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Variations in the Cuticular Chemical Profile of the Ant *Ectatomma brunneum* Smith, F. 1858 (Formicidae: Ectatomminae) Across Different Biomes in Brazil

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Abstract

The insect cuticle is composed of chemical compounds that evolved to prevent desiccation and provide defense against pathogens. These compounds were subsequently co-opted for chemical communication and, in social insects, are crucial for nestmate recognition and caste discrimination. Several factors can influence the cuticular chemical composition, making it a good biogeographical study tool, since it varies among populations, according to genetic and environmental factors. The use of cuticular compounds in studies of the social Hymenoptera is important for understanding the influence of the environment on the distribution and occurrence of species. This study aimed to determine whether environmental factors might be decisive in determining the cuticular chemical profiles of colonies of the ant *Ectatomma brunneum* (Smith). Samples were collected in different biomes of Brazil, for analysis using Fourier transform infrared-photoacoustic spectroscopy (FTIR-PAS). Similarly to other phenotypic characteristics, the cuticular chemical profile varied significantly among populations, confirming its potential as a tool to assess biogeographical differences. Furthermore, environmental factors were important determinants of the cuticular composition of this species of ant.

Introduction

The lipid layer, which constitutes the outermost portion of the insect cuticle, is composed of hydrocarbons, among other substances (Lockey, 1988). The cuticular hydrocarbons (CHs), including linear or branched alkanes and alkenes, are involved in chemical communication (Blomquist & Bagnères, 2010) and represent the dominant structural group in the epicuticle (Kather et al., 2011).

The cuticular chemical compounds provide species-specific chemical signatures, but also present intraspecific variability (Michelutti et al., 2017; Blomquist & Ginzel, 2021). In social insects, these compounds may vary according to the function of the individual in the colony, in addition to signaling reproductive status, age, and sex (Wyatt, 2003; Howard & Blomquist, 2005; Leonhardt et al., 2016; Soares et al., 2017; Firmino et al., 2020). Several studies have reported intraspecific variations in CHs composition between different insect populations (Panek & Gamboa, 2000; Cotoneschi et al., 2007; Buczkowski & Silverman, 2006; Khidr et al., 2013; Menzel et al., 2017).

These intraspecific differences can be explained by variations in factors that may be genetic (Howard & Blomquist, 2005), environmental (Menzel et al., 2017), or both

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(Hartke et al., 2019; Sprenger & Menzel, 2020). Genetic factors determine the biosynthesis of CHs and their phenotypic expression (Hartke et al., 2019), acting as the basis for variations (Sprenger & Menzel, 2020). On the other hand, environmental factors, such as climatic differences and the types of resources exploited by populations, are also important in shaping the cuticular chemical profile (Menzel et al., 2017).

The diet is one of the main environmental factors responsible for variation of the CHs composition (Bernardi et al., 2014; Mothapo & Wossler, 2016; Menzel et al., 2017; Sprenger & Menzel, 2020). The availability of resources varies with the environment, so the cuticular composition can be expressed differently between populations (Menzel et al., 2017). In addition, the CHs profile may be affected by temperature and/or humidity conditions, which can also vary between populations of the same species (Michelutti et al., 2018; Menzel et al., 2018).

Therefore, the cuticular chemical profile constitutes an important tool for evaluating geographic differences between populations (Cunha et al., 2017). Previous studies have already identified population-level variations in the cuticular chemical profile of different groups of social insects (Nielsen et al., 1999; Neves et al., 2012; Bernardi et al., 2014; Mothapo & Wossler, 2016). Hence, the cuticular chemical profile can provide helpful information not only about individual species, but also about the environments in which they live (Dapporto et al., 2004a).

The ant species *Ectatomma brunneum* (Smith) (Formicidae: Ectatomminae) occurs in the Neotropics and is distributed from Panama to Argentina, generally being found in forested areas (Brown, 1958). In Brazil, *E. brunneum* frequently occurs in areas with open vegetation, such as forest edges and clearings, as well as in crops, pastures, and areas of secondary vegetation (Kempf, 1972).

