UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

TRAITS OF SOCIAL IMMUNE MEMORY IN A FUNGUS-GROWING SUPERORGANISM

ARYEL CAMERO GOES

Rio Claro – SP
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Orientador: André Rodrigues

Coorientador: Pepijn Wilhelmus Kooij

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia).

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IMPACTO POTENCIAL DESTA PESQUISA

O presente estudo reforça algumas hipóteses sobre a capacidade de insetos sociais (formigas, abelhas e cupins) em se "lembrarem" sobre exposições prévias a patógenos. Isso nos sugere que a capacidade observada no sistema imunológico de seres vivos, como a memória imunológica ou respostas específicas contra patógenos já imunizados, evoluíram além do nível de células e moléculas biológicas. Os resultados deste estudo indicam que a formiga saúva-limão, *Atta sexdens*, é capaz de intensificar os comportamentos de higiene coletiva contra fungos patogênicos expostos previamente. Além disso, sugere que a colônia de formiga como um todo retêm essa habilidade por um período de 30 dias, devido à experiência. Tal descoberta é importante, pois os resultados abrem as portas para a investigação de traços do sistema imunológico fora do mundo celular, entendendo a sua evolução em organismos que vivem em grupo. Do ponto de vista aplicado, nossos resultados poderão auxiliar na elaboração de métodos efetivos no controle biológico das mesmas, uma vez que a memória contra patógenos pode burlar efeitos da aplicação de fungos para controle populacional; por exemplo, as formigas poderão lidar de maneira intensa e efetiva devido a experiência, se aplicada múltiplas vezes o mesmo patógeno.

POTENTIAL IMPACT OF THIS RESEARCH

This study supports previous hypotheses on the ability of social insects (ants, bees and termites) to remember prior challenges against microbial pathogens. It suggests that the capacity of the immune system, such as immune memory or specific responses against immunized pathogens, have evolved beyond the level of biological cells and molecules. Our results indicate that the leaf-cutting ant, *Atta sexdens*, is able of intensifying collective hygienic behaviors against previously exposed pathogenic fungi. In addition, it suggests that the ant colony as a whole retains such capacity for up to 30 days. This finding opens new research windows for investigating traits of immune system beyond the cellular level, understanding their evolution at the group level. Our study may also contribute to design od effective methods for the biological control of these ants, since such memory against pathogens can overcome effects of fungal application for population control, as these ants can deal faster and effectively to the same pathogen that was applied multiple times.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Rio Claro

CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: TRAITS OF SOCIAL IMMUNE MEMORY IN A FUNGUS-GROWING SUPERORGANISM

AUTOR: ARYEL CAMERO GOES
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“The boy with the thorn in his side, behind the hatred there lies a murderous desire for love. And if they don’t believe us now, will they ever believe us? And when you want to live, how do you start? Where do you go? Who do you need to know?”

The Smiths

RESUMO

O sistema imunológico é crucial para a defesa dos organismos contra patógenos. Da mesma forma, mecanismos imunológicos análogos evoluíram para combater pressões semelhantes em superorganismos. Reter informações de infecções anteriores e usá-las para regular a higiene são alguns dos mecanismos imunológicos convergentes encontrados em insetos sociais. Entretanto, ainda é deconhecido se esses mecanismos atuam de forma semelhante à memória imunológica. Neste estudo, avaliamos a memória imunológica no nível da colônia, desafiando a formiga cortadeira, *Atta sexdens*, com exposições homólogas e heterólogas aos fungos entomopatogênicos, *Metarhizium anisopliae* e *Beauveria bassiana*, e aos fungos micoantagonistas, *Fusarium oxysporum* e *Trichoderma spirale*. Contabilizando o comportamento das formigas, avaliamos a capacidade de *A. sexdens* de (i) intensificar a sua higiene coletiva, (ii) se a mesma ocorre rapidamente, (iii) quanto tempo dura essa capacidade nas colônias e (iv) o grau de especificidade para intensificar as respostas higienicas. A sanitização foi aprimorada para os quatro fungos inoculados, com incertezas acerca da velocidade de reação contra todos eles. A capacidade de provocar tais ações reguladas durou até 30 dias, quando expusemos o mesmo fungo pela terceira vez, mas não em colônias que o receberam após 60 dias. Em geral, as colônias mostraram um grau de especificidade imunológica ao diminuir a higiene quando expostas secundariamente a um fungo desconhecido. Nossos resultados destacam que *A. sexdens* possui algumas características de memória imunológica contra patógenos fúngicos, com base na dinâmica de suas respostas em nível de colônia sob contínuas infecções secundárias.

Palavras-chaves: Memória imunológica, Imunologia social, Patógenos, Especificidade, Attini.

ABSTRACT

The immune system is crucial for organisms to defend against pathogens. Likewise, analogous immune mechanisms evolved to confront similar pressures at the superorganismal level. Retain information from previous infections and use it to upregulate hygiene are some of the convergent immune mechanisms found in social insects. Whether it extends to immunological memory is still elusive. Here we evaluate immune memory at the colony-level by challenging the leaf-cutting ant *Atta sexdens* with homologous and heterologous exposures to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, and the mycoantagonistic fungi *Fusarium oxysporum* and *Trichoderma spirale*. By measuring ants' behaviors, we bounce the capacity of *A. sexdens* to (i) enhance their collective hygiene, (ii) whether it occurs faster, (iii) how long this capacity lasts in the colonies, and (iv) the degree of specificity to increase responses. Sanitization was enhanced to the four challenged fungi, with unclear signs of velocity against all of them. The capacity to elicit upregulated hygienic actions lasted for up to 30 days when we exposed the same fungus for a third time, but not in colonies that received it after 60 days. Overall, colonies showed a degree of immune specificity by decreasing hygiene when exposed secondarily to a distinct fungus. Our results highlight that *A. sexdens* have some traits of immunological memory against fungi pathogens, based on the dynamics of their colony-level responses under secondary infections.

Keywords: Immunological memory. Social immunity. Pathogens. Specificity. Attini ants.

FIGURES

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1. MANUSCRIPT

Traits of social immune memory in a fungus-growing superorganism

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ABSTRACT

The immune system is crucial for organisms to defend against pathogens. Likewise, analogous immune mechanisms evolved to confront similar pressures at the superorganismal level. Retain information from previous infections and use it to upregulate hygiene are some of the convergent immune mechanisms found in social insects. Whether it extends to immunological memory is still elusive. Here we evaluate immune memory at the colony-level by challenging the leaf-cutting ant *Atta sexdens* with homologous and heterologous exposures to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, and the mycoantagonistic fungi *Fusarium oxysporum* and *Trichoderma spirale*. By measuring ants' behaviors, we bounce the capacity of *A. sexdens* to (i) enhance their collective hygiene, (ii) whether it occurs faster, (iii) how long this capacity lasts in the colonies, and (iv) the degree of specificity to increase responses. Sanitization was enhanced to the four challenged fungi, with unclear signs of velocity against all of them. The capacity to elicit upregulated hygienic actions lasted for up to 30 days when we exposed the same fungus for a third time, but not in colonies that received it after 60 days. Overall, colonies showed a degree of immune specificity by decreasing hygiene when exposed secondarily to a distinct fungus. Our results highlight that *A. sexdens* have some traits of immunological memory against fungi pathogens, based on the dynamics of their colony-level responses under secondary infections.

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1.1 INTRODUCTION

During the lifetime of an organism, pathogens will continuously try to overcome host immunological barriers. Whenever behavioral strategies or external barriers are overcome, innate and adaptive immunity will act as decisive mechanisms for defense and reduction of damage (JANEWAY *et al*., 2005; SCHULENBURG *et al*., 2009). Due to a high likelihood of infection from the same pool of pathogens and by inhabiting dynamic environments, maintaining an immune memory is advantageous for hosts (KURTZ, 2005; GRAW *et al*., 2010). The information storage from previous challenges allows the immune system to elicit stronger and faster responses in future encounters to usual pathogens (KURTZ, 2005; PRADEU and PASQUIER, 2018). Long thought to be exclusive to the adaptive immune system of vertebrates, immunological memory has now been identified and highlighted in microorganisms, plants, invertebrates (NUÑEZ *et al*., 2016; MILUTINOVIĆ and KURTZ, 2016; REIMER-MICHALSKI and CONRATH, 2016), as well as a feature of the innate immune system (GOURBAL *et al*., 2018; NETEA *et al*., 2018).

