GABRIELA ZORZAL NEVES

IMPORTANCE OF CHEMICAL AND GEOGRAPHICAL DISTANCES IN DETERMINATION OF ANT'S AGGRESSIVE BEHAVIOR: FIRST INSIGHT IN THE *CECROPIA-AZTECA* SYSTEM

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ecologia, para obtenção do título de *Magister Scientiae*.

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(Orientador)

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SUMÁRIO

ABSTRACT

NEVES, Gabriela Zorzal, M.Sc., Universidade Federal de Viçosa, February, 2019. **Importance of chemical and geographical distances in determination of ant's aggressive behavior: first insight in the *Cecropia-Azteca* system.** Adviser: Ricardo Ildefonso de Campos.

Territorial animals respond less aggressively to intrusions by neighbors than by outsiders. This difference in behavioral responses is termed "dear-enemy phenomenon" and it determines recognition and aggressiveness in animal interactions. In social insects, the identification of non-nest mates is mainly performed through "colony odors", formed by hydrocarbon lipid compounds that cover the insects' cuticle (also called CHCs). The difference in the chemical composition of these compounds may be genetically influenced and influenced by the environment and/or by the diet of colony individuals. Myrmecophytic ants present an aggressive defensive behavior against plants natural enemies. These systems might be considered a promising model for studying nest-mate recognition and aggressiveness behaviors. Thus, the present study aims to experimentally test the importance of geographical and chemical distances on the aggressive behavior among individuals of *Azteca muelleri* ants inhabiting *Cecropia glaziovii* plants. We sampled a total of 50 ant colonies in three Atlantic Forest fragments located in the Viçosa municipality, state of Minas Gerais, Brazil. The distance in meters between sites were estimated using the GPS data provided from the two points of interest. After sampling, we performed aggression tests using five works from the same colony against five workers from other colony. Each colony was used only once. For the aggression tests we divided the 50 colonies in two distinct groups: same location group and different location group. Then, we observed the behaviors of ants in each aggression test and we classify them as non-aggressive and as aggressive. After the aggression tests, we identified picks of chemicals compounds (CHCs) and evaluated the concentrations of these components by chemical analysis. Our results showed a positive effect of geographical distance on ant aggressiveness and a significantly higher aggressiveness between pairs of ants from different site when compared to pairs of ants belonging to the same site. On the other hand, *A. muelleri* aggressiveness was not influenced by chemical cuticles profile and we also

did not detect a significant interaction between geographical distance and chemical distance. The *Azteca-Cecropia* system therefore exemplifies a relationship in which the geographic distance influences the aggressive behaviour. However, in this system the total of chemical compounds does not explain the aggression behaviour and only some these compounds may play a main role in the aggressive behaviour and non-nest mate recognition. Thus, it is necessary to investigate if these factors and others parameters such as genetic distance, may be influencing the aggressive behaviour in this species of *Azteca.*

RESUMO

NEVES, Gabriela Zorzal, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Importância das distâncias química e geográfica na determinação do comportamento agressivo de formigas: primeiro insight no sistema *Cecropia-Azteca***. Orientador: Ricardo Ildefonso de Campos.

Animais territoriais respondem menos agressivamente a intrusões de vizinhos do que de não-vizinhos. Essa diferença nas respostas comportamentais é denominada “fenômeno do inimigo íntimo” e determina o reconhecimento e a agressividade nas interações animais. Em insetos sociais, a identificação de parceiros que não pertencem ao seu ninho é realizada principalmente por meio de "odores da colônia", formados por compostos lipídicos de hidrocarbonetos que cobrem a cutícula dos insetos (também chamados de CHCs). A diferença na composição química destes compostos pode ser influenciada geneticamente e influenciada pelo ambiente e / ou pela dieta dos indivíduos da colônia. As formigas mirmecofíticas apresentam um comportamento defensivo agressivo contra os inimigos naturais das plantas. Esses sistemas podem ser considerados modelos promissores para o estudo de comportamentos de reconhecimento do companheiro de ninho e agressividade. Assim, o presente estudo tem como objetivo testar experimentalmente a importância das distâncias geográficas e químicas sobre o comportamento agressivo entre indivíduos de formigas *Azteca muelleri* que habitam plantas de *Cecropia glaziovii*. Para isso, amostramos um total de 50 colônias de formigas em três fragmentos de Mata Atlântica localizados no município de Viçosa, estado de Minas Gerais, Brasil. A distância em metros entre os locais foi estimada usando os dados de GPS fornecidos a partir dos dois pontos de interesse. Após as coletas, realizamos testes de agressividade utilizando cinco operárias da mesma colônia contra cinco operárias de outra colônia. Cada colônia foi usada apenas uma vez. Para os testes de agressividade, dividimos as 50 colônias em dois grupos distintos: grupo mesmo local e grupo locais diferentes. Após os testes de agressividade, identificamos os picos dos compostos químicos (CHCs) e avaliamos as concentrações desses componentes por meio de análises químicas. Nossos resultados mostraram um efeito positivo da distância geográfica sobre a agressividade de formigas