Some studies have already assessed the effects of environmental factors on the cuticular chemical profile of *E. brunneum*, such as the work of Bernardi et al. (2014), who investigated the importance of diet in determining the cuticular chemical composition of this ant. Firmino et al. (2020) suggested that variations in CHs among different *E. brunneum* populations were influenced by the effect of geographical distance in determining gene flows between populations, in addition to environmental conditions such as temperature, humidity, and available resources. In laboratory experiments, Duarte et al. (2019) observed the adjustment of the cuticular chemical composition of workers of this species in response to exposure to different temperature conditions. Although these studies showed that environmental factors can cause variations in the cuticular hydrocarbons profile of this species, no previous studies were performed with samples from populations originating from different biomes in Brazil. Therefore, this study aimed to determine whether environmental factors might be decisive in determining the cuticular chemical profiles of colonies of the ant *Ectatomma brunneum* (Smith) nesting in different biomes.

Materials and Methods

Colony collection

Analysis was made of the cuticular chemical profiles of a total of 118 foragers, collected in six regions of Brazil, covering four biomes (Figure 1): the Amazon biome, in Bragança, Pará state (01°03′13″ S; 46°45′56″ W); a transition area between the Cerrado and Atlantic Forest biomes, in Dourados, Mato Grosso do Sul state (22°13′16″ S; 54°48′20″ W); the Atlantic forest biome, in Itabuna, Bahia state (14°47′08″ S; 39°16′49″ W); transition areas between the Atlantic Forest and Cerrado biomes, in Pirassununga (22°00′00″ S; 45°25′42″ W) and Rio Claro, both in São Paulo state; and the Pantanal biome, in Porto Murtinho, Mato Grosso do Sul state (22°24′41″ S; 47°33′41″ W).

The Amazon biome consists mainly of dense or open ombrophilous forests, while the Atlantic Forest biome consists of semideciduous seasonal forests, as well as dense and mixed ombrophilous forests. The Cerrado is a Brazilian savanna biome, which may also occur in some transitional areas. In the present work, transitional areas between the Cerrado and Atlantic Forest were characterized by semideciduous seasonal forest, a typology characteristic of the Atlantic Forest, which generally occurs along river courses in the Cerrado, flanked by savanna regions. The Pantanal biome, in a vast floodplain, has a mosaic of vegetation types (IBGE, 2012).

For the collection of each ant, the researcher would walk 100 m away from the point where the previous forager had been collected, to ensure that the ants were not from the same colony. The number of ants collected from each region varied from 10 to 20, depending on the density of colonies and the foraging activities of the ants. The ants were collected with tweezers during their foraging activity, placed in 500 mL plastic containers, and sacrificed by freezing at -20 °C. The ants were kept cool in a Styrofoam box with ice during transport to the Behavioral Ecology Laboratory at the State University of Mato Grosso do Sul, where they were analyzed.

To standardize the age of the workers, only foragers were collected, since it has been found that cuticle compounds may vary according to age (Cuvillier-Hot et al., 2001).

Cuticular chemical profile analysis

The samples were analyzed using Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS), a technique that has been successfully applied to samples of social wasps and ants (Gibbs & Crowe, 1991; Antonialli-Junior et al., 2007; Neves et al., 2019; Firmino et al., 2020). This technique maintains the integrity of the sample and does not require any preparation or solvent use (Sguarizi-Antonio et al., 2017).

For chemical analysis, the moisture was removed from the samples for 12 h using a vacuum desiccator (model 0810, Nalgon), according to the methodology described by Sguarizi-Antonio et al. (2017). Only the gasters of the ants were used, since the goal was not to determine the diversity of