Superorganisms, such as ants, some termites, bees, and wasps (*sensu stricto* definition, WHEELER, 1911; BOOMSMA and GAWNE, 2018) possess analogous solutions to those found in the physiological immune system to fight infections (CREMER and SIXT, 2009; PULL and MCMAHON, 2020). For instance, they police nestmates to avoid intrusion of outsiders, apply chemical and physical defenses against pathogens, remove dead or infected individuals, and coordinate their social network to reduce infection (PULL and MCMAHON, 2020 and references therein). Beyond these mechanisms, the immune memory may also have evolved at the superorganismal level to suppress the likelihood of continuous threats (PRADEU, 2013; PULL and MCMAHON, 2020), as it evolved at the organismal level in several taxa (SCHMID-HEMPEL and EBERT, 2003; KURTZ, 2004; QUINTIN *et al*., 2014). For instance, some ant species enhance their grooming behavior toward nestmates (WALKER and HUGHES, 2009; KONRAD *et al*., 2018; WESTHUS *et al*., 2014) or mutualistic fungus (GOES *et al*., 2022) often contaminated with the same pathogen. Such plasticity may suggest the ability of some advanced social insects to retain information from past challenges, resembling a collective immune memory, benefiting the colony with plastic sanitary responses.

Fungus-growing superorganisms, known as ‘attine ants’ (Hymenoptera: Formicidae; subtribe Attina), have a good potential to possess a collective immune memory. Because they cultivate fungal symbionts as a source of food (MUELLER *et al*., 2005; VEGA and

BIEDERMANN, 2020), the foraging of fresh or dry plant material to manure the fungus brings a myriad of potentially antagonistic microbes to their nests (MUELLER *et al*., 2005; VAN BAEL *et al*., 2009; RORDRIGUES *et al*., 2005, 2008; SCOTT *et al*., 2010), requiring further hygienical strategies to protect and clean themselves and the fungal cultivar (CURRIE and STUART, 2001; NILSSØN-MOLLER *et al*., 2018; GOES *et al*., 2020). Considering the likelihood of the superorganism to be threatened across its lifetime (BOOMSMA *et al*., 2005), one could expect adaptive immune traits at group level to confront pressures from distinct pathogens and parasites in fungus-growing ants, i.e., collective immune memory. This would promote stronger and more efficient actions in secondary challenges to specific pathogens (GOES *et al*., 2020; PULL AND MCMAHON, 2020). Although some leaf-cutting ants indeed intensify hygienic responses under successive pathogen challenges (*Acromyrmex echinatior* (FOREL, 1899), WALKER and HUGHES, 2009; *Atta sexdens* (LINNAEUS, 1758), GOES *et al*., 2022), other aspects of the immune memory phenomenon are still underexplored, such as species-specific increased hygiene and the duration of this response in colonies (short or long-term). Since these aspects remain to be addressed, there is no assertation of immune memory in a group level for leaf-cutting ants or any other social insect.

Building up on previous studies (WALKER and HUGHES, 2009; GOES *et al*., 2022), we investigate whether the leaf-cutting ant *A. sexdens* possesses immune memory traits at the colony level. We inquired on four aspects of immunological memory based on Pradeu and Pasquier (2018): i) is the hygiene higher in secondary immune challenges? ii) does the increase occur in less time in secondary immune challenges? iii) how long do the capacity to elicit higher responses last? and iv) do higher hygiene only occur specifically to a previously encountered pathogen? Under the hypothesis that *A. sexdens* has a collective immune memory, successive infections to the same pathogen will elicit higher and faster hygienic actions in secondary challenges. Also, based on the age polyethism of *A. sexdens* that may affect the availability of experienced medium and major workers, the capacity to trigger enhanced hygiene may be affected by 30 to 60 days since the last infection (LACERDA *et al*., 2020). Lastly, if the collective immune memory has a specificity trait, then hygienic actions will increase when encountering a previously met pathogen but not when facing a secondary infection with a distinct pathogen (CONTRERAS-GARDUÑO *et al*., 2016). To evaluate these, we subjected *A. sexdens* colonies to homologous and heterologous challenges of two entomopathogens, i.e., *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) and *Metarhizium anisopliae* (Metschn.) Sorokin (1883), and two potentially antagonists to the fungus cultivated

by the ants, i.e., *Fusarium oxysporum* Schltdl. (1824) and *Trichoderma spirale* Bissett (1992), and measured group hygiene through time.

1.2 MATERIAL AND METHODS

Colony maintenance

We collected in total 80 incipient colonies of the leaf-cutting ant *A. sexdens* at the Itirapina Ecological Station, São Paulo, Brazil (-22.225662N -47.840134W) in the summer of 2021 and reared them at the Centro de Estudos de Insetos Sociais (CEIS, UNESP – Rio Claro). The fungus garden containing the queen, workers, and offspring was kept in an acrylic glass container (16.5 x 11 x 7 cm) with 1 cm of plaster at the bottom to maintain humidity. The container was connected to two smaller-sized containers (10 cm in diameter and 5 cm height, each) for the foraging area and the dump chamber. Each colony was allowed to settle and grown for four months before the start of the experiments. The colonies were maintained at 24 °C with daylight regime. Fresh leaves of *Mangifera indica* and *Syzygium* sp. were alternated daily.

Fungal cultures and conidial suspension

Aiming to investigate whether *A. sexdens* colonies show immunological memory traits, one strain of *Beauveria bassiana* LESF 477 and one of *Metarhizium anisopliae* LESF 206 were used to represent a threat to the ants, i.e., entomopathogens (LACERDA *et al*., 2010; LOPEZ and ORDUZ, 2003). One strain each of *Fusarium oxysporum* LESF 333 and of *Trichoderma spirale* LESF 117, used in a previous study (GOES *et al*., 2022), were chosen to represent a potential threat to the fungal cultivar, i.e., mycoantagonists (RODRIGUES *et al*., 2008; ROCHA *et al*., 2017). All strains used in this study were deposited in the collection of the Laboratory of Ecology and Fungal Systematics (LESF, UNESP, Rio Claro, SP). The fungi cultures were cultivated and maintained in Petri dishes containing Potato Dextrose Agar medium (PDA; Neogen, MI, USA), supplemented with 150 µg mL^{-1} of chloramphenicol (Sigma-Aldrich, MI, USA). The plates were incubated at 25 °C in the dark. Ten days prior to the experiments, the fungus was transferred to a new Petri dish with PDA and incubated at 25 °C for ten days.

The conidia suspensions were prepared by collecting hyphae and conidia from 7-day-old cultures (OSTI and RODRIGUES, 2018). We suspended the material in 0.05% Triton X

(diluted in water) in sterile 10 mL plastic tubes. To remove the mycelium from the conidia, we vortexed the material for one minute and then we filtered the suspensions using a 0.5 µm sterile filter membrane (Millipore, Sigma-Aldrich, MI, USA) for *B. bassiana,* and a 0.40 µm sterile cell strainer (Sigma-Aldrich, MI, USA) for the other fungal species. The concentration of conidial suspension was measured with a hemocytometer and diluted to approximately $1x10^6$ conidia mL^{-1}. For all inoculations, we employed a sterile 5 mL hand-sprinkler to expose the fungus garden surface with 0.5 mL of the conidial suspensions or 0.5 mL of 0.05% Triton X (sham solution) for the control treatment. Before reusing the sprinklers, we immersed both the nozzle and the tube in 96% EtOH for two days and cautiously washed them in distilled water. We checked the conidia viability by incubating them on Petri dishes with PDA at 25 °C for 48 hours. After incubation, we found >98% of viability in all cases.

