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Hygienic behavior and antimicrobial peptide expression of the leaf-cutting ant *Atta cephalotes* (Hymenoptera, Formicidae) to *Metharhizium anisopliae*

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Abstract

Leaf-cutting ants depend on mutualistic fungi to survive. An infection that massively affects the workers compromising the proper maintenance of the fungus, or that can attack the fungus garden, can be fatal to the colony. Thus, leaf-cutting ants have evolved a complex defense system composed of both innate individual immunity and collective immunity to protect the colony against potential threats. To characterize the collective and individual immunity of *Atta cephalotes* workers to *Metarizhium anisopliae* we assessed the hygienic behavior and the expression of antimicrobial peptides of *A. cephalotes* workers triggered by *Metarizhium anisopliae* spores. As a control challenge, workers were treated with water. Regardless of whether the challenge was with water or spore suspension, *A. cephalotes* workers displayed an immediate response characterized by an increase in time spent both self-grooming and collective grooming along with a reduction in time spent fungus-grooming. The individual immunity triggered the expression of abaecin as early as 24 hours post-infection, exclusively in workers challenged with *M. anisopliae*. In contrast, the level of expression of defensin remained constant. These results suggest that upon being challenged with a suspension of *M. anisopliae* spores, *A. cephalotes* workers deploy both collective and individual immunity to produce a response against the invader. However, when the spores of *M. anisopliae* are applied as liquid suspension collective immunity deploys a generic strategy, while individual immunity shows a specific response against this entomopathogen.

Keywords

abaecin, allogroming, defensin, expression, fungus grooming, gene, self-grooming

Introduction

Attine ants (Formicidae: Myrmicinae: Attini: Attina) comprise approximately 250 species that establish associations with mutualistic fungi as a source of nutrition (Ward et al. 2015; Branstetter et al. 2017). Leaf-cutting ants of genera *Atta* and *Acromyrmex* are among the most derived of the tribes, in which they cultivate *Leucoagaricus* fungi using fresh plant material as a substrate (Chapela et al. 1994; Mueller et al. 2001, 2005). In return, mutualist fungi produce gongylidia—specialized structures used to feed the queen and the brood (Hölldobler and Wilson 2010). To assure fungus garden productivity, the workers select, harvest, and process large quantities of suitable plant material (Littledyke and Cherrett 1978; Folgarait et al. 1996; Estrada et al. 2013; Rocha et al. 2017) and build complex nests underground to provide an optimum environment for fungus growth (Kleineidam and Roces 2000; Bollazzi and Roces 2010; Verza et al. 2017). For instance, the nest can have up to thousands of chambers inhabited by millions of individuals in species of *Atta* (Jonkman 1980; Moreira et al. 2004).

The social lifestyle of leaf-cutting ants could, in principle, make them prone to infections with hazardous microorganisms for workers or the fungus garden (Hughes et al. 2002) due to enhanced transmission between frequently interacting individuals with a similar genetic background that possibly are susceptible to the same pathogens (Wilson et al. 2003; Pie et al. 2004; Cremer et al. 2007). In this sense, workers are in permanent contact with fungi that can overgrow the mutualist (e.g., *Syncephalastrum racemosum* and *Trichoderma harzianum*) that inhabit the soil and within the plant tissue they cut (Rodrigues et al. 2009; Rocha et al. 2017). Furthermore, infection of the fungus garden by *Escovopsis*, a specialized parasite of the mutualist (Muchovej and Della Lucia 1990; Seifert et al. 1995; Currie et al. 1999; Currie and Stuart 2001), is frequently detected in the garden of leaf-cutting ants, but it only significantly compromises the garden if the ants cannot groom the mutualist or properly dispose of the waste (Heine et al. 2018).

To protect the colonies against infection, leaf-cutting ants have evolved a complex defense system composed of the innate immunity of individuals and collective immunity. Individual immunity involves physiologic mechanisms to clear potential threats, including the production of reactive oxygen species, encapsulation, and the production of antimicrobial peptides (Hoffmann 1995). In contrast, collective immunity is based on altruistic behaviors that result in avoidance, control, or elimination of parasitic infections (Siva-Jothy et al. 2005; Masri and Cremer 2014). Once a potential hazard is detected, the workers deploy hygienic behaviors of collective immunity including self-grooming, allogrooming (Fernández-Marín et al. 2003; Reber et al. 2011), and fungus grooming (Currie and Stuart 2001; Little et al. 2006; Cremer et al. 2007). The particles removed via hygienic behaviors are compacted, possibly sterilized in the infrabucal pocket, and dumped as pellets (Little et al. 2003, 2006). This hygienic behavior is complemented by chemical defenses mediated by secretions of exocrine glands and the metapleural gland that contain fungal and bacterial inhibitors

(Do Nascimento et al. 1996; Poulsen et al. 2003; Rodrigues et al. 2008; Fernández-Marín et al. 2015).

In addition to these mechanisms, *Acromyrmex* workers maintain a symbiosis with *Pseudonocardia*—an antibiotic-producing actinobacterium maintained in the cuticle. This association protects the fungus garden against infection with parasitic fungus *Escovopsis* (Currie et al. 2003; Poulsen et al. 2010; Cafaro et al. 2011). In contrast, *Atta* species have lost or reduced the symbiosis with *Pseudonocardia* (Currie et al. 2006; Mueller et al. 2008) suggesting that they rely more on collective immunity and chemical disinfection to protect the colony against invaders (Fernández-Marín et al. 2009).

