



REVISTA BRASILEIRA DE  
**Entomologia**  
A Journal on Insect Diversity and Evolution

[www.rbentomologia.com](http://www.rbentomologia.com)



Biology, Ecology and Diversity

## Chemical cuticular signature of leafcutter ant *Atta sexdens* (Hymenoptera, Formicidae) worker subcastes



Lohan Valadares\*, Fabio Santos do Nascimento

Universidade de São Paulo, Departamento de Biologia, Laboratório de Comportamento e Ecologia de Insetos Sociais, Ribeirão Preto, SP, Brazil

### ARTICLE INFO

#### Article history:

Received 24 March 2016

Accepted 23 June 2016

Available online 21 July 2016

Associate editor: Rodrigo Gonçalves

#### Keywords:

*Atta sexdens*

Cuticular hydrocarbons

Leafcutter ants

Worker subcaste

### ABSTRACT

In leafcutter ants the division of labour is associated to worker size variation clustered in four subcastes. In this work we used *Atta sexdens* Forel (1908) as a model to test the hypothesis that each subcaste expresses its own chemical signature comprised of cuticular lipids. To assess it, we extracted epicuticular compounds by using nonpolar solvent hexane and analysed the samples in a combined Gas Chromatography–Mass Spectrometer (GC–MS). We found 24 hydrocarbons with carbon chains ranging from 19 to 39 atoms most of them classified as linear and branched alkanes. No compound occurred in the cuticle of specific worker subcaste, however, the relative proportion pattern varied greatly among them. Our results suggest that although subcastes have similar chemical signatures, significant differences in their relative proportions may play an important role between nestmate and group identification.

© 2016 Sociedade Brasileira de Entomologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### Introduction

A key factor in the evolution of leafcutter ants (genus *Atta* and *Acromyrmex*) is the adaptation of task allocation system based on worker size variation fitted primarily to the utilisation of fresh vegetation as food resource to the symbiotic fungus that they cultivate (Wilson, 1980). In *Atta sexdens*, the distribution of activities related to foliar substrate treatment and fungus cultivation follow an assembly-line fashion performed by a total of four worker subcastes; large workers (foragers) forage, cut, and transport more efficiently leaf fragments, whereas small workers (gardeners) tend to remain inside the nest performing activities related to final treatment and implanting of foliar substrate particles (Della-Lucia et al., 1993; Wilson, 1980). Intermediate-size workers (generalists) perform a wide array of tasks from foliar substrate treatment to brood and queen caring. In addition, major workers, also referred as to soldiers, are virtually limited to defense (Wilson, 1980).

Although very organised, the division of labour in social insects do not follow a central control (Gordon, 2015). A single individual is able to perform a variety of tasks, but the probability of a leafcutter ant worker to perform a size-related task is high (Wilson, 1980). It has been evidenced that division of labour can be mediated by cuticular hydrocarbons (CHCs) that act as task decision cues (Greene and Gordon, 2003). CHCs are spread all over the outmost

cuticle layer of insects, the epicuticle. Due to the great diversity of these substances in eusocial insects combined with their exogenous and endogenous origins, they evolved to play an important role in nestmate recognition and social organisation (Greene and Gordon, 2003; Sturgis & Gordon, 2012; Tannure-Nascimento et al., 2007; Vander Meer and Morel, 1998; Wilgenburg et al., 2011;).

In leafcutter ants, some studies have characterised the cuticular composition of workers, most of them associating the influence of leaf substrate on their CHC profiles and nestmate recognition (Lambardi et al., 2004; Marinho et al., 2008; Martin and MacConnel, 1970; Valadares et al., 2015). However, to the best of our knowledge, the association between worker CHC profile and its four physical subcaste remains unclear. Within this context, we hypothesised that each subcaste of leafcutter ant genus *Atta* expresses its own chemical signature comprised of cuticular hydrocarbons. To test it, we used *A. sexdens* as a model, which is a broadly distributed species in the Neotropical ecosystems and considered to be one of the major herbivores of this region (Forti & Boaretto, 1997; Hölldobler and Wilson, 1990).

### Material and methods

#### Colonies

The present study was conducted with two laboratory populations (Colony 1 and Colony 2) collected from locations that were at least 50 km apart in Ribeirão Preto, São Paulo State, Brazil, during their initial phase (approximately 3 months old). They were

\* Corresponding author.