Homologous and heterologous challenge

Based on the fungal lifestyle, we divided our assays in two major groups: entomopathogens (*B. bassiana* and *M. anisopliae*) and mycoantagonists (*F. oxysporum* and *T. spirale*). To evaluate whether *A. sexdens* colonies respond faster and stronger towards secondary exposures of a given pathogen, we inoculated colonies with the same fungus species in the first and second challenge (homologous exposure; Figure S1a), with a 7-day interval between exposures. We used five colonies for each pathogen and five colonies to receive the sham solution for each major group (5 colonies for *B. bassiana* + 5 colonies for *M. anisopliae* + 5 colonies for control; 5 colonies for *T. spirale* + 5 colonies for *F. oxysporum* + 5 colonies for control; Figure S1a). To check whether the enhancement in hygiene through challenge is long lasting, we realized two homologous assays to each major group, with distinct colonies. One set of colonies received a third challenge of the same pathogen 30 days after the second exposure (entomopathogens/mycoantagonists, for each: n= 10 treatment colonies + 5 control colonies) and the other set received it after 60 days (entomopathogens/mycoantagonists, for each: n= 10 treatment colonies + 5 control colonies). We choose these time lags based on the age polyethism of major and medium workers of *A. sexdens* to allocate into gardening and foraging activities, i.e. nine weeks and four weeks, respectively (LACERDA *et al*., 2020). Since the switch of tasks by age affect the distribution of workers in laboratory colonies, the dynamic of social immune responses in late encounters may be affect by the availability of experienced and naïve workers to respond further challenges.

To evaluate whether the ants enhance their hygiene specifically to previously met pathogens, we subjected colonies to a heterologous challenge with 7-day intervals (heterologous exposure; Figure S1b). Thus, for each major group, five colonies received a first exposure of pathogen A, followed by a second challenge with pathogen B, and a reintroduction of pathogen A in a third challenge; and five colonies received the pathogens in contrasting order, i.e., pathogen B – pathogen A – pathogen B (entomopathogens/mycoantagonists, for each: n = 10 treatment colonies + 5 control colonies; Figure S1b). This experiment is important to reveal the degree of specificity in *A. sexdens* to enhance immune responses to a single or multiple pathogens. Simplified, stronger and faster responses would only be elicited towards a pathogen previously found, and not be affected by a second challenge of a different pathogen, denoting specific immune memory (KURTZ, 2004; 2005; CONTRERAS-GARDUÑO *et al*., 2016).

Experimental setup and procedure

On the challenge days, we disconnected the feeding arena of each colony and replaced it with a PVC-tee (2.5 cm in diameter) which led to a bifurcation of two isolated plastic bowls (10 cm in diameter and 8 cm height, each) with 1 cm layer of plaster on the bottom. We placed a piece of fungus garden of similar size (2.5-3 cm^3) from the main cultivar matrix in each chamber one day before the challenge. Only one chamber received the treatment (conidia or sham solution) and the other one was a negative control, i.e., without any form of treatment. Because we followed the initial defensive responses from zero to one-hour period, we removed all ants as possible from the fungus fragments. With soft forceps, we removed all the ants and brood from the piece while avoiding disrupting the whole garden structure. We left minima workers (i.e., gardening caste) to maintain the cultivar's health overnight, since it cannot be left unattended for hours (SOMERA *et al*., 2015). On the next day, before the start of the experiment, we carefully removed reminiscent workers from both garden pieces. For each colony, these procedures did not exceed more than 10 min to minimize fungus desiccation.

We applied the treatment solution in one of the two fungus pieces based on a previous raffle; we distinguished the challenge arena by a simple ink mark on the lid/side of the chamber. After that, we immediately connected the setup to the respective colony and started the video recording of both arenas. All treatments were performed at the same time of day, i.e., between 7 am to 11 am UTC -3, our time. After one hour, we disconnected the T-junction

and returned all the reminiscent workers to their respective colony. These manipulations were performed carefully to avoid losing the experienced workers or accidentally killing them. We never reused the fungus fragment for following exposures, but rather discarded it to avoid possible bias.

At the end of each challenge, the detached experimental chambers were soaked for two days in the neutral detergent Extran 5% (Merck KGaA, DA, Germany), washed, and air-dried for further use in the next challenges. We always replaced the plaster and surface sterilized the two arenas with UV light for 15 min. We cleaned the experimental chambers to remove any chemical and trace odors left by ants from the previous challenge. To avoid bias in ants' choices and actions based on spatial information retained by workers from the previous challenge, we switched the location of the treatment and negative control fragment in further exposures.

Video recording and scoring behaviors

The video recording started immediately after the attachment of the T-junction to the main colony. We followed in the progression of cleaning behaviors towards the challenged and non-challenged piece of the fungus cultivar, throughout a one-hour period. We recorded fully the first 10 min (i.e., lag time), which is the average time required for workers from the main arena to explore the experimental setup, detect, and recognize the situation offered. Then, we continued recording the arenas at 15, 30, 45, and 60 min, with video recordings of 15-20 seconds long. Evaluating the dynamic of defensive responses through these intervals is important to understand whether the colonies were faster in secondary exposures. We used two Sony HDR – CX150/B (9.0 megapixels) video cameras and allocated them to one arena each from the experimental setup. Thus, the colonies were recorded individually. We managed to record the fungus garden fragment and the whole area of the plastic bowls.

A single examiner (ACG) analyzed all the video recordings counting the number of i) defensive behaviors displayed by workers interacting with the fungus fragment, ii) the total number of ants on the fragment, and iii) the total number of workers in the arena. We used a hand-counter (VMC–4, Vonder, Brazil) to facilitate the counting. We chose the instantaneous scan sampling for assessments. In short, during the video, the examiner scored the behavior presented by each individual at the time it was observed (MARTIN and BATESON, 2007). The same individual was never observed twice. To avoid and reduce bias during the video assessment, another person (PWK) renamed the video files for a blind assignment (KARDISH

et al., 2015). Therefore, the examiner did not know which treatment the video recorded belonged to during the analysis. We scored the frequency of three hygienic behaviors from *A. sexdens'* social immunity (i.e., *fungus grooming*, *self* and *allogrooming*, as described previously in NILSSØN-MOLLER *et al*., 2018; GOES *et al*., 2022) and a singular defensive behavior seen in our study (*fungus rescue*):

i. *Fungus grooming*: when an ant was immobile at a fixed point of the fungus fragment. The antennae remained motionless and parallel to the fungus, with the ends touching the tips of the mandibles. The ant opened its mandibles and did retracting movements with the head, pulling off a tiny portion of fragment, or the glossa licked the fungus surface.

ii. *Fungus rescue*: when an ant used its mandibles to either cut or detached a large piece of the fungus fragment, pulling it off and carrying it out from the arena. We also scored this defensive behavior whenever an ant was found carrying the fungus piece inside the arena. The ant usually took the fragment back to the main arena, putting it back in the fungus garden matrix (personal observations). Therefore, we did not consider this behavior as *fungus weeding*, since the fragments were not discarded in the dump arena.

iii. *Allogrooming*: when one or more ants are nearby another nestmate, which remained motionless. The groomer ant(s) licked with its/their mouthparts opened, using the glossa, the motionless ant, moving slightly to lick the main body parts.

iv. *Self-grooming*: when a single ant remained at a portion of the fungus fragment and brushed the antennae on the front legs; or when the ant cleaned the antennae and the legs by passing them through the mouthparts, removing particles with their glossa, several times.

Statistical analysis

To evaluate the amount of cleaning behaviors applied in each exposure, we pooled fungus grooming, selfgrooming, and allogrooming into a single category called "number of cleaning responses". We analyzed the 'fungus rescue' behavior separately, because we did not consider this a cleaning strategy, but rather a defensive one. To assess whether the number of cleaning responses varied within exposure, we applied generalized linear mixed model analysis (GLMM; WINTER, 2013). The amount of cleaning responses were the response variable in the model, while the time intervals (5, 10, 15, 30, 45, and 60 minutes, as factor) and the interaction between treatment and exposure as fixed effects. The ant colony nested within exposure and it nested within time intervals were our random factors, since they

represent multiple measures without independence (SCHIELZETH and NAKAGAWA, 2013). We used this model for both the homologous and heterologous assays. The same were considered for the fungus rescue analysis, although this one was the response variable. To check whether responses were faster through challenges, we ran a separate GLMM model considering the time interval of the footage as our fixed effect, while exposure, colony, and time interval were the random factors. Because our data did not show homoscedasticity, we used a negative binomial distribution model, based on the good fitting of the test in residual diagnostics for hierarchical regression models (DHARMa package; HARTIG, 2020). For the responses seen at the negative control arena, we ran the non-parametric Wilcox test to evaluate whether the negative control arenas had a substantial number of ants and cleaning behaviors in comparison to the treatment arenas.