e uma agressividade significativamente maior entre pares de formigas de diferentes locais quando comparadas a pares de formigas pertencentes ao mesmo local. Por outro lado, a agressividade de *A. muelleri* não foi influenciada pelo perfil químico das cutículas e também não detectamos interação significativa entre a distância geográfica e a distância química. O sistema *Azteca-Cecropia*, portanto, exemplifica uma relação na qual a distância geográfica influencia o comportamento agressivo. No entanto, neste sistema o total de compostos químicos não explica o comportamento de agressão e apenas alguns destes compostos podem desempenhar um papel principal no comportamento agressivo e no reconhecimento de formigas de outra colônia. Assim, é necessário investigar se esses fatores e outros parâmetros, como a distância genética, podem estar influenciando o comportamento agressivo nesta espécie de *Azteca*.

1. Introduction

Territorial defence strategies are responsible for the establishment and maintenance of animal communities that lives in well-organized societies (Hölldobler and Lumsden, 1980). Generally, it is expected that territorial animals will respond less aggressively to intrusions by neighbors than by outsiders (Temeles, 1994). This difference in behavioral responses is termed “dear-enemy phenomenon” and it has been considered a key feature determining recognition and aggressiveness in animal interactions (Fisher, 1954; Temeles, 1990). Three mechanisms are considered the main responsible to recognition of “dear enemies”: i) environmental cues, ii) genetically based cues and iii) a form of learning called “habituation” (Hölldobler and Carlin, 1987; Hölldobler and Wilson 1990).

In social insects, the identification of non-nest mates is mainly performed through "colony odors", formed by hydrocarbon lipid compounds that cover the insects’ cuticle (also called CHCs) (Forel, 1929; Boulay et al., 2000). The difference in the chemical composition of these compounds is genetically influenced (Beye et al., 1997; Langen et al., 2000; Fournier et al., 2016), but they might also be influenced by the environment and/or by the diet of colony individuals (Wallis, 1962; Mabelis, 1979; Carlin and Hölldobler, 1986; Stuart, 1987). Ydenberg (1988) shows that, apart from chemical, physical and genetic species properties, habitat similarity may influence the individual recognition, even in cases of high kinship (eg. individuals of the same species).

The remarkable interaction between ants and myrmecophytes plants might be considered a promising model for studying nest-mate recognition and aggressiveness behaviors. It is well known that myrmecophytic ants present an aggressive defensive behavior against plants natural enemies, in exchange for shelter and food provided by the plants (Janzen, 1966, 1969; Vasconcelos, 1991; Fonseca, 1994; Del-Claro et al., 1996). *Azteca* Emery, 1893 (subfamily Dolichoderinae) is a genus of neotropical ants which present some obligatory mutualistic species, commonly found in myrmecophyte plants of the genus *Cecropia* (Emery 1893, 1912; Forel, 1929; Hölldobler and Wilson, 1990). Some studies show that *Azteca* workers behave aggressively against other insects that attack the plant in which they live (Janzen, 1969; Heil and McKay, 2003; Oliveira et al., 2015).