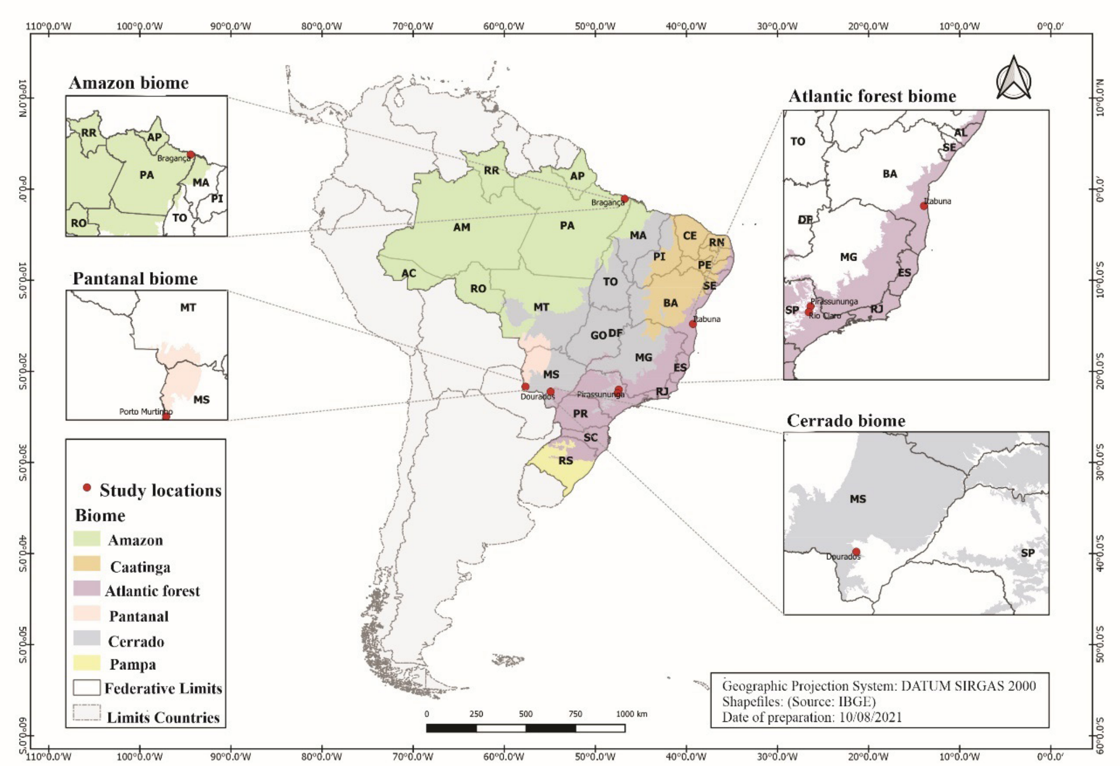

Fig 1. Sites in Brazil where the *Ectatomma brunneum* individuals were collected. The biomes represented were as follows: Amazon biome in Bragança (Pará state); transitional area between the Cerrado and Atlantic Forest biomes in Dourados (Mato Grosso do Sul state); Atlantic Forest biome in Itabuna (Bahia state); transitional areas between the Atlantic Forest and Cerrado biomes in Pirassununga and Rio Claro (São Paulo state); Pantanal biome in Porto Murtinho (Mato Grosso do Sul state).

compounds in each sample, but rather to compare the spectra of samples from different biomes. Furthermore, according to Cuvillier-Hot et al. (2001), this region of the ant has a higher concentration of hydrocarbons.

The samples were analyzed using a spectrophotometer (Nexus 670, Thermo Nicolet, Madison, USA) coupled to a photoacoustic detector (MTEC-300, Ames, Iowa, USA), following the methodology described previously (Neves et al., 2012, 2013). Identification was made of the peaks in the primary absorption regions related to hydrocarbons, as indicated by previous insect studies using this technique (Cunha et al., 2017; Sguarizi-Antonio et al., 2017; Firmino et al., 2020; Paula et al., 2020). The spectra were acquired between 400 and 4000 cm^{-1}, at a resolution of 8 cm^{-1}. A total of 64 readings were made for each gaster, which were averaged to obtain the final spectrum.

Statistical analysis

The main absorption peaks corresponding to functional groups were identified using Origin software. Discriminant function analysis (DFA) was applied to the data for the intensities of the most relevant absorption peaks to evaluate whether the populations from the different biomes differed in terms of the functional groups. This type of statistical analysis enables the identification of a set of variables most suitable for differentiating the groups evaluated (Quinn & Keough, 2002). The Wilks' lambda was used as a measure of the difference between groups, where values close to 0 indicated no overlap between groups. In contrast, values close to 1 indicated considerable overlap between groups and, consequently, a lack of significant differences between them (Manly, 2008).