We applied likelihood ratio tests with the 'anova' function to compare a model carrying the fixed effect of interest against a null model, i.e., without the aimed fixed effect. With that, we could determine which fixed factors significantly explained the variation of our response variable. In cases where we had a significant fixed factor and it contained more than two groups, we utilized post-hoc tests to compare them, i.e., Tukey test with Bonferroni adjusted *P*-value, from the 'emmeans' package (LENTH, 2016). All statistics were carried out in R 4.0.3 (R CORE TEAM, 2016).

1.3 RESULTS

Increased hygiene towards homologous challenges

Atta sexdens colonies substantially employed fungus grooming and selfgrooming when confronted with both treatment and sham solution, for entomopathogens or mycoantagonists. Both strategies were mostly observed in the treatment arena (Figure 1). Fungus rescue were seen in the negative control arena, where the fungus fragment did not receive any treatment (Figure S2). Ants tended to bring back the fungus fragment from the negative control arena to the main colony, 'planting' it in the main fungus garden (ACG personal observation). We did not observe allogrooming in any of the treatments and homologous and heterologous assays.

The number of ants in the negative control arenas was not substantially higher in comparison to those in the treatment arenas, for all the experiments (Wilcoxon's paired test, $P>0.05$ in all cases). We conclude that the number of ants did not negatively affect each other. Thus, we did not run further GLMM analysis with the negative control.

The time intervals and the effect between treatment (i.e., fungus or sham solution) within exposure significantly impact the amount of cleaning hygiene in the one-month and two-months homologous challenges with entomopathogens (GLMM one-month, $X^2 = 29.954$, df = 8, $P<0.01$; GLMM two-months, $X^2 = 10.874$, df = 8, $P<0.01$). In contrast, this effect was not observed in the one-month homologous challenges with mycoantagonists (GLMM one-month, $X^2 = 10.337$, df = 8, $P>0.05$), but only for the two-months (GLMM two-months, $X^2 = 33.092$, df = 8, $P<0.001$). Against homologous exposures of entomopathogens, there was significant increases in hygiene between the first/second and first/third exposures in the two-months assay (Tukey's test, $P<0.05$; Figure 2a). In the one-month assay, a significant increase only occurred between the first/third and second/third exposures (Tukey's test, $P<0.05$; Figure 2a). Ant workers seemed to upregulate hygiene against homologous exposures of mycoantagonistic fungi, though it was not statistically significant in most of the cases (Tukey's test, $P>0.05$; Figure 3a). Lastly, the average number of ants visiting the treatment arenas also varied through all the challenges, though not significantly (Figure 2c, 3c; for the negative control, see Figure S3c, S4c).

Lack of faster responses through homologous challenges

The number of hygienic responses was significantly affected by the time intervals for the entomopathogens (GLMM one-month, $X^2= 11.425$, df= 5, $P<0.01$), but not for the two-months assay (GLMM two-months, $X^2= 0$, df= 5, $P> 0.05$). Despite responses seemed to upregulate in the first minutes of secondary entomopathogenic challenges (Figure S5), such trend was not substantial in comparison to other exposures (Tukey's test, $P> 0.05$ for all). In the mycoantagonist homologous assays, time also had effect in hygiene (GLMM one-month, $X^2= 10.264$, df= 5, $P<0.01$; GLMM two-months, $X^2= 10.805$, df= 5, $P<0.01$), although there was no trend of faster responses through exposures (Tukey's test, $P>0.05$ for all; Figure S6).

The capacity for higher hygiene lasts up to 30 days but not for 60 days

Regarding the duration of adaptative responses, colonies triggered higher hygiene in a third challenge with the same entomopathogenic fungus after 30 days (Tukey's test, $P<0.05$; Figure 2a). In those colonies that received a third challenge 60 days later, responses decreased or maintained the same as the first and second exposures regardless of the fungus treatment (Tukey's test, $P> 0.05$; Figure 2a). In constrat, ant workers did not upregulate sanitization against a third challenge of the same mycoantagonistic fungus after 30 days (Tukey's test,

P>0.05; Figure 3a). Moreover, sanitization also tended to decrease or maintain the same in those colonies that received a third homologous challenge 60 days later (Figure 3a).

Hygienic responses decrease toward heterologous challenges

The effect of time and interaction between treatment and exposure was significant in the heterologous challenges, for both entomopathogenic (GLMM, X^2 = 29.503, df = 8, *P*<0.01) and mycoantagonistic fungi (GLMM, X^2 = 16.877, df = 8, *P*<0.05). A second challenge (i.e., heterologous challenge) with a different entomopathogenic fungus had a significant reduction in sanitization compared to the first and third exposure (Tukey's test, *P*<0.05; Figure 2b). Even so, the average number of ants in the arenas during the second challenge was similar to those in the first and third challenges (Figure 2c). Although it appears that there had been a reduction in the sanitization applied to the second challenge with another mycoantagonistic fungus, it was only significant when the colonies received a heterologous exposure of *T. spirale* (Tukey's test, *P*<0.05; Figure 3b). Lastly, the average number of ants in the arenas also remained similar through mycoantagonistic exposures (Figure 3c).

1.4 DISCUSSION

According to our data, laboratory colonies of *A. sexdens* improve hygienic behaviors when confronted with the same pathogen in repetitive exposures (Figures 2 and 3), consistent with our hypothesis and assumptions made in previous studies (WALKER AND HUGHES, 2009; GOES *et al*., 2022). Since colonies may re-encounter several pathogens throughout their lifespan (NAUG; CAMAZINE, 2002; SADD; SCHMID-HEMPEL, 2009a), enhanced and faster hygiene towards common challenges are traits expected of the immune memory (JANEWAY *et al*. 2005; PRADEU; PASQUIER, 2018). Although these flexible reactions come to a price in the immunological scenario (MCKEAN; LAZZARO, 2011), the benefits seem to be greater for social insects (SADD; SCHMID-HEMPEL, 2009b). For instance, higher responses result in improved removal of fungal contaminants, hence affecting positively group survival in ants (WALKER; HUGHES, 2009; WESTHUS *et al*., 2014). Our experimental settings did not address whether colonies have less mortality of workers or loss of fungus garden weight through challenges. However, we noticed that ants tend to avoid recovering inoculated fungus garden fragments (i.e., *fungus rescue* behavior; see Figure S2); instead, they applied cleaning strategies (Figure 1). Our data further suggests that enhanced hygiene may prevent or reduce the need to discard a piece of the mutualistic fungus that can

be cleaned up. The direct impact of plastic hygiene responses on colony survival and fungus garden protection can be accessed in future studies with multiple homologous challenges.

Whether the improvement of hygiene occurred in less time (i.e., faster) was not possible to be elucidated in our experiments. Faster task performance is reported in ants for thermal brood-caring (WEIDENMÜLLER *et al*., 2009) and the emigration process (LANGRIDGE *et al*., 2008), but little is known on how experience affects the speed of sanitary care. Apart of the conceived idea from multicellular immunology (JANEWAY *et al*., 2005; PRADEU; PASQUIER, 2018), fast induction of response at colony level would be expressed in a quicker detection of the contaminant, escalating hygiene behaviors in less time under secondary infections. Here cleaning adjustments remained similar or suffered an increase in half-hour under homologous challenges with entomopathogens in some cases (Figure S5), although not significantly. On the other hand, colonies challenged with mycoantagonists showed erratic patterns between exposures (Figure S6). Such lack of speed is not surprising since previous studies did not observe decrease in the time of detection and reaction against *Metarhizium* challenges for the clonal ant *Platythyrea punctata* (WESTHUS *et al*., 2014), nor faster removal of fungus-contaminated waste in *Myrmica rubra* (PEREIRA *et al*., 2020). In the light of the current knowledge and our results, it seems that ants do not rely on faster reactions when successively infected. They seemed to dedicate longer time to remove contaminants and intensifying the number of collective actions, instead (WESTHUS *et al*., 2014). Alternatively, the lag timing from the first worker exploring one of the arenas and recruiting additional one may have varied between colonies and exposures (see Figure S5 and S6). This might explain inconsistencies in the speed of hygiene observed, hampering any conclusion. More sophisticated experimental designs could evaluate how long it takes for *A. sexdens* or other social insects to detect and start recruiting ants to escalate hygiene in secondary challenges (see GERSTNER *et al*., 2011), and the role of naïve and experienced workers to execute this dynamic.

