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Behaviors in Fungus Garden Cultivation: Routes of Contamination of Leaf Cutting Ant Workers with Fat-Soluble Tracer Dye

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***Abstract* – Leafcutter ants cultivate in their nests a symbiont fungus used as a basis to their nutrition. To do so, vegetal material such as leaves and sprouts are cut by workers, which engage in a series of behaviors to incorporate them into the fungus garden, promoting their growth. For this reason, some crops end up being damaged by these insects. Toxic baits are used as a form of control. With this study, we investigate to show that behaviors of direct contact with baits lead to the contamination of workers. Through the experiments performed, we observed that the behaviors of holding and licking baits were the most frequent ones. We conclude that these behavioral acts are routes of entry for baits' toxic agents into the workers' body, causing their contamination.**

***Keywords* – Behavior, Leafcutter Ants, *Atta sexdens*, Toxic Baits.**

I. Introduction

Leafcutter ants are notorious for cutting leaves to grow the symbiont fungus, basis of their nutrition (Weber, 1972). During cultivation, they engage in a series of stereotyped and sequential behaviors aiming at the proper preparation of the vegetal material (Diniz and Bueno, 2009). Direct contact consists of behaviors performed by leafcutter ants involving the manipulation of the substrate during its processing and incorporation into the fungus garden (Brito et al., 2016).

Garrett et al., (2015) identified and described all behaviors performed by *Atta cephalotes* workers during the processing and incorporation of leaves, with the following sequence: After leaf discs are brought to the fungus garden, one worker or more hold the substrate with their jaws, elevating and stabilizing it; other workers engage in a series of behaviors such as licking, scraping and chopping the substrate: the surface and ends of leaf discs are licked by workers, which makes it clean and releases over all its extension fungicide and bactericide agents; sometimes it goes through a scraping process in which the workers' jaws open and close in the disc, possibly to increase the contact surface and facilitate access to the fungus, and then they are cut into smaller pieces; fragments of leaves have their edges chewed by workers, creating scars that facilitate the entry of digestive agents released by the fungus; in a less recurring and still little studied way, some workers deposit fecal fluids on the leaf fragment during its processing, which may relate to the decomposition process; after being cut, leaf fragments are collected by the workers and inserted into the fungus garden; finally, small workers inoculate hyphae in the fragments inserted into the fungus garden, facilitating the leaf decomposition process.

The abovementioned behaviors relate to the workers' morphological characteristics. According to Wilson, 1980, 1983, differences in the morphological characteristics of *Atta sexdensrubropilosa*, specifically in relation to the size of a worker's body, with variation in head size between 0.8mm and 3.0mm, relate to task division in the processing of the substrate. These results were confirmed by Andrade et al., (2002); Wilson, (1980); Lopes et al., (2004); Camargo et al, (2007); Dinizand Bueno, (2009); (2010) but focused on the size of workers and their respective roles: generalist workers, with heads 1.3 to 1.6mm wide, lick and chop leaves; smaller workers, with heads measuring between 0.8 and 1.2mm in width, chew the edges of leaves and inoculate the fragments in the fungus garden. Finally, hypha clumps are deposited on the surface of leaf fragments (Weber, 1956; Lopes et al., 2004).

Broadly speaking, both leaves and baits go through the same processing stages: physical treatment, which involves the behaviors of holding and licking the substrate; chemical treatment, in which workers deposit fecal fluid, press and chop the substrate; and incorporation, when workers incorporate substrate fragments into the fungus and inoculate the hyphae (Diniz and Bueno, 2009).

According to Silva et al. (2015), citrus pulp-based pellet processing compared to leaf processing takes more time due to its physical nature, being a more resistant material compared to leafs, going through a hydration process that enables its incorporation. Physical factors of the substrate to be processed have an influence on both processing time and number of workers involved in this process (Silva et al., 2015).In the conclusion of their study, Silva et al., (2015) showed that *Atta sexdensrubropilosa* workers, while cultivating the fungus garden with citrus pulp pellets, work more on its processing in comparison with leaves, causing the contamination of the colony's workers by the active ingredient, mainly garden and generalist workers.

Based on the above exposed, it can be considered that the preparation of substrate of the toxic-bait type and its incorporation into the fungus garden is related to the contamination of workers inside the colony through direct contact. To confirm this hypothesis that direct contact with the bait causes the contamination of workers, citrus pulp pellets were provided to laboratory colonies. Thus, behaviors performed by workers were identified and their frequencies were calculated in order to find the most recurrent behaviors during the processing and incorporation of citrus pulp pellets and which are involved in a more direct way with the contamination of workers.

