Deciphering the chemical phenotype in *Atta laevigata* (Smith, 1858) (Hymenoptera: Formicidae): A relationship between polymorphism and cuticular hydrocarbons

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**Abstract.** In the Attina subtribe the division of labor among workers is based on different tasks performed by morphological subcastes. Considering that cuticular chemical compounds play important roles as protection against water loss and mediates interactions between nestmates, and that studies on the cuticular chemical profile of ants of the genus *Atta* are still scarce, the aim of this study was to evaluate the relationship between morphological and chemical phenotypes in the subcastes of *Atta laevigata* (Smith, 1858) workers. The cuticular chemical composition of the different subcastes was assessed by Gas Chromatography coupled to Mass Spectrometry technique. The compounds found in the cuticle of the different subcastes had carbon chain size between 18 and 31 atoms, with higher content of linear alkanes in workers and soldiers and higher content of branched alkanes in gardeners and generalists, probably related to the type of tasks they perform in their colonies, since some workers perform more intra- or extranidal tasks in relation to others. The results show significant qualitative differences in the cuticular composition of the different subcastes, with a greater relationship between them according to the environmental restrictions that each subcaste is more subjected to due to the role played in the colony.

**Keywords.** Myrmicinae; Subcastes; Cuticular hydrocarbons; Leaf-cutting ants.

**INTRODUCTION**

Ants of the genus *Atta* (Fabricius, 1804) cut and transport parts of plants into the colonies, where the plant material will be used to cultivate the symbiont fungus that feed the colony (Hölldobler & Wilson, 2010). For this reason, they are popularly known as leaf-cutting ants. The success in the occupation of niches and maintenance of the colonies is due to the division of labor between workers that is made according to morphological polyethism, that is, in *Atta* the subcastes are morphologically distinct and the tasks performed by each worker in the colony are determined according to their morphological characteristics (Hölldobler & Wilson, 2010), nevertheless, there may be behavioral plasticity depending on whether the colony’s foundation is recent or if there has been some environmental disturbance that might require the relocation of subcaste for a specific task (Hölldobler & Wilson, 1990; Rodriguez, 2014; Gordon, 2016). However, in mature colonies fidelity to the task is advantageous to individuals, and can be reinforced with aggression (Hart & Ratnieks, 2001, 2002).

In colonies of *Atta sexdens* (Linnaeus, 1758), there are four subcastes: the gardeners are responsible for maintaining the interior of the colo-
ny, taking care of the offspring, queen and symbiont fungus; the generalists perform all kinds of tasks within the colony, such as sanitizing the colony and cutting plant material into smaller particles to provide as substrate for the symbiont fungus; the foragers spend much of their time outside the colony cutting and selecting plant material; and the soldiers in addition to foraging in search of resources are also responsible for the defense of the colony (Wilson, 1980). This scheme of division of labor based on morphological subcastes happens according to the needs of the colony, it is spatiotemporally stable, but it can vary in specific environmental conditions and depending on the age of the colony (Hölldobler & Wilson, 1990). These subcastes, in addition to morphological differences, may present differences in the cuticular chemical composition of compounds responsible for the interactions between individuals of their colonies, the so-called cuticular hydrocarbons (CHCs) (Valadares & Nascimento, 2016). In ants, hydrocarbons can be found in many parts of the body besides the cuticle. In colonies of Atta laevigata (Smith, 1858), these compounds can be produced by the post pharyngeal glands and mandibular glands (Hernández et al., 2002).

The CHCs profile can mediate the division of labor acting as signs for division of tasks in ant colonies (Greene & Gordon, 2003), however, these compounds also evolved to act as a barrier against desiccation and protection against pathogenic microorganisms (Martin & Drijfhout, 2009). The CHCs are organic molecules that have only carbons and hydrogens in their composition, and can be saturated as alkanes and unsaturated as alkenes and alkynes (Blomquist & Bagnères, 2010). Linear alkanes seem to be more important in protecting against parasites and preventing desiccation (Blomquist & Bagnères, 2010), while branched alkanes are probably more involved in nestmate recognition (Martin & Drijfhout, 2009).