The promoting factors evolved in recognition and aggressiveness among ant individuals have been debated in recent years but, they are still quite controversial (Brandt et al., 2009, Helanterä et al., 2011, Frizzi et al., 2015, Fournier et al., 2016). While some studies show that this behavior would only be mainly related to genetic characteristics (Fournier et al., 2016), others conclude that geographical and/or chemical distances are most important (Frizzi et al., 2015). Moreover, this literature is focused only on ground ant species (Frizzi et al., 2015 and references therein) and studies on arboreal ants are rare (see Newey et al., 2010) and even absent for 'obligatory' mutualistic ants (Heil and Mackey, 2003).

From the point of view of the interaction's natural history and the behavioral ecology, it is important to understand the recognition among individuals of *Azteca* in *Cecropia*. It is important then to elucidate the influence of geographical, chemical and genetic distances in the *Azteca* individual recognition, but it may also bring evidence for other ants' species. The present study aims to experimentally test the importance of geographical and chemical distances on the aggressive behavior among individuals of *Azteca muelleri* (Emery, 1893) ants inhabiting *Cecropia glaziovii* Snethl. plants. More specifically, we attempted to answer the following questions: i) Are there a positive effect of geographical and/or chemical distances on ant aggressiveness? ii) Is the effect of chemical distance on ant aggressiveness dependent on geographical distance?

2. Materials and Methods

Study area

We sampled the ant colonies in three Atlantic Forest fragments located in the Viçosa municipality, state of Minas Gerais, Brazil. The tree sampling sites were: "Mata do Paraíso" (MP), "Mata da Biologia" (MB) and "Mata do Seu Zé" (SZ). The Research Station Training and Environmental Education "Mata do Paraíso" (20º38'07 "S, 42º51'31" W) is a secondary forest reserve (56 years of regeneration process) with 195 ha and belonging to the Federal University of Viçosa (UFV). The "Mata da Biologia" (20°45'29.8"S 42°51'43.1"W) is located inside the Federal University of Viçosa campus and comprises an area of approximately 75 ha of forest under permanent protection undergoing a regeneration process of 93 years. Finally, our third site is private owned secondary Forest Fragment (years of regeneration unknown) on the border of the BR-120/356, located between Viçosa-MG and Coimbra-MG towns (20° 49'21.2 "S 42 ° 48'59.6" W; Fig. 1). The three sites are composed by secondary Atlantic Seasonal Semideciduous Montane Forests (Veloso et al., 1991). We calculated the distance between sites through online geographic calculator of the "Instituto Nacional de Pesquisas Espaciais" (INPE). In this calculator, the distance in meters were estimated using the GPS data provided from the two points of interest.

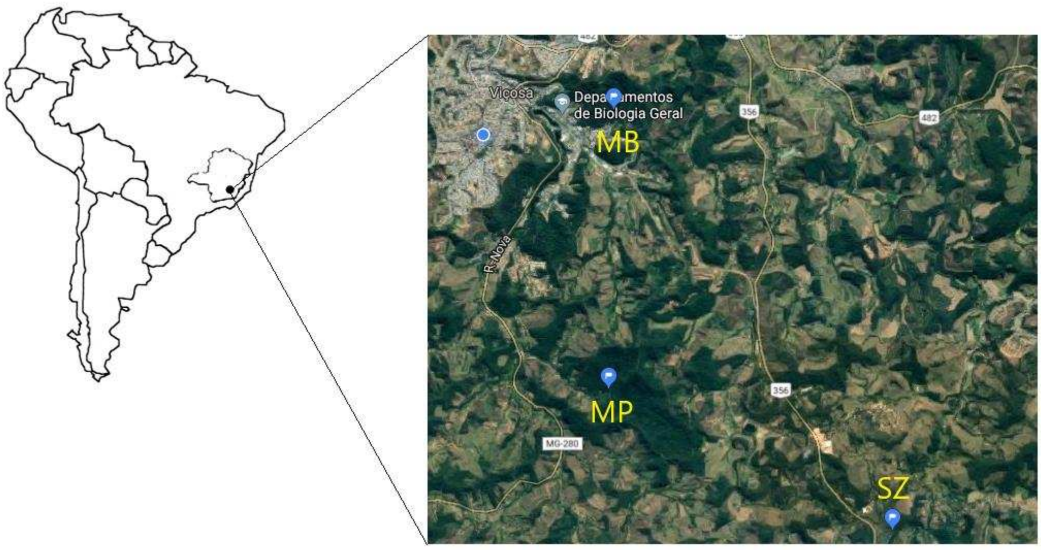

Fig. 1 Map showing the location of Viçosa municipality in South America and Brazil with the three localities under study. MB: "Mata da Biologia"; MP: "Mata do Paraíso"; SZ: "Mata do Seu Zé".

