



Aversive learning as a behavioural mechanism of plant selection in the leaf-cutting ant *Atta sexdens* (Hymenoptera: Formicidae)

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Abstract

The survival of leaf-cutting ants depends on their ability to select suitable resources for their fungal symbiont. Plant selection may be influenced by innate or acquired preferences. We investigated the learning processes involved in resource selection in *Atta sexdens* (LINNAEUS, 1758) and the role of symbiotic fungi. Subcolonies regularly fed leaves of one plant species (conditioning plant) were later tested for their preference between leaves of the conditioning plant and leaves of other plant species. In a second experiment, subcolonies fed with a plant species impregnated with fungicide (aversive conditioning) were subjected to a preference test between the conditioning plant (treatment) and another species (control), both without fungicide. In a third experiment, subcolonies subjected to aversive conditioning were allowed to choose between leaves treated or untreated with fungicide. In a fourth experiment, aversively conditioned subcolonies were allowed to choose between paper fragments treated with odours of aversive or nonaversive plants. In a fifth experiment, conditioned subcolonies were allowed to choose between leaves from a different plant impregnated or not with odours from aversive plants. In all five experiments, the number of transported fragments was statistically compared between treatment and control subcolonies. In a sixth experiment, fungus garden extracts of subcolonies treated or untreated with fungicide were analysed by gas chromatography coupled to mass spectrometry. Ants did not exhibit appetitive conditioning to suitable plants, but harmful resources induced delayed rejection and decreased preference, confirming the occurrence of aversive conditioning. Safe resources impregnated with odours from harmful resources were rejected by the ants, indicating that olfactory learning participates in the plant selection process. Harmful resources induced changes in the chemical profile of symbiotic fungi and caused a delayed rejection behaviour in worker ants. Odours of harmful plants are linked to food rejection behaviour through associative learning and inhibit future collection of these plants. Plant selection in *A. sexdens* seems to be based on recognition of resources that are harmful to symbiotic fungi rather than on recognition of adequate resources.

Key words: Olfactory conditioning, chemical communication, fungal garden, leaf-cutter ants, mutualism.

Received 12 August 2021; revision received 31 January 2022; accepted 10 February 2022

Subject Editor: Ivette Perfecto

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Introduction

Animals locate and exploit resources necessary for their survival through a process known as foraging (BREED & MOORE 2016). Foraging is collective when several individuals cooperate to find and transport food (AZEVEDO 2009, DUSSUTOUR & al. 2009). Cooperation occurs, for

instance, when nestmates share information about resource location; this behaviour is observed in social insects such as honey bees (VON FRISCH 1967, LALOI & al. 2000), termites (GAZAL & al. 2012, 2014), and ants (WILSON 1962, FARJI-BRENER & al. 2010, LANAN 2014, VIANA-BAILEZ

& ENDRINGER 2016). Among social insects, foraging involves exploring the environment, memorising routes, and identifying and transporting resources to the nest (HÖLLDOBLER & WILSON 1990, RIBEIRO & MARINHO 2011, NOMINATO 2015).

Most social insects live in perennial societies and face important environmental changes, to which they adapt owing to their plasticity in a broad range of behaviours (DUKAS 2004). The ability to exploit new food sources is probably one of the main adaptive responses to environmental variability (RAINE & CHITTKA 2008). The most widespread learning mechanism in the animal world is associative learning (DUKAS 2004), which allows animals to predict the occurrence of positive or negative events through the perception of signs that frequently occur spatially or temporally close to such events (SCHLEYER & al. 2018).

For associative learning to occur, the signs to be learned must be associated with events that either threaten (punishment) or promote (reward) their survival (ALCOCK 2011, PEREZ & al. 2016). Associative learning is classified as classical or Pavlovian when an innocuous stimulus (conditioned stimulus) starts to provoke a specific behavioural response in animals (conditioned response), after that same neutral stimulus has been previously paired (one or more times) with this specific behavior (PHAM-DELEGUE & al. 1993, DESMEDT & al. 2017). Another common associative learning process is operant or instrumental conditioning, whereby animal-specific behaviours are stimulated or inhibited via rewards (appetitive conditioning) or punishments (punitive conditioning) (SKINNER 1948). The probability of a given behavioural act increases with the number of times the act is succeeded by positive rewards and decreases with the number of negative rewards or punishments (PAPAJ & LEWIS 1993, PHAM-DELEGUE & al. 1993, SOUZA 2012).

Animals with a generalist diet that learn new behavioural acts through appetitive operant conditioning commonly exploit food resources more efficiently (ABRAMSON & al. 2016). Operant conditioning allows animals to predict the negative or positive consequences of behavioural acts (BREMBS 2003, SOUZA 2012). Most insects collect food resources for their own consumption, and the quality of the resource will define the foraging experience as positive or negative (HEIDECKER & LEUTHOLD 1984, CAMAZINE 1993).

Leaf-cutting ants have a generalist diet composed of resources located through the perception of olfactory signals emitted by plants (HÖLLDOBLER & WILSON 1990, ROCES 1990, RIBEIRO & MARINHO 2011), memorized from previous experiences (ROCES 1994). Leaf-cutting ant foragers eventually feed on sap during leaf cutting (LITTLEDYKE & CHERRETT 1976, QUINLAN & CHERRETT 1979, SILVA & al. 2003). Thus, nutrients and secondary metabolites present in leaves (HUBBELL & al. 1984, HOWARD 1987, 1988) can represent rewards or punishments, and the collection experience can be characterized as positive or negative at the individual level. However, the resource collected by foragers does not represent a conventional negative or positive reward in itself, as it is not the insect but the

symbiotic fungus that consumes the resource. A given resource may be suitable to foragers but harmful to the fungus (HERZ & al. 2008); such a collection experience must necessarily be characterized as negative for the ant colony. Some studies suggested that chemicals produced by damaged fungal cells can be used by ants as clues to assess the state of the fungus garden (NORTH & al. 1999, HERZ & al. 2008, GOES & al. 2020).

The introduction of a harmful resource into the nest may elicit a delayed rejection reaction (HERZ & al. 2008, ARENAS & ROCES 2016, 2017, GREEN & KOOLJ 2018). However, the physiological and behavioural bases of this possible mechanism have not yet been elucidated (HERZ & al. 2008, GREEN & KOOLJ 2018, GOES & al. 2020). In this study, we aimed to investigate whether learning processes participate in the resource selection mechanism of the leaf-cutting ant *Atta sexdens* (LINNAEUS, 1758) and understand the possible role of symbiotic fungi in this process.