The duration of immune memory is relevant for short and long-lived organisms with higher chances of recurrent exposures (KURTZ *et al*., 2005; GRAW *et al*., 2010). Considering that *Atta* colonies may live up to 15-25 years in nature (WEBER, 1972), they would benefit from long-term memory to elicit plastic responses during lifetime. We hypothesized that stronger actions occur due to the sum of individual experience to previous challenges, as observed in long-term memory for substrate choices in leaf-cutting ants (SAVERSCHEK *et al*., 2010; ARÊDES *et al*., 2022). Since workers will perform distinct

tasks accordingly to their age (SANTANA VIEIRA *et al*., 2010; LACERDA *et al*., 2020), this might cause a shift in the number of experienced workers available to respond in further exposures. Indeed, *A. sexdens* colonies had similar or higher hygiene after 30 days since the last exposure, losing such capacity after 60 days in homologous challenges (Figures 2a and 3a). The age polyethism of major and medium workers of *A. sexdens* change between the ninth week, for maintenance of the fungus garden, and the fourth week for foraging activities (LACERDA *et al*., 2020). In 60 days (eight and a half weeks), the task switch of experienced workers may have changed during this time, affecting the strength of the response. An alternative explanation could be a decrease in the average number of ants in the arenas. However, in both mycoantagonist and entomopathogen homologous assays the third exposure showed similar or above-average number of ants in comparison to previous ones (Figures 2c and 3c). Although this is the first study to evaluate the duration of plastic hygiene at the colony level, we still lack knowledge on how ants retain long-term information from past infections.

Specificity is another remarkable trait of immune memory. The traditional perspective is that immune memory is specific (KURTZ, 2005; PRADEU; PASQUIER, 2018). However, stronger responses may or may not come with specificity (SCHMID-HEMPEL; EBERT, 2003; KURTZ, 2004). A degree of specificity will be considered when faster and enhanced responses only occur to a previously encountered pathogen, as stated for immune priming (KURTZ, 2005; LITTLE; KRAAIJEVELD, 2004). Remarkably, our results indicate a similar phenomenon at the colony-level. *Atta sexdens* did not express stronger collective hygiene when confronted with a second distinct pathogen, regardless of the fungal species (Figures 2b and 3b). This highlights their capacity to discriminate between two distinct infection events, downregulating actions to a novel pathogen. Such effect was also reported for the invasive ant *Lasius neglectus* (KONRAD *et al*., 2018). In addition, it may suggest that enhanced hygiene was not induced by the exposure itself but by a prior infection to a particular fungus (discussed in GOES *et al*., 2022). Alternatively, the average number of ants in the arenas was not substantially reduced in the heterologous exposure (Figures 2c and 3c), thus not explaining the observed lower hygiene. Finally, the heterologous challenge did not seem to have influenced responses when we returned the same fungus exposed fourteen days ago (Figures 2b, and 3b). Thus, a novel infection might not interfere in optimal responses to previously seen pathogens (CONTRERAS-GARDUÑO *et al*., 2016).

Collateral damage (CREMER *et al*., 2019; PULL; MCMAHON, 2020) and costly allocation of resources (FRANK, 2000; SADD; SCHMID-HEMPEL, 2009b) are examples of social immunity tradeoffs for the colony's well-being (PULL; MCMAHON, 2020). Social immune behaviors can cause damage, since some antimicrobials may be toxic for the insect (TRAGUST *et al*., 2013a; PULL *et al*., 2018a) or actions not perfectly applied (TRAGUST *et al*., 2013b; PULL *et al*., 2018b). To cope with side effects, social insects have tolerance mechanisms to prevent or limit the negative impacts of infections or social immune responses (CREMER *et al*., 2019). As stated here with *A. sexdens*, triggering intense hygiene only to an experienced pathogen might play an important role to prevent unnecessary loss of fungus garden biomass by excessive fungus weeding (CURRIE and STUART, 2001) or overdose of antimicrobials (FERNÁNDEZ-MARÍN *et al*., 2009).

1.5 CONCLUSION

Both organisms and superorganisms must confront pathogens during the course of their life (PRADEU, 2013; PULL; MCMAHON, 2020). Defensive mechanisms evolved aside from molecular and cellular machinery, expressed as collective defenses to control multiple and diverse infections in organisms that live in groups (SIXT; CREMER, 2009; PULL; MCMAHON, 2020). By revealing similarities to the physiological immune system, social immunity became a research focus for potential evolutionary roots of immunological mechanisms. One of these is the immunological memory phenomenon at the group level. Despite many open questions highlighted here, this is the first study to reveal trends of a functional equivalent immune memory in *A. sexdens* laboratory colonies, based on their behavioral dynamics and specificity degree against multiple infections. Based on our findings, we encourage further investigation of immune memory traits in both fungus-farming and non-farming social insects. That would help clarifying in what extent immunological mechanisms evolved at the superorganismal level, and whether they influence the plasticity observed in social immunity.

1.6 FIGURES

Figure 1. The amount of cleaning responses, i.e., fungus grooming, allogrooming, and selfgrooming at the treatment arenas that received fungus treatment or sham solution (with) and the negative control arenas that did not receive inoculation (without). The number of cleaning for each treatment (median and quartiles 1 and 3), equals the sum of all responses given in all the exposures to that particular experiment. Cleaning responses occurred more often towards inoculated fungus fragments than in the fragment without inoculation. This pattern happened both in the (a) entomopathogens and (b) mycoantagonists assays. None of the comparisons was statistically significant. The dots above boxplots represent outliers.

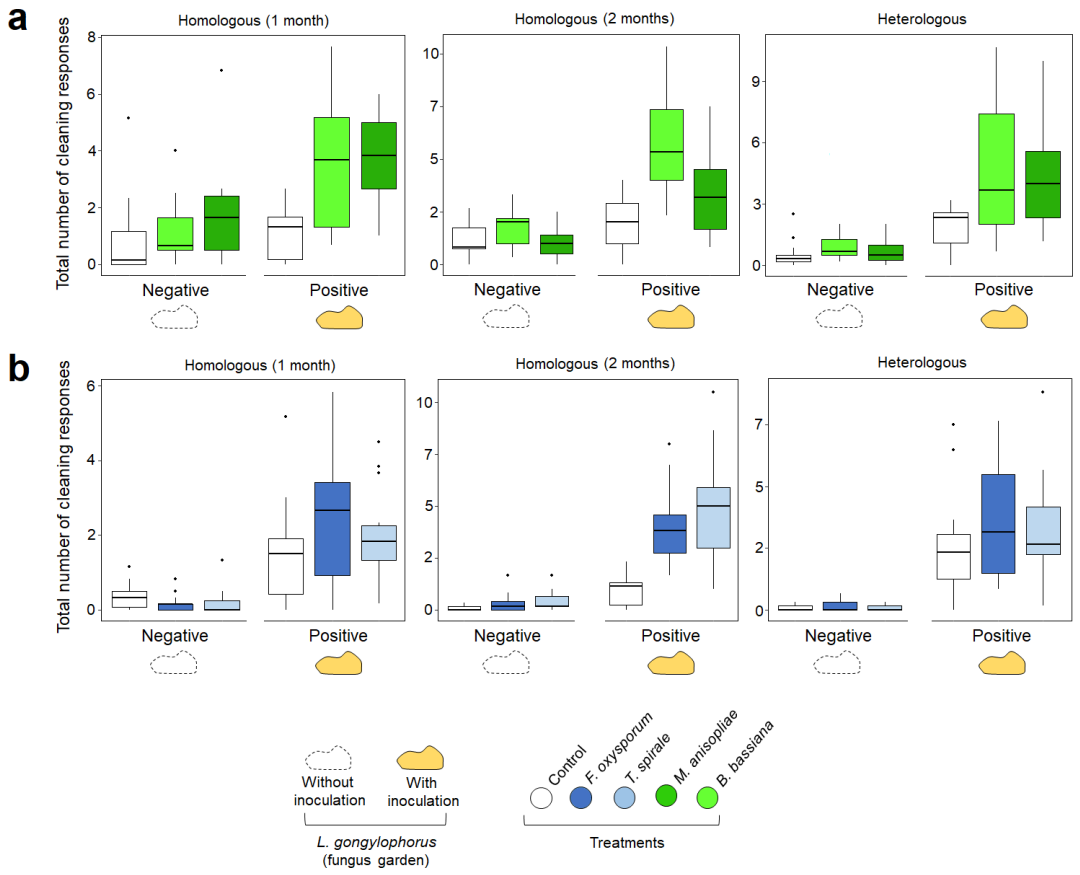


Figure 2. Number of cleaning responses, for each exposure, against the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* or the control solution. (a) Sanitization increased in secondary exposure of the same pathogen in the homologous challenges, but not statistically distinct from the first exposure. It was significantly higher in a third exposure after 30 days, but not after 60 days. (b) In contrast, colonies decreased significantly hygiene if they received a different fungus from the previous exposure in the heterologous challenge. (c) The heatmap indicates the average number of ants through exposures in their respective assays. Generally, colonies tended to increase or maintain the same average in the treatment arenas, for both homologous and heterologous assays. Numbers inside the boxes indicates the average value of the total number of ants. Colors from the heatmap represents average scale. The letter above plots indicates statistical differences (post-hoc at $\alpha = 0.05$). The abbreviation 'n.s' means that there was no statistical difference. The dots in boxplots represent outliers.