Biological system

We used the plant species *Cecropia* glaziovii which is a fast-growing tree usually found in regeneration fragments of Semi deciduous Seasonal Forests (Berg and Rosseli, 2005). *Cecropia glaziovii* has its distribution mainly influenced by altitude (600-1,500 meters) (Berg and Rosseli, 2005). This species is usually associated with *Azteca muelleri* (Dolichoderinae) ants (Davidson et al., 1991) which is very aggressive ant species (Davidson & McKey, 1993). We identified the plants based on leaf characteristics, according to Berg and Rosseli (2005) and the *A. muelleri* ants were identified morphologically by the taxonomist Msc. Julio Chaul.

Ant sampling

We collected 14 plants of C. glaziovii in the MP, 14 in the MB and 22 in the SZ, totaling 50 plants which were used in all experiments. We considered each plant and the resident ants as a single colony. We previously marked the plants in GPS and measured height and diameter using a tape measure. In addition to these measurements, we observed the presence of Müllerian bodies and workers in the plants. For the ants' collection, we cut the plant internodes and collected the queens and workers present in each internode. After the collection we stored the ants in plastic pots with perforated cover. In addition, we observed the presence of dead queens, the contact between internodes and the presence of immatures and fungi in each collected plant. We kept the ants in a breeding room at 25ºC and controlled humidity at the Laboratório de Ecologia de Formigas (LEF) of the UFV.

Aggression test

We performed aggression tests using five works from the same colony against five workers from other colony. Each colony was used only once. For the aggression tests we divided the 50 colonies in two distinct groups. First, we used ants from 28 different colonies but the same location (same location group). Second, we performed aggression tests using ants from 22 different colonies and from different locations (different location group). The ants were placed in an arena (25 cm^2) during six minutes, one for acclimation and five for observations. Then, we observed the behaviors of ants in each aggression test and the classification followed the protocol of aggressiveness by Giraud et al. (2002) (Table 1). Before the observations, we transformed the behaviour values in an aggression index using the frequency distribution of aggression score levels.

Aggressiviness index = value of interaction (0,1,2,3,4,5) x frequency

Value	Behavior	Aggression level
0	Ignores, has fisical contact and shows no interest.	Non-aggressive
1	Antennation, repeated antenna taps on the other ant.	Non-aggressive
2	Evasion, retract to the opposite direction after contact.	Non-aggressive
3	Dorsal flexion of the gaster and opening of the mandibule.	Aggressive
4	Aggression, pulls the head or other part of the body.	Aggressive
5	Fight, prolonged aggression, stabbing the jaw in one body part of the other ant.	Aggressive

Table 1: Table with the aggressiveness values, the behaviors referring to each value and the aggression level (aggressive or non-aggressive) of each behavior.

Chemical analysis

After the aggression test, we frozen the used ant individuals. Therefore, we have frozen five workers from 50 colonies from the previously described locations. Subsequently, we subjected the ants to the cuticular hydrocarbons extraction using apolar solvent hexane. In this extraction we immersed 5 workers in 50µl of hexane for two minutes. For the quantification, we injected the obtained extract (1µl) into a gas chromatography (GC) equipment following the protocol: initial temperature of 100ºC, heating ramp 10ºC per minute, final temperature of 280ºC (maintained for 10 minutes), temperature of the injector at 250°C. To quantify the difference in hydrocarbon profiles between A. muelleri colonies, we used the internal standardization method (Trans, trans-farnesol ($C_{15}H_{26}O$)) (Fischer, 2006) to evaluate the concentrations of the components of interest. For the qualification, we injected the same extracts into a chromatography equipment coupled to a mass spectrometer (GCMS) for the compounds identification based on the Kovats Index, derivatizations and database.