To assess whether there was spatial autocorrelation for the different profiles of CHs, Moran's I method was used for each functional group, using the geographic coordinates of each sampled location for the analyses. All the statistical analyses were performed using Statistica 14 software (Tibco, 2020).

Results

The average mid-infrared absorption spectra for all the populations from the different biomes are shown in Figure 2. From these spectra, 17 functional groups with more intense signals were selected, with the vibrational modes identified from data reported in the literature (Lin-Vien et al., 1991).

The discriminant analysis found twelve statistically significant peaks that were more relevant for the separation of the groups (Table 1). The most important peak for group separation was at 1650 cm^{-1}, attributed to the presence of alkenes, which was more intense in the spectra for the samples from Porto Murtinho (Pantanal biome) (Table 1).

Table 1. List of 17 functional groups and corresponding vibrational modes selected for statistical analysis, obtained by FTIR-PAS, for ants of the species *Ectatomma brunneum* collected in six regions representing different biomes in Brazil.

Peak	Wavenumber (cm^{-1})	Functional group	Vibrational mode
1	895^*	=C-H Alkenes	Deformation out of plane
2	953	=C-H Alkenes	Deformation out of plane
3	$1030^{\#}$	-C-O-C Ether	Asymmetric Stretching
4	1076	-O-C-C Saturated Ester	Stretch
5	$1115^{\#}$	-O-C-C Aromatic Ester	Stretch
6	1157	-C-N Amines II Saturated	Stretch
7	1242^*	-C-C-O Saturated ester (Acetate)	Stretch
8	1377^*	$-C-CH_3$ Alkenes	Symmetrical deformation
9	1454^*	$-C-CH_2$ Alkenes	Scissors folding mode
10	1550^*	-N-H Amines II	Deformation in the plane
11	$1650^{\#}$	-C=O Alkenes	Stretch
12	2854^*	$-C-H\ (CH_2)$ Methylene	Symmetrical Stretch
13	2874^*	$-C-H\ (CH_3)$ Methyl	Symmetrical Stretch
14	2931^*	$-C-H\ (CH_2)$ Methylene	Asymmetric Stretching
15	2962	$-C-H\ (CH_3)$ Methyl	Asymmetric Stretching
16	$3093^{\#}$	=C-H Alkenes	Stretch
17	3294	≡C-H Alkynes	Stretch

*Significant peaks and #most significant peaks.

The intensities of the selected peaks varied among the cuticular chemical profiles of individuals from different areas, suggesting quantitative variation in their cuticle chemical compositions (Figure 2). According to the discriminant analysis, the cuticular compositions of the samples from the different areas presented significant differences (Wilk's lambda = 0000198, F = 46.27586, $p < 0.0001$) (Figure 3). Moran's I indicated no spatial autocorrelation for any of the 17 peaks ($p > 0.05$ in all cases).

