ECOLOGY, BEHAVIOR AND BIONOMICS

Allogrooming, Self-grooming, and Touching Behavior as a Mechanism to Disperse Insecticides Inside Colonies of a Leaf-Cutting Ant

Tarcísio Marcos Macedo Mota Filho[1] · Roberto da Silva Camargo[1] · Luis Eduardo Pontes Stefanelli[1] ·
José Cola Zanuncio[2] · Alexandre dos Santos[3] · Carlos Alberto Oliveira de Matos[4] · Luiz Carlos Forti[1]

Abstract

Toxic baits, containing the active ingredients sulfluramid or fipronil, are the main methods to control leaf-cutting ants of the genera *Atta* Fabricius, 1805, and *Acromyrmex* Mayr, 1865 (Hymenoptera: Formicidae). However, the insecticide dispersion among members of the colony during the control needs further studies. We studied whether the behaviors of allogrooming, self-grooming, and contact among individuals spread the insecticides among those of the colony. The insecticides sulfluramid and fipronil (0.1% and 1.0% (w/w)) were applied topically in groups of workers of *Atta sexdens* (Linnaeus, 1758), and the social interactions among them with or without insecticide were studied. In addition, toxic baits (sulfluramid or fipronil) were provided to colonies and their behavioral acts were observed. At the end of the experiment, colony mortality, number and mass of dead workers, and mass of wet waste were compared between ant nests receiving baits and ants with topical application. In the topical application, behavioral analysis showed higher interaction between ants in the colonies and touch and allogrooming behaviors as the most frequent in those that received the concentrations of sulfluramid. In the baits, the behavior of licking the pellet and allogrooming was more frequent. Colony mortality was faster for those that received topical application, especially with the insecticide fipronil (0.1%). However, the number and mass of dead workers was similar between topical application and toxic baits. In the toxic baits, the licking behavior of the bait pellets and subsequent allogrooming probably dispersed the insecticides. In the topical application, the route of the insecticide occurred by excessive touches among workers, with subsequent allogrooming. Thus, allogrooming, self-grooming, and touching among workers increased the dispersion of insecticides among members of the ant colonies.

Keywords *Atta* · Contamination routes · Dispersion insecticides · Social interactions · Trophallaxis

✉ Tarcísio Marcos Macedo Mota Filho
tarcisio972010@hotmail.com

1 Depto de Produção Vegetal, Faculdade de Ciências Agronômicas, Univ Estadual Paulista - FCA/UNESP, Botucatu, São Paulo, Brazil

2 Depto de Entomologia/BIOAGRO, Univ Federal de Viçosa - UFV, Viçosa, Minas Gerais, Brazil

3 Lab de Fitossanidade (FitLab), Instituto Federal de Mato Grosso - IFMT, Cáceres, Mato Grosso, Brazil

4 Campus Experimental de Itapeva, Univ Estadual Paulista - UNESP, Itapeva, São Paulo, Brazil

Introduction

Leaf-cutting ants of the genera *Atta* Fabricius, 1805, and *Acromyrmex* Mayr, 1865 (Hymenoptera: Formicide), occur exclusively in the Neotropical region as the main pests in forest plantations, agriculture, and livestock (Vinha et al. 2020). Chemical control with toxic baits, containing the active ingredients sulfluramid or fipronil, is the main method to control these insects (Della Lucia et al. 2014; Zanetti et al. 2014). The contamination of workers occurs through direct contact with the active ingredient contained in toxic baits during fungus garden cultivation (Britto et al. 2016; Camargo et al. 2017a). During this process, the insecticides contaminate 50% of the workers (Forti et al. 2019) but the way this happens with the other members of a colony is not well-known.

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The dispersion of insecticides in baits to other members of the colony might occur by trophallaxis (Rust et al. 2004; Moreira et al. 2006, 2010; Buczkowski 2019). Trophallaxis is a behavior associated with fluid ingestion and exchanging, stored during the worker foraging and regurgitated among members of the colony. This behavior plays an important role in exchanging nutrients, symbionts, pheromones, and information among individuals of the colony (Hölldobler and Wilson 1990; Moreira et al. 2015; Meurville and LeBoeuf 2021). There is no reduction in this social behavior even among individuals contaminated with toxic substances (Souza et al. 2008).