After the chromatography reading we identified 25 important picks of chemicals (the ones which appeared in more than 50% of chromatograms) per ant's nest (groups of 5 ants analyzed in one sample). We then compared the concentrations of these 25 chemical compounds among the same ant pairs used in the aggressiveness tests. For this purpose we calculated a similarity index (using Bray-curtis for quantitative data) between each pair of ants used in the aggressiveness tests. This number was termed hereafter as "chemical distance" and then used in our statistical models (see statistical analyses).

Statistical analysis

To investigate the effects of geographical and chemical distances on ant aggressiveness and the interaction between two variables we performed a generalized linear model (GLM). We used geographical and chemical distances as explanatory variables and the ant aggressiveness index as response variable. We used backward-elimination multiple regression to identify a minimally adequate model (Crawley, 2005). We specified a Gaussian Model and we performed residual analyses for all models and checked for the distribution of errors. Distance was log transformed to achieve normality of residuals and homoscedasticity. Finally, to compare ant's chemical similarity among sampling sites we used a Non-metrical Multi-dimensional Scaling (NMDS). NMDS ordinations were based on the Bray-Curtis index of dissimilarity between sites as we used continuous data (chemical distances).

For testing the effect, the sampling site on ant aggressiveness we performed an ANOVA model with site as explanatory variable and aggressiveness index as response variable. For this model we used a Gaussian Model and we performed residual analyses for all models and checked for the distribution of errors. We used a post-hoc Tukey test to determine which pair of means differed significantly.

3. Results

Out from our 25 encounters tests between the pairs of ants, workers showed aggressive behavior in 55,55% of total tests. The tests between individuals of the same sites showed aggressive behavior in 33,33% of cases. In contrast, the ants belonging to different sites were aggressive in 83,33% of tests. As expected we find a positive effect of geographical distance on ant aggressiveness (Fig. 2, $F_{2,22}$=8.9014, p=0.007). We also find that the levels of aggressiveness were significantly higher between pairs of ants from different sites when compared to pairs of ants belonging to the same site (Fig. 3, $F_{3,21}$=9,498; p<0.001).

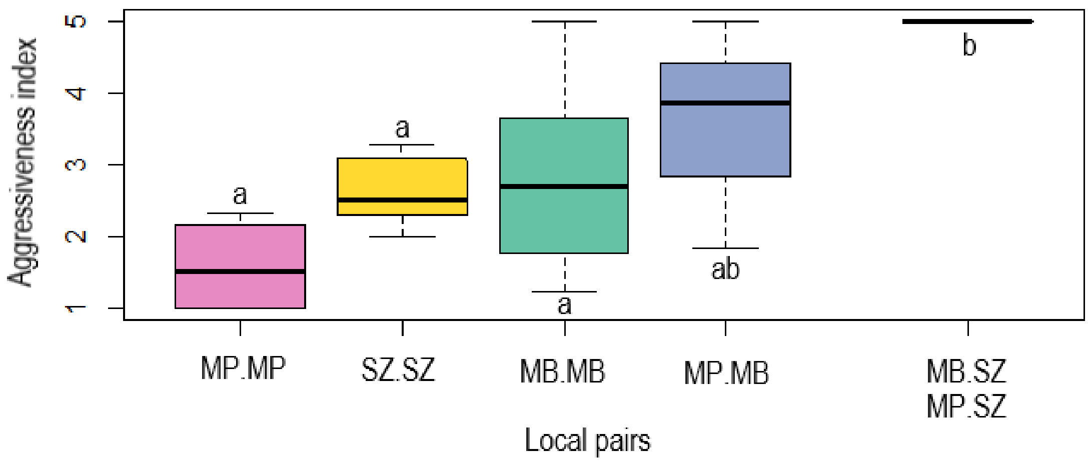

Fig. 2 Results between aggressiveness index and the local pairs. The letters indicate mean significant differences (Tukey's test) between the local pairs. Two of the different local treatments (MB.SZ and MP.SZ) differ from the other pairs (p<0.05). Abreviativos: MB: "Mata da Biologia"; MP: "Mata do Paraíso"; SZ: "Mata do Seu Zé".

