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

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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.

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

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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, dismounted, excavated soil with approximately 2 m^2.

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

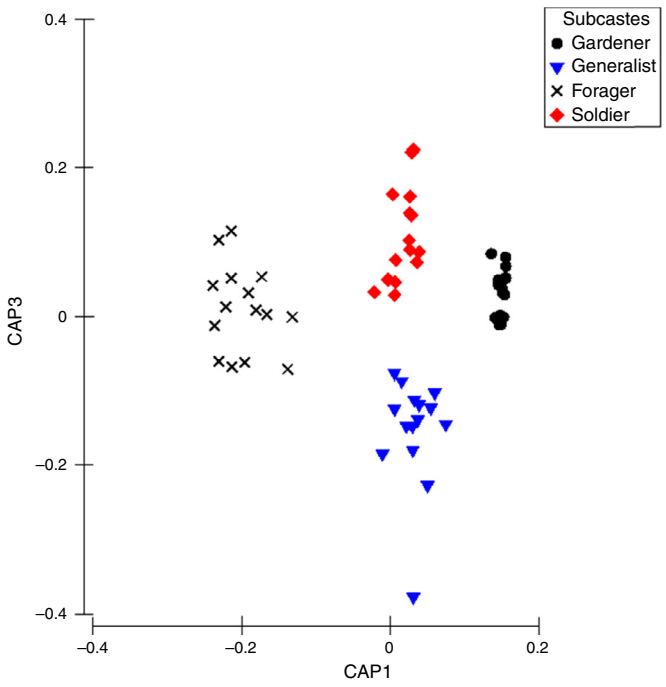

Fig. 1. Result of Canonical Analysis of Principle Coordinates carried out with cuticular hydrocarbons of four *Atta sexdens* subcastes, Colony 1.

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_{36}, 7,11-DiMeC_{39}) and two linear alkanes ($C_{25}H_{52}$, $C_{27}H_{56}$). 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