E-mail: [valadareslohan@gmail.com](mailto:valadareslohan@gmail.com) (L. Valadares).

kept in artificial conditions, with temperature and humidity kept at approximately 25 °C and 75%, respectively, until the age of two years when the experiment was conducted. At the time, the colonies had approximately 5 L of fungus garden. We also collected individuals under natural conditions (Colony 3) from a nest characterised by a big amount of voluminous, dismantled, excavated soil with approximately 2 m<sup>2</sup>.

### Subcaste identification

Before the chemical procedures we collected and measured the head width (HW) of 50 workers. After measurements, the individuals were rank-ordered in rows and it were chosen workers with HW around 1.0 mm, 1.4 mm, 2.2 mm and >3.0 mm, respectively classified as gardeners, generalists, foragers, and soldiers (Wilson, 1980). The specimens were then fixed with an entomological pin with a subcaste identification label and used as reference to collect individuals for chemical analyses.

### Chemical analyses

For chemical analysis we used 15 individuals per subcaste for each colony. Cuticular waxes were extracted by subjecting each individual to an appropriate volume of the nonpolar solvent hexane for 2 min. In order to obtain well concentrated samples, we previously determined the best volume of hexane to extract the compounds of the smallest subcaste (gardener). Then, we made a correlation between the solvent volume used in gardeners and the HW of the other subcastes. Thus, we subjected workers in 50, 100, 130, and 200 µL of hexane for each one of the respective subcastes: gardeners, generalists, foragers and soldiers. The samples were ran in a combined Gas Chromatography–Mass spectrometry GC–MS (Shimadzu, model QP2010 plus) equipped with silica capillary column and helium as carrier gas at 1 mL/min. The oven temperature was initially set to 150 °C, increasing 3 °C/min until it reached 280 °C (maintained for 10 min) and again 10 °C/min until reach 300 °C (maintained for 15 min).

The identification of the compounds was based on their mass spectra and with the aid of a standard solution of synthetic hydrocarbons (Sigma Chemical Co.), as well as Wiley and NIST Library database. The identification of the position of alkene double bounds was made through derivatisation method of hexane extracts from 20 foragers per subcaste, with Dimethyl Disulphide (DMDS) (Carlson, 1989). The extracts were dried with nitrogen and re-suspended in 200 µL of hexane. Subsequently, 200 µL of DMDS (Sigma–Aldrich) and 100 µL of iodine solution (dissolved in diethyl ether, 6% p/v) were added. The vials were then purged with nitrogen, closed, and agitated at ambient temperature for 24 h. Thereafter, the mixture was diluted in hexane and 5% sodium thiosulfate solution, thereby extracting the organic phase, which was subsequently dried with sodium sulphate and analysed in the GC–MS.

### Statistical analysis

Multivariate analysis was conducted with a single factor PerMANOVA using 9999 permutations. Compounds that were present in less than eight individuals per group, as well as compounds contributing less than 0.5% to the total compounds were excluded from analysis. Post hoc pairwise tests were conducted to compare the CHC profiles of worker subcastes. Canonical analysis was performed to classify individuals in their groups. Principal Coordinate analysis was conducted to demonstrate the main compounds responsible for significant discretisation of the groups. These calculations were

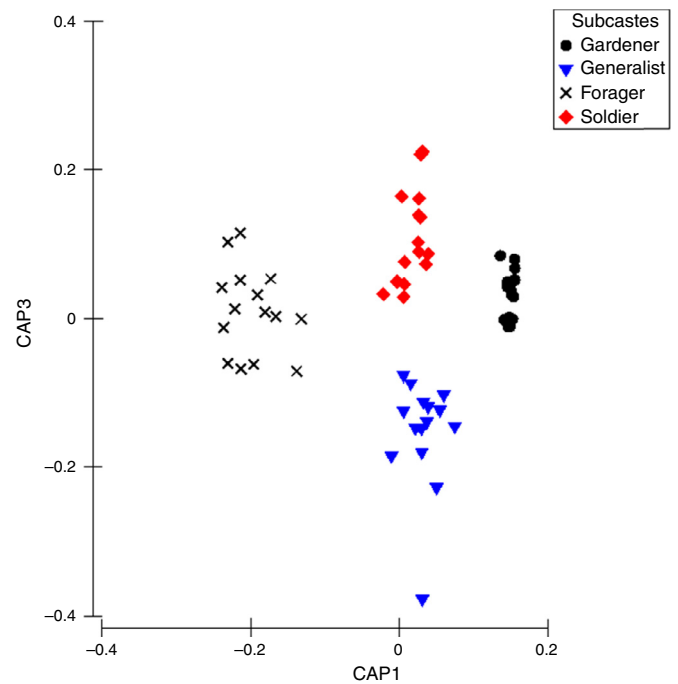


Fig. 1. Result of Canonical Analysis of Principle Coordinates carried out with cuticular hydrocarbons of four *Atta sexdens* subcastes, Colony 1.

carried out using the statistical software Primer version 6 (Primer-e Ltd).

