UNIVERSIDADE DE SÃO PAULO

FACULDADE DE FILOSOFIA, CIÊNCIAS E LETRAS DE RIBEIRÃO PRETO

PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

Unraveling the interactions between Neotropical butterflies and ants: the role of chemical communication

Desvendando as interações entre borboletas Neotropicais e formigas: o papel da comunicação química

VERSÃO CORRIGIDA

Amalia Victoria Ceballos González

Tese apresentada à Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo, como parte das exigências para obtenção do título de Doutor em Ciências, obtido no Programa de Pós-Graduação em Entomologia.

Ribeirão Preto - SP
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ABSTRACT- Interactions between organisms of different species are widely represented across various taxa in nature. Ant-butterfly interactions stand out in Lycaenidae and Riodinidae. Associations between ants and butterflies generally occur, but not exclusively, in the larval stage. Given the chemotaxis of ants, caterpillars have evolved diverse strategies and organs enabling them to explore ant resources and services. Neotropical myrmecophilous butterfly species within Riodinidae have historically received less attention compared to their Lycaenidae counterparts from other regions. This thesis explores life cycles, chemical strategies involving cuticular hydrocarbons (CHCs) as well as the effects of specialized caterpillar organs called Tentacle Nectary Organs (TNOs) and Anterior Tentacle Organs (ATOs) in two riodinid species. The goals of the present work were: 1) Describe and characterize the interactions between two myrmecophilous butterflies and their attendant ants, focusing on aspects such as life cycle, diversity of host plants, attendant ants, and geographical distribution; 2) Identify and compare cuticular hydrocarbons (CHCs) from ants, host plants, and caterpillars; and 3) Identify the effects of ATOs and TNOs on the behavior of attendant ants for one riodinid caterpillar. The natural history data show that the two species studied use many plants as food sources, exhibit contrasting myrmecophily, and can be found in various environments throughout South America. These data may be useful for addressing future ecological, behavioral, or evolutionary issues. The results concerning CHCs indicate that facultative caterpillars exhibit a distinct chemical profile compared to that of plants and ants, with slight influence from the food source and no significant impact from the attending ant species. Additionally, TNOs and, to a lesser extent, ATOs were effective in sustaining ant attention behavior. Here we demonstrate that the close interactions established by caterpillars with ants exert a significant influence on the distribution and selection of host plants, as well as on the behavioral evolution, diversity, and chemical strategies of the caterpillars. Although these results are for caterpillar-ant interactions, they can be extended to other myrmecophilous systems.

KEYWORDS: Anterior tentacle organs, cuticular hydrocarbons, life cycle, myrmecophily, *Nymphidium lisimon,* Riodinidae, *Synargis calyce,* tentacle nectary organs.

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RESUMO- As interações entre organismos de diferentes espécies estão amplamente representadas em diferentes táxons na natureza. As interações entre formigas e borboletas se destacam em Lycaenidae e Riodinidae. As associações entre formigas e borboletas ocorrem geralmente, mas não exclusivamente, no estágio larval. Uma vez que as formigas são organismos predominantemente orientados quimicamente, as lagartas exibem diversas estratégias e órgãos que lhes permitem explorar os recursos e serviços das formigas. As espécies de borboletas mirmecófilas Neotropicais de Riodinidae historicamente têm recebido pouca atenção em comparação com as Lycaenidae de outras regiões. Nesta tese, são explorados tanto os ciclos de vida e as estratégias químicas envolvendo hidrocarbonetos cuticulares (HCs) quanto os efeitos dos órgãos especializados de lagartas chamados Órgãos Nectários Tentaculares (TNOs em inglês) e Órgãos Tentaculares Anteriores (ATOs em inglês) em duas espécies de Riodinidae. Os objetivos do presente trabalho incluíram: 1) Descrever e caracterizar as interações entre duas borboletas mirmecófilas e suas formigas assistentes, focando em aspectos como ciclo de vida, diversidade de plantas hospedeiras, formigas assistentes e distribuição geográfica; 2) Identificar e comparar os hidrocarbonetos cuticulares (HCs) de formigas, plantas hospedeiras e lagartas; e 3) Identificar os efeitos de ATOs e TNOs no comportamento das formigas assistentes para uma lagarta de Riodinidae. Os dados de história natural mostram que as duas espécies estudadas usam muitas plantas como fontes de alimento, apresentam mirmecofilia contrastante e podem ser encontradas em vários ambientes na América do Sul. Esses dados podem ser úteis para abordar questões ecológicas, comportamentais ou evolutivas futuras. Os resultados sobre HCs indicam que as lagartas facultativas apresentam um perfil químico distinto, principalmente influenciado pela fonte de alimento e não afetado pelas formigas atendentes. Além disso, os TNOs e, em menor medida, os ATOs foram eficazes em sustentar o comportamento de atenção das formigas. Aqui demonstramos que as interações próximas estabelecidas pelas lagartas com as formigas exercem uma influência significativa na distribuição e seleção das plantas hospedeiras, bem como na evolução comportamental, diversidade e estratégias químicas das lagartas.

Embora esses resultados sejam para interações entre lagartas e formigas, eles podem ser estendidos a outros sistemas mirmecófilos.

PALAVRAS-CHAVE: Ciclo de vida, hidrocarbonetos cuticulares, mirmecofilia, *Nymphidium lisimon, ó*rgãos nectários tentaculares, órgãos tentaculares anteriores, Riodinidae, *Synargis calyce.*

GENERAL INTRODUCTION

Biodiversity as we know it can be understood almost entirely as a result of the evolution of species interactions (Thompson, 1999). Interspecific interactions present a vast array of forms and are ubiquitous in the natural world, serving as a driving force for evolutionary changes in the species involved with them (Bronstein, 1994; Thompson, 1999; Otto and Nuismer, 2004). Interactions can be antagonistic, where one organism uses another as a resource and causes a negative effect, for example, in interactions such as parasitism or predation (Leung and Poulin, 2008). Others may follow the way of commensalism, where individuals of one species receive a benefit, but individuals of the other interacting species are neither harmed nor benefited (Mathis and Bronstein, 2020). There are also interactions where both participants benefit mutually, in a relationship called mutualism (Leung and Poulin, 2008; Martin and Schwab, 2012). Some examples of mutualistic interactions include insects that use plant nectar and, in return, transfer pollen between flowers, enabling pollination; and soil fungi that receive nutrients from plants in exchange for assimilating nitrogen (Boucher, 1982; Pound, 1893; Bronstein, 1994; Smith and Read, 2008; Stadler and Dixon, 2008).