Material and methods

Ant subcolonies

Twenty-four queenless subcolonies were established in 500 mL plastic pots, each comprising 800 workers, 200 larvae and pupae, and 50 g of fungus garden. Insects and fungus garden material were obtained from four 4-year old colonies of *Atta sexdens* (identified using FERNÁNDEZ & al. 2015) maintained under laboratory conditions.

Subcolonies were placed in plastic trays (46 × 28 × 12 cm) that served as a foraging arena and were kept in a room at 25 °C (with variations of ± 2 °C) and 80% relative humidity (± 10%). A 50 mL cup placed in each tray was used by ants as a dump chamber. The foraging arena was supplied daily with leaves of *Acalypha wilkesiana* (MÜLLER ARGOVIENSIS, 1866) (used as regular food throughout).

The subcolonies were used in experiments after their foraging behaviour became similar to that of queenright colonies, which occurred about 48 h after their establishment (LOECK & al. 1994, JACCOUD & al. 1999).

Preliminary test: Spontaneous preference

On the sixth day after preparation of subcolonies, the food (*A. wilkesiana* leaves) of each one of the 24 subcolonies was replaced by two Petri dishes (diameter = 3.5 cm) containing each 20 leaf fragments (diameter = 5 mm). One Petri dish contained leaves of *Rosa alba* (LINNAEUS, 1753) and the other leaves of *Ligustrum japonicum* (THUNBERG, 1780). Fragments transported from each plate were counted 2 h later or when all fragments had been collected from one of the plates. The following days, *A. wilkesiana* leaves were placed in the foraging arena of all colonies as regular food.

Experiment 1: appetitive conditioning

Conditioning phase: Seven days after the preliminary test, *A. wilkesiana* leaves were removed from the trays of the 24 subcolonies, and half of the subcolonies

were fed *Rosa alba* leaves (rose group, n = 12) and the other half *Ligustrum japonicum* leaves (privet group, n = 12) for five days.

Preference test: At the end of the conditioning phase, the food of subcolonies was replaced by two Petri dishes. One of the plates contained 20 leaf fragments of *R. alba* and the other 20 leaf fragments of *L. japonicum*. The number of fragments transported from each plate was recorded for 2 h. The following day, regular food was returned to the subcolonies.

Experiment 2: aversive conditioning

Conditioning phase: Seven days after the completion of Experiment 1, *Acalypha wilkesiana* leaves were removed from the arena of both groups (rose and privet) and replaced by a Petri dish (diameter = 9 cm). The Petri dish contained 40 leaf fragments of *Rosa alba* leaves (rose group) or *Ligustrum japonicum* (privet group) impregnated with a 0.02% aqueous solution of cycloheximide (Sigma®, St. Louis, USA) (1 µL / fragment). Thus, the rose group was exposed to cycloheximide-treated *R. alba* leaves, whereas the privet group was exposed to cycloheximide-treated *L. japonicum* leaves.

In this experiment, the fungicide cycloheximide represents a stimulus that, in low concentrations, is innocuous and undetectable to ants but lethal to fungi and harmful to the colony (RIDLEY & al. 1996, HERZ & al. 2008, SOUSA & al. 2018).

Preference test: At 24 h after fungicide treatment, a Petri dish containing 20 fragments (diameter = 5 mm) of *Rosa alba* leaves and another containing 20 fragments of *Ligustrum japonicum* leaves, both without fungicide, were placed in the centre of the foraging arena. Fragments transported into the nest as well as those later discarded from the nest were counted after 2 h. The experimental method was based on a study on *Acromyrmex lundii* (see HERZ & al. 2008).

Experiment 3: fungicide perception

Seven days after being subjected to aversive conditioning, the ants received, instead of regular food, one plate containing 20 leaf fragments impregnated with 0.02% cycloheximide solution together with a second plate containing 20 leaf fragments impregnated with distilled water (1 µL / fragment). The rose group was exposed to fragments of *Rosa alba* and the privet group to fragments of *Ligustrum japonicum*. Transported and discarded fragments were quantified after 2 h.

Experiment 4: signals used for recognition of unsuitable resources

Preparation of leaf extracts: Leaves of *Rosa alba* and *Ligustrum japonicum* (5 g each) were placed separately in a beaker containing 60 mL of distilled water, stirred with a glass rod for 5 min, and left to stand for 24 h at 10 °C in the dark. Macerates were filtered through filter paper, and the resulting aqueous extracts were used immediately after preparation.

Rosa alba and *Ligustrum japonicum* leaf extracts were also prepared using pentane (Tedia®, Fairfield, USA) as solvent.

Preference test 1: At 14 days after the aversive conditioning experiment, the conventional food of subcolonies was replaced by two Petri dishes (diameter = 3.5 cm) containing 20 pieces of filter paper (20 mm² each). Fragments from one plate were impregnated with *Rosa alba* aqueous extract and those from the other with *Ligustrum japonicum* aqueous extract (1 µL / fragment). The number of fragments transported by worker ants was quantified 2 h later.

Preference test 2: An almost identical preference test was performed 21 days after the completion of the aversive conditioning experiment. The difference was that in this test ants were presented with pieces of filter paper impregnated with pentane extracts instead of aqueous extracts.

Experiment 5: preference changes induced by aversive resource odours

At 28 days after aversive conditioning, the regular food resource of subcolonies was replaced by two Petri dishes (diameter = 3.5 cm) containing 20 leaf fragments from the species to which the group had not been exposed in the aversive conditioning phase. In other words, the rose group (n = 12) was presented with *Ligustrum japonicum* leaves and the privet group with *Rosa alba* leaves. Fragments from one plate were impregnated with an aqueous extract (1 µL / fragment) of the plant resource used for aversive conditioning (aversive odour treatment), and those from the other plate were impregnated with distilled water (odour-free control). The number of fragments transported from each plate to the fungus garden was quantified after 2 h.

Experiment 6: chemical analysis of fungus garden odours

Fungus garden extracts: Six subcolonies had their regular food removed and were fed 1 g of *Rosa alba* leaves per day for five days. On the sixth day, three samples of fungus garden (circa 200 mg) were collected from each subcolony, placed separately in a vial containing 3 mL of hexane (Tedia®), and kept at rest for 48 h at 4 °C. On the same day of collection, half of the subcolonies (n = 3) were fed 40 *R. alba* leaf fragments treated with an aqueous solution of cycloheximide, and the other half (n = 3) were fed fragments impregnated with distilled water (1 µL / fragment) (control). After 48 h, fungus garden samples were collected once again and processed as described above.

Samples were incubated in an ultrasonic bath (Kondortech®, São Carlos, Brazil) for 10 min to facilitate extraction of chemical compounds. Supernatants from triplicate samples were transferred to a single vial and evaporated under argon gas. Then, 90 µL of hexane and 10 µL of a standard solution of eicosane diluted to 1100 ng / µL in hexane were added to the samples.

