and the number of females (Wright et al, 2020). However, in the context of intra- and heterospecific competition, morphological and behavioural heterogeneity across group members allows adaptive collective responses required for the gathering, maintenance, and protection of resources in an ever-changing environment (Jeanson, 2019). For instance, in the stingless bee *T. angustula*, small-sized workers engage in foraging activities while large workers (soldiers) guard the nest entrance tube against robber bee species (Grueter et al, 2011).

In social insects, the division of labour is generally based on age differences between workers (age polyethism), implying that young individuals will often perform intranidal tasks (i.e., brood care and nest maintenance), while older workers will engage in riskier tasks outside the nest (i.e., foraging and colony defence) (Seeley, 1982;

Robinson, 1987; Withers et al, 1993; Durst et al, 1994). However, in some species, workers present discrete differences in body size (physical castes) that are linked to their behavioural repertoire (alloethism), often leading to limited flexibility and behavioural specialisation on specific tasks (reviewed by Robinson & Jandt, 2020). For instance, in many species of ants, termites, and stingless bees, colony defence is performed by soldiers - workers with enlarged body size that are more efficient than smaller ones to deter or defend the nest against intruders (Wilson, 1980; Holldobler & Wilson, 1990; Grueter et al, 2011). Soldiers are considered highly specialised workers (Beshers & Fewell, 2001; Hölldobler and Wilson 1990, but see Baudier et al, 2019) and consequently express a reduced behavioural repertoire compared to their smaller sisters (Rettenmeyer, 1963; Wilson, 1980; Powell et al, 2005; 2006).

Among ants, the leaf-cutting ants (genus *Atta*) show the highest degree of morphological differentiation among workers (Wilson, 1980; Holldobler & Wilson, 1990): soldiers may be up to 300 times heavier than small-sized workers (minors) (Weber, 1972; Wilson, 1980). Overall, *Atta* minors tend to stay inside the nest engaging in tasks related to brood care and farming of the fungal crop (Wilson, 1980; Camargo et al, 2007 but see Whitehouse and Jaffé (1996) and Hughes & Goulson, 2001). Intermediate-sized workers, on the other hand, tend to perform energetically demanding tasks, such as foraging for fresh leaves to feed the symbiotic fungus, load transportation, and excavation of underground tunnels and chambers (Wilson, 1980; Camargo et al, 2007). The largest *Atta* workers, traditionally named “soldier”, are thought to represent a specialised defensive subcaste (Wilson, 1980; Holldobler & Wilson, 1990). However, the relationship between body size and aggression has been poorly investigated in *Atta* and so far, it is not well understood how colonies employ alloethism into their defensive strategies.

Outside the nest, Whitehouse and Jaffé (1996) demonstrated that soldiers were more likely to respond aggressively to a simulated vertebrate threat while minors were more likely to respond to the presence of other ants (Whitehouse & Jaffé, 1996), suggesting that the nature of the threat influences the number of differently-sized workers engaged in defence. However, experiments under controlled conditions are required to clarify whether any subcaste responds differently to the presence of non-nestmates (NNMs). Here, we investigated how body size affects the decision to attack an enemy as well as the persistence of the attack against intra- and heterospecific ant intruders. We divided workers into three distinct subcastes (minors, majors, and soldiers) and reared them in sub-colonies containing two individuals of each subcaste, as well as brood and fungus garden, and measured the aggression of workers towards NNMs.

METHODS

Animals

Ants for behavioural experiments were obtained from four stock colonies of *A. sexdens* established in the Laboratório de Etologia, Ecologia e Evolução dos Insetos Sociais (LEEEIS), at the Universidade de São Paulo, São Paulo, Brazil. Colonies were fed three times a week, mainly with leaves and flowers of *Acalypha* spp. During the experiments, the colonies had an approximate volume of 6 L of fungus garden and had been kept for at least 6 years under controlled conditions (approx. 25°C and a 12:12h LD cycle).