There are countless examples of animals, plants, and fungi that establish associations with ants (Fiedler, 1995; Kronauer and Pierce, 2011; Hölldobler and Kwapich, 2022). There are many explanations for this as ants are dominant insects in terms of abundance, biomass, and biodiversity, with over 15,700 described species and many more to discover (Hölldobler and Wilson, 1990; Schultheiss et al., 2022). Additionally, they are the main predators, herbivores, or seed dispersers in many ecosystems (Schultheiss et al., 2022). As if that were not enough, they have an incredible geographic range, being found in nearly all terrestrial ecosystems (Hölldobler and Wilson, 1990). This success may be due to ants being social insects with one of the most complex systems of organization and chemical communication (Hölldobler and Wilson, 1990). Close associations between ants and other organisms are known as myrmecophily, and in these associations, myrmecophilous organisms typically have adaptations that allow them to benefit from the association with ants (Hinton, 1951; Atsatt, 1981; Cottrell, 1984; Marquis and Koptur, 2022).

It is known that myrmecophily in insects is widely represented in orders such as Hymenoptera, Diptera, Coleoptera, and Lepidoptera. However, many myrmecophilous organisms are cryptic, suggesting that this phenomenon may extend to numerous other taxa (Casacci et al., 2019). Therefore, an estimated 100,000 myrmecophilous species of invertebrates exist (Thomas et al., 2005). Representatives of the order Lepidoptera are well known for establishing close relationships with ants, especially species within Lycaenidae and Riodinidae (Pierce et al., 2002; Casacci et al., 2019; Marquis and Koptur, 2022). These families comprise almost 30% of known Papilionoidea species (Pierce et al., 2002). In Lycaenidae, over 70% of species are associated with ants, and in Riodinidae, 20% of species, the latter being exclusively found in the Neotropics (Marquis and Koptur, 2022). Associations between butterflies and ants can be obligate with a single ant species, or facultative with two or more ant species (Pierce et al., 2002). In obligate relationships, the presence of ants is generally crucial for caterpillars to complete their life cycles; in some cases, females do not even oviposit whether ants are not present (Fiedler, 1994; Pierce and Elgar, 1985; Pierce and

Dankowicz, 2022; Marquis and Koptur, 2022). In facultative relationships, attending ants are not essential for caterpillars to complete their life cycles, and often the presence of ants is occasional (DeVries, 1991a; Pierce et al., 2002; Casacci et al., 2019; Marquis and Koptur, 2022; Pierce and Dankowicz, 2022).

Thomann (1901) was among the pioneers to investigate the relationships between butterflies and ants, proposing a mutualistic hypothesis wherein ants protect caterpillars from natural enemies and, in return, caterpillars provide ants with food secretions. Lenz (1917) put forward the "appeasement" hypothesis, suggesting that caterpillars offer appeasement rewards to ants through their myrmecophilous organs, thus avoiding attacks. Currently, we know that associations between caterpillars and ants can vary widely, ranging from mutualistic to commensal, and even parasitic interactions. Some organisms produce secretions (trophobionts) and are actively attended by ants, while others are non-trophobiotic and do not receive constant attention from ants (Marquis and Koptur, 2022). Other research on ant-butterfly interactions has revealed uncommon interactions that would typically be classified as mutualism. For instance, Hojo et al. (2015) demonstrated that caterpillars of the butterfly *Arhopala japonica* (Murray, 1875) not only supply the ants *Pristomyrmex punctatus* Smith, 1860 with a food reward but also a substance that manipulates their behavior. After consuming the caterpillar secretions, *P. punctatus* ants exhibit reduced locomotor activity, rendering them more likely to attend to the caterpillars on subsequent occasions. Experiments revealed that caterpillar secretions can influence the levels of biogenic amines (dopamine) in worker ants (Hojo et al., 2015).

Associations between butterflies and ants typically occur during the larval stage, and despite being mediated by a combination of chemical, visual, and acoustic cues, the chemical communication likely plays the most significant role in establishing the ant-caterpillar connection (Pierce et al., 2002). This is because, although ants can utilize a variety of signals, including tactile, vibrational, or visual cues (Hölldobler and Wilson, 1990), chemical communication plays a crucial role (Adams et al., 2020). Worker ants have, on average, seven different glands that release chemical compounds, forming the basis of the communication system within their colony (Adams et al., 2020). The chemical compounds released by these glands play a crucial role in ant behaviors, eliciting alarm responses, enabling the recognition of colony members, being utilized during nestmate recruitment, and also being released during trail tracking (Lenoir et al., 2001; Von Thienen et al., 2014). Particularly, the cuticular hydrocarbons (CHCs), the main substances covering the outer layer of the cuticles of most insects, serve in social insects such as sexual recognition pheromones, caste recognition, dominance signals, fertility signals, among others (Blomquist and Ginzel, 2021). Within colonies, discriminating between nestmates and non-nestmates is crucial for maintaining social integrity (Hamilton, 1987). CHCs also play a significant role in this recognition, being colony-specific (Howard and Blomquist, 2005). However, many myrmecophilous organisms, such as caterpillars, have managed to exploit these complex communication systems to their advantage (Casacci et al., 2019; Adams et al., 2020).