The first barrier of individual immunity in leaf-cutting ants is a hard exoskeleton reinforced with a biomineral armor that protects workers from invaders in most cases (Siva-Jothy et al. 2005; Li et al. 2020). However, specific pathogens such as entomopathogenic fungus *Metarizhium anisopliae* can penetrate the worker's cuticle and reach the hemocoel (Hajek and St. Leger 1994; Moino Jr et al. 2002). In insects, constitutive mechanisms of innate immunity such as profenol oxidase cascade, phagocytosis, and reactive oxygen species production act to neutralize the microorganism as the first line of defense (Nappi and Christensen 2005; Evans et al. 2006; Buchon et al. 2014). The expression of antimicrobial peptides (AMPs) is induced after this generic response. These molecules eliminate the microorganisms via pore formation or metabolism disruption (Bulet et al. 2004; Siva-Jothy et al. 2005; Haine et al. 2008). The genome of leaf-cutting ants, *Atta cephalotes* and *Acromyrmex equinatior* contains sequences that code to abaecins, hymenoptaecins, and defensins (Zhang and Zhu 2012). Furthermore, abaecin and hymenoptaecin are expressed as a part of the immune response of *Ac. equinatior* workers against *M. anisopliae* (Yek et al. 2013).

The collective immunity of *Acromyrmex* against *M. anisopliae* has been previously described (Richard and Errard 2009; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Abramowski et al. 2011; Yek et al. 2013; Tranter et al. 2015; Nilsson-Møller et al. 2018; Calheiros et al. 2019). In contrast, the collective immunity of *Atta* has been less explored (Fernández-Marín et al. 2006; Fernández-Marín et al. 2009; Walker and Hughes 2011) and the research has been primarily centered on describing metapleural gland grooming. While this evidence highlights the relevance of chemical disinfection in *Atta* species, it does not assess the full spectrum of workers' behavior upon a challenge with an entomopathogen. Furthermore, studies that simultaneously assess innate and collective immunity responses against an entomopathogen in leaf-cutting ants are scarce (Yek et al. 2013). To characterize the collective and individual immunity of *A. cephalotes* workers to *M. anisopliae*, we assessed workers' hygienic behavior before and after a challenge with *M. anisopliae*. We evaluated the time spent by workers executing self-grooming, allogrooming, metapleural gland grooming, fecal fluid grooming, and the production of infrabuccal pellets. In addition, we evaluated the expression of antimicrobial peptides abaecin and defensin as effectors of individual immunity. We hypothesized that upon a challenge with *M. anisopliae*, workers deploy collective and individual immunity to clear the entomopathogen.

Methods

Leaf-cutting ant nest collection and immune challenge preparation

Between January and December of 2019, twelve mature nests of *A. cephalotes* were selected and collected in Santiago de Cali, Valle del Cauca, Colombia (3°22'33.24"N, 76°32'0.24"W). At least 400 g of fungus accompanied by workers was extracted per nest. The collected material was kept undisturbed for at least one week in 10 L plastic containers connected to a waste chamber (Valderrama et al. 2006). Microcolony arrangements were established to carry out the assays based on these source colonies. The composition of the microcolonies, as well as their number, will be described for each experiment.

Metarhizium anisopliae was isolated from commercial product BIO-MA (Bioproteccion SAS, Colombia). Initially, 90 g of product was resuspended in 90 ml of sterile water. Serial dilutions of this suspension were then cultured in potato dextrose agar (PDA) (BD, USA) to obtain axenic cultures. From these cultures, liquid suspensions of conidia at a concentration of 10^7 conidia/ml were prepared as a treatment.

Experiment 1: Behavioral response to *M. anisopliae* exposure

Five mature nests of *A. cephalotes* were selected to assess the behavioral response to *M. anisopliae*. Six microcolonies composed of 15 medium workers (cephalic width 1.4–1.8 mm) and 0.5 g of mutualistic fungus were established from each nest. The microcolonies were randomly assigned to treatment with spores of *M. anisopliae* or treatment with water. Each microcolony was placed in a glass box (10 × 15 × 9 cm) with walls coated with Fluon plus (Bioquip, USA). The workers were left undisturbed for 90 minutes to adapt to the new environment. Then video recordings of the one-hour basal period were taken. Next, the microcolonies assigned to spore treatment were sprayed with approximately 400 µl of the spore suspension evenly distributed between fungus and workers. Simultaneously, Control microcolonies were sprayed with 400 µl of water. Immediately after the treatment was applied, the activity of each microcolony was video recorded for 2 hours. The recordings were acquired with a GoPro Hero 5 Black edition (GoPro, USA) camera coupled with a macro lens (PolarPro, USA) at 60 photograms per second and 1080 megapixels. At the end of the video recording, the 15 ants of each microcolony were transferred to Petri dishes and given an agar diet (Bueno et al. 1997). The infrabuccal pellets produced by the workers were counted after 24 hours (Fig. 1A).