Workers of *Atta sexdens* Linnaeus, 1758 (Myrmicinae), perform trophallaxis with low frequency than other ants that feed on nectar such as *Camponotus rufipes* Fabricius, 1775 (Formicinae), and *Neoponera villosa* Fabricius, 1804 (Ponerinae) (Paul and Roces 2003). These last species collect fluids (nectar) during foraging and store them in a well-developed crop adapted to this strategy (Eisner 1957; Paul and Roces 2003). Leaf-cutting ants ingest liquids during cutting leaves and gongylida harvesting in the fungus garden, but their crop is not adapted to storing large volumes of liquid (Caetano 1990). The fluid intake rate of *C. rufipes* workers of 6.7 µl/min is much higher than that of *A. sexdens*, which does not exceed 0.6 µl/min (Paul and Roces 2003). The hyphae of the fungus *Leucocoprinus gongylophorus* Heim, 1957, that grow in the fungus chambers inside ant nests are the main food source for leaf-cutting ants (Schultz et al. 2005) and they rarely feed on liquid substances (Littledyke and Cherrett 1976; Garrett et al. 2016).

The contamination of ant workers with insecticides inside the colony is not mainly by trophallaxis. The intoxication through direct contact with toxic baits during their processing and incorporation into the fungus garden and the hygienic behaviors such as self-grooming and allogrooming and contact among contaminated and non-contaminated workers are the most important dispersing mechanism of insecticides to other members of the colony (Andrade et al. 2002; Camargo et al. 2017a; Forti et al. 2020). Interactions among *A. sexdens* workers, such as self-grooming, allogrooming, and contact among contaminated and uncontaminated ants, were the main routes dispersing a fat-soluble substance to approximately half of the nestmates (Camargo et al. 2017a; Catalani et al. 2020). However, the dispersion of insecticides in toxic baits through these behavioral acts has not been tested.

Given the above, the following question arises: how is the insecticide dispersed in leaf-cutting ant colonies? Our hypothesis is that the contact of workers contaminated with the insecticide during self-grooming, allogrooming, and touching provides dispersion of the insecticide within the colony. To test this hypothesis, *A. sexdens* workers were topically contaminated with two concentrations of the insecticides sulfluramid and fipronil (0.1% and 1% (w/w)), and whether the social interactions between contaminated and uncontaminated workers disperse insecticides and cause colony suppression was studied.

Material and methods

Colonies studied

The colonies of *A. sexdens* used were approximately 6 months old with 350.0 cm^3 of fungus garden and collected in March 2020 in Botucatu, São Paulo, Brazil. These colonies were kept in the Laboratory of Social Insect Pests of the Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP) in Botucatu at 24 ± 2 °C, RH of 80%, and a photoperiod of 12 h of light. Each colony was kept inside a container (length: 15 cm, width: 15 cm, and height: 15 cm) with a fungus garden, and they received *Acalypha* spp. (leaves and stems).

Experimental setup

The hypothesis is that social interactions, allogrooming, and self-grooming, besides touching among workers, disperse the insecticides inside colonies of the leaf-cutting ant *A. sexdens* and lead to their suppression. Workers were contaminated with two concentrations (0.1% and 1.0% (w/w)) of the insecticides fipronil and sulfluramid, and commercial toxic baits containing these compounds were provided in foraging chamber, and social interactions between individuals of the colonies were minutely studied.

The number of contaminated individuals was based on the amount of active ingredient contained in 0.3 g of toxic bait (Supplementary Material 1). This is equivalent to 3 and 30 medium-size workers (head width from 1.2 to 2.2 mm) topically contaminated with 1.0 µl of fipronil solution dissolved in vegetable oil at the concentrations of 0.1% and 1% (w/w), respectively, or 60 and 600 workers contaminated with 1.0 µl of sulfluramid solution dissolved in vegetable oil at concentrations of 0.1% and 1% (w/w), respectively. The control treatments consisted of the same number of individuals exposed to the insecticides fipronil and sulfluramid; however, they were contaminated with only 1 µl of vegetable oil. In addition, bait consisting of citrus pulp (96%) and soybean oil (4%) was also used as a control treatment (Forti et al. 2019, 2020).