The strategies employed by caterpillars and other myrmecophiles to overcome the chemical recognition barrier of ants vary considerably depending on the specificity of their association (von Beeren et al., 2012). In nonspecific associations where a single plant is visited by several ant species, the least costly strategy for the caterpillar is to mimic the surroundings (Akino et al., 2004; Lima et al., 2021). It has been demonstrated that cuticular chemical correspondence between caterpillars of *Biston robustum* (Butler, 1879) and their host plants is efficient in helping caterpillars to escape attacks from various ant species (Akino et al., 2004). Recent studies have shown a high degree of similarity between CHC profiles of some facultative myrmecophilous caterpillar species of Lycaenidae and their host plants (Lima et al., 2021). Butterfly caterpillars can also employ chemical insignificance by reducing the compounds on their cuticular profile (Inui et al., 2015; Barbero, 2016). An example of this is found in the caterpillars of *Arhopala zylda* (Corbet, 1941), which carry only traces of seven CHCs on their cuticular profile, less than a third of the number of compounds forming the chemical profile of the other two *Arhopala* species, and are generally ignored by ants visiting host plants (Inui et al., 2015). Another strategy is chemical mimicry, where caterpillars acquire the ants' cuticular profile through contact, diet, or active biosynthesis of CHCs (Dettner & Liepert, 1994; Akino et al., 1999; Lenoir et al., 2001). For example, caterpillars of *Phengaris rebeli* (Berger, 1946) manage to mimic the chemical profile of *Myrmica schencki* (Viereck, 1903) ants. Due to these similarities in the chemical profile, caterpillars of this species are taken into ant nests, where they are subsequently fed by ants, effectively becoming cuckoos (Akino et al., 1999). Studies conducted by Lima et al. (2021) suggest that there may be an additional strategy, chemical conspicuousness. In this strategy, caterpillars exhibit a distinct cuticular profile compared to attending ants or host plants, and similar to that of other caterpillars coexisting with them (Lima et al., 2021).

In addition to the strategies involving CHCs, most myrmecophile butterfly caterpillars have specialized organs that release substances mediating ant-caterpillar interactions (Fig 1). Some of these organs provide nutritive rewards, while others release substances that modify ant behavior (Malicky, 1970; Henning, 1983; Cottrell, 1984; DeVries, 1988; Fielder, 1988; Casacci et al., 2019). For instance, the dorsal nectary organ (DNO) in Lycaenidae (Malicky, 1970) and the tentacle nectary organs (TNOs) in Riodinidae (DeVries, 1988) provide sugars and amino acids to attending ants. The tentacle organs (TOs) in Lycaenidae (Malicky, 1970) and the anterior tentacle organs (ATOs) in Riodinidae (Ross, 1966; DeVries, 1988) appear to produce volatiles acting as chemical messengers, modifying ant behavior (DeVries, 1991a). The perforated cupola organs (PCOS) are found in both butterfly families and secretes appeasement substances or food for ants (Malicky, 1970). Even some butterfly pupae (Riodinidae) have specialized structures for producing sounds, called vibratory papillae, which are suggested to be involved in emitting signals that promote ant-pupa interactions (DeVries, 1990; DeVries, 1991b; Casacci et al., 2019).

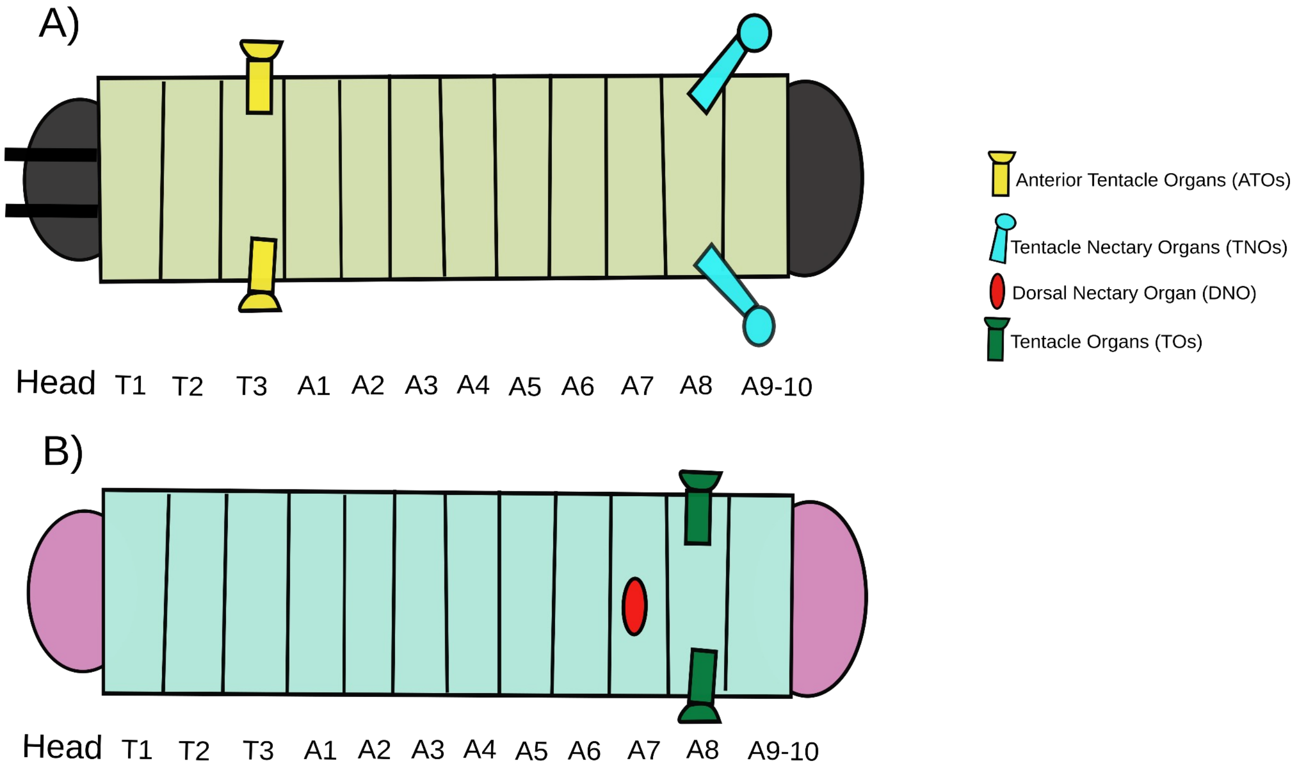


Fig.1. A) Drawing of a typical caterpillar of Riodinidae showing the location of anterior tentacle organs (ATOs) on the third thoracic segment, and tentacle nectary organs (TNOs) on the eighth abdominal segment. B) Drawing of a typical caterpillar of Lycaenidae showing the location of the dorsal nectary organ (DNO) on the seventh abdominal segment and the tentacle organs (TOs) on the eighth abdominal segment (Modified from DeVries 1991c).