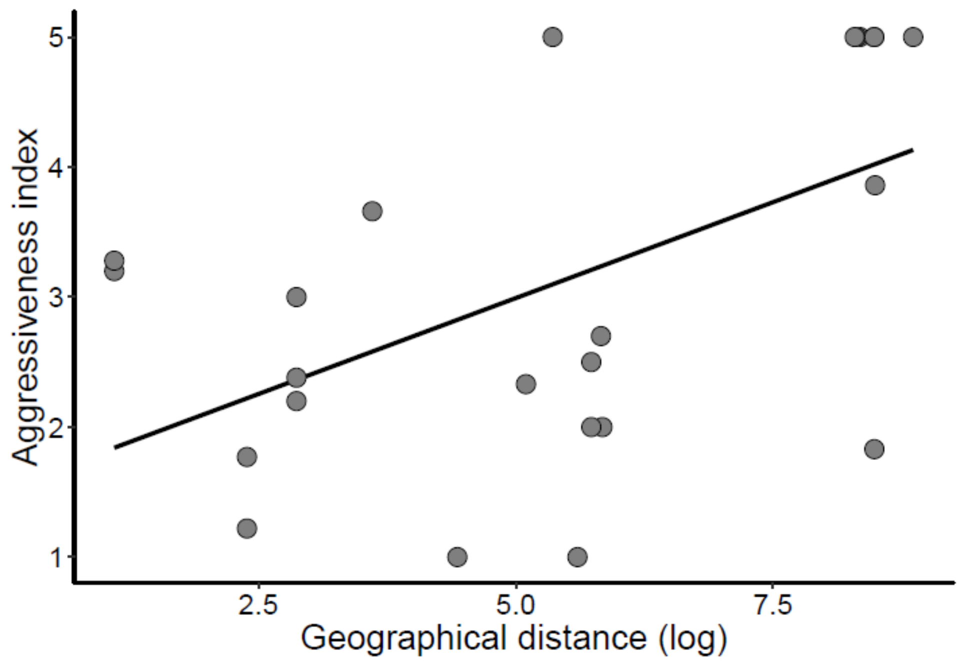

Fig. 3 Correlation between the logarithmized geographical distance in meters and aggressiveness index. Each point represents a pair of locality. The geographical distance between the pairs of localities influence the aggressiveness ($p<0.05$).

On the other hand, *A. muelleri* aggressiveness was not influenced by chemical cuticle profile (Fig. 4, $F2,2=0.0065$; $p=0.936$). We also did not detect a significant interaction between geographical distance and chemical distance ($F4,20=0.4161$; $p=0.526$). Finally, we find no difference in ant's chemical cuticles composition among the tree sampled sites (Fig. 5)

4. Discussion

In this study, we focused on testing the importance of geographical distance and chemical distance on aggressiveness. We demonstrated that geographical distance influences on this behavior, whereas the chemical distance does not. We also showed that the differences in chemical cuticular compounds may be influenced by several factors, such as diet, shelter, habits and colony structure. For instance, the cuticular composition of soil ants may be more environmentally influenced than in plant ants such as Azteca, once there is a more stable environment inside the plant's trunk whereas the soil is a much more dynamic, changeable ecosystem.

In our results, the geographical distance may be also reflecting the genetic distance, as spatially closer individuals tend to be more related (Stuart, 1987; Tsutsui et al., 2003; Fournier et al., 2016). Thus, the low level of aggression toward neighbours can be related to a higher degree of genetic kinship. This pattern is consistent with the lowest dispersal distance of Azteca species owing to their superior efficiency at finding host plants (Bruna et al., 2011). Based on this assumption, it is reasonable to believe that founder Azteca queens commonly colonize plants that are closer to their mother-nest which could so far explain why here the ants from the same next sites behaved less aggressively with themselves than to the other two sites. However, these arguments must be taken with care since we do not measure genetic kinship here.