The cuticular hydrocarbon profile varies between species, different colonies (Blomquist & Bagnères, 2010), and in some species such as A. sexdens, which is polymorphic, it may also vary according to the different morphological subcastes of workers (Valadares & Nascimento, 2016), those who perform intranidal tasks have more branched alkanes, while those who perform extranidal tasks have more linear alkanes. Since some compounds may be more effective in avoiding desiccation (Blomquist & Bagnères, 2010), others in mediating interactions (Greene & Gordon, 2003) and even being involved in more than one role (Blomquist & Bagnères, 2010), the subcastes may differ in CHCs composition depending on how exposed to the external conditions of the nest they are (Wagner et al., 2001) or even how much they interact with other workers.

Even in species whose organization of colonies is different from the genus Atta, such as Pogonomyrmex barbatus (Smith, 1858), workers who perform different types of tasks, have differences in the cuticular hydrocarbons profile (Wagner et al., 1998). These differences are likely related to the exchange of signals between nestmates who need to know who they are interacting with to optimize their decision-making (Greene & Gordon, 2003). Therefore, considering the importance of cuticular hydrocarbons to maintain the cohesion and mediation of interactions in ant colonies and the scarcity of data on how the variation of these compounds occurs in species whose division of labor is based on morphological subcastes, especially in the genus Atta, the aim of this study was to assess the relationship between the morphological phenotype and the chemical profile in the subcastes of workers of A. laevigata.

MATERIAL AND METHODS

Workers of A. laevigata were collected using forceps (active sampling), near the scouts and on the foraging trails of eight colonies of the species in the vicinity of the municipality of Dourados, Mato Grosso do Sul, Brazil (22°13’24.39”S; 54°54’44.53”W), with a minimum distance of 5 km from each other. The colonies were located in an urban area and in a forest area. All ants were collected in the hot and humid season, which comprises the months of April to November, in the forest area there was a wide diversity of plants having characteristics of a seasonal semideciduous forest, which was in good conservation state. On the other hand, in the urban area, the diversity of plants and vegetation cover was lower, and there were plant species characteristic of the Brazilian savannah and other invasive species used in urbanization. After collection, the ants were transported to the laboratory in plastic containers. From each colony, 15 individuals from each subcaste were collected, totaling 60 workers per colony. To identify the subcastes, we measured the cephalic capsule with the aid of an electronic caliper, ants with the width of the cephalic capsule varying between 0.8-1.0 mm were classified as gardeners, generalists had a cephalic capsule ≥ 1.4 mm, foragers had a cephalic capsule ranging from 2.0-2.2 mm, the soldiers had a cephalic capsule width > 3.0 mm. This classification was made following the criteria previously established by Wilson (1980). The species were identified with the help of a specialist. Finally, the ants were killed by freezing for extraction of the cuticular chemical compounds.

The Gas Chromatography coupled to Mass Spectrometry technique was used to assess the composition of the cuticular chemical profile for each subcaste. The cuticular chemical compounds of the whole body were extracted using 2 ml of hexane (HPLC grade), for 3 minutes and the extracts were dried under fume hood for solvent evaporation. The dry extracts were solubilized in 400 μl of hexane. The samples were analyzed using a gas chromatograph (CG-2010 Plus, Shimadzu, Kyoto, Japan) coupled to a mass spectrometer (CG-EM Ultra 2010, Shimadzu, Kyoto, Japan). The conditions of analysis were the same as described in the study of Duarte et al. (2019). The chromatograms were recorded by the software GCMS Real Time Analysis. The peak area of each compound was determined by manual integration of each total ion chromatogram (TIC). Then all areas were transformed into relative percentage areas. To identify the analyzed compounds, the calculated retention in-
Table 1. Relative abundance (%) of cuticular hydrocarbons identified in the subcastes of the ant Atta laevigata.