Atta sexdens workers were removed from their colonies and separated by size category, based on head width. The pronotum of the workers were marked with a small dot of ink made with a pen (Edding®) with excellent adhesion, quick drying, and good visibility (Camargo et al. 2007). The workers remained for 2 h in plastic cups with the edges

greased with Fluon (fluoroethylene resin), a product that prevents ants from escaping, and according to treatments, 1.0 μl of insecticide was applied topically on the workers' pronotum with a Hamilton™ (5.0 μl) micro syringe, which were then released into their respective colonies.

Experiment

The treatments were toxic baits—fipronil (0.01% w/w) and sulfluramid (0.2% w/w)—topical application of fipronil 1.0%, fipronil 0.1%, sulfluramid 1.0%, and sulfluramid 0.1% and the controls in *A. sexdens* colonies. Subsequently, the waste and leaf remain were removed from the waste and foraging chambers and the colonies did not receive the plant substrate for 24 h. After that period, each colony received 0.3 g of toxic bait or contaminated individuals in the foraging chamber. The fungus garden container was closed with a transparent glass lid; after that, the behaviors of the workers inside the colonies were observed.

The observations lasted for 6 h at regular intervals of 30 min, and followed by scanning observation (Martin and Bateson 1986) to observe a group of workers, and the behavior of each ant was recorded. The behavioral act frequency of each ant worker was quantified. The behavioral acts observed for topical application were (i) touching—touches among the contaminated worker with others; (ii) self-grooming—self-grooming of the contaminated worker; (iii) allogrooming—mutual grooming of contaminated worker by other workers; (iv) touching 2—touches among workers without insecticide; (v) self-grooming 2—self-grooming of workers without insecticide; (vi) allogrooming 2—mutual grooming among workers without insecticide (Supplementary Material 2, 3). The behavioral acts observed for bait application were (i) transporting the pellets to the fungus garden; (ii) holding the pellet; (iii) licking the pellet; (iv) fragmenting the pellet; (v) incorporating the pellet; (vi) self-grooming; and (vii) allogrooming (Supplementary Material 4).

Twenty-four hours after the first day of evaluation, the plant substrate was supplied in the foraging chamber to maintain the growth of the symbiotic fungus. The ant mortality was observed daily, and at the end of the experiment (death of the colonies), the number of dead workers, the total mass of dead workers, and the mass of wet waste from the garbage chamber were evaluated.

Statistical analysis

The four treatments related to topical application were compared with their controls using the Wilcoxon rank sum test with continuity correction (Mann–Whitney *U* test). Posteriorly, these same data related to topical application were submitted to the Kruskal–Wallis test. A multiple comparison was performed between the different treatments for the most frequent behavioral acts performed by the workers and also those of the number of dead workers, the weight of dead workers, and the weight of wet waste were performed with the paired Wilcoxon rank sum test with the adjustment of the *P* value through the false discovery rate method (Benjamini and Hochberg 1995) ($\alpha = 0.05$). The data of the bait treatments were subjected to the Kruskal–Wallis test and multiple comparison of medians with pairwise Wilcoxon rank sum test with continuity correction.

The survival function was calculated using the Kaplan–Meier estimator (also known as the product limit) (Kaplan and Meier 1985) which is an adaptation of the empirical survival function:

$S_{(t)}$ – number of individuals that survived until time *t*/ total number of individuals.

This function implies in the absence of censorship and presence of incomplete or partial information (Colosimo and Giolo 2006) as a staircase-like function with steps in the time when the individual death occurred. The size of the steps ($n =$ sample size) was multiplied by the number of ties in case they occur.

The hypothesis that there were no differences between treatments in survival functions was tested with the log-rank or Mantel–Haenszel tests. The *P* values were adjusted (Benjamini and Hochberg 1995) to control the false discovery rate (the expected proportion of false discoveries among the rejected hypotheses) and which is one of the most powerful methods to adjust the *P* values.

The survival package from the R version 4.0.0 environment was used for statistical computing and graphing (R Core Team 2020).