The video recordings obtained from each microcolony for the basal period and the two hours after treatment were divided into 10-minute segments. For each of these segments, three minutes of footage were randomly selected to record the behavior of ten workers. The workers were digitally labeled to score the time spent for each one of them in the execution of five hygienic behaviors associated with collective immunity: self-grooming, allogrooming, fecal fluid grooming, fungus grooming, and metapleural gland grooming, defined according to the literature as follows:

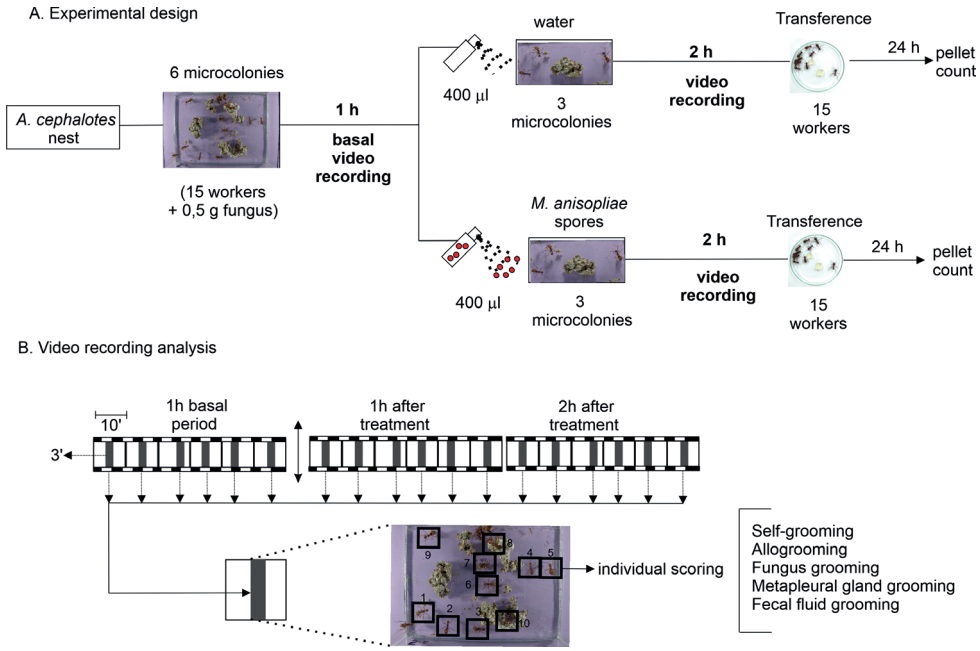

Figure 1. Description of A. Experimental design and B. Video recording analysis used to assess the behavioral response of *Atta cephalotes* workers to *M. anisopliae*

Self-grooming

The antennae are pulled through the antenna cleaners on the front legs, then the ant cleans the legs and the antenna cleaners, by pulling the legs through the mouthparts, removing particles with the glossa (Nilsson-Møller et al. 2018).

Allogrooming

One or more grooming ants approached a recipient worker. The antennae of the grooming ants are pointed towards a specific point of the receiving ant or are moving and lightly tapping the receiver. The maxillae and lower labium mouthparts are open, with the glossa emerging to lick the receiver ant (Nilsson-Møller et al. 2018).

Fungus grooming

The ant stops leg movements at a fixed point on the fungus garden. The antennae are motionless and parallel pointed towards the mutualistic fungus, and the tip of the antennae are almost touching each other, close to the tips of the mandibles. The maxillae and lower labium mouthparts are open, with the glossa emerging to lick the fungus (Currie and Stuart 2001; Nilsson-Møller et al. 2018).

Metapleural gland grooming

The ant leans to one side to reach one of its front legs to rub the meatus of the metapleural gland. The other front leg is simultaneously licked by the glossa. The ant leans to the opposite side and switches legs and repeats the same motion with the opposite legs. (Fernández-Marín et al. 2006; Nilsson-Møller et al. 2018).

Fecal fluid grooming

The ant bends its gaster and head towards each other to apply a droplet of fecal fluid to the mouthparts. the ant pulls the front legs through the mandibles, one at a time. Subsequently, the ant moves the antennae through the antenna cleaners located on the tibia-tarsus joint of the front legs (Nilsson-Møller et al. 2018).

This procedure was repeated until the observation was completed for ten labeled workers in each segment of three minutes. Finally, the video recordings were analyzed independently by two observers blinded to the treatments. (Fig. 1B).

Experiment 2: Worker's survival and colonization by *M. anisopliae*

The pathogenicity of the *M. anisopliae* strain was confirmed by assessing the percentage of colonization. Here, four colonies were chosen to extract 60 individuals that were randomly assigned to a challenge either with water or a spore suspension. After the challenge, workers were transferred to sterile Petri dishes with diet agar in groups of 10 ants. The number of living workers was recorded every 24 hours for ten days. The dead ants were then removed and disinfected in sodium hypochlorite (0.7%) solution followed by three washes with sterile water. Finally, the corpses were transferred to Petri dishes lined with absorbent paper moistened with sterile water. After five days of incubation, the corpses were assessed under a stereomicroscope SMZ-745 (Nikon, Japan) to determine colonization by detecting mycelial growth from inside the intersegmental sections (Moino Jr et al. 2002).