| RT (min) | Cl   | Compounds                        | Forager          | Soldier         | Generalist        | Gardener         |
|---------|------|----------------------------------|------------------|-----------------|-------------------|------------------|
| 12.38   | 1873 | 3-Methylcholesta-12-en-25-ol     | 12.5 ± 6.97*     | 6.81 ± 4.70     | 24.71 ± 9.25*     | 35.6 ± 11.19*    |
| 14.73   | 1960 | 4-Methylcholesta-12-en-25-ol     | 0.75 ± 1.19      | 0.35 ± 0.45     | 1.56 ± 1.68       | 2.28 ± 1.96      |
| 22.90   | 2266 | Tetracosane                       | 2.26 ± 3.66      | 0.24 ± 2.21     | 7.78 ± 9.94       | 3.72 ± 5.96      |
| 23.57   | 2281 | 3-Triicosene                      | 0.78 ± 1.13      | 0.27 ± 0.33     | 1.88 ± 1.51       | 2.43 ± 1.89      |
| 26.19   | 2387 | Tetraicosene                      | 5.61 ± 3.50      | 3.78 ± 2.35     | 15.67 ± 8.1*      | 25.98 ± 8.04*    |
| 28.69   | 2486 | Pentaicosene                      | 2.79 ± 1.92      | 2.65 ± 1.94     | 3.58 ± 2.20       | 4.56 ± 2.99      |
| 29.90   | 2533 | 13-Methylpentacosane              | 0.32 ± 0.40      | 0.30 ± 0.38     | 1.22 ± 1.20       | 1.92 ± 1.52      |
| 31.16   | 2585 | 5,9,11-Dimethylpentacosane        | 0.67 ± 0.55      | 0.87 ± 0.55     | 0.49 ± 0.60       | 0.95 ± 1.25      |
| 33.52   | 2700 | Heptacosane                       | 49.06 ± 9.82*    | 55.28 ± 7.01*    | 27.78 ± 11.83*    | 15.19 ± 6.72*    |
| 35.83   | 2783 | 5,11,13-Dimethylheptacosane       | 3.65 ± 1.53      | 4.67 ± 1.25     | 1.62 ± 1.81       | 0.24 ± 0.77      |
| 38.06   | 2886 | 4,8-; 14,16-; 14,18-Dimethylheptacosane | 20.79 ± 6.17* | 22.54 ± 6.88* | 13.59 ± 5.42* | 6.88 ± 4.32 |
| 42.36   | 3084 | 4,8-; 14,16-; 14,18-Dimethylheptacosane | 0.82 ± 1.27 | 1.74 ± 1.89 | 0.13 ± 0.51 | 0.26 ± 1.85 |

RT = Retention time; C.I. = Calculated index; * = Major compounds.

Statistical analyses

For the data obtained by GC-MS, the relative percentage abundance of the peaks of cuticular compounds present in the samples was used. To evaluate whether there are significant differences between the cuticular chemical composition of the different subcastes, a Discriminant Function Analysis was performed with the relative percentage areas of the peaks of the compounds present in the samples. In this analysis, the Wilk’s Lambda statistic is used as a measure of difference between groups, in which values close to 0 indicate that the groups do not overlap, while values close to 1 indicate that there is high overlap between the groups, and that the differences between them should not be significant. The value of “p” must be less than or equal to 0.05 so that the differences between the evaluated groups are considered significant.

To evaluate which subcastes are chemically more related, we applied a Cluster Analysis using the mean relative percentage abundance of the compounds. This analysis provides the Cophenetic Correlation Coefficient that can be used to estimate the consistency of cluster patterns. Values close to 1 indicate better representativeness. These analyses were performed using the software R (R Core Team, 2017), for the Discriminant Function Analysis the used package was “rrcov”, and for the Cluster Analysis the used package was “vegan”.