Results

Touching behavior, followed by allogrooming and self-grooming, was the most frequent behavior with both concentrations of the insecticides (fipronil and sulfluramid) and in the control (Fig. 1 and Supplementary Material 5, 6). Behaviors carried out by workers of *A. sexdens* contaminated with different concentrations of the insecticides sulfluramid and fipronil did not differ from their respective control (Fig. 1 and Supplementary Material 5). The ant behaviors, in general, differed between the insecticide concentrations, being more frequent in colonies that received the application of 0.1% and 1.0% of sulfluramid (Fig. 1 and Supplementary Material 6). The behavioral acts performed during the preparation of the pellets to be incorporated into the fungus garden were similar between the toxic baits and the control (Fig. 2 and Supplementary Material 7). The survival of *A. sexdens* colonies in the treatments fipronil 0.1% ($p = 0.001$), fipronil 1% ($p = 0.001$), sulfluramid 0.1% ($p = 0.006$), and sulfluramid 1% ($p = 0.010$) and toxic bait containing fipronil

Fig. 1 Frequency of behaviors of *Atta sexdens* (Hymenoptera: Formicidae) with topical application of two concentrations of the insecticides fipronil (Fip.) and sulfluramid (Sulf.) and the respective control. There are no differences between the medians of the treatments and their respective controls. Medians followed by different letters differ by the pairwise Wilcoxon rank sum test with the adjustment of the *P* values ($\alpha = 0.05$). Behaviors: (1) self-grooming; (2) allogrooming; (3) touching; (4) self-grooming 2; (5) allogrooming 2; (6) touching 2; and (7) total
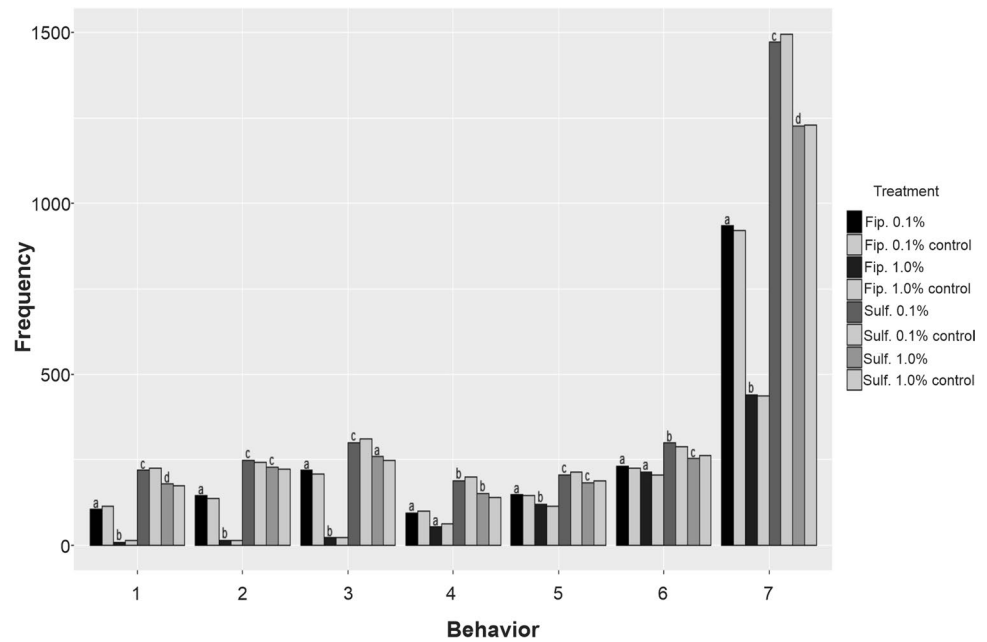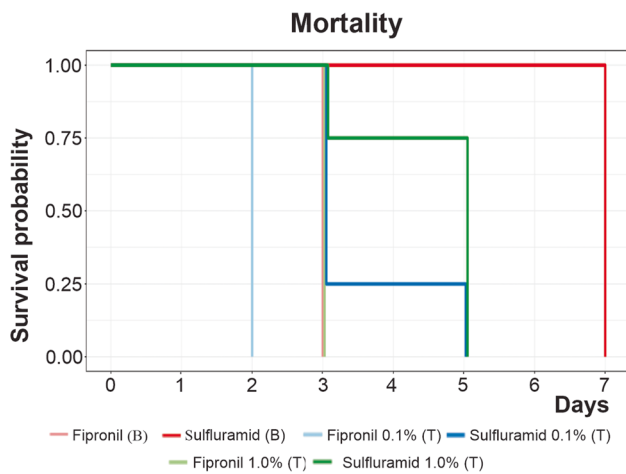