Various studies have focused on exploring associations between caterpillars and ants. For example, the genera *Phengaris* and *Niphanda* are models for studying myrmecophily, chemical communication, biodiversity, conservation, and the evolution of these associations (Casacci et al., 2019; Marquis and Koptur, 2022). However, our understanding of the relationships between myrmecophilous butterflies and ants in the Neotropics is limited compared to other regions (Kaminski et al., 2012). Additionally, while there are several well-documented species of Lycaenidae, this is not the case for Riodinidae (Barbero, 2016; Casacci et al., 2019). For example, there is no literature available exploring the CHCs of myrmecophilous caterpillars of Riodinidae, their host plants, and their attendant ants (Casacci et al., 2019). On the other hand, although some studies have investigated the specialized organs of caterpillars that mediate interactions with ants, such as the ATOs (Riodinidae), there is still no consensus on their function and effects on ants, making further studies relevant (Gnatzy, 2017; Casacci et al., 2019).

Studies on butterflies of Riodinidae have the potential to provide important information on various aspects of evolutionary biology, phenotypic plasticity, ecological patterns, and myrmecophily in general (D'Abrera, 1994; DeVries, 1991c). For instance, it is suggested that myrmecophily is responsible for the transition of riodinids from monophagy to polyphagy (DeVries, 1994); however, studies exploring the diversity of host plants of myrmecophilous species, as well as the diversity of attendant ants interacting with them and influencing the selection of these host plants are scarce (Kaminski et al., 2013; DeVries and Chacon, 1992). Considering this, the present thesis addresses the relationship between riodinid butterflies and associated ants, focusing on chemical communication. The study models include the butterfly species *Nymphidium lisimon* (Stoll, 1790), whose larvae are polyphagous and have an obligate association with the ant species *Wasmannia auropunctata*

(Roger 1863), and *Synargis calyce* (Felder & Felder, 1862), a polyphagous species with a facultative association with various ant species.

GENERAL OBJETIVES

Chapter 1: Natural history and distribution of two riodinid species with contrasting myrmecophilous strategies

Interactions between ants and other organisms are abundant in nature. However, we are still far from understanding and knowing all of them, especially in the tropics. Caterpillars of Lycaenidae and Riodinidae are widely known for establishing relationships with ants, although riodinids have historically received less attention. Furthermore, few studies have focused on exploring the diversity of host plants, attending ants, and complete distribution for myrmecophilous species of this family. Here, we present aspects of the life cycle, field notes, diversity of host plants and attending ants, as well as the distribution of two myrmecophilous riodinids that have different interaction strategies. We consider that this data is a fundamental basis for establishing future lines of research, in ecological, behavioral, or evolutionary aspects, as well as promoting strategies for species conservation.

General Objective

- Describe and characterize the interactions between two myrmecophilous butterflies and their attendant ants, focusing on aspects such as life cycle, diversity of host plants and attendant ants, and geographical distribution.

Chapter 2: Influence of host plants and tending ants on the cuticular hydrocarbon profile of a generalist myrmecophilous caterpillar

It is well known that insect cuticular hydrocarbons (CHCs) play a fundamental role in communication among individuals in ant societies and in species that live in symbiosis with them, such as myrmecophilous caterpillars. However, it is still poorly understood the influence of biotic environmental factors on the CHC profiles of myrmecophiles, which could shed light on the different chemical strategies used by caterpillars (especially facultative species) to interact with ants. Thus, we reared *S. calyce* caterpillars (Lepidoptera: Riodinidae) under different conditions and subsequently conducted chemical analyses of the cuticular profiles of these caterpillar groups, host plants, and tending ant species. We expected the caterpillar profiles to be primarily influenced by their food source rather than the contact established with tending ants.

General Objective

- Investigate the cuticular profiles of myrmecophilous butterfly caterpillars, attending ants, and host plants in order to gain a comprehensive understanding of the biotic influence on caterpillar chemical profiles and the potential chemical strategies employed by these caterpillars.

Chapter 3: The role of ant-organs of myrmecophilous riodinid caterpillars and their impact on ant attendant behavior.

Ants play a crucial role in protecting myrmecophilous organisms against natural enemies such as predators and parasitoids. Among Riodinidae caterpillars, TNOs and ATOs are specialized structures that mediate interactions with ants. Elucidating the individual effect of these organs or any potential synergy between them, can offer a comprehensive understanding of the costs and benefits that myrmecophily brings to both members of the myrmecophilous system. In this chapter, our objective was to construct an ethogram of behavioral sequences in interactions between myrmecophilous caterpillars *S. calyce* (Lepidoptera: Riodinidae) and one of the attendant ant species, *Camponotus crassus* Mayr, 1862 (Hymenoptera: Formicidae). Subsequently, we manipulated the ATOs and TNOs through occlusion to determine their role in sustaining the interactions between attendant ants and caterpillars. Our hypothesis was that the TNOs and ATOs would facilitate and sustain ant-attending behavior.