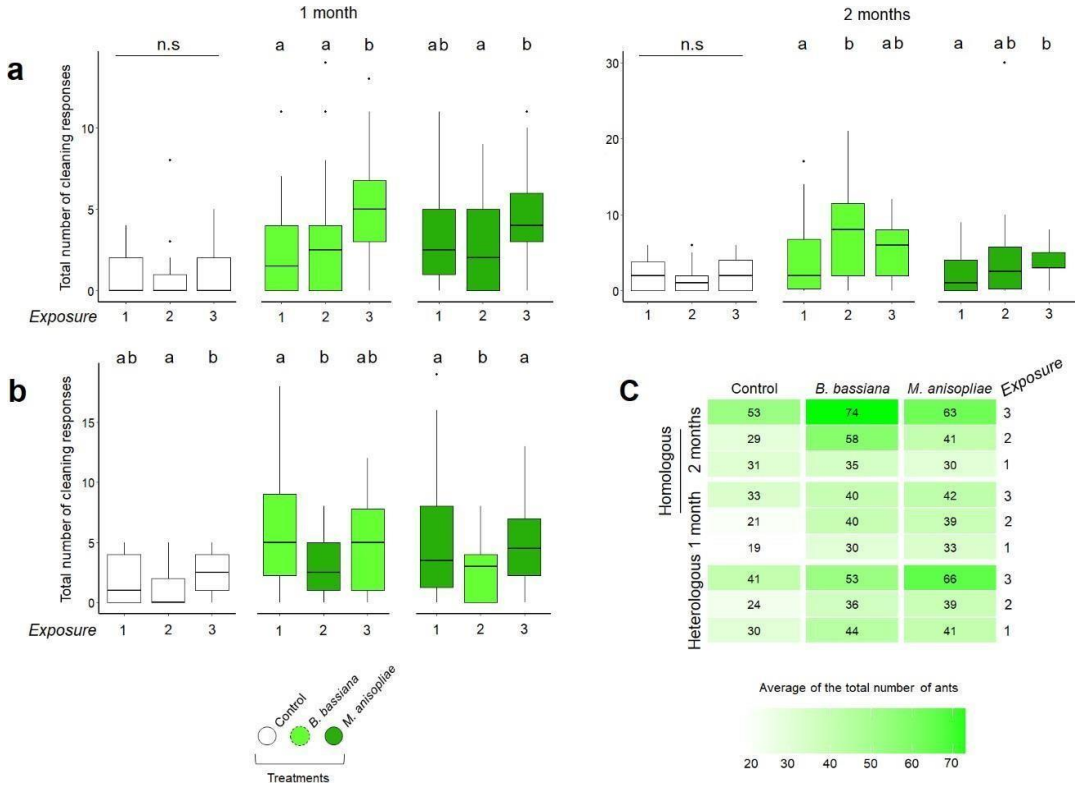
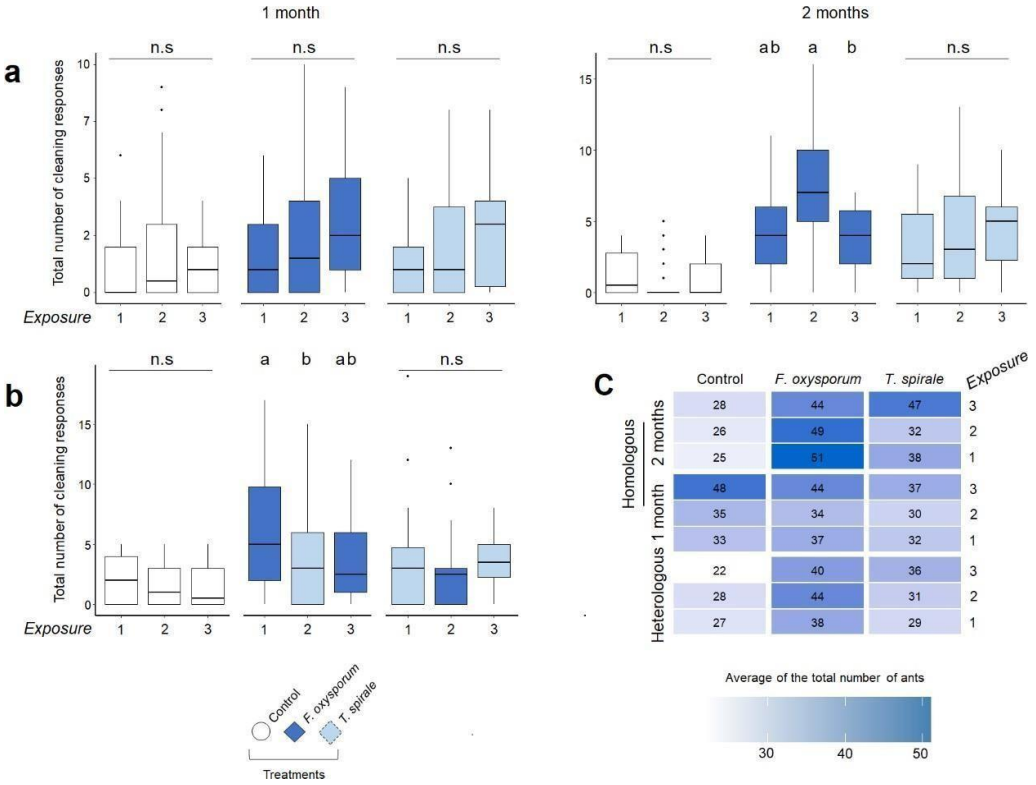


Figure 3. Number of cleaning responses, for each exposure, against the mycoantagonistic fungi *Fusarium oxysporum* and *Trichoderma spirale* or the control solution. (a) Sanitization showed a non-significant trend to increase in secondary exposures to the same pathogen in the homologous challenges, and slightly increased in the third exposure after 30 days and decreased after 60 days since the last exposure. (b) Colonies decreased hygiene if they received a different fungus from the previous exposure in the heterologous challenge, but significantly only in the *F. oxysporum* group. (c) The heatmap indicates the average number of ants through exposures in their respective assays. Generally, colonies tended to increase or maintain the same average in the treatment arenas, for both homologous and heterologous assays. Numbers inside the boxes indicates the average value of the total number of ants. Colors from the heatmap represents average scale. The abbreviation 'n.s' means that there was no statistical difference. The dots in boxplots represent outliers.



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2 SUPPLEMENTARY MATERIAL

Figure S1. Experimental design of the homologous and heterologous challenges. The bioassays were divided into two major groups according to pathogen lifestyle: entomopathogens (*Beauveria bassiana* and *Metarhizium anisopliae*) and mycoantagonists (*Fusarium oxysporum* and *Trichoderma spirale*). (a) To evaluate the strength and speed of sanitization to a given fungus, homologous challenges were introduced to colonies containing two exposures with a 7-day interval, and a third delayed challenge. The delayed challenge divided the homologous bioassays into two further groups to evaluate how long the collective immune memory traits last in colonies: one set received the third challenge within a 30-day interval after the second exposure and the second set received it within a 60-day interval. (b) To pursue the degree of specificity in collective immune memory, heterologous challenges were applied in colonies within 7-day intervals. Ants were considered to be naïve in challenges where they had never entered in contact with the pathogen, hence, predicted to trigger a lower sanitization response.