Morphological worker subcastes: small, major, and majors (soldiers)

We classified individuals into three distinct subcastes based on head widths (HWs): minor ($HW \cong 1.0$ mm), major ($HW \cong 2.0$ mm), and soldier ($HW > 3.0$ mm). HWs were measured using as reference the longitudinal-axis distance at the level of the eyes, a procedure that represents a standard for ant taxonomy (Wilson, 1980). Prior to the experiments, we collected and measured with an ocular scale the head capsule of 25 individuals that were rank ordered according to body size. These specimens were pinned with a subcaste identification label and used as reference for collecting individuals for behavioural experiments. At the end of the experiment, each focal ant was photographed and had their HW measured at nearest 0.01 mm, using the image processing software ImageJ (ImageJ 1.51j8, Wayne Rasband, National Institute of Health, USA). The mean and standard deviation of focal ants' HWs were: minor (0.94 ± 0.25), major (2.14 ± 0.36), and soldiers (3.25 ± 0.27).

Behavioural assay

To prevent the effects of age polyethism, focal ants (*A. sexdens*) for behavioural experiments were generally collected outside of the fungus chambers, and care was taken to collect only individuals with brownish red cuticular colour, a characteristic of intermediate aged individuals (lighter and darker cuticle colours indicate young and old individuals, respectively (Camargo et al, 2007; Norman et al, 2014). We reared a total of 45 sub-colonies, each containing two individuals of each subcaste ($N = 2$ minors + 2 majors + 2 soldiers = 6 individuals) placed in small plastic pots of 250 mL (H: 6 cm, W: 9 cm). To simulate the 'within-colony' environment, in each sub-colony we placed two brood items and approximately 1 cm³ of fresh symbiotic fungus. For acclimation, ants were kept undisturbed in such an environment for 24 h prior to the beginning of the experiment. In nature, the number of fungus chambers in *A. sexdens* mature colonies

varies between 77 to 1027 and are found between 1 and 2 meters deep (Autuori, 1942; Pretto, 1996).

The experiment consisted of introducing a single major NNM (or nestmate in the control group) in a testing sub-colony for two minutes. For each type of experimental treatment (heterospecific, intraspecific, control), we tested a total of 15 sub-colonies ($3 * 15 = 45$). Heterospecific and intraspecific treatments involved introducing heterospecific (*Atta laevigata*) and intraspecific (*A. sexdens*) NNMs, respectively. NNM colonies and stock colonies were reared together in the same laboratory and thus in the same conditions (see detailed above). To distinguish focal ants from NNMs, we paint a dot on the thorax or gaster of NNM subjects, using a water-based acrylic paint (Corfix, Brazil). Since we predicted that behavioural response of NNMs could influence the aggressive response of tested subjects, we cut off the antennae of all NNMs (and nestmates in the control group) after a rapid anaesthesia on CO₂ (Valadares, 2018). After recovery, NNMs were kept undisturbed for at least 20 minutes before introducing them to sub-colonies. During the experiments, antennae-less NNMs generally stayed motionless with mandibles and antennae pointed upwards and were never observed to initiate a fight, however, when being attacked they often responded aggressively to the attacker. Each trial was entirely videotaped using the webcam C920 HD PRO (Logitech, Switzerland), and the pronounced size difference between individuals were enough to distinguish subcastes during quantification of behavioural responses, without the need to paint marked them. In each trial, we quantified the number of bites displayed by group (subcolony). Behavioural quantifications were carried out at the subcaste level since focal ants were not paint-marked. Behavioural documentation was done blind in respect to experimental manipulations, although size differences between subcastes were obvious. Experimental treatments were presented in a random order and

since all tested subjects were sacrificed after testing, all data about individuals are independent from each.