Fungus garden volatiles: A plastic bottle (diameter = 5 cm) containing 1 g of Amberlite® (St. Louis, USA) XAD4 resin and a glass tube containing 150 mg of Tenax® (Bellefonte, USA) trapping agent (TA) resin (Supelco®, Bellefonte, USA) were placed in the fungus garden chamber of each subcolony. Amberlite® bottles were collected after 48 h and their contents transferred to a test tube containing 5 mL of ethyl acetate (Tedia®). Samples were ultrasonicated (Kondortech®) for 10 min, the supernatant collected into a new tube, and the liquid fraction evaporated to 500 µL under argon gas.

Tenax® TA samples were collected after 48 h and analysed for volatile compounds by thermal desorption.

Chromatographic analysis: An aliquot (1 µL) of fungal extract from each subcolony was injected into a Shimadzu® (Tokyo, Japan) GC-2010 gas chromatograph coupled to a Shimadzu® GCMS-QP2010 plus mass spectrometer. Chromatographic conditions were as follows: column pressure of 100 kPa, gas flow rate of 1.5 mL / min, initial column temperature of 80 °C maintained for 1 min, final column temperature of 280 °C maintained for 10 min, injector temperature of 280 °C, and detector temperature of 280 °C.

Tenax® TA tubes were connected directly to a Shimadzu® GC-2010 gas chromatograph coupled to a Shimadzu® GCMS-QP2010 Plus mass spectrometer and a Shimadzu® TD-20 thermal desorption system. Amberlite extracts (1 µL) were also analysed on this system. Operating conditions were column pressure of 95 kPa, gas flow rate of 1.62 mL / min, initial column temperature of 48 °C for 5 min, final column temperature of 230 °C for 5 min, injector temperature of 200 °C, and detector temperature of 300 °C.

Separation was achieved using a fused-silica DB-5 column (J&W Scientific, Folsom, USA) (30 m × 0.250 mm × 0.25 µm). Helium was used as carrier gas. Ionisation was achieved by electron impact at 70 eV. Data were recorded as total ion chromatograms.

Compounds were identified by comparing their mass spectra with the National Institute of Standards and Technology library database (GCMSsolution version 4.20). The relative abundance of each compound was quantified from its peak area.

Statistical analysis

Data from Experiments 1 to 5 (number of transported and discarded leaf fragments) were tested for homogeneity of variance and normality (Shapiro-Wilk test). As normality and homogeneity of variance assumptions were not met, treatment and control values were compared by Kruskal-Wallis tests (SigmaPlot version 12.5).

Chromatographic peak area data from Experiment 6 were subjected to arcsine square root transformation followed by discriminant analysis to compare the fungal chemical composition of fungicide-treated and untreated subcolonies (Statistica version 12.0). Compounds identified in less than 70% of the samples or that represented less than 0.3% of total compounds were excluded from analysis.

Permutational multivariate analysis of variance (PERMANOVA) was performed to identify differences in fungal chemical profiles before and after cycloheximide treatment. A total of 9999 permutations were used.

Results

Preliminary preference test

The number of *Rosa alba* (72 ± 6.7%) and *Ligustrum japonicum* (90 ± 2.5%) fragments transported by workers did not differ significantly (Kruskal-Wallis test: $H_1 = 1.7$, $P = 0.189$).

Experiment 1: appetite conditioning

During the conditioning phase, both food resources were regularly collected by ants. During the test phase, when new and previously experienced resources were simultaneously offered to ants, no preference was detected. Subcolonies previously fed with *Rosa alba* collected 78 ± 9.1% of the *R. alba* fragments and 82 ± 7.9% of the *Ligustrum japonicum* fragments ($H_1 = 0.3$, $P = 0.579$). Subcolonies previously fed with *L. japonicum* collected 81 ± 7.3% of the *R. alba* fragments and 82 ± 8.1% of the *L. japonicum* fragments ($H_1 = 0.2$, $P = 0.643$).

Experiment 2: aversive conditioning

In the conditioning phase, subcolonies of both groups transported all of the plant fragments with fungicide in less than 30 min, incorporated the material into the fungus garden, and showed no signs of rejection within the first 24 h.

In the preference test, subcolonies of the rose group collected fewer fragments of *Rosa alba* than of *Ligustrum japonicum* (40 vs 87%, $H_1 = 10.5$, $P < 0.001$, Fig. 1), and 42% of the collected *R. alba* fragments were rejected after 2 h, whereas *L. japonicum* fragments were not rejected ($H_1 = 9.1$, $P = 0.003$). Privet group subcolonies foraged only 3% of the *L. japonicum* fragments and 100% of the *R. alba* fragments ($H_1 = 20.5$, $P < 0.001$, Fig. 1). *Ligustrum japonicum* fragments were rejected in their entirety within

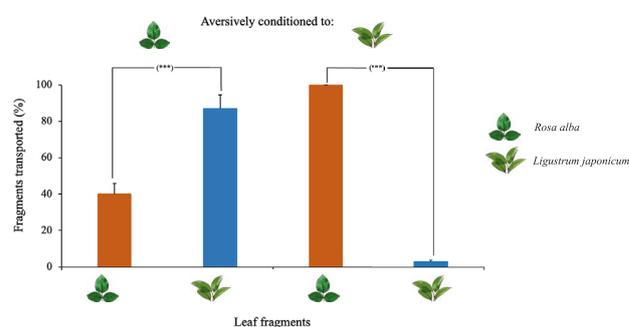


Fig. 1: Preference test after aversive conditioning. Percentage of *Rosa alba* (n = 20) and *Ligustrum japonicum* (n = 20) leaf fragments transported to the nest by *Atta sexdens* workers (n = 12) at 24 h after being aversively conditioned to leaves of *R. alba* (left) or *L. japonicum* (right) impregnated with the fungicide cycloheximide. *** $P < 0.001$ (Kruskal-Wallis test).

2 h, but *R. alba* fragments were not rejected ($H_1 = 9.5$, $P = 0.002$).

Experiment 3: fungicide perception

Subcolonies aversively conditioned to fungicide-treated leaves did not discriminate between fungicide-treated and fungicide-free leaves.