RESULTS

In total, 12 peaks related to cuticular hydrocarbons were detected in the samples of all subcastes of A. laevigata with carbon chain ranging between 18 and 30 atoms. Linear alkanes, branched alkanes, alkenes and alkadienes were identified (Table 1). All subcastes share the same peaks, therefore, there is no qualitative variation (Table 1).

 Regarding the relative proportion, the samples of foragers and soldiers present higher percentage of linear alkanes, followed by branched alkanes and, to a lesser extent, alkenes and alkadienes. Generalists, had a higher percentage of branched alkanes, followed by linear alkanes, alkenes and finally alkadienes. Gardeners presented higher percentage of branched alkanes, followed by alkenes, a lower proportion of linear alkanes and finally the alkadienes (Fig. 1; Table 1).

The Discriminant Function Analysis shows that there are significant quantitative differences between the chemical composition of the cuticle of the subcastes with Wilk’s Lambda = 0.115; F = 16.92; p < 0.01. The first canonical root explains 94.7% of the results and the second 4.7%, totaling 99.7% (Fig. 2). The dispersion diagram shows a greater overlap between the values of the samples of soldiers and foragers. The Cluster Analysis demonstrates that the cuticular chemical composition of foragers and soldiers are relatively more similar to each other than those of generalists and gardeners who grouped in another branch with a Cophenetic Correlation Coefficient = 0.83 (Fig. 3). Thus, reinforcing the data obtained by discriminant analysis.

DISCUSSION

Our results show significant variation between the compositions of the cuticle of the different subcastes of A. laevigata workers. These results corroborate those of Valadares & Nascimento (2016), who reported that the hydrocarbon profile varies according to the task performed by each worker subcaste of A. sexdens. Similar results were observed by Wagner et al. (2001), who found that workers of P. barbatus who perform different groups of tasks differ in the composition of cuticular hydrocarbons. Colonies of eusocial insects can only function if groups of individuals can recognize who they are and
what their roles within the colony are, and the main means of communication are the CHCs. Thus, the recognition of worker subcastes allows an organization within the colony (Sprenger & Menzel, 2020).

In all samples, 12 peaks with carbon chain composition ranging from 18 to 30 atoms were detected. Therefore, it is not possible to observe qualitative differences between groups, only quantitative ones. This result reinforces the theory that intracolonial variations are smaller than differences between species (Sprenger & Menzel, 2020). On the other hand, although Valadares & Nascimento (2016) found a similar variation in the size of chains in *A. sexdens* samples, the number of peaks and/or compounds varied between the two species.

The cluster analysis (Fig. 3) indicates that the composition of the cuticle is related to the type of task and environmental factors which subcastes are submitted to according to the function the workers perform, and since the groupings between ants that spend more time outside the nest, have cuticle composition closer than those that perform tasks inside the nest. Indeed, behavioral studies with leaf-cutting ants show that foragers and soldiers of the genus *Atta* spend more time performing tasks outside the nest than gardeners and generalists (Hölldobler & Wilson, 2010).

As a consequence, workers who perform tasks outside the nest may have a cuticular composition that makes them more resistant to exposure to heat. Larger *A. laevigata* ants have a higher survival rate when exposed to high temperatures than smaller ants (Bouchebti et al., 2015). In addition, *A. laevigata* ants make long trails to food sources, leaving foragers and soldiers exposed to solar radiation for longer (Moreira et al., 2004).

Menzel et al. (2018) comparing the CHCs of queens, nurses and foragers of *Temnothorax ambiguus* (Emery, 1895) and *Temnothorax longispinosus* (Roger, 1863) suggest that some differences in the proportion of classes of CHCs, like more dimethyl-alkanes in workers or less monomethyl-alkanes in queens, are more determined by environmental factors than by morphoanatomical dif-
ferences between the castes (queens and workers) and subcastes (nurses and foragers). Thus, foragers and nurses may differ from the queens, because they are more exposed to the conditions that can lead to some thermal stress in these species. Duarte et al. (2019) demonstrated that temperature variations can directly affect the composition of CHCs of different ant species, being the chemical plasticity an important factor for the survival of individuals.