II. Material and Methods

The colonies used in this experiment were collected in the city of Botucatu and stored in the Laboratory of Social Insects and Pests [*Laboratório de Insetos Sociais-Praga*] (LISP) of the College of Agronomical Sciences of UNESP, Botucatu.

For the observations, three colonies with capacity to store 1L of fungus were used. For humidity maintenance, they contained a 1cm layer of plaster in the central compartment where the fungus was. In addition, they had connections with containers reserved to foraging and waste deposition in their opposite ends (Lopes, 2004). Room temperature was maintained at 22±2°C approximately, with relative humidity around 70± 20%.

For the observations, with the aid of an eyepiece micrometer stereo microscope, individuals of varied sizes were taken from their colonies for measurement of their heads' width. Castes were defined according to what Wilson, (1980) proposed. Observations were made based on the behavior of garden workers (head width between 0.8 and 1.2mm) and generalist workers (head width between 1.3 and 1.6mm), respectively categorized as small and medium workers.

The substrate provided to the colonies for observations was composed of pellets made of citrus pulp bran and formulated without active principle, with standardized surface of 62.86mm^2.

Each of the 3 colonies were observed over 8 hours so as to record behaviors performed in the preparation and incorporation of citrus pulp-based pellets. Observations were made every ten minutes, and behaviors related to the processing of citrus pulp-based pellets, mutual cleaning and self-cleaning were calculated. The observation system was done adaptively to the scanning type described by Martin and Bateson,(1986), in which a group of individuals is scanned quickly and the behavior of each one of them is registered at regular intervals.

The behaviors performed by *Atta sexdensrubropilosa* workers during the processing and incorporation of citrus pulp-based pellets into the fungus garden were classified and, then, the frequencies of occurrence of these acts and their percentages were calculated. These calculations were also made according to the size category of the workers that performed the behaviors.

A contingency table was prepared with application of Chi-squared test taking into consideration the workers' size. Residues were judged through standard normal distribution (Conover, 1999; Conover and Iman, 1979). The analysis of standardized residues assessed whether the frequency of behaviors performed by small and medium workers met normal standards. With the Chi-squared test it was possible to obtain that behaviors with Z values between -1,96 and 1,89 fit into the standard occurrence (Table 2); Z values < -1,96 (italics) are identified as scarcity of significant occurrences of the behaviors (Table 2) and Z values > 1.89 (bold) are identified as excess of significant occurrences of the behaviors (Table 2).

To test the hypothesis that the 8 behaviors (Licking the pellet on the surface of the fungus, Holding the pellet on the surface of the fungus, Chopping the pellet, Chewing the sides of the pellet, Incorporating pellet fragment into the fungus, Incorporating hypha into the pellet fragment deposited on the fungus, Self-cleaning and Mutual cleaning) have the same distribution, a "nonparametric Anova" was conducted (Kruskal-Wallis). The Kruskal-Wallis nonparametric test rejected the hypothesis that all behaviors would have the same distribution = 20.373 P value > 0.05 (Conover,1999; Conover and Iman, 1979).

In order to verify whether there was difference between the frequencies of occurrence of behaviors, analyses of paired comparisons were performed through Conover test for multiple comparison of independent samples and Bonferroni P values adjustment method (Conover, 1999; Conover and Iman, 1979). In Table 3, P values < 0.05 (bold) represent significant difference between the frequency of occurrence of behaviors, with one of them having higher frequency of occurrence compared to the other.

An additional experiment was carried out so as to study the action of the post-pharyngeal gland in the digestion of fat compounds present in the citrus pulp-based pellets. The formulation of this substrate contained a tracer dye that dyes the gland during the absorption of fat molecules. To perform this experiment, 3 colonies were used, with application of citrus pulp pellets containing 10% of Sudan III tracer dye. Twenty-four hours after the application, the colonies were opened, and 50 workers were collected from each colony, totaling 150 workers. These workers were dissected and categorized according to the presence of the dye in the post-pharyngeal gland, thus dyed and non-dyed. A Chi-squared test was applied to results, where H_0 proportions were equal between what was observed and expected, with α=5% (Table 4). The analyses were performed using the R 3.1.1 program (R Core Team, 2015).