Statistical analyses

All statistical analyses were done using R (3.3.3) in RStudio (1.1.383). We first investigated the effects of the response variables (i) percentage of attacks and (ii) number of bites only between experimental treatments (heterospecific, intraspecific, and the control). Since both intraspecific treatment and the control evoked low levels of aggression (see results), quantification of aggression between subcastes (minor, major, and soldiers) were analysed separately and focused only on heterospecific interactions. In all cases, we included *colony identity* as covariate to control for possible differences between colonies.

To investigate the effects of body size on the aggressive behaviour of ants, we analysed the *percentage of attacks* between treatments (heterospecific, intraspecific, and the control) (model 1) and between subcastes (minor, major, and soldiers) (model 3) using a generalized linear model (GLM) with a binomial distribution and log-link function, where the frequency of bites in each trial was set to 0 (in absence of attacks) or 1 (when nestmates attacked intruders at least once). To analyse the dependent variable measured as count data (*number of bites*) between treatments (model 2) and subcastes (model 4), we first used the Shapiro-Wilk test to test the normality of data distribution. Since data distribution did not show a normal distribution, we used a GLM with a negative binomial distribution to account for data overdispersion (model 2). We estimated overdispersion by obtaining the ration between the residual deviance and the degrees of freedom, as well as by using the overdispersion test from the AER R

package. Post-hoc Tukey tests were based on 95% confidence interval of estimated marginal means (EMMs) computed with the R package ‘emmeans’. For all cases we used an alpha level of 0.05. The documentation of the analyses can be found as supplementary material file (R markdown file).

RESULTS

During social interactions, we found that *A. sexdens* resident workers were far more aggressive towards heterospecific intruders (*A. laevigata*) than toward conspecifics and nestmates (the control) (Figure 1 and 2). Regarding the percentage of attacks across treatments, only the heterospecific treatment differed from the other two treatments (intraspecific and the control) ($F_{(5,129)} = 16.32$, $P < 0.001$) (Table A1 and A2): we registered attacks in 14 heterospecific trials (93.3 % of cases), while intraspecific intruders and nestmates evoked lower percentages of attacks (21.9 % and 13.6 % of the cases, respectively) and no statistical difference was found between them (Figure 1) (Table A1 and A2). We also found a significant difference in the number of bites across treatments ($F_{(5,129)} = 9.668$, $P < 0.001$) (Table A3) , and post-hoc comparisons showed again that only the heterospecific treatment differed from the other two treatments (intraspecific and control) ($P < 0.0001$ for both comparisons) (Figure 2) (Table A4). Intraspecific treatment and the control did not differ with respect to number of bites ($P = 0.480$) (Table A4). Because intraspecific interactions evoked low levels of aggression, quantification of aggression between subcastes were focused only on heterospecific interactions.

In heterospecific interactions, minors were far more aggressive than majors and soldiers (Figure 1 and 3). Analysis of the percentage of attacks showed no statistical difference between subcastes – that is, the probability to attack is not related to body size (Table A5). However, we found that the number of bites differed between subcastes (Figure 3) ($F_{(5,39)} = 5.532$, $P < 0.001$) (Table A6): minors displayed a larger number of bites towards NNMs (87 occurrences, $M + SD = 6.69 + 1.32$), than majors (26 occurrences, $M + SD = 1.62 + 0.45$) and soldiers (19 occurrences, $M + SD = 1.18 + 0.57$). Post-hoc comparisons showed that only minors differed from the other two subcastes (majors and soldiers) ($P < 0.001$ for both comparisons) (Figure 3) (Table A7). Indicating that minors are more persistent fighters than larger subcastes. Interestingly, minors never attacked nestmates, while majors and soldiers did (Figure 1).