### Results

The epicuticular extracts of *A. sexdens* workers presented a total of 24 hydrocarbons with carbon chains ranging from 19 to 39 atoms, most of them classified as branched alkanes (61.2%) and linear alkanes (38.1%), but also a low proportion of alkenes was found (0.68%) (Table 1). The chemical profiles of individuals were significantly different according to their colonies (PerMANOVA, Pseudo- $F=48.537$ ,  $p=0.0001$ ). Euclidean calculations in addition to Centroid Distance calculations classified individuals collected under natural conditions (Colony 3) as the most chemically different (Table 2).

A significant variation on CHC worker profile was found in relation to their subcastes (PerMANOVA, Pseudo- $F=25.568$ ,  $p=0.0001$ , Figs. 1–3). According to Principal Coordinate Analysis (PCO) the main compounds responsible for significant separation of subcastes were two branched alkanes (4,8,12-TriMeC<sub>36</sub>, 7,11-DiMeC<sub>39</sub>) and two linear alkanes (C<sub>25</sub>H<sub>52</sub>, C<sub>27</sub>H<sub>56</sub>). These compounds were among the most abundant ones (Table 1). No compound occurred exclusively in the cuticle of workers. However, the concentration pattern varied greatly in the subcastes, which suggests similar chemical signatures as to the variety of compounds but with great differences in their relative proportions (Fig. 4). Trimethylalkanes were more concentrated in foragers, making up 35% of total compounds, while linear alkanes were generally more concentrated on both gardeners and soldiers. Overall, generalists presented a high relative concentration of both classes of compounds, and interestingly, they presented all 24 identified hydrocarbons (Table 1; Fig. 4).

### Discussion

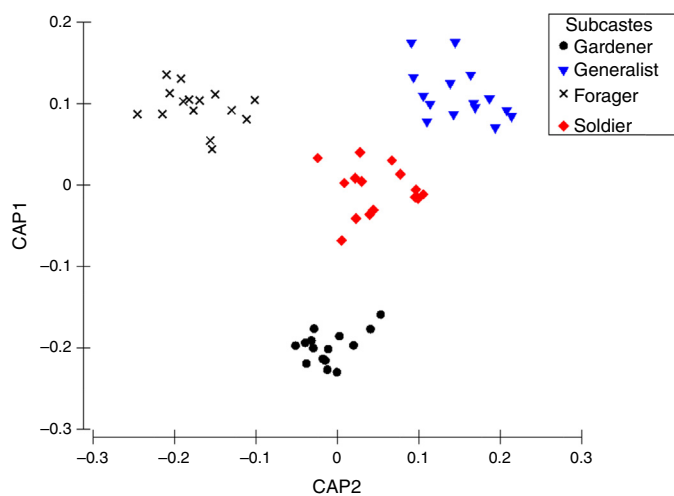
This study demonstrates that there is a variation in the cuticular hydrocarbon composition of *A. sexdens* workers in relation to its four subcastes. This variation seems to be more influenced by the concentration profile of most abundant hydrocarbons than to

**Table 1**  
Relative proportions (Mean  $\pm$  standard deviation) of cuticular hydrocarbons of four *Atta sexdens* subcastes, colonies 1, 2, and 3.