III. Results

Processing and incorporation of citrus pulp pellets involve several stages, which, in general, are very similar to those of leaves: Holding the substrate on the surface of the fungus, licking the substrate, scraping the substrate, chopping the substrate, depositing fecal fluids, inserting the substrate into the surface of the fungus and inoculating hyphae into the substrate fragment inserted into the fungus.

Some behaviors above described were not observed in the experiment, such as scraping the substrate and depositing fecal fluids. The sequence of observed behaviors happened as follows: the pellets were carried inside the colony by workers and then deposited on the surface of the fungus garden. Some workers held the substrate on the surface of the fungus while other workers licked it. After this stage, the workers chopped the pellets, which, in turn, were chewed, making them soft and wet. Afterwards, the workers incorporated pellet fragments already processed into the fungus garden and deposited hyphae on their surface.

The behavior with higher frequency of observation was that of holding the pellet on the surface of the fungus, accounting for 49.67% (Table 3) of all performed behaviors. The most frequently recorded size category performing this behavior was that represented by medium workers, responsible for 95.83% (Table 2) (Z_I1= 1.869867; Z_I2= 6.335226; Z_I3= -5.16939), followed by small ones 4.17% (Z_I1= -4.91235;Z_I2= -3.89842;Z_I3= -2.85388).

The behavior of licking the pellet was second in frequency, accounting for 26.79% (Table 1) of all performed behaviors. Medium workers were those that most frequently performed this act, with 89.65% (Table 2) (Z_I1= 7.794788; Z_I2= -6.81778; Z_I3= -2.28958), followed by small ones 10.35% (Z_I1=2.633788; Z_I2= 1.031894; Z_I3= 1.393092).

The behaviors of chopping the pellet, chewing the sides of the pellets and incorporating the pellet fragment into the fungus had, respectively, frequencies of 16.56%, 3.44% and 2.5% (Table 3). It was possible to observe that medium workers had a higher frequency in the performance of the behavior of chopping the pellet 92.69% (Table 2) (Z_I1=-8.77159; Z_I2=2.050101; Z_I3=7.527665), while small ones performed 7.31% of this act (Z_I1=-3.49144; Z_I2=1.988575; Z_I3=0.094049). About the activity of chewing the sides of the pellet, medium workers had higher frequency performing this act, with 94.32% (Table 2) (Z_I1=-2.51003; Z_I2=-2.36097; Z_I3=6.115607), while small ones performed 5.68% (Z_I1=-0.76143; Z_I2= 0.194791; Z_I3= -1.1237). The behavior of incorporating the pellet fragment into the fungus was performed with higher frequency by medium workers 73.44% (Table 2) (Z_I1=-2.663; Z_I2= -0.18344; Z_I3=-0.53046), while small ones performed 26.56% of this act (Z_I1=-0.41797; Z_I2= 5.307338; Z_I3=4.498051).

Finally, the behavior observed with the lowest frequency was that of incorporating hyphae into the pellet fragment deposited on the fungus, with 1.1% (Table 1). Only in this activity, small workers performed this behavior with higher frequency, with 88.89% (Table 2) (Z_I1=27.3306; Z_I2= -1.08545; Z_I3=2.717899), while medium ones performed 11.11% (Z_I1=*-2.48335*; Z_I2= -*4.42169*; Z_I3=*-2.25726*).

A Kruskal-Wallis test was conducted to verify whether there was difference of frequency between the eight studied behaviors. The Kruskal-Wallis nonparametric test rejected the hypothesis that all behaviors would have the same distribution $\chi^2_{7\ g.l.}$ = 20.373 P value < 0.05. Table 3 shows that some behaviors had significant difference when compared to other behaviors, such as licking the pellet on the surface of the fungus, holding the pellet on the surface of the fungus, self-cleaning and mutual cleaning.

According to the analyses, licking the pellet on the surface of the fungus had significant difference and higher frequency of occurrence in relation to the behavior of incorporating hyphae into the pellet fragment deposited on the fungus (Table 3). The behavior of holding the pellet on the surface of the fungus had significant difference and higher frequency of occurrence in relation to the behaviors of chewing the sides of the pellet, incorporating the pellet fragment into the fungus and incorporating hyphae into the pellet fragment deposited on the fungus (Table 3). The behavior of chopping the pellet had significant difference and lower frequency of occurrence in relation to the behavior of mutual cleaning (Table 3). The behaviors of self-cleaning and mutual cleaning had significant differences and higher frequencies of occurrence in relation to the behaviors of chewing the sides of the pellet, incorporating pellet fragment into the surface of the fungus and incorporating hyphae into the pellet fragment deposited on the fungus (Table 3).