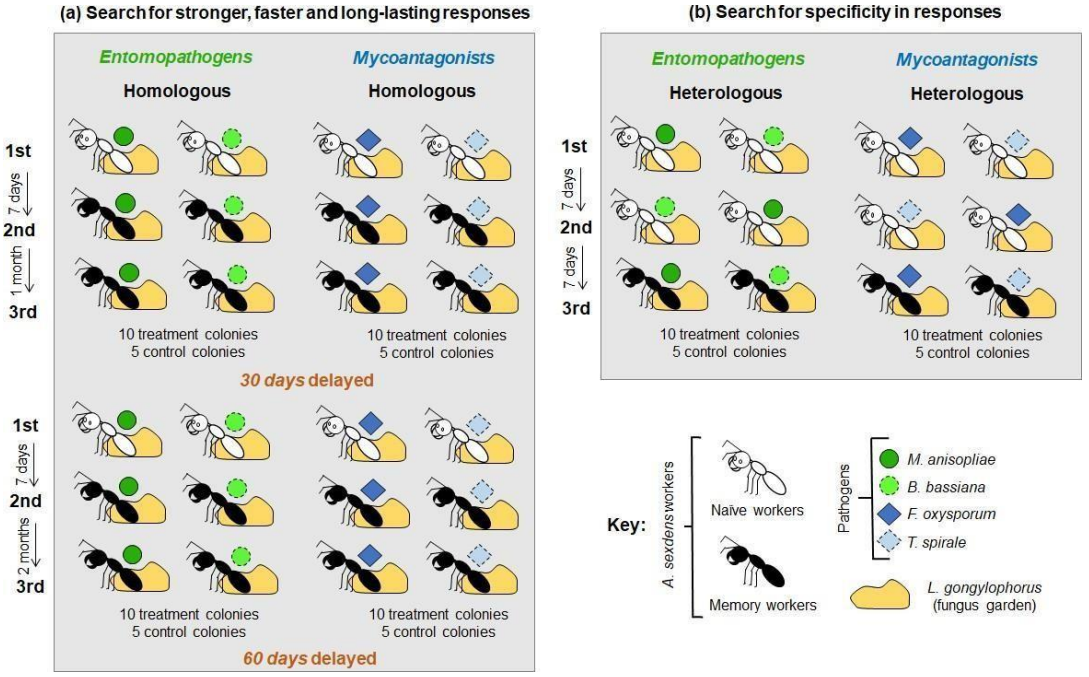

Figure S2. The amount of fungus rescue responses at the treatment arenas that received fungus treatment or sham solution (positive) and the negative control arenas that did not receive inoculation (negative). The number of fungus rescue for each treatment (median and quartiles 1 and 3), equals the sum of all rescue responses given in all the exposures to that particular experiment. Fungus rescue occurred often towards non-inoculated fungus fragments than in those that received inoculation. This pattern happened either in the (a) entomopathogens or (b) mycoantagonists assays. The dots above boxplots represent outliers.

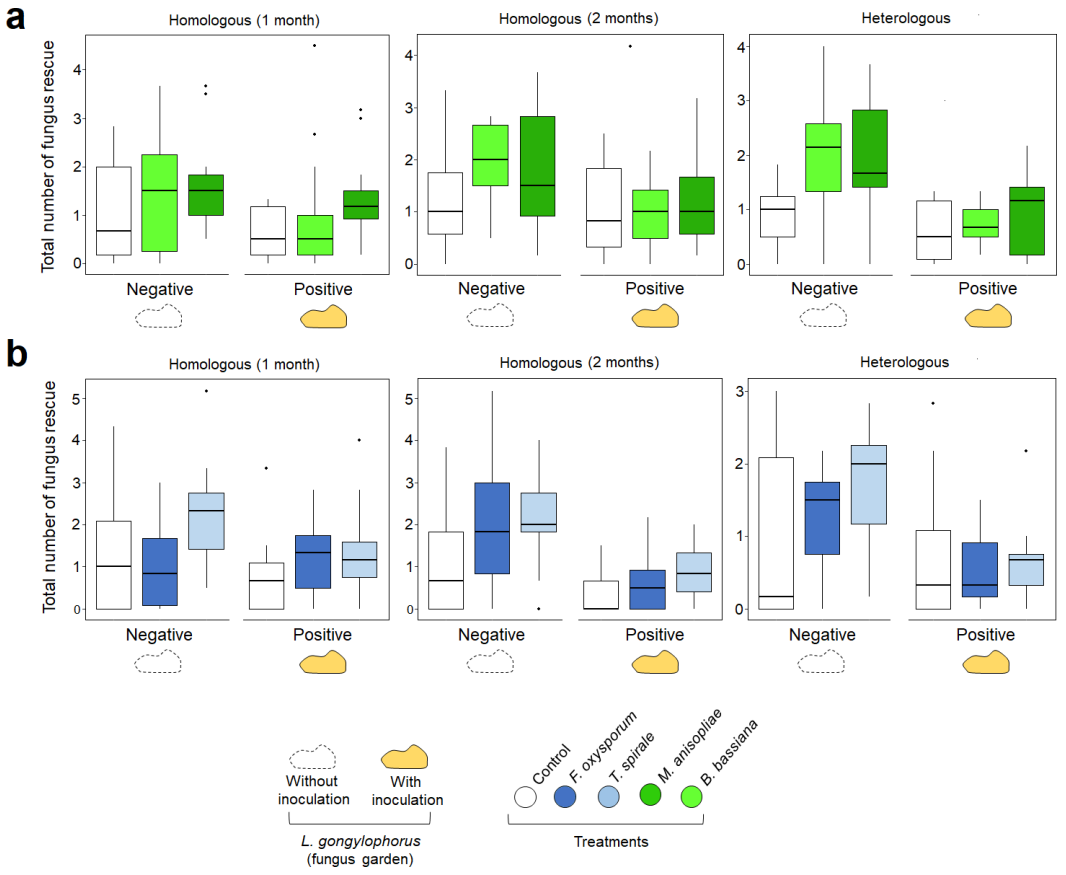

Figure S3. Number of cleaning responses at the control negative arenas, for each exposure, against the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* or the control solution. Sanitization seemed not to follow a trend to increase in secondary exposures to the same pathogen either in the (a) homologous challenges and (b) heterologous challenges. (c) Through exposures, the average number of ants tended to increase or maintain the same, for both homologous and heterologous assays. The abbreviation 'n.s' means that there was no statistical difference. The dots above or below boxplots represent outliers.

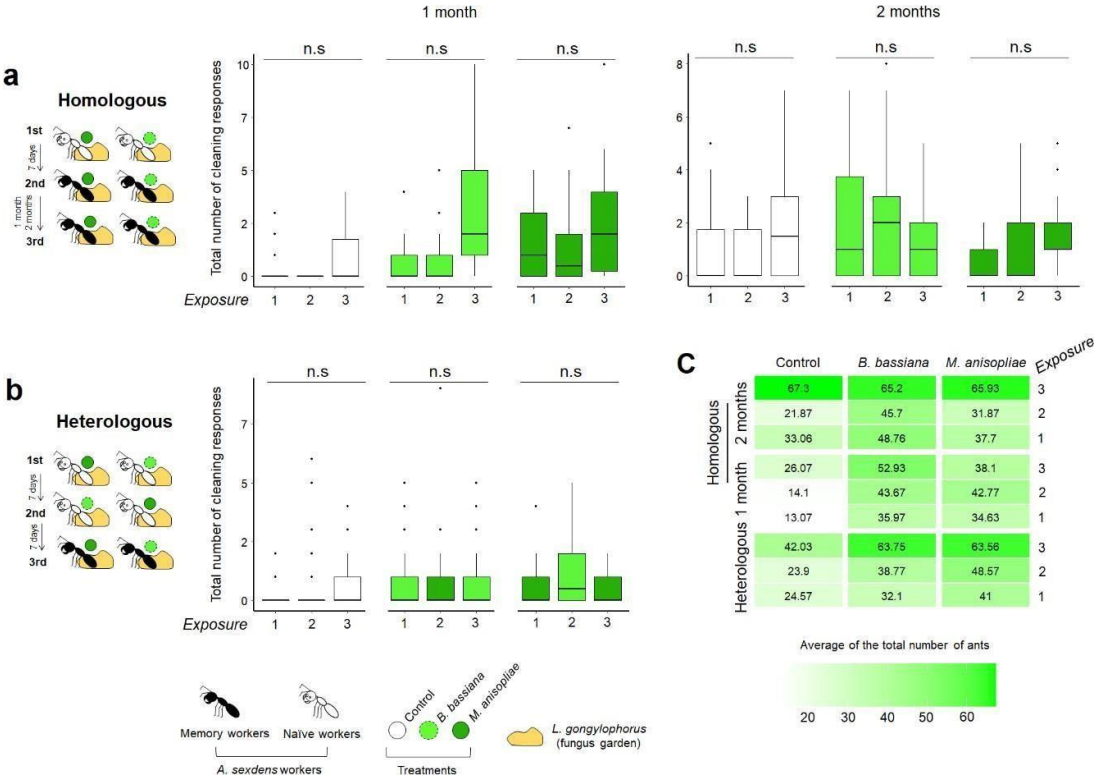

Figure S4. Number of cleaning responses at the control negative arenas, for each exposure, against the mycoantagonistic fungi *Fusarium oxysporum* and *Trichoderma spirale* or the control solution. Sanitization did not show a tendency to increase in secondary exposures to the same pathogen either in the (a) homologous challenges and (b) heterologous challenges. (c) Through exposures, the average number of ants tended to increase or maintain the same, for both homologous and heterologous assays. The abbreviation 'n.s' means that there was no statistical difference. The dots above boxplots represent outliers.