The cuticular compounds whose primary function is most related to cuticle waterproofing are the linear alkanes. In the samples of foragers and soldiers subcastes, linear alkanes are the ones with the highest relative abundance. In the study by Wagner et al. (2001), with P. barbatus, subcastes who spend long periods outside the nest, such as foragers and scouts also have a higher proportion of linear alkanes in their cuticles compared to workers responsible for most intranidal tasks.

In the cuticle of ants, the greater abundance of branched alkanes and alkenes increases the vulnerability of the insects to desiccation because they considerably decrease the cuticular breakpoint (Conte & Hefetz, 2008). Linear chain alkanes have the highest melting temperature and highly branched alkanes have the lowest melting temperatures (Conte & Hefetz, 2008).

The nest architecture in Atta plays an important role in protecting against environmental variations, with the nests of A. laevigata being the deepest of the genus, reaching 7 m deep and the accumulation of loose soil along the fungal chambers allows good thermal insulation (Moreira et al., 2004). Therefore, ants that care for fungal gardens are less subject to climatic variation, which could explain the lower relative abundance of linear alkanes of these workers.

Another possible explanation for the greater abundance of linear alkanes in soldiers is the rate of parasitism of forids in this group of workers. Some studies report that forids such as Neodohrniphora erthali (Brown, 2001), Apocephalus attophilus (Borgmeier), Apocephalus vicosae (Disney), Neodohrniphora tonhascai (Brown, 2001) parasitize major workers of A. laevigata (Erthal & Tonhasca, 2000; Bragança et al., 2002; Bragança & Medeiros, 2006; Bragança, 2007) and may lay eggs on ant’s gaster, posterior or right side of the head (Bragança & Medeiros, 2006). Therefore, the higher content of linear alkanes in the cuticle of these workers could make the cuticle more resistant to the attacks of these flies, since these compounds are related to protection against parasites and desiccation (Martin et al., 2011).

On the other hand, gardeners presented a high proportion of branched alkanes, alkenes and alkadienes and, according to Wilson (1980), these workers are responsible for the care of the queen, the hyphae, and also providing the plant particles to the symbiont fungus, constantly interacting with other workers from whom they receive material to be processed (Hölldobler & Wilson, 2010). As this subcaste spends most of its time within the colony, it is expected that they present high content of these compounds that act as communication signals between individuals (Blomquist & Bagnères, 2010). The aggregation of CHCs for communication can also explain the richness of branched hydrocarbons, because their high molecular diversity compared to linear chain alkanes enables greater informative content (Conte & Hefetz, 2008).

In addition, ants that have greater contact with fungi are likely to have a greater influence on their composition, since Valadares & Nascimento (2016) suggest that the intimate relationship between offspring and fungus can shape the profile of hydrocarbons from both fungi and the leaf-cutting ant A. sexdens.

Finally, branched alkanes, alkenes and alkadienes stand out in the samples of all groups of workers, probably because their colonies are numerous and in order to maintain their cohesion it is necessary that the chemical profiles of their cuticles are rich in compounds that optimize their interactions, even though the CHCs may have multifunction (Blomquist & Bagnères, 2010).

**CONCLUSION**

The results of this study demonstrate that there is significant variation between the cuticular chemical profiles of the different subcastes of A. laevigata. Linear alkanes represented a higher percentage in foragers and soldiers, probably due to the need for waterproofing due to the greater need for exposure to temperature variation. In addition, there are higher contents of branched alkanes, alkenes and alkadienes in gardeners and generalists, which are those who perform more intranidal tasks and constantly interact with other workers, immature and queen at the same time. The climatic stability provided by the nest allows generalists and gardeners to maintain greater abundance of these important compounds especially for communication between nestmates. Therefore, the differences found in the chemical phenotype are directly related to the environmental conditions that each subcaste is subjected to due to the role it performs the most in the colony.

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