Rose group subcolonies foraged $42 \pm 9.2\%$ of the contaminated *Rosa alba* fragments and $27 \pm 7.9\%$ of the non-contaminated fragments (treated with distilled water) ($H_1 = 1.5$, $P = 0.223$), and privet group subcolonies foraged $37 \pm 9.2\%$ of the contaminated *Ligustrum japonicum* fragments and $27 \pm 6.9\%$ of the noncontaminated fragments ($H_1 = 1.0$, $P = 0.322$). However, rose group subcolonies rejected $33 \pm 6.9\%$ of fungicide-treated fragments and $18 \pm 2.7\%$ of fungicide-free fragments within 2 h of transportation ($H_1 = 1.2$, $P = 0.271$). Similarly, privet subcolonies rejected $38 \pm 7.3\%$ of the contaminated fragments and $37 \pm 4.2\%$ of the noncontaminated fragments ($H_1 = 0.05$, $P = 0.831$).

Experiment 4: signals used for recognition of unsuitable resources

Perception test 1: Subcolonies aversively conditioned to fungicide-treated *Rosa alba* leaves collected fewer paper fragments impregnated with *R. alba* aqueous extract than paper fragments impregnated with *Ligustrum japonicum* aqueous extract ($H_1 = 15.1$, $P < 0.001$, Fig. 2). Likewise, subcolonies conditioned against *L. japonicum* collected fewer fragments impregnated with *L. japonicum* extract ($H_1 = 10.7$, $P = 0.001$, Fig. 2).

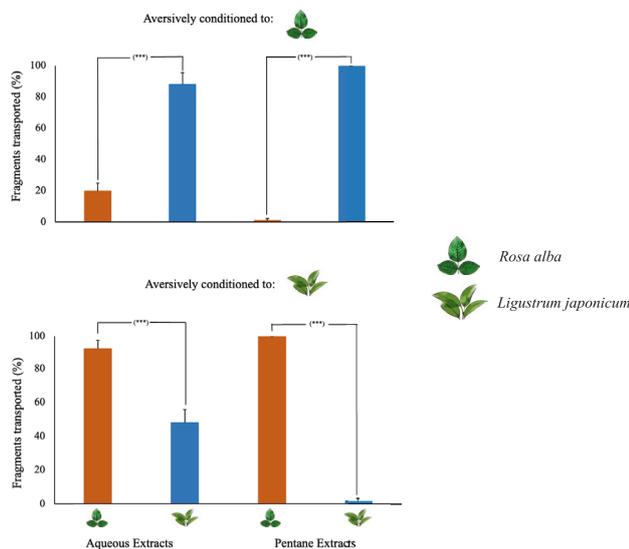


Fig. 2: Plant odour discrimination after aversive conditioning. Percentage of filter paper fragments impregnated with extracts of *Rosa alba* ($n = 20$) or *Ligustrum japonicum* ($n = 20$) transported to the nest ($n = 12$) by *Atta sexdens* workers previously fed a diet of *R. alba* (above) or *L. japonicum* (below) leaves treated with the fungicide cycloheximide. Left, aqueous extracts; right, pentane extracts. *** $P < 0.001$ (Kruskal-Wallis test).

Perception test 2: When using pentane extracts, we observed that rose subcolonies collected fewer paper fragments impregnated with *Rosa alba* odours ($H_1 = 22.2$, $P < 0.001$, Fig. 2) and privet subcolonies collected fewer fragments impregnated with *Ligustrum japonicum* odours ($H_1 = 21.5$, $P < 0.001$, Fig. 2).

Experiment 5: preference changes induced by aversive resource odours

Subcolonies of the rose group foraged fewer *Ligustrum japonicum* fragments impregnated with *Rosa alba* odours than fragments treated with water ($H_1 = 19.7$, $P < 0.001$, Fig. 3). Similarly, privet group subcolonies collected fewer *R. alba* fragments impregnated with *L. japonicum* odours than fragments treated with distilled water ($H_1 = 16.1$, $P < 0.001$, Fig. 3).

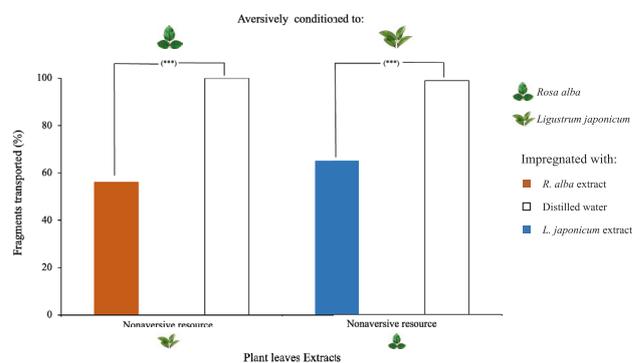


Fig. 3: Preference change induced by aversive resource odours. Percentage of nonaversive leaf fragments ($n = 20$) impregnated with odour extracts of aversive resources or with distilled water transported by *Atta sexdens* subcolonies aversively conditioned to *Rosa alba* (left) ($n = 12$) or *Ligustrum japonicum* (right) ($n = 12$). *** $P < 0.001$ (Kruskal-Wallis test).

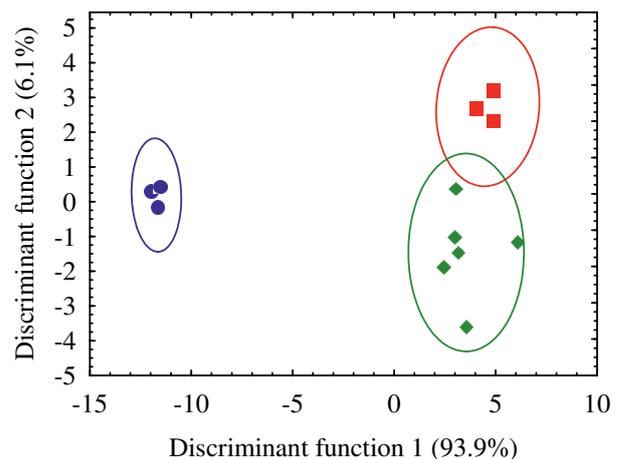


Fig. 4: Discriminant analysis of the chemical profile of fungus gardens of *Atta sexdens* subcolonies. \blacklozenge , fed for five days with *Rosa alba* ($n = 6$); \bullet , fed for five days with *R. alba* and two days with *R. alba* + cycloheximide (treatment) ($n = 3$); and \blacksquare , fed for five days with *R. alba* and two days with *R. alba* + distilled water (control) ($n = 3$). Ellipses represent confidence intervals ($P < 0.05$).

Experiment 6: chemical analysis of fungus garden odours and composition

Discriminant analysis separated fungus garden chemical profiles into three groups. The chemical profile of fungus gardens at 48 h after fungicide treatment differed significantly from that obtained before fungicide treatment and from that of the control ($F_{12,8} = 11.0$, $P < 0.001$, Fig. 4). Quantitative data of compounds identified after solvent extraction are shown in Table S1 (see Appendix, as digital supplementary material to this article, at the journal's web pages).