Moreover, we found no influence of the chemical cuticular compounds on ant aggressiveness. The *A. muelleri* chemical profile was also independent from geographical distance. Despite no studies have addressed this issue for ant-plants, similar results were found in studies with soil ants such as *Myrmica* (Fürst et al., 2012), *Pheidole* (Fournier et al., 2016) and *Solenopsis* (Morel et al., 1990). One reason to explain this issue it is the colony social structure (number of queens and nests under the queen's domain) (Crosland, 1990; Dimarco et al., 2010; Helanterä et al., 2011) and the colony physical and functional structure (workers activity, individuals number, colony age) would be a factor influencing ant aggressiveness, and so far much more important than genetic distance or chemical differences (Kleineidam et al., 2017). Thus, the SZ ant's may be more aggressive against distant net mates because of the polydomic colony structure (Zinck et al., 2007). This site seems to have satellite nests, once we not found queens in all colonies.

Some studies showed that nest mate recognition is correlated with specific compounds presents in the chemical profile and not to whole cuticular profile (Dani et al., 2005; Martin et al., 2008). These compounds are individual chemicals belonging to some classes of chemicals (Guerrieri et al., 2009; Krasnec & Breed, 2013; Fournier et al., 2016; Neupert et al., 2018). More specifically, these authors suggest that Z9-alkenes and/or methyl-branched alkanes interferes with recognition and aggressive behavior while linear alkanes are not responsible for these reactions. Therefore, it is necessary investigate the presence of these compounds in *A. muelleri* chemical profile and which of these compounds influence the recognition and aggressiveness in *Azteca* ants.

5. Conclusion

Over the last years, many studies focused on investigate the ecological factors influencing animal aggressiveness (e.g. Wilson, 1971). Such influence can be due to genetic distances (Martin et al., 2008; Fournier et al., 2012; Fournier et al., 2016), chemical distances (Carlin and Hölldobler, 1986; Stuart, 1987) or both (Langen et al., 2000); the geographical distance (Frizzi et al., 2015); or the colony social structure (Crosland, 1990; Dimarco et al., 2010; Helanterä et al., 2011). These variety of results point out that such determinant factors are still very controversial.

The *Azteca-Cecropia* system therefore exemplifies a relationship in which the geographic distance influences the aggressive behaviour. However, in this system the total of chemical compounds does not explain the aggression behaviour and only some these compounds may play a main role in the aggressive behaviour and non-nest mate recognition. Thus, it is necessary to investigate if these factors and others parameters such as genetic distance, may be influencing the aggressive behaviour in this species of *Azteca*. For this, it is possible to carry out a microsatellite analysis in order to assess the kinship between these ants.

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APPENDIX I

Molecular tests

In order to confirm morphological identification of Azteca muelleri we performed a molecular approach. We submitted fifteen individuals from different colonies to sequencing of the mitochondrial gene region encoding cytochrome oxidase I (COI) protein by PCR method (Hebert et. al, 1999). We preserved the specimens in 96% ethanol and the DNA was extracted by the technique described by Doyle and Doyle (1987). The quality and quantity of the recovered DNA were verified using the NanoDrop Lite spectrophotometer (Thermo ScientificTM). We amplified the COI following primers and protocols described by Folmer et al. (1994). The COI fragments sequencing was performed by Macrogen (Seoul). Sequences were determined by both DNA tapes, visualized and aligned in the Geneious program version 8.1.5 (http://www.geneious.com, Kearse et al., 2012). We compared obtained fragments with the Genbank database to confirm molecular identification. The variability between the sequences was evaluated with the aid of Mega7 software (Sudhir Kumar, Glen Stecher, and Koichiro Tamura, 2015; MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Molecular Biology and Evolution).

Ant species identification - Molecular analysis

From 15 individuals sequenced, only eight allowed clear readings of chromatograms. Alignments of these included 545 base pairs, with 1 variable site and none parsimoniously informative site. Showing no significant variation between analyzed individuals and thus indicating a strong probability to that these individuals actually belong to the same species. Comparison of sequences obtained with the Genbank database allowed only the identification at the genus level, since there are no records of the COI gene available for the Azteca mulerii species. These results corroborate with the morphological identification, once the ants were group into the same genus, but also denotes a gap in molecular studies with this species.