DISCUSSION

In this study, we investigated how body size affects both the decision to attack an enemy as well as the persistence of the attack, using as model a species from the ant genus (*Atta*) showing the highest degree of size differentiation among workers. We found that the decision to attack is not related to body size, however, attack persistence is far higher in minors than larger subcastes (majors and soldiers) (Figure 1 and 3). This defensive strategy differs from what have been observed in other taxa. For instance, in the fruitfly *Drosophila silvestris* (Boake, 1989), in crayfish (Graham et al, 2020), in dogs (Mikkola et al, 2021), in mountain gorillas *Gorilla beringei beringei* (Wright et al, 2020), and even in human males (Hartin et al, 2020) body size has been positively

correlated with levels of aggression. In ants, this defensive strategy differ from other polymorphic ant genus in which larger workers are more aggressive than small ones, i.e., *Pheidole pallidula* (Detrain & Pasteels, 1992), *Cataglyphis niger* (Nowbahari et al, 1999), *Oecophylla smaragdina* (Newey et al, 2009), *Eciton* spp. (Lalor & Hughes, 2011) and *Acromyrmex echinator* (Larsen et al, 2014). In *Acromyrmex*, the sister genus of *Atta*, minors were found to be less aggressive than medians and majors (Larsen et al, 2014). It is important to note, however, that body size differentiation in *Acromyrmex* is relatively small compared to *Atta*, and a soldier subcaste is only found in *Atta* species. Larsen et al (2014) hypothesised that less aggressive behaviour of minors could be due to brain miniaturisation and consequently less space to house receptors and neurons. However, our results do not support this hypothesis in *Atta*, since we found that only the intensity of the attack differed between subcastes but the probability to attack was not related to body size (i.e., all subcastes engaged in attacks in similar proportions).

Despite being the smallest individuals in the colony, previous works illustrate *Atta* minors as an important defensive subcaste. For instance, during foraging, minors hitchhike on leaf-fragments transported back to the nest by larger nestmates to protect them against phorid attacks (Lutz, 1929; Bragança et al, 1998; Linksvayer et al 2002). Under natural conditions, minors were found to patrol foraging trails for threats, being highly attracted to alarm pheromone sources (> 90% of visits to possible dangerous areas were performed by minors) (Hughes & Goulson, 2001). In addition, minors are also more sensitive than their larger nestmates to substrate-borne vibrations resulting from stridulation (Roces & Hölldobler 1995), which is a signal used by ants as an alarm (Markl 1965; Roces & Hölldobler 1995). Combined, these findings suggest that minors have an important and perhaps crucial role characterised by responding promptly to the

249 presence of NNMs with high levels of aggression and alarm recruitment, both inside
250 and outside of the colony.

251 Previous work has shown evidence that *Atta* colonies use the soldier caste to
252 defend the nest against large organisms and recruit numerous small ants in response to
253 conspecific and interspecific ant threats (Whitehouse & Jaffé, 1996). Despite being
254 recruited, the role of small ants in battle sites may go beyond that of fighting because
255 there was proportionally more small ants present than those seen fighting (Whitehouse
256 & Jaffé, 1996). This suggests that minors and soldiers might present different response
257 thresholds to the stimuli associated with different kinds of threat. Although the
258 following argument does not support recruitment of small-sized ants to conflicting areas
259 outside the nest as reported previously (Whitehouse & Jaffé, 1996) - and in our case we
260 are not measuring recruitment but instead motivation to fight - it is reasonable to
261 consider that the social context of the nest, where minors perform most of the tasks
262 (nursing the brood and the queen, final processing of foliar substrate, and caring for the
263 fungal hyphae) (Wilson, 1980), might then constitute a strong incentive for them to
264 respond to intruders, lowering their response threshold and increasing their aggressive
265 response towards the presence of foreign ants.