Kovats Index	Compound	Gardener (n = 45)	Generalist (n = 45)	Forager (n = 45)	Soldier (n = 45)
1613	Z-9-C <sub>19</sub>	0.35 $\pm$ 0.77	1.39 $\pm$ 2.29		0.26 $\pm$ 0.57
2500	Z,Z-C <sub>23:2</sub>		0.51 $\pm$ 0.87		13.3 $\pm$ 7.29
2600	n-C <sub>25</sub>	12.7 $\pm$ 5.7	9.95 $\pm$ 3.95	6.91 $\pm$ 2.28	13.3 $\pm$ 7.29
2700	n-C <sub>26</sub>	0.78 $\pm$ .53	0.75 $\pm$ 0.46	0.48 $\pm$ 0.38	1.69 $\pm$ 0.81
2900	n-C <sub>27</sub>	14 $\pm$ 4.51	13.6 $\pm$ 4.41	10 $\pm$ 3.87	16.2 $\pm$ 6.75
3034	n-C <sub>29</sub>	3.58 $\pm$ 1.89	2.97 $\pm$ 1.61	2.21 $\pm$ 1.9	2.89 $\pm$ 3.13
3060	9-MeC <sub>30</sub>	0.48 $\pm$ 0.52	0.53 $\pm$ 0.56	0.44 $\pm$ 0.66	0.19 $\pm$ 0.34
3100	7,11-DiMeC <sub>30</sub>	8.33 $\pm$ 1.6	6.69 $\pm$ 2.17	6.17 $\pm$ 2.69	8.14 $\pm$ 2.2
3134	n-C <sub>31</sub>	1.26 $\pm$ 0.54	1.09 $\pm$ 0.41	0.74 $\pm$ 0.93	0.69 $\pm$ 0.61
3165	9-MeC <sub>31</sub>	5.72 $\pm$ 1.3	4.32 $\pm$ 1.02	3.55 $\pm$ 1.51	5.66 $\pm$ 1.5
3234	3,7,11-TriMeC <sub>31</sub>	2.81 $\pm$ 3.68	2.06 $\pm$ 3.11	1.49 $\pm$ 1.78	1.53 $\pm$ 2.21
3250	9-MeC <sub>32</sub>	10.2 $\pm$ 2.85	8.8 $\pm$ 3.39	6.23 $\pm$ 3.68	5.81 $\pm$ 1.78
3300	4,8,12-TriMeC <sub>32</sub>	16.8 $\pm$ 3.15	14.3 $\pm$ 4.66	11.1 $\pm$ 5.22	12 $\pm$ 3.48
3310	n-C <sub>33</sub>	2.78 $\pm$ 3.11	2.58 $\pm$ 3.69	2.31 $\pm$ 3.34	1.77 $\pm$ 2.7
3330	Unknown	4.64 $\pm$ 3.86	3.48 $\pm$ 2.57	1.87 $\pm$ 1.41	2.65 $\pm$ 2.11
3368	9-MeC <sub>33</sub>	6.02 $\pm$ 1.22	5.09 $\pm$ 1.28	3.62 $\pm$ 1.27	4.78 $\pm$ 1.49
3400	3,7,11-TriMeC <sub>33</sub>	0.17 $\pm$ 0.34	0.3 $\pm$ 0.45	3.25 $\pm$ 3.19	
3424	n-C <sub>34</sub>	0.89 $\pm$ 0.61	0.87 $\pm$ 0.35	0.33 $\pm$ 0.53	0.39 $\pm$ 0.41
3445	Unknown	0.6 $\pm$ 0.69	0.77 $\pm$ 1.07	0.32 $\pm$ 0.33	0.29 $\pm$ 0.45
3500	4,8,12-TriMeC <sub>34</sub>	4.39 $\pm$ 1.09	4.15 $\pm$ 1.55	3.03 $\pm$ 1.3	3.34 $\pm$ 1.1
3570	n-C <sub>35</sub>	0.1 $\pm$ 0.22	0.4 $\pm$ 0.32		0.39 $\pm$ 0.5
3753	3,7,11-TriMeC <sub>35</sub>		1.37 $\pm$ 1.57	3.22 $\pm$ 3.09	1.78 $\pm$ 2.02
3965	4,8,12-TriMeC <sub>36</sub>	2.45 $\pm$ 2.7	8.2 $\pm$ 4.57	17.4 $\pm$ 6.77	6.85 $\pm$ 4.1
	7,11-DiMeC <sub>39</sub>		5.22 $\pm$ 3.72	15.2 $\pm$ 7.87	3.87 $\pm$ 2.13

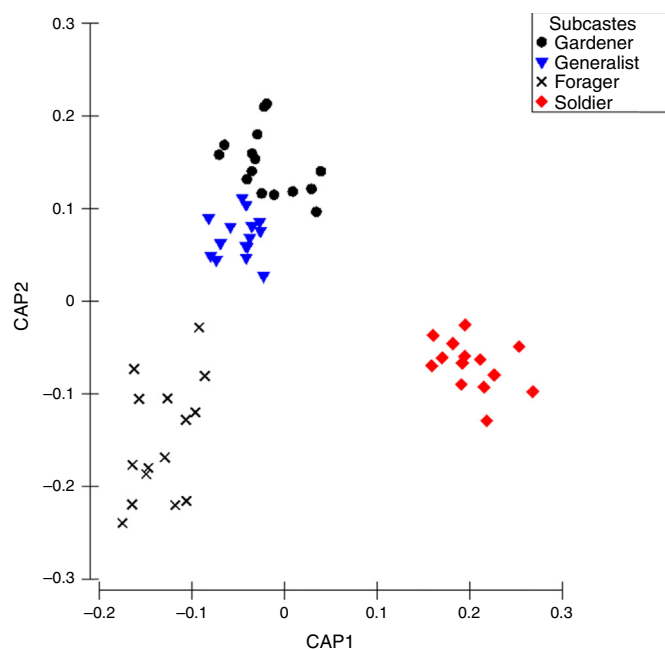
**Table 2**  
Chemical distance, centroid distance, and pairwise tests carried out with the cuticular hydrocarbon profiles of four *A. sexdens* subcastes.