Regarding the additional experiment with Sudan III tracer dye (Table 4), it was possible to observe that in a higher concentration of this substance, there was significant difference between workers with the dyed and the non-dyed post-pharyngeal gland. A total of 88.7% of sampled workers had their post-pharyngeal gland dyed, and 11.3% had no dye, which was statistically significant (X^2 =44.85, p< 0.05).

IV. Discussion

With the results obtained in this study, we corroborate the hypothesis that direct contact with baits causes the contamination of workers, considering the behavioral repertoire of workers performed during the processing of citrus pulp-based pellets and, then, the frequency of workers dyed with fat-soluble tracer dye (Figure 1).

The citrus pulp-based pellet processing experiment provided that the most recurrent behavioral act was that of holding the pellet on the surface of the fungus. According to Garrett et al., (2015), about leaf processing, the act of holding fragments or leaf discs promotes their stability, decreasing movement and causing some tension for cut or other processing behaviors, which might reduce energy expenditure.

The second most frequent behavior was that of licking the citrus pulp pellet (Table 2). Garrett et al., (2015) showed that there is in leafcutter ants the presence of antibiotic and antifungal properties in the saliva of workers coming from metapleural and mandibular glands secretions, which are transferred to leaves when they lick their surface, promoting the asepsis that benefits the symbiont fungus. It was also proposed that the act of licking the leaf promotes the removal of epicuticular wax (Guerrett et al., 2015). In addition, Silva et al.,2015 inferred that the act of licking becomes frequent in citrus pulp-based pellets, as it hydrates and then softens them, facilitating their processing.

The third most frequent behavior was that of chopping the bait. This behavioral act was the only one observed in *Atta cephalotes* in the processing of leaves responsible for breaking this substrate into fragments (Garrett et al., 2015). We can infer that by breaking the substrates into smaller fragments workers can perform other processing behaviors more easily.

Concerning the table of standardized residues and frequency of behaviors in relation to the size of the workers that performed them (Table 2), we observed that out of the six behavioral acts related to direct contact five had higher frequency of occurrence on the part of medium workers, and these behaviors were: holding the pellet on the surface of the fungus, licking the pellet on the surface of the fungus, chopping the pellet, chewing the sides of the pellet and incorporating pellet fragment into the fungus. Small-sized workers performed with higher frequency the behavior of incorporating hyphae into the pellet fragment deposited on the fungus in relation to medium ants.

With the results obtained through the nonparametric Anova (Table 3), it was possible to observe that the acts with significant difference in relation to the frequency of occurrence were: holding the pellet on the surface of the fungus, licking the surface of the pellet, self-cleaning and mutual cleaning. Based on that, we conjectured that these behaviors relate significantly to the contamination of workers in the studied colonies.

About the post-pharyngeal gland experiment, the pellets provided to the colonies were prepared with the same basis of ingredients of toxic baits but, in this case, without the presence of the toxic agent that causes the workers' death. Twenty-four hours after the citrus pulp pellets prepared with Sudan III dye were introduced into the colonies, the workers dissected after the processing of this substrate presented in their majority dyed post-pharyngeal glands (Table 4). Decio (2016) showed that the lumen of the gland contained "liquids of oily and yellowish aspect" and that they might come from the workers' food. Thus, there is a chance for the behaviors performed during the processing of pellets being responsible for the presence of dye in the post-pharyngeal gland and, during the digestion of toxic baits, processing behaviors and the gland becoming routes of entry for toxic agents present in the baits of the workers' bodies, causing their contamination.

With the conduction of this study, we conclude that during the process of citrus pulp pellets the two behaviors that occurred most frequently and that showed significant difference in relation to the other behaviors were: holding the pellets on the surface of the fungus and licking the pellets. In this way, when toxic baits are used as a form of control of leafcutter ants, these two behaviors, along with other factors, can be involved more significantly with the contamination of workers. Thus, it has been proved in this study that direct contact with the baits causes the contamination of workers.

V. Conclusion

We conclude that during the process of citrus pulp pellets the two behaviors that occurred most frequently and that showed significant difference in relation to the other behaviors were: holding the pellets on the surface of the fungus and licking the pellets.