Fig. 2 Behaviors of *Atta sexdens* (Hymenoptera: Formicidae) during the preparation of toxic baits with the insecticides fipronil and sulfluramid and that in the control to be incorporated into the fungus garden. Medians followed by different letters differ by the pairwise Wilcoxon rank sum test with the adjustment of the *P* values ($\alpha = 0.05$). Behaviors: (1) transporting the pellet; (2) holding the pellet; (3) licking the pellet; (4) fragmenting the pellet; (5) incorporating the pellet; (6) self-grooming; (7) allogrooming; and (8) total
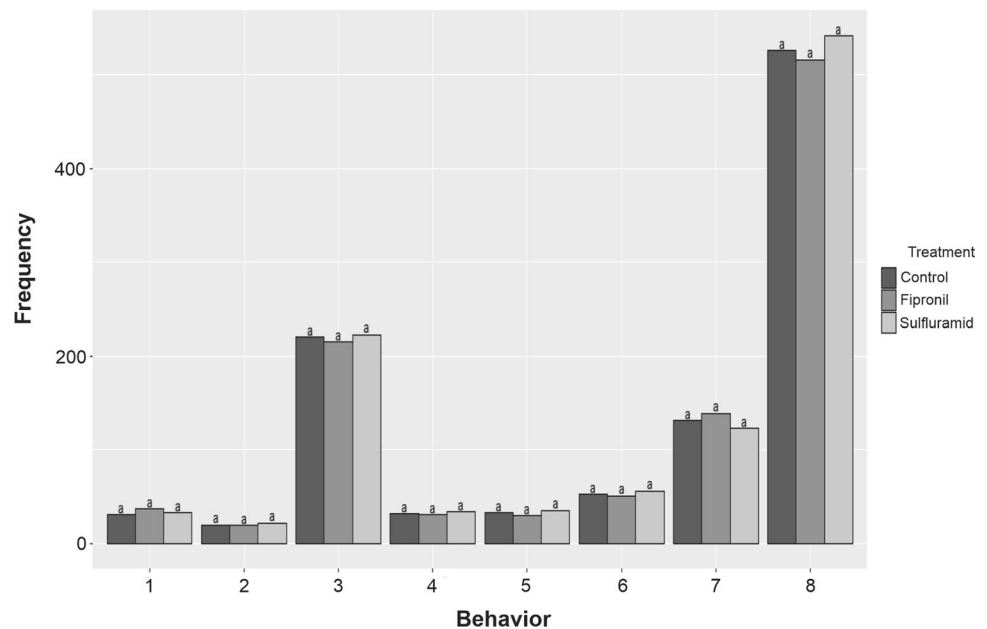
($p = 0.001$) or sulfluramid ($p = 0.001$) was lower than that of its respective control. The survival (2 days) of the colonies that received workers contaminated with 0.1% fipronil was shorter than those that received workers contaminated with the other concentrations and formulation of the insecticides fipronil and sulfluramid (Fig. 3 and Supplementary Material 8, 9). On the other hand, the longest survival time (7 days) was in colonies that received baits containing the insecticide sulfluramid and differed from all other treatments. The survival curves of the ant nests with the two concentrations of sulfluramid (0.1% and 1.0%) did not differ and those with

this insecticide also did not differ from the concentration of fipronil 1.0% (Fig. 3 and Supplementary Material 8, 9). The survival (3 days) of colonies that received bait containing fipronil is statistically identical to those that received workers topically contaminated with 1% fipronil (Fig. 3 and Supplementary Material 8, 9).

The number and mass of dead workers were similar between the two concentrations and formulations of the fipronil and the sulfluramid insecticides (Table 1). In addition, the ants did not produce wet residues (waste) in the colonies that received contaminated workers with the two

Fig. 3 Survival curves of *Atta sexdens* (Hymenoptera: Formicidae) colonies as a function of different concentrations (topical application (T) and toxic baits (B)) with insecticides fipronil or sulfluramid

concentrations of the insecticides (Table 1). The production of wet residues (waste) was higher in the control and similar in the colonies that received the toxic baits. In the five control treatments, there were no dead individuals and the production of wet residues (residues) was similar ($p = 0.3581$).