Six compounds were accountable for the separation of groups: n-hexadecanoic acid, 9,12-octadecadienoic acid, triacontanal, heneicosane, 8,12-dimethyldotriacontane, and 2,6,10-trimethylpentadecane (Fig. 4). Fungus gardens decreased concentrations of n-hexadecanoic acid (31.16%) and 9,12-octadecadienoic acid (24.65%) as well as increased concentrations of nonacosane (38.89%) and hentriacontane (33.65%) after fungicide exposure.

Fungus garden volatiles captured with both types of resins did not differ quantitatively or qualitatively between treated and untreated subcolonies (Tenax TA®: pseudo-F = 0.4248, $P = 0.5969$; Amberlite XAD4: pseudo-F = 0.1181, $P = 0.5061$).

Discussion

Leaf-cutting ants may show preferences for plant species or plant parts that favour fungus garden development (CHERRETT 1972, HUBBELL & WIEMER 1983, HUBBELL & al. 1984, HOWARD 1987, 1988). The preliminary experiment demonstrated that *Atta sexdens* workers show no innate preference for *Rosa alba* or *Ligustrum japonicum* leaves. These plant species are often used to maintain laboratory nests, confirming their lack of toxicity to ants and suitability as fungus garden substrates (MARINHO & al. 2005, CAMARGO & al. 2008, HERZ & al. 2008, NAGAMOTO & al. 2009, TEREZAN & al. 2010, TATAGIBA-ARAÚJO & al. 2012).

The first experiment showed that previous foraging experiences with a given plant do not induce a preference for the given resource in a choice situation with another plant species. Subcolonies of *Atta sexdens* collected similar amounts of previously known and new resources. This suggests that learning processes do not take place when leaf-cutting ants forage suitable plant species.

However, the second experiment demonstrated that new resources that are harmful to the fungus garden are regularly collected but rejected a few hours later. Furthermore, aversion or reduction of ants' preference to this resource is manifested in future foraging experiences.

This aversive behavioural response of *Atta sexdens* was also observed in *Acromyrmex lundii* (GUÉRIN-MÉNEVILLE, 1838), *Atta colombica* (GUÉRIN-MÉNEVILLE, 1844), and *Acromyrmex ambiguus* (EMERY, 1888) in studies using a similar method (HERZ & al. 2008, SAVERSHEK & al. 2010, SAVERSHEK & ROCES 2011), which suggests the existence of a rejection mechanism common to all leaf-cutting ants for resources that are inappropriate to the fungus garden. The rejection of resources that are harmful to the fungus

would be a long-lasting behavioural response, which, once learned, can take up to 18 weeks to be reverted (e.g., *Atta colombica*, see SAVERSHEK & al. 2010).

Such a behavioural modification can be explained by associative learning processes (GOLDSTEIN 1962, TERNENT & GARSHELIS 1999, CONOVER & LYONS 2003, DESMEDT & al. 2017). Collection and incorporation of leaf fragments into the fungus garden are behavioural ant responses that may be inhibited by the effects of the fungicide cycloheximide, representing an indirect punitive stimulus. It must be emphasized that, at low concentrations, cycloheximide is innocuous and undetectable to ants (RIDLEY & al. 1996, HERZ & al. 2008). In an experiment designed to assess the effects of the fungicide on worker ants by a 24 h exposure of individuals to pellets impregnated with cycloheximide, mortality was only significant at a concentration five times higher than that used in the current study (SOUSA & al. 2018). This was confirmed by Experiment 3, when ants were not able to discriminate between resources with and without fungicide. Therefore, the punitive stimulus must arise from another substance caused by the effects of the fungicide on the fungus garden. This stimulus would trigger in some workers the rejection of resources that are harmful to the colony. Such a response would be similar to the hygienic behaviour of *Apis mellifera*, whereby some workers discard contaminated substrates after detecting the odour of dead offspring (MASTERMAN & al. 2000). In leaf-cutting ants, the removal of waste from the nest is usually performed by ants of different sizes (small, medium, and large) specialized in this task (CALHEIROS & al. 2019). In *Atta colombica*, there is a strict division of labour between foragers and waste workers, with no task switching (HART & RATNIEKS 2002). However, in *Acromyrmex echinatior* (FOREL, 1899), it was found that in situations of sudden waste accumulation, 20% of ants that participate in waste disposal may become foragers (WADDINGTON & HUGHES 2010). Foragers are more flexible in their task-choice behaviour, and it is believed that foragers seek out work when within the nest (TRIPET & NONACS 2004).

Therefore, it is possible that foragers that took part in disposal might have associated the odours of plant waste through Pavlovian conditioning, leading to rejection acts (removal of harmful substrates from the fungus garden and nest), causing aversion in future foraging experiences (DUPUY & al. 2006, GUERRIERI & D'ETTORRE 2010, MATSUMOTO & al. 2012).

Animals can associate odours, sounds, and images perceived during negative situations and subsequently predict the spatial or temporal proximity of the negative event by identifying these cues in future experiences (WUSTMANN & al. 1996). Aversive conditioning was demonstrated in *Camponotus aethiops* (LATREILLE, 1798): The ants were conditioned to open the mandible (conditioned response) when exposed to a neutral odour (conditioned stimulus) that had been previously presented during application of heat to the ant body (unconditional stimulus), activating a mandible-opening reflex (unconditional response) (DESMEDT & al. 2017).

In environments with high plant diversity, it is of high selective value for leaf-cutting ants to discriminate between suitable and unsuitable plants for fungal cultivation (SAVERSCHEK & ROCES 2011). The capability of recognising the odour of plants that are harmful to the fungus garden has been reported in leaf-cutting ants, but the physiological and behavioural mechanisms that regulate aversion behaviour have not yet been elucidated (SAVERSCHEK & ROCES 2011).

The aversion shown by worker ants (Experiments 4 and 5) to food resources impregnated with the odour of aversive resources (leaf fragments treated with fungicide) confirmed that odour aversive conditioning is a mechanism used by *Atta sexdens* to identify and reject unsuitable resources. The efficiency of this selectivity mechanism depends on quick installation of the learning process to reduce damage to the fungus garden. Foraged plant fragments are chewed by gardener ants and incorporated into the fungus garden in less than 48 h (HERZ & al. 2008). In our experiments, rejection of harmful resources was observed 24 h after foraging, indicating that the metabolic processes that generate substances to trigger rejection responses are rapid. In the ant *Linepithema humile* (MAYR, 1868), necrophoresis is triggered in just 1 h after nestmate death in response to chemical changes that occur on the cuticle of the dead worker (lack of dolichodial and iridomyrmecin) (CHOE & al. 2009). Persistent memorisation of odours of harmful resources, associated with rejection acts learned by associative conditioning, is of high selective value for the fungus-ant complex (HERZ & al. 2008).