Experiment 3: Gene expression associated with innate immunity in workers

To evaluate gene expression, 12 microcolonies from three nests were selected. The microcolonies were composed of 100 medium workers and 5 g of mutualistic fungus. Six microcolonies were randomly assigned to the challenge with spore suspension, and six microcolonies were assigned to a sterile water control. Each microcolony was placed on a glass box, and the corresponding treatment was applied. Twenty ants per microcolony were collected before and at 24, 48, and 72 hours after applying the challenge. An additional sample of 20 workers challenged with *M. anisopliae* was collected to assess the efficiency of the primers at 24 h. The collected ants were kept in liquid nitrogen until RNA isolation.

RNA extraction and relative gene expression

The RNA extractions were performed using the SV Total RNA Isolation System (Promega, USA) following the manufacturer's instructions. The RNA quantity and integrity were assessed by agarose gel (2%) electrophoresis and analysis with a Nanodrop™ spectrophotometer (ThermoFisher, USA). The RNA extracted from the ants was reverse transcribed with cDNA synthesis kit ProtoScript First Strand cDNA Synthesis (New England Biolabs, USA).

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Previously reported primers were used for *abaecin* and *ribosomal protein* L18 (rpL18) (Chérasse et al. 2018). For the *defensin* and *NADH dehydrogenase* (NADH), primers were designed by Primer-BLAST (NCBI) using *A. cephalotes*-specific sequences available in the Gen Bank (NCBI) for *defensin* (BK008405.1) and *NADH* (XM_012205180.1). The primers were designed to span an exon-exon junction, thus avoiding the amplification of the contaminating genomic DNA (Table 1).

Table 1. List of the specific primers used for expression assays.

Gene	Primer name	Sequence (5'-3')	Amplicon size (bp)	Efficiency (%)	Reference
abaecin	Aba-f	ATCTTCACTCTGCTCTTGGC	156	103	Chérasse et al. 2018
	AbaM-r	AATGAGGAAATCTGATCTTCGG			
defensin	DG2-f	TGAAGCTGTTCGCTATCCTCG	112	90	This study
	DG2-r	GGATCCTCGATGGTAGTCAGTTC			
ribosomal protein	CRL18-f	TCCCCAAGTTGACGGTATG	140	97	Chérasse et al. 2018
(rpL18)	CRL18M-r	TCCCTGCATCAAGACTGTAC			
NADH	NAC1-f	AGAGCAGATGGATCTCGACG	122	100	This study
	NAC1-r	AATTCGAAGTTGGGACCCTCA			

Quantitative PCR was carried out in a CFX96 *Touch Real-Time PCR Detection System* (Bio-Rad, USA) with a reaction mix containing 4 µl of cDNA, 5 µl de 2 × SsoFast EvaGreen (Bio-Rad, USA), and 0.5 µl of each primer (10 µM). The amplification conditions were 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s; melt curves were run after 40 amplification cycles while increasing the temperature from 60 to 95 °C; each sample was assessed in duplicate. A no templated control was included as a negative control for each primer.

The primer's efficiencies were determined using standard curves as previously described (Pfaffl 2001; Moreira et al. 2017). The efficiencies (E) and the mean C_t (threshold cycle) were used to calculate relative expression (RE) of gene targets abaecin and defensin compared to housekeeping genes rpL18 and NADH equation 1 (Chérasse et al. 2018):

$$\mathrm{RE} = \frac{\sqrt{\left[(E_{rpL18})^{Ct\ sample}\ (E_{NADH})^{Ct\ sample}\ \right]}}{(E_{target})^{Ct\ sample}} \quad (1)$$

The fold change in expression of each target at different time points was estimated by dividing the relative expression values at 24, 48, and 72 h by the relative expression at 0 h.

Statistical analyses

All the statistical analyses were performed using R version 4.0.2 (Core-Team 2020)

The consistency of the observations recorded by the observers was contrasted while calculating a two-way mixed-effects intraclass coefficient (ICC) using the *psych* package (Revelle 2022). The time spent by all the workers in a microcolony in each hygienic behavior was summed for the basal period, the first hour, and the second hour post-challenge. The resulting data were $\log_{10}$ transformed to satisfy the assumptions of normality and homoscedasticity. Independent mixed linear models were calculated for each behavior using the nlme package (Pinheiro and Bates 2022). For the time spent in self-grooming, allogrooming, fecal fluid grooming, and fungus grooming, the fixed factors were: (i) treatment (spraying with water or infection with *M. anisopliae* spores), (ii) time (baseline period, the first and second hours after the application of the challenge), and (iii) the interaction between time and treatment. For the variable production of infrabucal granules, treatment was set as the only fixed factor was the treatment. The nest was set as a random factor for all models.

The impact of treatment on worker survival was assessed via Cox regression using the *Survival* package (Therneau and Grambsch 2000; Therneau 2022). The treatment (*M. anisopliae* challenge or control challenge), nest, and interaction between treatment and nest were considered fixed effects.