Colony combinations	Chemical distance	Centroid distance	Pairwise test (p value)
Colony 1 vs. Colony 2	5.57	3.24	0.0001
Colony 1 vs. Colony 3	6.33	4.65	0.0001
Colony 2 vs. Colony 3	6.16	4.36	0.0001
Subcaste Combinations	Chemical distance	Centroid distance	Pairwise test (p value)
Gardener vs. Generalist	5.22	2.99	0.0001
Gardener vs. Forager	6.96	5.54	0.0001
Gardener vs. Soldier	5.21	3.05	0.0001
Generalist vs. Forager	5.51	3.05	0.0001
Generalist vs. Soldier	4.78	1.62	0.0025
Forager vs. Soldier	5.68	3.45	0.0001



**Fig. 2.** Result of Canonical Analysis of Principle Coordinates carried out with cuticular hydrocarbons of four *Atta sexdens* subcastes, Colony 2.

the qualitative differences, because no substance occurred exclusively in the cuticle of any worker groups. Similar results were found in the weaver ant *Camponotus textor* where only a single compound occurred exclusively in the cuticle of different size



**Fig. 3.** Result of Canonical Analysis of Principle Coordinates carried out with cuticular hydrocarbons of four *Atta sexdens* subcastes, Colony 3.

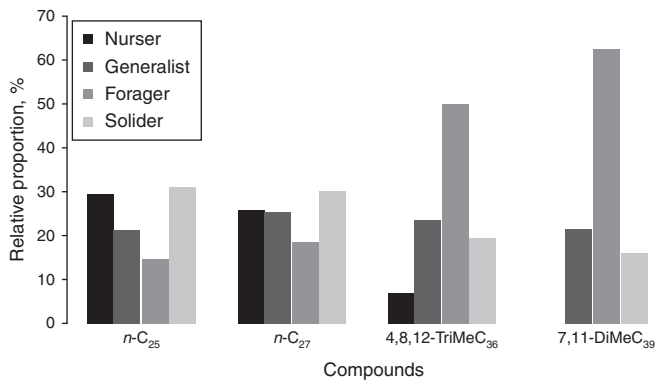


Fig. 4. Distribution pattern of four cuticular hydrocarbons among *Atta sexdens* worker subcastes.

workers (Campos et al., 2012). According to Wilson (1980), the most specialised workers in *A. sexdens* have head capsule width less than 1.2 mm and larger than 2.4 mm, excluding generalists with its morphological traits more likely to the basal Attine species. Interestingly, our analyses using three different colonies indicated generalists as the most chemically different subcaste (Table 2). This may be explained because this worker group has an intermediate size and performs a wide array of activities inside and outside the nest. In our results, the presence of three trimethylalkanes that was also reported by Martin and MacConnel (1970) for the same species reflect the most derived position of genus *Atta* inside the fungus-growing Attini tribe (Schultz and Brady, 2008). Indeed, the presence of methylalkanes and dimethylalkanes is an ancestral trait in ants, whereas the presence of trimethylalkanes seems to be a derived one (Wilgenburg et al., 2011). Furthermore, branched hydrocarbons are likely species-specific compounds involved in nestmate recognition (Martin and Drijfhout, 2009), and linear hydrocarbons seems to be more related to protection against parasites and desiccation (Blomquist and Bagnères, 2010).