Discussion

Topical application

The social interactions, allogrooming, self-grooming, and touching among workers dispersed the insecticides inside the colonies of the leaf-cutting ant *A. sexdens*, leading to their suppression. The higher social interaction in the colonies that received the concentrations of 0.1% and 1.0% sulfluramid increased the numbers of contaminated workers, 60 and 600, respectively. The social interactions of *A. sexdens* as a dispersal mechanism of insecticides is similar

to that reported for insect species with social, semi-social, and even solitary habits (Rust and Saran 2006; Buczkowski et al. 2008; Akhtar and Isman 2013). For example, the black carpenter ant, *Camponotus pennsylvanicus* DeGeer (Hymenoptera: Formicidae), through social interactions dispersed the insecticide fipronil to nestmates (Buczkowski 2019); the German cockroach *Blattella germanica* Linnaeus, 1767 (Dictyoptera: Blattellidae), dispersed the insecticide indoxacarb to their nestmates (Buczkowski et al. 2008); and the larvae of *Heortia vitessoides* Moore (Lepidoptera: Crambidae) dispersed the insecticide avermectin for groupmates (Liang et al. 2019). However, dispersion of insecticides through social interactions by leaf-cutting ants needs further studies with only one addressing this type of dispersion among *A. sexdens* workers. The self-grooming, allogrooming, and touching behavior among contaminated and noncontaminated workers dispersed a lipid-soluble substance to approximately half of their nestmates (Camargo et al. 2017a).

The ant workers that acquired the insecticide at its application point transfer it to other members of the population (Soeprono and Rust 2004; Buczkowski and Wossler 2019). Subsequently, the insecticide collected during random touches was ingested and absorbed by the post-pharyngeal gland during self-grooming and allogrooming behaviors. This occurs when ants clean themselves and others using their mouthpieces, antennae, and legs, consequently poisoning the entire colony (Vander Meer et al. 1985; Britto et al. 2016; Camargo et al. 2017a).

The social interactions of leaf-cutting ants, including behavioral studies on other social insects typically focused on the positive effect of cleaning behaviors (allogrooming and self-grooming) on individuals exposed to pathogens (Soroker et al. 1995; Hughes et al. 2002; Theis et al. 2015), does not protect them from insecticides. The allogrooming and self-grooming are effective to removing parasites as *Metarhizium* from ant cuticle (Hughes et al. 2002) which makes these insects practically immune to this and other

Table 1 Number of dead workers (number of dead), mass of dead workers (mass of dead), and mass of waste in *Atta sexdens* (Hymenoptera: Formicidae) colonies that received the two concentrations (toxic baits (B) and topical application (T)) with the fipronil and sulfluramid insecticides. Medians followed by different letters per column differ by the Kruskall-Wallis test ($\alpha = 0.05$)

Treatment	Number of dead	Mass of dead (g)	Mass of waste (g)
Fipronil (B)	885.5	3618	1611a
Sulfluramid (B)	901.5	3675	0.977a
Fipronil 0.1% (T)	875.5	3581	-
Fipronil 1.0% (T)	876.5	3633	-
Sulfluramid 0.1% (T)	843.0	3578	-
Sulfluramid 1.0% (T)	903.0	3759	-
Control	-	-	6.348b
p-value	0.871	0.982	0.018

microorganisms through exposure and contact (Walker and Hughes 2009). However, these hygienic behaviors are not efficient to controlling insecticide dispersion and, on the contrary, they increase its dispersion among the nestmates (Bueno et al 2001; Theis et al. 2015; Camargo et al. 2017a). The dispersion of insecticides probably occurred due to the high frequency of touching among workers followed by self-grooming and allogrooming.

Bait application

The behavior of licking pellets of both toxic baits corroborates results with behavioral acts performed by *Acromyrmex subterraneus* Forel, 1893, and *Atta sexdens rubropilosa* Forel, 1908, respectively, during incorporation of bait pellets in their fungus (Silva et al. 2015; Forti et al. 2019). Licking behavior by leaf-cutting ants is due to the secretions produced in their mandibular glands with antibiotic and antifungal properties and incorporated to the fungus garden, and it promotes the asepsis of the substrate and benefiting the symbiont fungus growth (Fernández-Marín et al. 2006; Britto et al. 2016; Garrett et al. 2016). Besides, the licking behavior hydrates the pellets, facilitating their processing for later incorporation into the fungus garden (Silva et al. 2015), but this behavior is an important contamination route of leaf-cutting ant workers. It does not decrease when a harmful substrate is provided; on the contrary, its frequency increases to improve substrate asepsis (Sousa et al. 2017), favoring the contamination of the symbiotic fungus (Silva et al. 2015; Garrett et al. 2016; Camargo et al. 2017b).