The fold change in the expression of antimicrobial peptides, *abaecin*, and *defensin* was logarithmic transformed ($\log_{10}$), and independent linear mixed models were calculated. The treatment (*M. anisopliae* challenge or control challenge), time (24, 48, and 72 h), and interaction between treatment and time were considered fixed factors. The nest was considered as a random effect.

The *Anova* function of the CAR package (Fox and Weisberg 2018) was used in all models calculated to evaluate whether the fixed factors affected the response variables. Tukey's multiple comparisons were performed using the *multcomp* package when fixed factors significantly affected the response variable (Hothorn et al. 2016).

Results

The ICC was 0.76, thus suggesting that the behavioral observations were consistent between observers. Self-grooming was a behavior in which workers invested more time before and after the application of challenges. In the basal status, workers spent a median

of 1000 seconds dedicated to self-grooming; the investment in fungus grooming was seen for a median of 160 s; the time invested in allogrooming and fecal fluid grooming was under 100 s (Fig. 2 A–D). The metapleural gland grooming was detected only once in the 5440 s of footage; hence, the time invested in this behavior is negligible.

Workers reacted to the treatments by increasing their investment in self-grooming: However, the time after challenge ($F = 49.54$, $p < 0.0001$, Df 9)—but not the nature of the treatment itself ($F = 0.66$, $p = 0.4$, Df 9)—influenced their behavior. In the first hour after the challenge, workers duplicated the time investment in self-grooming behavior (Tukey test $p < 0.0001$). The time investment in self grooming decreased in the second hour, but it was higher than in the basal status (Tukey test $p < 0.0001$) (Fig. 2A).

A similar tendency was observed for the time invested in allogrooming. Workers increased the time investment in this behavior depending on the time after the treat-

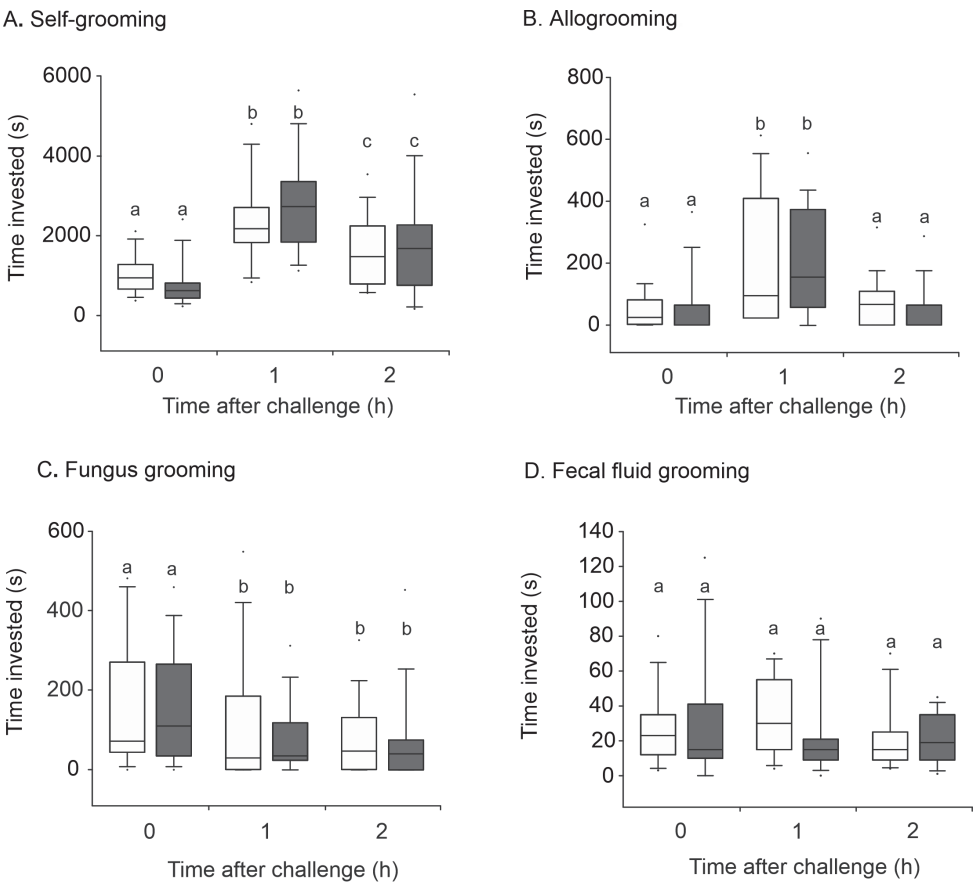

Figure 2. Time invested by *Atta cephalotes* workers in prophylactic behavior during the basal period (0 h) the first hour, and the second hour after challenge with water (white boxes) or *Metarizhium anisopliae* spores (gray boxes) **A** self-grooming **B** allogrooming **C** fungus grooming, and **D** fecal fluid grooming. Each box represents the sum of the time in seconds invested in each behavior from 10 workers ($n = 5$ nests, 60 workers per nest). Different letters indicate significant differences ($p < 0.05$).

ment (F = 6.32, p = 0.0028, Df 9) no matter whether they were challenged with water or *M. anisopliae* conidia (F = 0.16; p = 0.68, Df 9) (Fig. 2B). Workers almost duplicated the time invested in allogrooming in the first hour after challenges. This variable returned to basal levels in the second hour (Tukey test p = 0.83).