General Objectives

- Describe the interactions betwee*n S. calyce* (Riodinidae) caterpillars and their associated ants using ethograms to document behavioral patterns.
- Conduct experiments to explore the functions of Tentacle Nectary Organs (TNOs) and Anterior Tentacle Organs (ATOs) in the myrmecophilic relationship between *S. calyce* caterpillars and ants.

REFERENCES

Adams, R. M., Wells, R. L., Yanoviak, S. P., Frost, C. J., & Fox, E. G. (2020) Interspecific eavesdropping on ant chemical communication. Frontiers in Ecology and Evolution, 8, 24. https://doi.org/10.3389/fevo.2020.00024.

Akino, T., Knapp, J. J., Thomas, J. A., & Elmes, G. W. (1999). Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. Proceedings of the Royal Society of London. Series B: Biological Sciences, 266, 1419-1426. https://doi.org/10.1098/rspb.1999.0796.

Akino. T., Nakamura. K.I., & Wakamura, S. (2004) Diet-induced chemical phytomimesis by twig-like caterpillars of Biston Robustum Butler (Lepidoptera: Geometridae). Chemoecology 14:165–174. https://doi.org/10.1007/s00049-004-0274-4.

Atsatt, P. R. (1981). Lycaenid butterflies and ants: selection for enemy-free space. The American Naturalist, 118(5), 638-654. https://doi.org/10.1086/283859.

Barbero, F. (2016) Cuticular lipids as a cross-talk among ants, plants and butterflies. International Journal of Molecular Sciences, 17,1966. https://doi.org/10.3390/ijms17121966.

Blomquist, G. J., & Ginzel, M. D. (2021) Chemical ecology, biochemistry, and molecular biology of insect hydrocarbons. Annual Review of Entomology, 66, 45-60. https://doi.org/10.1146/annurev-ento-031620-071754.

Boucher, D. H., James, S., & Keeler, K. H. (1982) The ecology of mutualism. Annual Review of Ecology and Systematics, 13, 315-347. https://doi.org/10.1146/annurev.es.13.110182.001531.

Bronstein, J. L. (1994) Conditional outcomes in mutualistic interactions. Trends in Ecology & Evolution, 9, 214-217. https://doi.org/10.1016/0169-5347(94)90246-1.

Casacci, L.P., Bonelli. S., Balletto, E., & Barbero, F. (2019) Multimodal signaling in myrmecophilous butterflies. Frontiers in Ecology and Evolution 7:454. https://doi.org/10.3389/fevo.2019.00454

Cottrell, C.B. (1984) Aphytophagy in butterflies: its relationship to myrmecophily. Zoological Journal of the Linnean Society, 79, 1–57. https://doi.org/10.1111/j.1096-3642.1984.tb02318.x.

D'Abrera, B. (1994) Butterflies of the neotropical region. Part VI. Riodinidae. Victoria, Hill House, IX p 216.

Dettner, K., & Liepert, C. (1994) Chemical mimicry and camouflage. Annual Review of Entomology, 39(1), 129-154. https://doi.org/10.1146/annurev.en.39.010194.001021.

DeVries, P. J. (1988). The larval ant-organs of *Thisbe irenea* (Lepidoptera: Riodinidae) and their effects upon attending ants. Zoological Journal of the Linnean Society, 94, 379-393. https://doi.org/10.1111/j.1096-3642.1988.tb01201.x.

DeVries, P. J. (1991a). Mutualism between *Thisbe irenea* butterflies and ants, and the role of ant ecology in the evolution of larval-ant associations. Biological Journal of the Linnean Society, 43, 179-195. https://doi.org/10.1111/j.1095-8312.1991.tb00592.x.

DeVries, P. J. (1991b) Detecting and recording the calls produced by butterfly caterpillars and ants. Journal of Research on the Lepidoptera, 28, 258-262.

DeVries, P. J. (1991c). Evolutionary and ecological patterns in myrmecophilous riodinid butterflies. Ant-plant interactions. Oxford University Press, Oxford, 143-156.

DeVries, P. J. (1994) Patterns of butterfly diversity and promising topics in natural history and ecology. La Selva: ecology and natural history of a Neotropical rain forest. University of Chicago Press, Chicago, 187-194.

DeVries, P. J., Chacon, I. A., & Murray, D. (1992) Toward a better understanding of host use and biodiversity in riodinid butterflies(Lepidoptera). Journal of Research on the Lepidoptera, 31, 103-126.

Fiedler, K., & Maschwitz, U. (1988) Functional analysis of the myrmecophilous relationships between ants (Hymenoptera: Formicidae) and lycaenids (Lepidoptera: Lycaenidae) II. Lycaenid larvae as trophobiotic partners of ants—a quantitative approach. Oecologia, 75, 204-206. https://doi.org/10.1007/BF00378598.

Fiedler, K. (1994) Lycaenid butterflies and plants: is myrmecophily associated with amplified hostplant diversity? Ecological Entomology 19, 79–82. https://doi.org/10.1111/j.1365-2311.1994.tb00393.x

Fiedler, K. (1995) Associations of lycaenid butterflies with ants in Turkey. Die Tagfalter der Türkei unter Berücksichtigung der Angrenzenden Länder 1, 437–50.

Gnatzy, W., Jatho, M., Kleinteich, T., Gorb, S. N., & Hustert, R. (2017) The eversible tentacle organs of *Polyommatus* caterpillars (Lepidoptera, Lycaenidae): Morphology, fine structure, sensory supply and functional aspects. Arthropod Structure & Development, 46, 788-804. https://doi.org/10.1016/j.asd.2017.10.003.

Hamilton, W.D. (1987). Kinship, recognition, disease and intelligence. In Animal Societies: Theories and Facts, Y. Ito, J.L. Brown, and J. Kikkawa, eds. (Tokyo: Japan Scientific Societies Press), pp. 81–102.

Henning, S. F. (1983) Chemical communication between Iycaenid larvae (Lepidoptera: Lycaenidae) and ants (Hymenoptera: Formicidae). Journal of the Entomological Society of Southern Africa, 46, 341-366. https://hdl.handle.net/10520/AJA00128789_3496.