The behavior transport, fragmentation, incorporation, and holding of the pellet were the lesser frequent ones during the bait preparation. The transporting behavior of substrate demands the most energy expenditure by leaf-cutting ants (Garrett et al. 2016) and it is their first direct contact with the active ingredient in the toxic baits. In addition, depending on the distance from the pellet collection site to the nest, this behavior is that with the longest contact period between the ant worker and the insecticide. Holding stabilizes the pellets, reducing movement and tension for cutting or other processing behaviors and the energy expenditure (Garrett et al. 2016). The pellet fragmentation behavior reduces this substrate into tiny parts (Garrett et al. 2016) making it easier for smaller ant workers to perform other processing behaviors (Bos et al. 2011). Thus, there is a possibility that these behaviors provide direct contact among workers and the insecticide in toxic baits.

The allogrooming and self-grooming as the second and third most frequent behavior, respectively, indicate that direct contact with toxic baits does not cause contamination of all members of a colony (Forti et al. 2019). Allogrooming performed by workers and associated with the recognition of nestmates is triggered when a substance is recognized in

the nestmate tegument (Soroker et al. 1995; Hughes et al. 2002). This also occurs with self-grooming, because ants increase this behavior in response to the exposure to harmful substances (Bos et al. 2011; Theis et al. 2015; Camargo et al. 2017b).

The insecticide dispersion among the workers probably occurred due to high allogrooming and self-grooming intensity as found for *A. sexdens* and *A. sexdens rubropilosa* (Bueno et al. 2001; Barbieri et al. 2009; Camargo et al. 2017b; Forti et al. 2019). Thus, these behaviors are related to insecticide dispersion among nestmates, when applied in toxic baits.

Topical application vs. bait comparison

The 100% mortality of the colonies with toxic baits and topical application and the shortest survival period (2 days) with the 0.1% concentration of fipronil were probably due to this insecticide action. The fipronil is of the chemical group phenyl pyrazole, acting in the central nervous system, blocking the transmission of signals from nerve cells and inhibiting the neurotransmitter GABA (gamma-aminobutyric acid), leading to knockdown effect (Tomlin 2000). Besides, the fipronil is toxic at low amounts and easily transferable (Gandra et al. 2016; Buczkowski and Wossler 2019) by contact and ingestion among ants (Tomlin 2000). Probably, social interactions increase the contamination rate with faster dispersion of the insecticide between ant workers.

The delayed mortality of *A. sexdens* colonies at 5 and 7 days with the concentrations of sulfluramid (0.1% and 1.0%) confirms the delayed action of this insecticide, of the chemical group of fluoroaliphatic sulfones (Schnellmann and Manning 1990). This delay is due to its mechanism of action, when this molecule is broken in the insect body, becoming a main component called DESFA (perfluorooctanesulfonamide). This molecule acts in the oxidative phosphorylation process (aerobic respiration), interrupting the production of adenosine triphosphate (ATP) in the mitochondria (Schnellmann and Manning 1990; Laranjeiro and Zanuncio 1995). The movement and aggression behaviors of ant workers with sulfluramid intoxication symptoms are due to their low energy levels, which gradually decrease and leading to death (Schnellmann and Manning 1990; Britto et al. 2016). Thus, the effect of the insecticide on colony suppression is delayed even with a large number of ants contaminated.

The similar numbers and weight of dead workers between the fipronil and sulfluramid concentrations in the baits, despite the differences in the colony survival and mechanism action of the insecticides, confirm the efficiency of these insecticides to controlling leaf-cutting ant colonies (Nagamoto et al. 2004; Gandra et al. 2016). The higher quantity of wet waste produced in the colonies with the application of toxic baits and the null production in those with workers

contaminated topically are due to the fact that workers interacted, through touching, allogrooming, and self-grooming behaviors and, consequently, became contaminated, when they detect contaminated ones, leading to the breakdown of the colony as a whole.

Social interactions, allogrooming, and self-grooming, besides touching behavior among workers, are responsible for the fast dispersal of insecticides between nestmates as observed in our experiment. We thus corroborate the hypothesis that social interactions promote the contamination of nestmates, serving as a model for further studies on the contamination of workers with the active ingredients of insecticides.

Declarations

Conflict of Interest The authors declare no competing interests.

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