Workers showed a significant reduction in the time invested in fungus grooming after challenge (F = 6.4, p < 0.0026, Df 9) independent of whether they were challenged with water or spores (F = 0.78, p < 0 .37, Df 9) (Fig. 2C). In the first hour after the challenge, workers reduced the time investment in fungus grooming by approximately 60% (Tukey test p = 0.0228). This reduction was maintained in the second hour after treatment (Tukey test p = 0.010). In contrast, workers did not modify the time invested in fecal fluid grooming after the challenge neither in response to the time after treatment (F = 2.02, p = 0.16, Df 9) nor to nature of the treatment (F = 0.54, p = 0.58, Df 9) (Fig. 2D). Finally, workers treated with *M. anisopliae* showed ten-fold more infrabuccal pellets than control workers treated with water 24 h after the challenge (F = 124.80, p < 0.0001) (Fig. 3).

The workers' survival decreased progressively in control-treated workers and workers treated with spores over the ten-day observation period. However, the workers treated with *M. anisopliae* spores showed higher mortality from day four than workers treated with water (z = 2.80, p = 0 .005) (Fig. 4). Furthermore, the colonization assay showed that 80% of worker's corpses were effectively colonized by *M. anisopliae*; no signs of colonization were observed in water-treated workers (data not shown).

The expression of abaecin was affected by treatment (F = 6.2, p = 0.03). Workers treated with *M. anisopliae* showed an increase in the expression of abaecin as early as

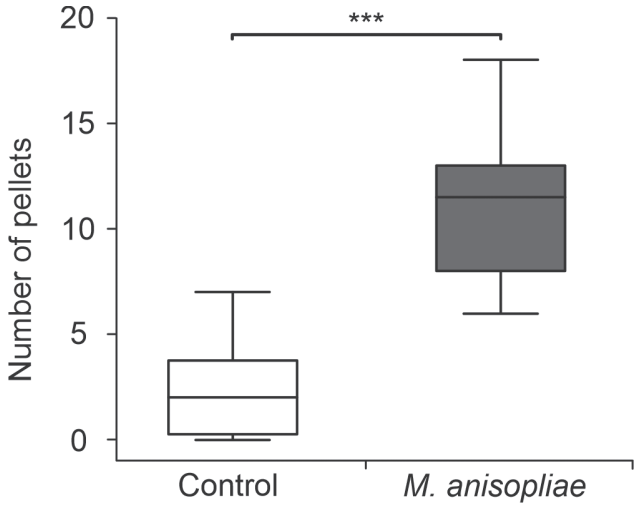

Figure 3. Production of infrabuccal pellets 24 hours after control challenge with water or spores of *Metarhizium anisopliae*. (n = 5 nests, 60 workers assessed per nest). Significant differences are marked with asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001).

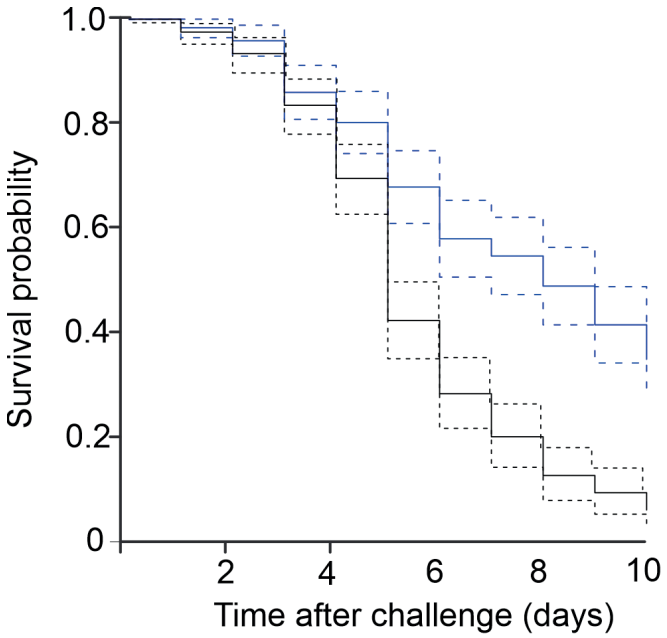

Figure 4. The survival probability of *Atta cephalotes* workers upon control challenge with water (blue line) or spores of *Metarhizium anisopliae* (black line). Blue and black dashed lines represent the 95% confidence interval. n = 4 nests with 60 workers assessed per nest.

24 h after the challenge. The expression of abaecin increased by nearly 200-fold at 48 h versus immediately after the challenge (Fig. 5A). In contrast, the expression of abaecin remained similar across time points in workers treated with water. The expression of defensin increased at 48 h after the challenge in workers treated with a spore suspension; however, no significant differences were detected versus expression before treatment (Fig. 5B).

Discussion

The results indicated that under laboratory conditions, *A. cephalotes* workers reacted to a potential hazard by increasing their time investment in self-grooming and allogrooming and reducing their interaction with the fungus. These hygienic behaviors were complemented by the production of infrabuccal pellets. However, the results suggest that workers could not discriminate an innocuous challenge from an actual hazard. Hence, the time invested in hygienic behaviors was similar between workers treated with water and workers treated with spores of *M. anisopliae*. Furthermore, workers were unable to clear the spores completely under this experimental setup—the invader reached the hemocoel triggering a rise in the expression of abaecin. Finally, infection with *M. anisopliae* decreased the workers' survival.