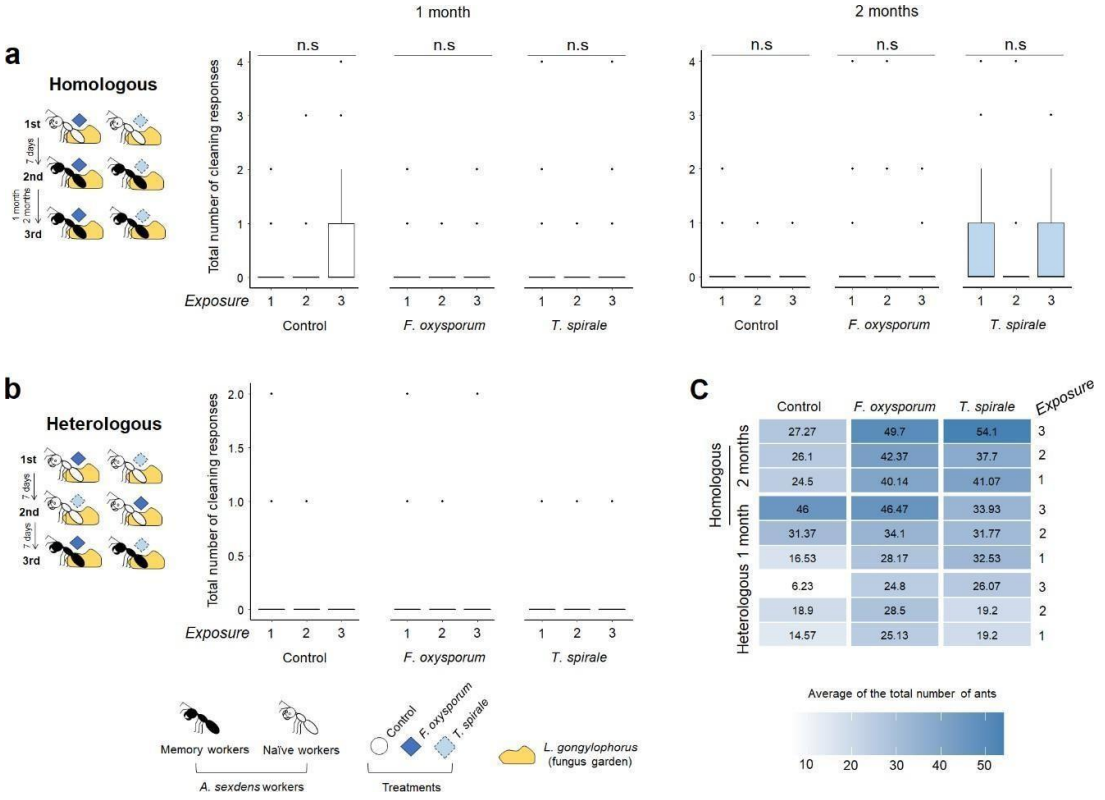

Figure S5. Behavioral responses over time in entomopathogenic assays. The graphics represent the mean ± SE proportion of the total number of behaviors, i.e., fungus grooming, rescue, and selfgrooming for each treatment. The line colors represent distinct exposures. Overall, the third exposure in the 30-day homologous elicited more behaviors in the first 15 minutes towards *Metarhizium anisopliae* and *Beauveria bassiana*, though not statically significant in comparison to others. In the 60-day homologous, the third exposure had a lower response over time in comparison to the previous one. In the heterologous experiments, the second and non-specific challenge elicited a lower number of behaviors, but with the exception to *B. bassiana.*

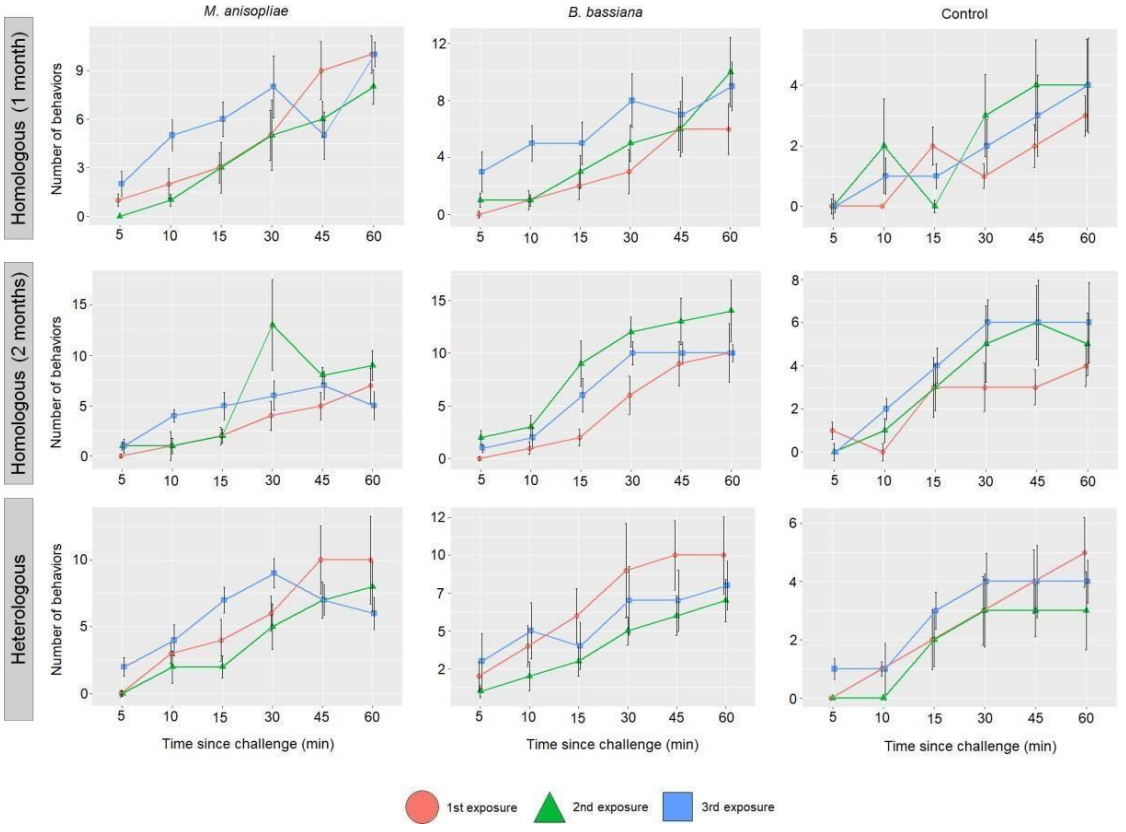

Figure S6. Behavioral responses over time in mycoantagonist assays. The graphics represent the mean ± SE proportion of the total number of behaviors, i.e., fungus grooming, rescue, and selfgrooming against each treatment. The line colors represent distinct exposures. Overall, the third exposure in the 30-day homologous elicited more behaviors in the first 15 minutes towards *Fusarium oxysporum* and *Trichoderma spirale*. There are no clear patterns of faster responses in the 60-day homologous experiment, with the exception to the second exposure towards *F. oxysporum.* In the heterologous experiments, the second and non-specific challenge tended to be similar in the number of behaviors applied in comparison to the others challenges.