Volatile samples captured by the aeration method (resins) did not differ between treated and untreated subcolonies. SOUSA & al. (2020), in using the solvent extraction method for washing fungal fragments (dichloromethane), also did not detect volatile differences between subcolonies treated or not with cycloheximide. However, chemical analysis of extracts obtained by the hexane solvent wash method revealed significant alterations in the chemical profile of the fungus garden at 48 h after incorporation of the harmful resource. As we did not detect differences in volatiles captured by the aeration method between cycloheximide-treated and untreated subcolonies, the chemical changes detected in extracts probably correspond to compounds that would likely be propagated via contact.

Some of these chemical changes can be used as cues by ants and act as a trigger of rejection behaviour (RIDLEY & al. 1996, NORTH & al. 1999). Rejection triggers could be a secondary metabolite (GOES & al. 2020) or gustatory stimulus derived from fungus degradation (SAVERSCHEK & ROCES 2011). Such chemical compounds would be transmitted between workers by grooming and trophallaxis (RIDLEY & al. 1996) or detected by foragers through contact with the damaged fungus or other workers during waste disposal (NORTH & al. 1999, WADDINGTON & HUGHES 2010). Compounds that differed quantitatively between fungal extracts from treated and untreated subcolonies are the most suitable for future tests aiming to identify which compounds are responsible for triggering the substrate rejection behaviour.

Gardener ants are the most susceptible to associating a harmful substrate with fungus damage and would likely be responsible for interrupting the processing and incorporation of harmful leaves into the fungus garden (ARENAS & ROCES 2016). However, forager ants also learn to reject plants when exposed to colony-dump odours (ARENAS & ROCES 2017, 2018). In our experiments, we found that the experience of collecting suitable plants does not increase the preference for this resource in future collections. However, the experience of gathering resources that are unsuitable for the fungus garden reduces the foraging of this resource. Furthermore, we found that foragers associate the odour of the collected plant with the negative effect exerted on the fungus. Therefore, we believe that the plant selection mechanism is based exclusively on aversive learning processes. When harmful substrates are incorporated into the fungus garden, they lead to fungal degradation, and compounds resulting from this process would serve as cues, which trigger in some ants the behavioural response of removing fungi and substrates from the contaminated area (probably an innate behaviour). Accumulation of waste in galleries would act as a triggering stimulus for the disposal behavioural response in waste workers (more predisposed to perform this task) and in some foragers. These foragers would associate the plant's odour, by Pavlovian associative learning, to the act of disposing of the resource and would probably be responsible for the rejection of these plants in future collections. Thus, leaf-cutting ants would be capable of complex collective behaviours that arise from the combination of individual responses and interactions with the symbiotic fungus.

Conclusions

The leaf-cutting ant *Atta sexdens* foraged indiscriminately among plants suitable and unsuitable to the fungal symbiont, but unsuitable plants caused chemical changes to the fungus garden that triggered late rejection behaviour in worker ants. The odours of harmful plants are linked to the rejection behaviour by associative learning and determine aversion to these plants in future foraging experiences. Plant selection in leaf-cutting ants seems to be based on recognition of plants that are harmful to the fungus garden rather than on recognition of adequate plants.

Acknowledgements

We thank the Rio de Janeiro State Research Foundation (FAPERJ) for supporting the project, the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF / FAPERJ) for awarding a grant to the first author, and the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for granting a scholarship to the second author.

References

ABRAMSON, C.I., DINGES, C.W. & WELLS, H. 2016: Operant conditioning in honey bees (*Apis mellifera* L.): the cap pushing response. – Public Library of Science One 11: art. e0162347.