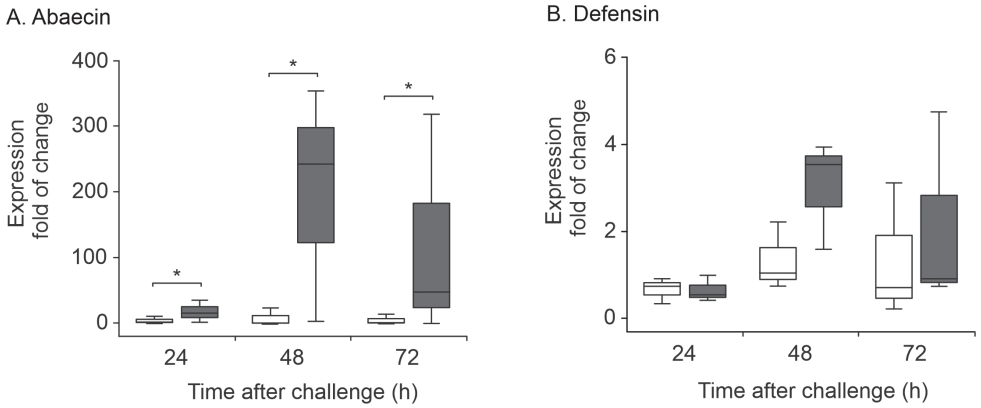

Figure 5. Expression of antimicrobial peptides in workers of *Atta cephalotes* upon challenge with water (white boxes) or spores of *Metharhizium anisopliae* (gray boxes) **A** abaecin **B** defensin. n = 3 nests; 20 workers assessed per time point. Significative differences are marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Workers of *A. cephalotes* combined self-grooming and allogrooming to remove the contaminants applied directly to their bodies, thus suggesting that this is the first line of defense against a potential hazard. Self-grooming has been identified as a proactive behavior widely extended in social insects including ants, wasps (Sumana and Starks 2004), bees (Morfin et al. 2020), and termites (Yanagawa et al. 2008). In leafcutter ants, this behavior is deployed by several species in response to challenges applied directly to the workers and to those applied to the mutualist fungi (Wilson 1976; Wheeler 1984; Morelos-Juárez et al. 2010; Reber et al. 2011; Walker and Hughes 2011; Bos et al. 2012; Nilsson-Møller et al. 2018; Bos et al. 2019), thus suggesting the relevance of this behavior to remove contaminants.

Allogrooming complements self-grooming hence allowing two or more individuals to clean the body of a third party in places that this one cannot reach, thus clearing potential hazards (Hughes et al. 2002; Reber et al. 2011). However, the time investment in self-grooming was 10-fold higher than the time invested in allogrooming. The difference in the time investment between those behaviors is probably explained because allogrooming depends on the proportion of clean and contaminated workers. In carpenter and leaf-cutting ants, if the treatment is applied simultaneously to the majority of individuals in a group, then the investment in this behavior decreases versus the investment observed when there is a proportion of clean workers (Bos et al. 2012; Da Silva Camargo et al. 2017). In this situation, clean workers dedicate time to clean other workers in the group.

The workers did not alter the investment in fecal fluid grooming, thus suggesting that this behavior is not deployed in response to challenges with water or *M. anisopliae*. In agreement with those findings, *Ac. echinatior* workers perform fecal fluid grooming to prepare plant material for degradation in (Kooij et al. 2016). Similarly, fecal fluid

grooming is performed by *Ac. octospinosus* minor workers when they incorporate plant material into the mutualist garden (Forti et al. 2020), and also by workers that make contact with the mutualist (Nilsson-Møller et al. 2018). Finally, it has been observed in foundress queens of *Ac. octospinosus* during the preparation of plant material to incorporate into the fungus garden (Fernández-Marín et al. 2003).

The deployment of hygienic behaviors led to the production of infrabuccal granules. An increase in infrabuccal pellet production has been previously described in *Acromyrmex* and *Tachymirmex* workers after exposure to *Escovopsis* and *Penicillium* (Fernández-Marín et al. 2006; Little et al. 2006). In line with these findings, the infrabuccal pocket has been described as a structure that compacts and possibly sterilizes the material removed by the workers during hygiene (Little et al. 2006). Furthermore, the final disposition of the pellets in the waste chambers helps isolate the potential hazard, thus protecting the workers and the fungus garden from further contamination (Currie and Stuart 2001; Little et al. 2003).

Atta cephalotes workers respond to the challenges applied in this study by modifying the time invested in the hygienic behaviors, but they could not produce a differential response between an innocuous challenge and an actual threat with an entomopathogen. This finding contrasts with previous reports, which show that leaf-cutting ants deploy a differential response against innocuous substances like talc and an actual threat (Fernández-Marín et al. 2006; Morelos-Juárez et al. 2010; Yek et al. 2013; Tranter et al. 2015; Nilsson-Møller et al. 2018). This finding cannot be attributed to the innocuity of the strain of *M. anisopliae* because the colonization assay and the survival curve showed that the strain of *M. anisopliae* has a virulence mechanism that allows it to reach the hemocoel of its host and significantly impacts its viability.