266 In *Pheidole pallidula*, minors are responsible for detecting intruders and
267 recruiting larger nestmates with powerful mandibles that effectively kill the intruder
268 (Detrain & Pasteels, 1992). Since *Atta* minors are the most abundant individuals in the
269 nest (Simas et al, 2002), it is tempting to hypothesise that the signals emitted (ie,
270 chemical cues and/or stridulation) during agonistic interactions involving minors
271 stimulate larger workers to engage in fights, because although minors responded more
272 aggressively to the presence of foreign ants in our experiment, they are most probably
273 less efficient than larger nestmates to subdue competitors. Thus, minors would act as

274 amplifiers of signals consequently inducing aggression in other workers. The integration
275 of these different size-related behavioural traits might constitute a very efficient
276 defensive mechanism, especially in a within-colony context, in which minors are more
277 numerous than larger ants (Simas et al, 2002). In fact, amplification of signals by
278 minors have been described at least during foraging activities (Evison et al, 2008),
279 where they were responsible for > 80% of trail pheromone markings on foraging trails
280 then consequently increasing foraging efficiency. In this way, foragers (majors) can
281 focus on food transport while minors are responsible for maintenance of pheromone
282 trails (Evison et al, 2008).

283 We observed a low level of aggression towards conspecifics NNMs (Figure 1, 2,
284 and S2). Both tested subjects and NNMs were collected from the same laboratory and
285 had been kept under the same conditions for many years (see Methodology). The
286 process of nestmate recognition is mediated by cuticular hydrocarbons (CHCs), a
287 diverse class of chemical compounds spread at the outmost cuticular layer of the insects
288 (Blomquist, 2010). The composition of CHCs is influenced by both genetic and
289 environmental factors, which makes the profile of these compounds subject to temporal
290 changes according to seasonal variation, nesting substrate, food and the presence of
291 symbionts and pathogens (Blomquist, 2010). Since the environmental conditions
292 (temperature, humidity, LD) of the laboratory was controlled and stable over time, and
293 colonies were fed with the same foliar substrates, a homogenization of the cuticular
294 chemical profile of the colonies is thus expected, and consequently lower levels of
295 aggression between them. Indeed, it has been shown in *Atta sexdens* that the foliar
296 substrate shared by different colonies makes their profile more similar than those who
297 fed on a different source, with aggression levels between colonies correlating with the

degree of differentiation of these chemical profiles (Valadares et al, 2015; Valadares & Nascimento, 2017).

CONCLUSIONS

Our results suggest that task specialisation may not be the most important explanation of how polymorphic species are organized, but that on the contrary task integration between workers of different size may allow a better efficiency and coordination of the collective response. This was shown in a foraging context (Evison et al, 2008) and we propose that during colony defense this is also the case. Superorganism allegories insisting on compartmentalisation of tasks between different groups of workers (division of labour) may actually impair the perception of the fluid interactions that occur between subcastes to improve communication. In this respect, if we keep the analogy, minor workers in *Atta* colonies could be the circulating hormones that unites the functioning of all part, although in our opinion this should convince researchers that interactions and individual behaviour and experience and how they contribute to collective behaviour is a worthier object of study (Anderson and Mc Shea, 2001; Lihoreau et al. 2021).

Author contributions

LV conceived and designed the experiment. LV performed the statistical analyses. All authors interpreted the results and wrote the manuscript.

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Figure 1 – Percentage of attacks by different *A. sexdens* subcastes registered in each trial (subcolony) towards nestmates (control), conspecifics (intraspecific) and heterospecific non-nestmates (*A. laevigata*).

Figure 2 – number of bites that *A. sexdens* resident workers displayed each trial (subcolony) against nestmates (control), conspecifics (intraspecific) and heterospecific non-nestmates (*A. laevigata*). The bottom and the top of the boxes are the 25th and 75th percentiles, the line inside the box is the 50th percentile (median). Error bars are the 95 % confidence interval.

Figure 3 – number of bites that *A. sexdens* subcastes displayed in each trial (subcolony) against heterospecific non-nestmates (*A. laevigata*). The bottom and the top of the boxes are the 25th and 75th percentiles, the line inside the box is the 50th percentile (median). Error bars are the 95 % confidence interval.