- ALCOCK, J. 2011: Comportamento animal: uma abordagem evolutiva. – Artmed, Porto Alegre, 606pp.
- ARENAS, A. & ROCES, F. 2016: Gardeners and midden workers in leaf-cutting ants learn to avoid plants unsuitable for the fungus at their worksites. – *Animal Behaviour* 115: 167-174.
- ARENAS, A. & ROCES, F. 2017: Avoidance of plants unsuitable for the symbiotic fungus in leaf-cutting ants: Learning can take place entirely at the colony dump. – *Public Library of Science One* 12: art. e0171388.
- ARENAS, A. & ROCES, F. 2018: Appetitive and aversive learning of plants odors inside different nest compartments by foraging leaf-cutting ants. – *Journal of Insect Physiology* 109: 85-92.
- AZEVEDO, D.L.O. 2009: O papel das rotas e da obtenção de informações sobre a eficiência no forrageamento de *Dinoponera quadricaps* em ambiente natural. – Master thesis, Universidade Federal do Rio Grande do Norte, Natal, 83 pp.
- BREED, M.D. & MOORE, J. 2016: *Animal behaviour*. – Academic Press, New York, NY, 552 pp.
- BREMS, B. 2003: Operant conditioning in invertebrates. – *Current Opinion in Neurobiology* 13: 710-717.
- CALHEIROS, A.C., RONQUE, M.U.V. & OLIVEIRA, P.S. 2019: Social organization and substrate specialization in the leaf-cutting ant *Acromyrmex subterraneus* (Formicidae: Myrmicinae). – *Journal of Insect Behavior* 32: 267-280.
- CAMARGO, R.S., FORTI, L.C., LOPES, J.F.S. & MATOS, C.A.O. 2008: Growth of populations and fungus gardens of *Atta sexdens rubropilosa* (Hymenoptera, Formicidae) response to foraged substrates. – *Sociobiology* 52: 633-643.
- CAMAZINE, S. 1993: The regulation of pollen foraging by honey bee: how foragers assess the colony's need for pollen. – *Behavioral Ecology and Sociobiology* 32: 265-272.
- CHERRETT, J.M. 1972: Some factors involved in the selection of vegetable substrate by *Atta cephalotes* (L.) (Hymenoptera: Formicidae) in tropical rain forest. – *Journal of Animal Ecology* 41: 647-660.
- CHOE, D.H., MILLAR, J.G. & RUST, M.K. 2009: Chemical signals associated with life inhibit necrophoresis in Argentine ants. – *Proceedings of the National Academy of Sciences of the United States of America* 106: 8251-8255.
- CONOVER, M.R. & LYONS, K.S. 2003: Reducing or delaying egg depredation by punishing free-ranging predators for opening eggs. – *Applied Animal Behaviour Science* 83: 177-185.
- DESMEDT, L., BARACCHI, D., DEVAUD, J.M., GIURFA, M. & D'ETTORRE, P. 2017: Aversive learning of odor-heat associations in ants. – *Journal of Experimental Biology* 220: 4661-4668.
- DUKAS, R. 2004: Evolutionary biology of animal cognition. – *Annual Review of Ecology, Evolution, and Systematics* 35: art. 347374.
- DUPUY, F., SANDOZ, J.C., GIURFA, M. & JOSENS, R. 2006: Individual olfactory learning in *Camponotus* ants. – *Animal Behaviour* 72: 1081-1091.
- DUSSUTOUR, A., DENEUBOURG, J.L., BESHES, S. & FOURCASSIÉ, V. 2009: Individual and collective problem-solving in a foraging context in the leaf-cutting ant *Atta colombica*. – *Animal Cognition* 12: 21-30.
- FARJI-BRENER, A.G., AMADOR-VARGAS, S., CHINCHILLA, F., ESCOBAR, S., CABRERA, S., HERRERA, M.I. & SANDOVAL, C.A. 2010: Information transfer in head-on encounters between leaf-cutting ant workers: food, trail condition or orientation cues? – *Animal Behaviour* 79: 343-349.
- FERNÁNDEZ, F., CASTRO-HUERTAS, V. & SERNA, F. 2015: Formigas cortadoras de hojas de Colombia: *Acromyrmex* & *Atta* (Hymenoptera: Formicidae). – Universidad Nacional de Colombia, Bogotá, 352 pp.
- GAZAL, V., BAILEZ, O. & VIANA-BAILEZ, A.M. 2014: Mechanism of trail following by the arboreal termite *Nasutitermes corniger* (Isoptera: Termitidae). – *Zoological Science* 31: 1-5.
- GAZAL, V., BAILEZ, O., VIANA-BAILEZ, A.M., AGUIAR-MENEZES, E.L. & MENEZES, E.B. 2012: Decayed wood affecting the attraction of the pest arboretum termite *Nasutitermes corniger* (Isoptera: Termitidae) to resource foods. – *Sociobiology* 59: 287-295.
- GOES, A.C., BARCOTO, M.O., KOOLJ, P.W., BUENO, O.C. & RODRIGUES, A. 2020: How do leaf-cutting ants recognize antagonistic microbes in their fungal crops? – *Frontiers in Ecology and Evolution* 8: art. 95.
- GOLDSTEIN, M.L. 1962: Aversive-conditioning methodology in animal research. – *Psychological Reports* 11: 841-868.
- GREEN, P.W.C. & KOOLJ, P.W. 2018: The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants. – *Chemoecology* 28: 101-107.
- GUERRIERI, F.J. & D'ETTORRE, P. 2010: Associative learning in ants: conditioning of the maxilla-labium extension response in *Camponotus aethiops*. – *Journal of Insect Physiology* 56: 88-92.
- HART, A.G. & RATNIEKS, F.L.W. 2002: Waste management in the leaf-cutting ant *Atta colombica*. – *Behavioral Ecology* 2: 224-231.
- HEIDECKER, J.L. & LEUTHOLD, R.H. 1984: The organization of collective foraging in the harvester termite *Hodotermes mossambicus* (Isoptera). – *Behavioral Ecology and Sociobiology* 14: 195-202.
- HERZ, H., HÖLLDOBLER, B. & ROCES, F. 2008: Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. – *Behavioral Ecology* 19: 575-582.
- HOWARD, J.J. 1987: Leafcutting ant diet selection: the role of nutrients, water and secondary chemistry. – *Ecology* 68: 503-515.
- HOWARD, J.J. 1988: Leafcutting ant diet selection: relative influence of leaf chemistry and physical features. – *Ecology* 69: 250-260.
- HÖLLDOBLER, B. & WILSON, E.O. 1990: *The ants*. – Harvard University Press, Cambridge, MA, 746 pp.
- HUBBELL, S.P., HOWARD, J.J. & WIEMER, D.F. 1984: Chemical leaf repellency to an attine ant: seasonal distribution among potential host plant species. – *Ecology* 65: 1067-1076.
- HUBBELL, S.P. & WIEMER, D.F. 1983: Host plant selection by an Attini ant. In: JAISSON, P. (Ed.): *Social insects in the tropics*. – University of Paris, Paris, pp. 133-154.
- JACCOUD, D.B., HUGHES, W.O.H. & JACKSON, C.W. 1999: The epizootiology of a *Metarhizium* infection in mini-nests of the leaf-cutting ant *Atta sexdens rubropilosa*. – *Entomologia Experimentalis et Applicata* 93: 51-61.
- LALOI, D., BAILEZ, O., BLIGHT, M., ROGER, B., PHAM-DELEGUE, M.H. & WADHAMS, L. 2000: Recognition of complex odors by restrained and free-flying honeybees *Apis mellifera*. – *Journal of Chemical Ecology* 26: 2307-2319.
- LANAN, M. 2014: Spatiotemporal resource distribution and foraging strategies of ants (Hymenoptera: Formicidae). – *Myrmecological News* 20: 53-70.
- LITLEDYKE, M. & CHERRETT, J.M. 1976: Ingestão direta de seiva vegetal de folhas cortadas pelas formigas cortadeiras *Atta cephalotes* (L.) e *Acromyrmex octospinosus* (REICH) (Formicidae, Attini). – *Bulletin of Entomological Research* 66: 205-217.
- LOECK, A.E., ROSENTHAL, M.D. & GUSMÃO, L.G. 1994: Mini formigueiro: método de criação de formigas cortadeiras na ausência da rainha. – *Anais da Sociedade Entomológica do Brasil* 23: 359-362.