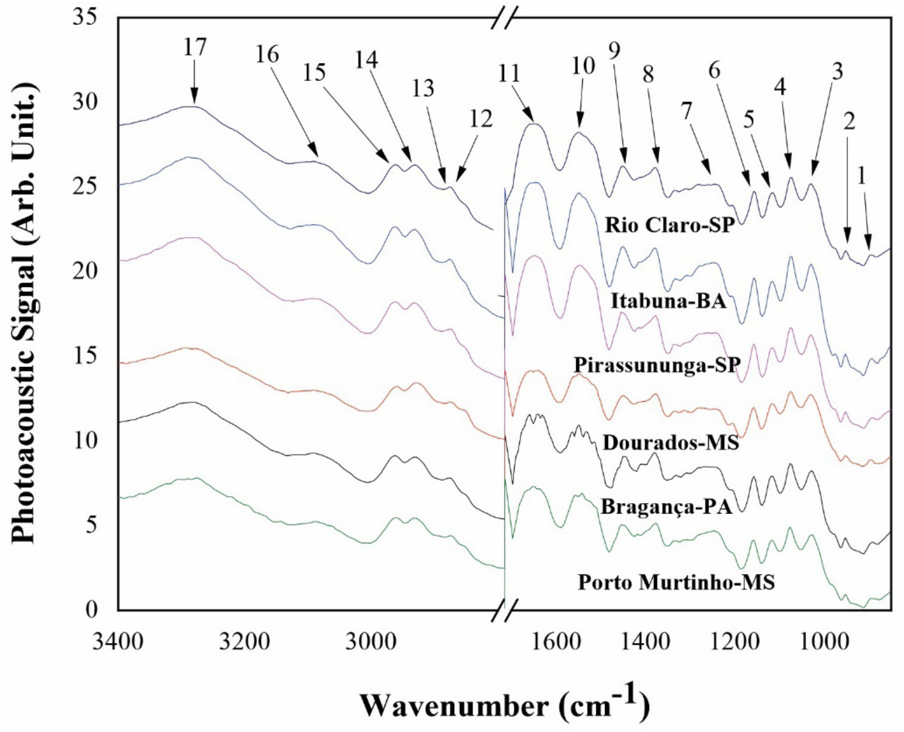

Fig 2. Mean FTIR-PAS spectra for *Ectatomma brunneum* samples from six regions representing the following biomes: Amazon biome in Bragança (Pará state); transitional area between the Cerrado and Atlantic Forest biomes in Dourados (Mato Grosso do Sul state); Atlantic Forest biome in Itabuna (Bahia state); transitional areas between the Atlantic Forest and Cerrado biomes in Pirassununga and Rio Claro (São Paulo state); Pantanal biome in Porto Murtinho (Mato Grosso do Sul state).

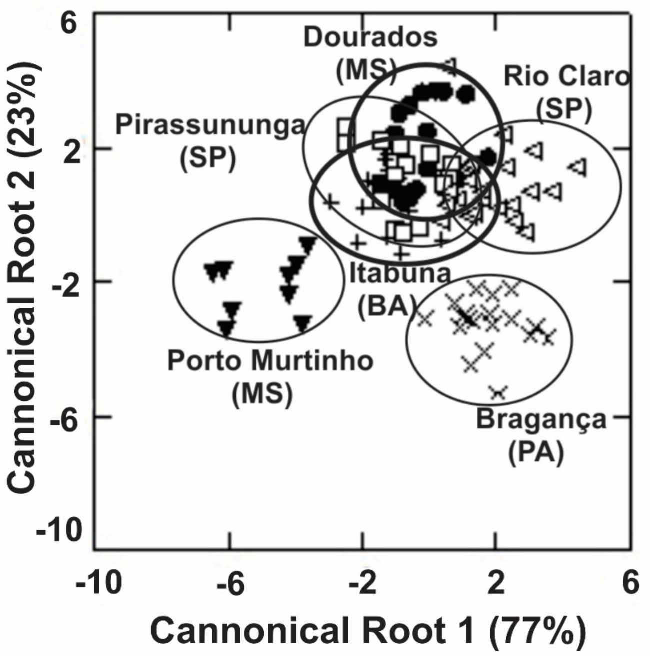

Fig 3. Scatter plot showing the differentiation of the cuticular chemical profiles identified by FTIR-PAS in the ant species *Ectatomma brunneum*, using individuals collected in six regions representing different biomes in Brazil.

Discussion

The results validated the hypothesis that environmental factors were decisive in determining the cuticular chemical profiles of the *E. brunneum* colonies. Visual inspection of the average absorption spectra revealed variations among the chemical profiles and intensities of the signals for the different populations (Figure 2). The 17 functional groups, with their corresponding wavenumbers and selected vibrational modes, mainly represented hydrocarbons present in the ant cuticle, although amines and carbonyls were also identified (Table 1).