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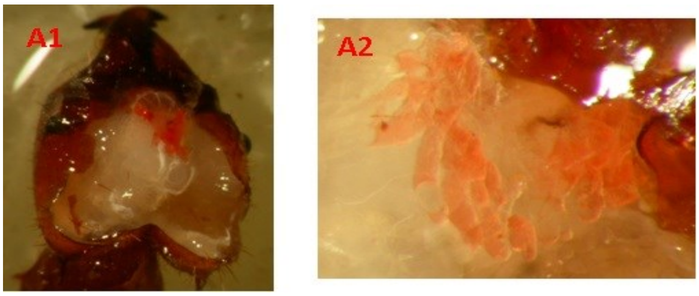

Fig. 1. A1- Worker dissected with dyed post-pharyngeal gland(Sudan III dye) andA2- Detail of dyed post-pharyngeal gland. (60 and 120 X magnification)

Table 1. Total frequency and total percentage of behaviors performed by workers in the analyzed colonies.

Behaviors	Absolute frequency	%
Licking the pellet on the surface of the fungus	686	26.79
Holding the pellet on the surface of the fungus	1272	49.67
Chopping the pellet	424	16.56
Chewing the sides of the pellet	88	3.44
Incorporating the pellet fragment into the fungus	64	2.5
Incorporating hypha into the pellet fragment deposited on the fungus	27	1.1
Total	2561	100

Table 2. Standardized residues in the contingency of behaviors performed by *Atta sexdens rubropilosa* during preparation and incorporation of the substrate composed of citrus pulp pellets.

Behaviors	Head width (mm)	Observed frequency	%	Standardized residues		
				$Z_{I}1$	$Z_{I}2$	$Z_{I}3$
Licking the pellet on the surface of the fungus	0.8 - 1.2	71	10.35	**2.633788**	1.031894	1.393092
	1.3 - 1.6	615	89.65	**7.794788**	*-6.81778*	*-2.28958*
Total		686	100			
Holding the pellet on the surface of the fungus	0.8 - 1.2	53	4.17	*-4.91235*	*-3.89842*	*-2.85388*
	1.3 - 1.6	1219	95.83	1.869867	**6.335226**	*-5.16939*
Total		1272	100			
Chopping the pellet	0.8 - 1.2	31	7.31	*-3.49144*	**1.988575**	0.094049
	1.3 - 1.6	393	92.69	*-8.77159*	**2.050101**	**7.527665**
Total		424	100			
Chewing the sides of the pellet	0.8 - 1.2	5	5.68	-0.76143	0.194791	-1.1237
	1.3 - 1.6	83	94.32	*-2.51003*	*-2.36097*	**6.115607**
Total		88	100			
Incorporating pellet fragment into the fungus	0.8 - 1.2	17	26.56	-0.41797	**5.307338**	**4.498051**
	1.3 - 1.6	47	73.44	*-2.663*	-0.18344	-0.53046
Total		64	100			
Incorporating hypha into the pellet fragment deposited on the fungus	0.8 - 1.2	24	88.89	**27.3306**	-1.08545	**2.717899**
	1.3 - 1.6	3	11.11	*-2.48335*	*-4.42169*	*-2.25726*

Table 3. Likelihood value of the multiple comparison of independent samples analysis, with Conover test, at 5% significance.

Behaviors	Licking the pellet on the surface of the fungus	Holding the pellet on the surface of the fungus	Chopping the pellet	Chewing the sides of the pellet	Incorporating pellet fragment into the fungus	Incorporating hypha into the pellet fragment deposited on the fungus	Self-cleaning	Mutual cleaning
Holding the pellet on the surface of the fungus	1	*	*	*	*	*	*	*
Chopping the pellet	1	0.24123	*	*	*	*	*	*
Chewing the sides of the pellet	0.17906	**0.00282**	1	*	*	*	*	*
Incorporating pellet fragment into the fungus	0.05383	**0.00092**	0.43498	1	*	*	*	*
Incorporating hypha into the pellet fragment deposited on the fungus	**0.00671**	**0.00014**	0.05383	1	1	*	*	*
Self-cleaning	0.5815	1	0.07274	**0.00092**	**0.00031**	**5.3e-05**	*	*
Mutual cleaning	0.32435	1	**0.03985**	**0.00053**	**0.00019**	**3.3e-05**	1	*

Bold values represent significant difference of the frequency of occurrence between behaviors.

Table 4. Total frequency of workers with Sudan III tracing dye present in the post-pharyngeal gland. Chi-squared test applied to results.

Colonies	Dyed workers	Non-dyed workers	Total
1	45	5	50
2	46	4	50
3	42	8	50
Total	133	17	150
(%)	88.67	11.33	100