Hinton, H.E. (1951) Myrmecophilous Lycaenidae and other Lepidoptera—a summary. Proceedings of the London Entomological & Natural History Society 1949–1950, 111–175

Hojo, M. K., Pierce, N. E., & Tsuji, K. (2015) Lycaenid caterpillar secretions manipulate attendant ant behavior. Current Biology, 25, 2260-2264. https://doi.org/10.1016/j.cub.2015.07.016.

Hölldobler, B., & Kwapich, C. (2022) The Lycaenidae: mutualists, predators, and parasites. The guests of ants: how myrmecophiles interact with their hosts. Harvard University Press, Massachusetts, pp 149–219

Hölldobler, B., & Wilson, E.O. (1990) The Ants. The Ants. The Belknap Press of Harvard University Press, Cambridge p 732.

Howard, R. W., & Blomquist, G. J. (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annual Review Entomology, 50, 371-393.

Inui, Y., Shimizu-Kaya, U., Okubo, T., Yamsaki, E., & Itioka, T. (2015) Various chemical strategies to deceive ants in three *Arhopala* species (Lepidoptera: Lycaenidae) exploiting *Macaranga* myrmecophytes. PLoS One, 10, e0120652. https://doi.org/10.1371/journal.pone.0120652.

Kaminski, L. A., & Carvalho-Filho, F. S. (2012) Life history of *Aricoris propitia* (Lepidoptera: Riodinidae)—A myrmecophilous butterfly obligately associated with fire ants. Psyche: A Journal of Entomology, 2012. https://doi.org/10.1155/2012/126876.

Kaminski, L. A., Mota, L. L., Freitas, A. V., & Moreira, G. R. (2013) Two ways to be a myrmecophilous butterfly: natural history and comparative immature-stage morphology of two species of *Theope* (Lepidoptera: Riodinidae). Biological Journal of the Linnean Society, 108, 844-870. https://doi.org/10.1111/bij.12014.

Kronauer, D. J., & Pierce, N. E. (2011) Myrmecophiles. Current Biology, 21, R208-R209. https://doi.org/10.1016/j.cub.2011.01.050

Lenoir, A., d'Ettorre, P., Errard, C., & Hefetz, A. (2001) Chemical ecology and social parasitism in ants. Annual Review of Entomology, 46, 573-599. https://doi.org/10.1146/annurev.ento.46.1.573.

Lenz F. 1917. Kleinere Mitteilungen. Der Erhaltungsgrund der Myrmekophilie. Zeit. Induk. Abstamm. Vererbungsl. 18,44–48

Leung, T. L., & Poulin, R. (2008) Parasitism, commensalism, and mutualism: exploring the many shades of symbioses. Vie et Milieu/Life & Environment, 107-115.

Lima, L.D., Trigo, J.R., & Kaminski, L.A. (2021) Chemical convergence between a guild of facultative myrmecophilous caterpillars and host plants. Ecological Entomology 46, 66–75. https://doi.org/10.1111/een.12941.

Malicky, H. (1970) New aspects of the association between lycaenid larvae (Lycaenidae) and ants (Formicidae, Hymenoptera). Journal of the Lepidopterists´ Society, 24, 190-202.

Marquis, R.J., & Koptur, S. (2022) Caterpillars in the middle: Tritrophic interactions in a changing world. Springer. pp. 319-391

Martin, B. D., & Schwab, E. (2012) Symbiosis:"Living together" in chaos. Studies in the History of Biology, 4, 7-25.

Mathis, K. A., & Bronstein, J. L. (2020) Our current understanding of commensalism. Annual Review of Ecology, Evolution, and Systematics, 51, 167-189. https://doi.org/10.1146/annurev-ecolsys-011720-040844.

Pierce, N. E., & Elgar, M. A. (1985) The influence of ants on host plant selection by Jalmenus evagoras, a myrmecophilous lycaenid butterfly. Behavioral Ecology and Sociobiology, 16, 209-222. https://doi.org/10.1007/BF00310983

Pierce, N. E., Braby, M. F., Heath, A., Lohman, D. J., Mathew, J., Rand, D. B., & Travassos, M. A. (2002). The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). Annual Review of Entomology, 47, 733-771. https://doi.org/10.1146/annurev.ento.47.091201.145257

Pierce, N. E., & Dankowicz, E. (2022). Behavioral, ecological and evolutionary mechanisms underlying caterpillar-ant symbioses. Current Opinion in Insect Science, 52, 100898. https://doi.org/10.1016/j.cois.2022.100898.

Pound, R. (1893) Symbiosis and mutualism. The American Naturalist, 27, 509-520.

Otto, S. P., & Nuismer, S. L. (2004) Species interactions and the evolution of sex. Science, 304, 1018-1020. https://doi.org/10.1126/science.109407.

Ross, G. N. (1966) Life-history studies on Mexican butterflies. IV. The ecology and ethology of *Anatole rossi*, a myrmecophilous metalmark (Lepidoptera: Riodinidae). Annals of the Entomological Society of America, 59, 985-1004. https://doi.org/10.1093/aesa/59.5.985.

Schultheiss, P., Nooten, S. S., Wang, R., Wong, M. K., Brassard, F., & Guénard, B. (2022) The abundance, biomass, and distribution of ants on Earth. Proceedings of the National Academy of Sciences, 119, e2201550119. https://doi.org/10.1073/pnas.220155011.