As reported by Lockey (1988), in addition to CHs, compounds such as esters, alcohols, fatty acids, paraffins, acylglycerides, phospholipids, and glycolipids are also present in insect cuticles. Bernardi et al. (2014), who used the same technique applied to samples of the same species, found a greater number of functional groups in the cuticular profile, although many functional groups were in common with this study. Therefore, the results were indicative of both qualitative and quantitative variation of these functional groups in spectra for samples of the same species. Such variations in the cuticular composition for the same species have been described previously (Foitzik et al., 2007; Leonhardt et al., 2016), showing that the compound profile can adjust in response to the specific environmental pressures to which different populations are exposed.

The most important peak for separating the groups was at 1650 cm^{-1}, corresponding to the presence of alkenes, with the highest intensity for the samples from Porto Murtinho (Pantanal biome). This peak has been found in other studies (Ferveur & Jallon, 1996), with strong evidence that the associated compounds are involved in chemical communication (Gibbs, 2002). Using the FTIR-PAS technique, Tofolo et al. (2014) also reported a peak at 1650 cm^{-1} for samples of the ant *Ectatomma opaciventre* (Roger).

A peak at 1030 cm^{-1} was more intense for the samples from Dourados and Pirassununga (transition areas of the Cerrado and Atlantic Forest biomes) (Table 1, Figure 2). A peak at 1115 cm^{-1} was more intense for the samples from Bragança and Porto Murtinho (in the Amazon and Pantanal biomes, respectively) (Table 1, Figure 2). These peaks could be attributed to the presence of esters, which were detected previously by Tofolo et al. (2014) in samples of *E. opaciventre* and by Bernardi et al. (2014) in a study of the effect of diet on the cuticular composition of *E. brunneum*.

A peak at 3093 cm^{-1} was more intense for the samples from Rio Claro and Bragança, in the transitional Atlantic Forest/Cerrado and Amazon Forest biomes, respectively (Table 1, Figure 2), with the results showing quantitative variations among the samples from different biomes.

In investigations of the intraspecific variation of the CHs composition for the same ant species, Firmino et al. (2020) and Pereira et al. (2019) observed significant differences between populations, using analysis by gas chromatography coupled with mass spectrometry (GC-MS). The data corroborated the present results, reinforcing the utility of the FTIR-PAS technique for assessment of intraspecific differences in the CHs composition.

The findings demonstrated the occurrence of intraspecific variations in the cuticular chemical profiles of ants of this species, which could reflect adaptations to local conditions (Ridley, 2006). In other work using the FTIR-PAS technique, Cunha et al. (2017) and Neves et al. (2019) found significant intraspecific differences in the cuticular chemical profiles of the wasp *M. consimilis*, which were attributed to variations in the environmental conditions to which the populations were exposed.

The significant differences found between samples from different areas (biomes) suggested that the cuticular composition could be used as a valuable biogeographic tool. This contrasted with the findings of Santos and Nascimento (2017), in a study of orchid bees (*Euglossa cordata* (Linnaeus)) from different biomes of the Neotropical region, where no correspondence was observed between the cuticular profiles of the bees and the biomes in which they occurred.

The present results indicated that environmental factors strongly influenced the cuticular compositions of the samples of this species, since the groupings appeared to be influenced to a greater extent by the biome (including the transitional areas) than the geographic distances between the populations. This is shown in Figure 3, revealing three groupings of collection locations: (1) Itabuna, Pirassununga, Rio Claro, and Dourados, (2) Porto Murtinho, and (3) Bragança. Using Moran's I statistic indicated that geographic distances alone could not explain the differences and similarities among the samples.

It was evident that some types of samples were grouped in ways that did not reflect the geographic distances between the collection points (Figures 1 and 3). The groupings occurred as a function of the environment in which the colonies were nesting. The first grouping (Itabuna, Pirassununga, Rio Claro, and Dourados) could probably be explained by the fact that the individuals were all in the Atlantic Forest biome. It should be noted that although Dourados is considered to be in the Cerrado biome, the collection area was in a transition zone between Cerrado and Atlantic Forest, explaining its grouping with the other samples influenced by the Atlantic Forest biome (Figures 1 and 3). Meanwhile, the samples from Porto Murtinho, in the Pantanal biome, and Bragança, in the Amazon biome, formed separate groups, with the last biome seeming to be the most different from the others (Figure 3).