It is also possible that the application of the entomopathogen as a suspension of spores hinders the detection of specific cues by the workers. Although the mechanism that mediates the recognition of hazards in leaf-cutting is not well understood, microorganisms might release semiochemicals, volatile compounds detected by ants as cues of danger, thus triggering a prophylactic response (Davis et al. 2013; Goes et al. 2020). Thus, it is possible that those semiochemicals are diluted in suspension, thus hindering workers from detecting the threat. In this line of evidence, similar to the findings reported here, workers of *Atta colombica* did not generate a differential response to a suspension of spores of *M. anisopliae* and a challenge with a solution of detergent in water (Walker and Hughes 2011). Furthermore, workers of *Formica selysi* challenged with different concentrations of a suspension of *Metarhizium bruneum* spores at a concentration of 10^7 equivalent to the one used in this study workers produced a response similar to the one observed in control. In contrast, they deployed a differential response when they were challenged with a concentration of 10^8 spores/ml (Reber et al. 2011), thus supporting the idea that the concentration of the microorganisms is essential to its recognition and the scaling of the behavioral response against it.

Workers of *A. cephalotes* did not deploy the grooming of the metapleural gland in response to the challenge with *M. anisopliae*. This evidence is in contrast with a previous report showing that *A. cephalotes* workers increase the frequency of this behavior

up to 150 times in response to a challenge with dry spores of *Penicillium*. This indicates that this is the primary mechanism of response against fungal threats among *A. cephalotes* (Fernández-Marín et al. 2006). The differences between these findings can be explained by the strategy used to deliver the challenge to the workers. Dry spores may elicit metapleural gland grooming because they are easily detected by ants whereas the cues released by the microorganisms are diluted in suspension, thus hindering recognition. Nevertheless, among workers of *Ac.echinatior*, the inoculation of the fungus garden with dry *Escovopsis* spores did not increase the frequency of metapleural grooming thus indicating that another factor is necessary to trigger this behavior (Nilsson-Møller et al. 2018). Recently, *Tachymirmex* workers have been shown to increase the frequency of metapleural gland grooming upon challenge with germinated spores of *Metarhizium* and *Escovopsis* versus the challenge with ungerminated conidia, thus suggesting that the status of the microorganisms also influences the workers' response (Bonadies et al. 2019).

The evidence shows that although treatment with *M .anisopliae* spores, significantly impacts workers' viability, the treatment with water also caused a reduction in this parameter. Previously, it has been shown that *A. cephalotes* workers lose viability when isolated on Petri dishes, even when left untreated (Valencia-Giraldo et al. 2021), possibly because they do not consume the agar diet. In contrast, colonies maintain their viability under laboratory conditions six weeks after the extraction (Valencia-Giraldo, personal observation). These findings suggest that experimental conditions may alter the health status of workers. Hence it cannot be ruled out that this factor may influence workers' behavior avoiding the discrimination between a sham challenge and the challenge with an entomopathogen.

In terms of individual immunity, evidence shows that workers of *A. cephalotes* increase the expression of the antimicrobial peptide abaecin. The peak in the expression of abaecin at 48 h is consistent with the dynamics of invasion of *M. anisopliae*, which reaches the hemocoel between 24 and 48 h after hosts exposure (Moino Jr et al. 2002). In insect hosts, abaecin has shown antibacterial activity (Casteels et al. 1990; Otvos 2002; Rahnamaeian et al. 2015). However, the expression of this AMP in response to fungal infection has also been reported in *Apis mellifera* (Bull et al. 2012) and *Ac. echinatior* (Yek et al. 2013) suggesting that this peptide might protect against *M. anisopliae*.

In contrast, the expression of defensin was not altered in response to infection with *M. anisopliae.* This finding contrasts with evidence showing that expression of defensin in response to infection with *M. anisopliae* is increased in social insects including *A. mellifera* (Bull et al. 2012) and *Lasius neglectus* (Konrad et al. 2012) as well as in solitary insects like *Aedes aegypti* and *Locusta migratoria* (Cabral et al. 2020; Jiang et al. 2020). This finding may be explained by reducing genes related to individual immunity observed in *A. cephalotes* (Suen et al. 2011). However, the IMD and toll pathway genes responsible for the signaling transduction that lead to defensin expression and are functional in this species (Schlüns and Crozier 2007; Suen et al. 2011). Therefore, the explanation for the low defensin expression during the *Metarhizium* challenge remains unclear.

Conclusion

The results show for the first time that *A. cephalotes* workers deploy mechanisms of collective and individual immunity upon challenge with *M. anisopliae* spores. Under this particular experimental setup A. *cephalotes* workers cannot discriminate between a hazardous stimulus and an innocuous one; hence, they deploy a generic behavioral response independent of the level of threat posed by the challenge. In contrast, once *M. anisopliae* reaches the hemocoel, individual immunity recognizes the danger and triggers the expression of abaecin, possibly as a defense mechanism against the invader.

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