Smith, S. E., & Read, D. J. (2010) Mycorrhizal symbiosis. Academic Press, Great Britain p 787

Stadler, B., & Dixon, A. F. G. (2008) Mutualism: ants and their insect partners. Cambridge university press, Cambridge Uk, p 219

Thomann, H. (1901) Schmetterlinge und Ameisen: Beobachtungen über eine Symbiose zwischen *Lycaena argus* L. und *Formica cinerea* Mayr. Jaresbericht Naturforsch. Ges. Graub¨undens 44, 1–40

Thomas. J.A., Schönrogge, K., & Elmes, G.W. (2005) Specialisations and host associations of social parasites of ants. Evolutionary ecology. In: Rolff J, Fellowes M, Holloway G (eds) Symposium of the Royal Entomological Society XXI, pp 475–514. https://doi.org/10.1079/9780851998121.047

Thompson, J. N. (1999) The evolution of species interactions. Science, 284, 2116-2118. https://doi.org/10.1126/science.284.5423.2116.

von Beeren, C., Pohl, S., & Witte, V. (2012) On the use of adaptive resemblance terms in chemical ecology. Psyche: A Journal of Entomology, 2012. https://doi.org/10.1155/2012/635761.

von Thienen, W., Metzler, D., Choe, D. H., & Witte, V. (2014) Pheromone communication in ants: a detailed analysis of concentration-dependent decisions in three species. Behavioral Ecology and Sociobiology, 68, 1611-1627. https://doi.org/10.1007/s00265-014-1770-3.

CHAPTER 1

NATURAL HISTORY AND DISTRIBUTION OF TWO RIODINID SPECIES WITH CONTRASTING MYRMECOPHILOUS STRATEGIES

Abstract

Interactions between species are numerous and represents a driving force behind the incredible biodiversity of our planet. In the natural world, there are numerous examples of organisms interacting with ants. Lycaenidae and Riodinidae butterflies have a large number of species that establish close interactions with ants. The myrmecophilous species of Riodinidae are exclusively confined to the Neotropics and have received less attention compared to their sister family, Lycaenidae. The ecology, evolution, and behavior of myrmecophilous species are greatly influenced by their interactions with ants, hence the importance of studies on the nature of the interaction, as well as the diversity of attending ants and their effect on the distribution and selection of host plants by butterflies. Here we present aspects of the life cycle, field notes, diversity of host plants and attendant ants, as well as geographical distribution of two myrmecophilous riodinid species that exhibit contrasting myrmecophilous strategies. We found that the species studied present five larval instars, and both *N. lisimon* and *S. calyce* exhibit functional tentacle nectary organs TNOs. However, field and laboratory observations suggest that only *S. calyce* presents anterior tentacle organs ATOs. Both species are widely distributed in South America, from Venezuela to Brazil, with a broad range for host plants and attending ants regarding the facultative caterpillars. We consider that both species are interesting models for studying various aspects of myrmecophily such as phenotypic plasticity, evolution, and ecology. Efforts in studying the natural history of the species contribute to understanding general patterns of myrmecophily, its implications in host plant selection, biogeography, and also to highlight conservation demands of the species.

Key Words: Attendant ants, distribution, host plants, myrmecophily, *Nymphidium lisimon*, Riodinidae, *Synargis calyce.*

Introduction

The Riodinidae butterflies exhibit notable peculiarities, one of which is their nearly exclusive confinement to a geographic region (Hall and Harvey, 2002). Riodinids constitute more than 8% of all butterflies, with almost 93% of the species occurring in the Neotropics, contrasting with only 7% occurring in the Paleotropics (Espeland et al., 2015). Additionally, these butterflies display a wide variety of morphological, ecological, and behavioral features (Hall and Harvey, 2002; Espeland et al., 2015; Seraphim et al., 2018). Besides Lycaenidae, Riodinidae have complex associations with ants, particularly during the larval stage, and in many cases, various specialized organs promote these associations (Hinton, 1951; Pierce et al., 1987; DeVries, 1991b; Fiedler, 1991; DeVries et al., 1992; Pierce et al., 2002; Campbell and Pierce, 2003). Within Riodinidae, most species that interact with ants are found within Nymphidiini (Hall and Harvey, 2002).

The ant associations may involve obligate relationships with a particular ant genus or species, where typically the caterpillars cannot complete their life cycle without the ants. In contrast, in facultative relationships, caterpillars can interact with many ant species and occasionally may be found in the field without ants (DeVries, 1991a; Pierce et al., 2002; Casacci et al. 2019; Marquis and Koptur, 2022; Pierce and Dankowicz, 2022). Facultative relationships appear to be the dominant strategy in both Lycaenidae and Riodinidae, however, there are a few taxa that have developed obligate myrmecophily with ants (see Casacci et al. 2019; Marquis and Koptur, 2022). All myrmecophilous riodinids possess Tentacle Nectary Organs (TNOs), which secrete amino acids and carbohydrates exploited by attendant ants. Additionally, most species have another pair of tentacle organs, the Anterior Tenacle Organs

(TNOs), which appear to be involved in chemical stimuli delivery (DeVries, 1991a, 1991b; Casacci et al. 2019).

Caterpillars that are myrmecophilous generally present another notable particularity: they have a greater diversity of host plants than their non-myrmecophilous counterparts (Pierce & Elgar, 1985; Fiedler, 1994). This greater diversity can be explained because the selection of host plants in myrmecophilous caterpillars is influenced by the presence of their attendant ants (Marquis & Koptur, 2022). Furthermore, there is evidence that female myrmecophilous butterflies can use the presence of attendant ants as cues for oviposition (Pierce & Elgar, 1985; Casacci et al., 2019). As a consequence, many of the plants used by myrmecophilous caterpillars have extrafloral nectaries, as they are constantly visited by ants (DeVries, 1991b). Therefore, understanding patterns in the use of host plants in myrmecophilous caterpillars requires an analysis of the relationships between ants and plants (DeVries et al., 1992).

From a general perspective, Riodinidae is the least understood of all butterfly families (DeVries, 1997; Seraphim et al., 2018). While notes and life cycle studies on some myrmecophilous riodinid species can be found (Callaghan, 1981; Ross, 1966; Callaghan, 1986; Horvitz et al., 1987; Callaghan, 1988; DeVries, 1988; DeVries, 2000; Kaminski, 2008; Kaminski and Carvalho-Filho, 2012; Kaminski et al., 2013; Torres and Pomerantz, 2016; Kaminski and Lima, 2019; Mota et al., 2020; Kaminski et al., 2020; Kaminski et al., 2021; Pérez-Lachaud et al., 2021; Guedes and Kaminski, 2023), exploration into other aspects, such as species distribution or the high diversity usage of host plants and interactions with attendant ants, has been largely neglected (Kaminski et al. 2013).