Several studies have shown that the environment has a strong influence on CHs (Liang & Silverman, 2000; Buczkowski & Silverman, 2006; Sorvari et al., 2008). Menzel et al. (2018) and Michelutti et al. (2018) discussed the ways that environmental factors can cause variation in the cuticular chemical profile composition between populations. The climate is one of the most important environmental factors determining the cuticular chemical profile (Dapporto et al., 2004a). It has been shown that the temperature to which insects are exposed can lead to adjustments of cuticle composition (Menzel, 2017; Michelutti et al., 2018). Therefore, since different biomes have distinct climatic regimes, differences in cuticular chemical profiles may be related, at least in part, to differences in temperature and relative humidity conditions.

All the biomes were characterized as tropical, with the Cerrado biome being distinguished by having two distinct seasons: a prolonged dry season, lasting approximately five months, and a rainy season for the rest of the year (Eiten, 1994). The climate of the Atlantic Forest biome also has two seasons, but with high temperatures and abundant rainfall throughout the year, due to humid air masses from the Atlantic Ocean (Marques et al., 2021). The Amazon biome has high average temperatures and two seasons, one dry and the other wet, with high relative humidity of the air, averaging 88% during the rainy season and 77% during the dry season (Coutinho, 2016). The Pantanal biome is also characterized by high temperatures, but features a flood (wet) season quite distinct from the dry season, with waters arriving from the surrounding higher regions (Coutinho, 2016). Therefore, the differences in average temperatures and, especially, relative humidity, as well as the time of year and the number of months defining each season in each of the biomes, can result in the ant populations being exposed to conditions of temperature and relative humidity that induce adjustments of the cuticular profiles.

Another important and potentially determinant environmental factor is the diet. Studies such as those by Liang and Silverman (2000), Buczkowski and Silverman (2006), and Bernardi et al. (2014) showed that dietary variations influence the cuticular compound profile. Bernardi et al. (2014) investigated the same species of ant from this study. After removing it from its natural environment and housing it in the laboratory, they verified changes in the chemical signature in the first 30 days in the ants treated under feeding control. Buczkowski and Silverman (2006) reported that regional variations found in the chemical compositions of the cuticles of *Linepithema humile* (Mayr) ants were due, in part, to their diets.

In addition, the observed variations in the CHs could be influenced by other specific environmental factors, such as the type of plant material composing the substrate of the nests, as observed in social wasps (Sguarizi-Antonio et al., 2017), as well as the type of soil, reinforcing that several environmental factors can contribute to determining the cuticular chemical composition.

It is essential to consider the importance of genetic factors in establishing the cuticular chemical profiles. For example, in an investigation of the variation of cuticular chemical profiles between populations of the social wasp *Polistes dominula* (Christ), Dapporto et al. (2004b) found a correlation between cuticular composition and genetic factors. Hence, similarities between neighboring populations can be attributed to the genetic factors they share (Howard & Blomquist, 2005), as well as environmental factors (Buczkowski & Silverman, 2006). Therefore, interpretation of the results obtained here should not exclude the possibility that combinations of exogenous factors and genetic aspects such as differences in allele frequencies, phenotypic plasticity, and gene flow (Ridley, 2006) acted together in determining the cuticular chemical profiles (Dapporto et al., 2004b; Cunha et al., 2017).

It could be concluded from the results that, in common with other phenotypic traits, the cuticular chemical composition varies significantly across populations, confirming its utility as a tool to assess biogeographic differences.

Authors' Contributions

W.F. Antonialli-Junior: Conceptualization, resources, supervision, visualization, review & editing.
M.C. Paula-Silva: Conceptualization, investigation, data curation, writing-original draft, visualitation.
E.F. Neves: Conceptualization, formal analysis, investigation, data curation, writing-original draft.
S.E. Lima-Junior: Methodology, formal analysis, investigation, visualization.
L.H.C. Andrade: Methodology, resources, formal analysis, writing-original draft, writing-review & editing.
S.M. Lima: Methodology, resources, formal analysis, writing-original draft, writing-review & editing.

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