- MARINHO, C.G.S., DELLA LUCIA, T.M.C., GUEDES, R.N.C., RIBEIRO, M.M.R. & LIMA, E.R. 2005: Beta-eudesmol-induced aggression in the leaf-cutting ant *Atta sexdens rubropilosa*. – *Entomologia Experimentalis et Applicata* 117: 89-93.
- MASTERMAN, R., SMITH, B. & SPIVAK, M. 2000: Evaluation of brood odor discrimination abilities in honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. – *Journal Insect Behavior* 13: 87-101.
- MATSUMOTO, Y., MENZEL, R., SANDOZ, J.C. & GIURFA, M. 2012: Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. – *Journal of Neuroscience Methods* 211: 159-167.
- NAGAMOTO, N.S., CARLOS, A.A., MOREIRA, S.M., VERZA, S.S., HIROSE, G.L. & FORTI, L.C. 2009: Differentiation in selection of dicots and grasses by the leaf-cutter ants *Atta capiguara*, *Atta laevigata* and *Atta sexdens rubropilosa*. – *Sociobiology* 54: 127-138.
- NOMINATO, F.C. 2015: Análise do efeito de inseticidas neonicotinóides sobre a orientação e coleta de alimento de operárias de *Apis mellifera* L., 1758 (Hymenoptera: Apidae). – Master thesis, Universidade Estadual Paulista, São Paulo, 57 pp.
- NORTH, R.D., JACKSON, C.W. & HOWSE, P.E. 1999: Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens rubropilosa*, regarding choice of substrate for the fungus. – *Physiological Entomology* 24: 127-133.
- PAPAJ, D.R. & LEWIS, A.C. 1993: Insect learning: ecology and evolutionary perspectives. – Chapman & Hall, New York, NY, 398 pp.
- PEREZ, M., NOWOTNY, T., D'ETTORRE, P. & GIURFA, M. 2016: Olfactory experience shapes the evaluation of odour similarity in ants: a behavioural and computational analysis. – *Proceedings of the Royal Society B-Biological Sciences* 283: art. 20160551.
- PHAM-DELEGUE, M.H., BAILEZ, O., BLIGHT, M.M., MASSON, C., PICARD-NIZOU, A.L. & WADHAMS, L.J. 1993: Behavioural discrimination of oilseed rape volatiles by the honeybee *Apis mellifera* L. – *Chemical Senses* 18: 483-494.
- QUINLAN, R.J. & CHERRETT, J.M. 1979: The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). – *Ecologia Entomology* 4: 151-160.
- RAINE, N.E. & CHITTKA, L. 2008: The correlation of learning speed and natural foraging success in bumble-bees. – *Proceedings of the Royal Society B-Biological Sciences* 275: 803-808.
- RIBEIRO, M.M.R. & MARINHO, C.G.S. 2011: Seleção e forrageamento em formigas-cortadeiras. In: DELLA LUCIA, T.M.C. (Ed.): *Formigas Cortadeiras da bioecologia ao manejo*. – Editora UFV, Viçosa, pp. 189-200.
- RIDLEY, P., HOWSE, P.E. & JACKSON, C.W. 1996: Control of the behaviour of leaf-cutting ants by their symbiotic fungus. – *Experientia* 52: 631-635.
- ROCES, F. 1990: Olfactory conditioning during the recruitment process in a leaf-cutting ant. – *Oecologia* 83: 261-262.
- ROCES, F. 1994: Odour learning and decision-making during food collection in the leaf-cutting ant *Acromyrmex lundi*. – *Insectes Sociaux* 41: 235-239.
- SAVERSCHKEK, N., HERZ, H., WAGNER, M. & ROCES, F. 2010: Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. – *Animal Behaviour* 79: 689-698.
- SAVERSCHKEK, N. & ROCES, F. 2011: Foraging leafcutter ants: olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. – *Animal Behaviour* 82: 453-458.
- SCHLEYER, M., FENDT, M., SCHULLER, S. & GERBER, B. 2018: Associative learning of stimuli paired and unpaired with reinforcement: evaluating evidence from maggots, flies, bees, and rats. – *Frontiers in Psychology* 9: art. 1494.
- SILVA, A., BACCI, M. Jr., SIQUEIRA, C.G., BUENO, O.C., PAGNOCCA, F.C. & HEBLING, M.J.A. 2003: Survival of *Atta sexdens* workers on different food sources. – *Journal of Insect Physiology* 49: 307-313.
- SKINNER, B.F. 1948: 'Superstition' in the pigeon. – *Journal of Experimental Psychology* 38: 168-172.
- SOUSA, K.K.A., CAMARGO, R.D.S., FORTI, L.C. & CALDATO, N. 2018: Effects of cycloheximide on the mortality of *Atta sexdens* leaf-cutting worker ants. – *Revista Brasileira de Entomologia* 62: 169-171.
- SOUSA, K.K., CATALANI, G.C., GIANETI, T., CAMARGO, R., CALDATO, N., RAMOS, V.M. & FORTI, L.C. 2020: A volatile semiochemical released by the fungus garden of leaf-cutting ants. – *Florida Entomologist* 103: art. 1.
- SOUZA, J.M.F. 2012: Neuroproteômica: proteínas relacionadas à aprendizagem em abelhas e rato. – Master thesis, Universidade de Brasília, Brasília, 70 pp.
- TATAGIBA-ARAÚJO, G., VIANA-BAILEZ, A.M. & BAILEZ, O. 2012: Increasing attractiveness of baits with venom gland extract for *Atta sexdens rubropilosa* (FOREL) (Hymenoptera: Formicidae). – *Neotropical Entomology* 41: 232-236.
- TEREZAN, A.P., ROSSI, R.A., ALMEIDA, R.N.A., FREITAS, T.G., FERNANDES, J.B., SILVA, M.F.G.F., VIEIRA, P.C., BUENO, O.C., PAGNOCCA, F.C. & PIRANI, J.R. 2010: Activities of extracts and compounds from *Spiranthera odoratissima* St. HIL. (Rutaceae) in leaf-cutting ants and their symbiotic fungus. – *Journal of the Brazilian Chemical Society* 21: 882-886.
- TERNENT, M.A. & GARSHELIS, D.L. 1999: Taste-aversion conditioning to reduce nuisance activity by black bears in a Minnesota military reservation. – *Wildlife Society Bulletin* 27: 720-728.
- TRIPET, F. & NONACS, P. 2004: Foraging for work and age-based polyethism: the roles of age and previous experience on task choice in ants. – *Ethology* 110: 863-877.
- VIANA-BAILEZ, A.M. & ENDRINGER, F.B. 2016: Plasticidade do comportamento de forrageamento em formigas cortadeiras. – *Oecologia Australis* 20: 11-19.
- VON FRISCH, K. 1967: *The dance language and orientation of bees*. – Harvard University Press, Cambridge, MA, 592 pp.
- WADDINGTON, S.J. & HUGHES, W.O.H. 2010: Waste management in the leaf-cutting ant *Acromyrmex echinator*: the role of worker size, age and plasticity. – *Behavioral Ecology and Sociobiology* 64: 1219-1228.
- WILSON, E.O. 1962: Chemical communication among workers of the fire ant *Solenopsis saevissima* (F. SMITH) I. The organization of mass-foraging. – *Animal Behaviour* 10: 134-138.
- WÜSTMANN, G., REIN, K., WOLF, R. & HEISENBERG, M. 1996: A new paradigm for operant conditioning of *Drosophila melanogaster*. – *Journal of Comparative Physiology A* 179: 429-436.