In addition to the influence of internal factors on task allocation (e.g., worker age, body size, and genetic factors), we hypothesised that in *Atta sexdens* the differences found in worker chemical cuticular profiles may act as task decision cues. Pheromones that determine the global situation of a colony seems to be involved in task decision in social insects, as demonstrated in the harvest ant *Pogonomyrmex barbatus* where colony organisation arises from the responses of individuals to local cues, and individuals regulate foraging using interactions mediated by CHCs between returning and outgoing foragers (Greene and Gordon, 2003). Further studies approaching the influence of abiotic factors, such as temperature and humidity, as well as investigations on the direct evidence of CHCs on nestmate recognition and task allocation are required to a better understanding on their reliability as chemical cues in leaf-cutter ants.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgments

The authors thank National Council of Scientific and Technological Development (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp 2010/10027-5) for financial support. The authors also thank two anonymous referees for their valuable suggestions on the previous version of this manuscript.

## References

- Blomquist, G.J., Bagnères, A.G., 2010. Structure and analysis of insect hydrocarbons. In: Blomquist, G.J. (Ed.), *Insect Hydrocarbons*. Cambridge University Press, Cambridge, pp. 19–34.
- Campos, M.C.G.C., Campos, M.L.G., Turatti, I.C., Nascimento, F.S., 2012. Cuticular hydrocarbon variation of Castes and Sex in the Weaver Ant *Camponotus textor* (Hymenoptera: Formicidae). *Sociobiology* 59, 1025–1035.
- Carlson, D.A., 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61, 1564–1571.
- Della-Lucia, T.M.C., Folwer, H.G., Araújo, M.S., 1993. Castas de formigas cortadeiras. In: Della-Lucia, T.M.C. (Ed.), *As Formigas Cortadeiras*. Folha de Viçosa, Viçosa, pp. 43–53.
- Forti, L.C., Boaretto, M.A.C., 1997. *Formigas Cortadeiras: Biologia, Ecologia, Danos e Controle*. Botucatu.
- Gordon, M., 2015. From division of labor to collective behavior of social insects. *Behav. Ecol. Sociobiol.*, 1–8.
- Greene, M.J., Gordon, D.M., 2003. Cuticular hydrocarbons inform task decisions. *Nature* 423, 423–432.
- Hölldobler, B., Wilson, E.O., 1990. *The Ants*. Belknap Press of Harvard University Press, Cambridge.
- Lambardi, D., Chegia, B., Turilazzi, S., Boomsma, J.J., 2004. Diet-induced aggression among colonies of the leafcutter ant *Acromyrmex echinatior* Fore1 (Hymenoptera Formicidae). *REDIA, LXXXVII* 21, 219–221.
- Marinho, G.C.S., Della-Lucia, M.T.C., Ribeiro, M.M.R., Magalhães, S.T.V., Guedes, R.N.C., Jham, G.N., 2008. Interference of b-Eudesmol in nestmate recognition in *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Bull. Entomol. Res.* 98, 467–473.
- Martin, S., Drijfhout, F., 2009. A review of ant cuticular hydrocarbons. *J. Chem. Ecol.* 32, 1151–1161.
- Martin, M.M., MacConnel, J.G., 1970. The alkanes of *Atta colombica*. *Tetrahedron* 26, 307–319.
- Schultz, T.R., Brady, S.G., 2008. Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci. U.S.A.* 14, 5435–5440.
- Sturgis, S.J., Gordon, M.D., 2012. Nestmate recognition in ants (Hymenoptera: Formicidae): a review. *Myrmecol. News* 16, 101–110.
- Tannure-Nascimento, I.C., Nascimento, F.S., Turatti, I.C., Lopes, N.P., Trigo, J.R., Zucchi, R., 2007. Colony membership is reflected by variations in cuticular hydrocarbon profile in a neotropical paper wasp, *Polistes satan* (Hymenoptera, Vespidae). *Genet. Mol. Res.* 6, 390–396.
- Valadares, L., Nascimento, D., Nascimento, F.S., 2015. Foliar Substrate Affects Cuticular Hydrocarbon Profiles and Intraspecific Aggression in the Leafcutter Ant *Atta sexdens*. *Insects* 6, 141–151.
- Vander Meer, R.K., Morel, L., 1998. Nestmate recognition in ants. In: Vander Meer, R.K., Breed, M., Wintson, M., Espelie, K.E. (Eds.), *Pheromone Communication in Social Insects*. Westview Press.
- Wilgenburg, E., Symonds, M.R.E., Elgar, M.A., 2011. Evolution of cuticular hydrocarbon diversity in ants. *J. Evol. Biol.* 24, 1188–1198.
- Wilson, E.O., 1980. Caste and division of labor in leaf-cutter ants (Hymenoptera, Formicidae: *Atta*). I: The overall pattern in *A. Sexdens*. *Behav. Ecol. Sociobiol.* 7, 143–156.