We selected the riodinid species *Synargis calyce* (Felder & Felder, 1862) and *Nymphidium lisimon* (Stoll, 1790), based on their distinct myrmecophilous strategies, with *S. calyce* being a facultative species and *N. lisimon* an obligate species (Callaghan, 1986; Kaminski, 2021). Additionally, both species are widely distributed in South America and serve as interesting study models due to their high ecological plasticity. In the case of *N. lisimon*, it utilizes a wide variety of host plants, while *S. calyce*, besides the diversity of host plants, is also attended by several species of ants.(Hall, 2018; Kaminski, 2021). In the literature, there are data on the life cycle for *S. calyce* and preliminary information on some instars of *N. lisimon*. However, there are no studies compiling the diversity of host plants or attendant ants (Callaghan, 1986, 1988).

In this study, we present life cycle data, field observations, distribution, host plant records, and attendant ant records of these two species of riodinids that exhibit contrasting interaction strategies with ants. Comprehensive natural history information on species can contribute to unraveling the universe of myrmecophily in butterflies, establishing patterns that can be extended to other groups and may help promoting strategies for species preservation.

Material and Methods

Collection and life cycle of species

We conducted collections at the University of São Paulo (21.1637° S, 47.8592° W), Ribeirão Preto Campus, Brazil to study the life cycles of two riodinids.

Synargis calyce: Attempts to mate adults in captivity were unsuccessful. Instead, we collected approximately 30 eggs from the host plant *Senegalia pollyphyla* (DC.) Britton & Rose (Fabaceae) in different locations in the field and raised them in the laboratory. The caterpillars (n=20) were placed individually in a 250 ml plastic container along with shoots of the host plant where the eggs were found.

Nymphidium lisimon: Adults were collected in the field and brought to the laboratory. In the laboratory, the adults were allowed to mate and over 100 eggs were obtained and 20 were selected for life cycle monitoring. The caterpillars were also placed individually in a 250 ml plastic container with shoots of the host plant *S. pollyphyla*, as a large number of caterpillars were observed in the field on this host plant.

We used a current terminology for inmature stage morphology of Riodinidae based on Kaminski, et al. (2021). To determine the duration of the different larval instars of the two species, containers with larvae were checked daily for the exuviae of larval head capsules, which are indicative of molting. Additionally, we conducted daily monitoring of each individual, which was essential for noting the changes, comparing characteristics with the literature (Callaghan, 1986, 1988), and taking pictures in the laboratory using a stereomicroscope (Leica M125C, Leica Mycrosystems, Germany) with a coupled image capture system. All caterpillars were maintained at a controlled temperature of 25 °C with a photoperiod of 12 hours of light and 12 hours of darkness. Field observations were also conducted on attendant ants, host plants, and parasitoids attacking these butterflies species. This study was conducted between 2021 and 2023 during the months of January to May and October to December. Collections were made in the morning, with an effort of 4 hours per day.

Distribution records

The distribution data of the two species were obtained from searching the database *Google Scholar* for published studies using the following terms: *Synargis calyce, Nymphidium lisimon* and Riodinidae butterflies. We also compiled data from museums and collections accessible through the Global Biodiversity Information Facility online database (https://www.gbif.org), and citizen records with a research level in iNaturalist (https://www.inaturalist.org/), including the project Ant-Butterfly Interactions (Kaminski, 2021). The distribution data for *N. lisimon* from Hall (2018) were also utilized. Besides the collection sites, data on host plants and attendant ants were also gathered for immature stage records.

Results

Natural history and Life cycle

Synargis calyce- In the study location, the species is abundant between May and October (AVCG personal observations). Adult females were observed laying eggs after 12:30 h on plants patrolled by attendant ants like *Camponotus crassus* Mayr, 1862*, Paratrechina longicornis (*Latreille, 1802)*, Camponotus atriceps* Smith, 1858 and *Camponotus renggeri (*Emery, 1894). In the laboratory, gravid females collected in the field did not lay eggs on plants if ants were not present (AVCG personal observations). Egg-laying is typically individual, with only a few eggs per plant. Additionally, females have been observed ovipositing directly on treehoppers (Lima et al., 2023).

Egg: Color lilac when laid, changing to bright bronze before hatching. It has a semi-spherical shape with a concave upper surface, which internally has a small convex region. The egg is covered by small cells forming hexagonal patterns (Fig 1A).The egg stage lasted approximately six days (length: 1mm).

First instar: Black head and orange-yellow body. Numerous setae distributed throughout the body. (Fig 1B). Duration six days (length of the whole body: 2mm).

Second instar: Black head and thorax with two black horns, each with two setae at the end. TNOs are already visible on the eighth abdominal segment. Both the thorax and abdomen are green-yellow (Fig 1C). Duration five days (length of the whole body: 4mm).

Third instar: Black head. Thorax, and abdomen are green with small brown spots. The TNOs are more developed (Fig 1D). Duration five days (length of the whole body: 7-8mm).

Fourth instar: Black head and green thorax with horns with a light brown area in the middle. Abdomen is green with dark brown spots (Fig 1E). Duration five days (length of the whole body: 10-11mm)

Fifth instar: Black head and green thorax with black horns, but with a large cream area in the middle of them. V-shaped figure of dark and light brown colors between T2 and T3. There is a brown spot between the TNOs (Fig1 F). Duration nine days (length of the whole body:19-20mm).

Pupa: Irregular shape, convex dorsally, and light brown-grayish. The end part of the abdomen is light brown with darker longitudinal lines (Fig 1G). Duration eight days (length of the whole body: 16mm).

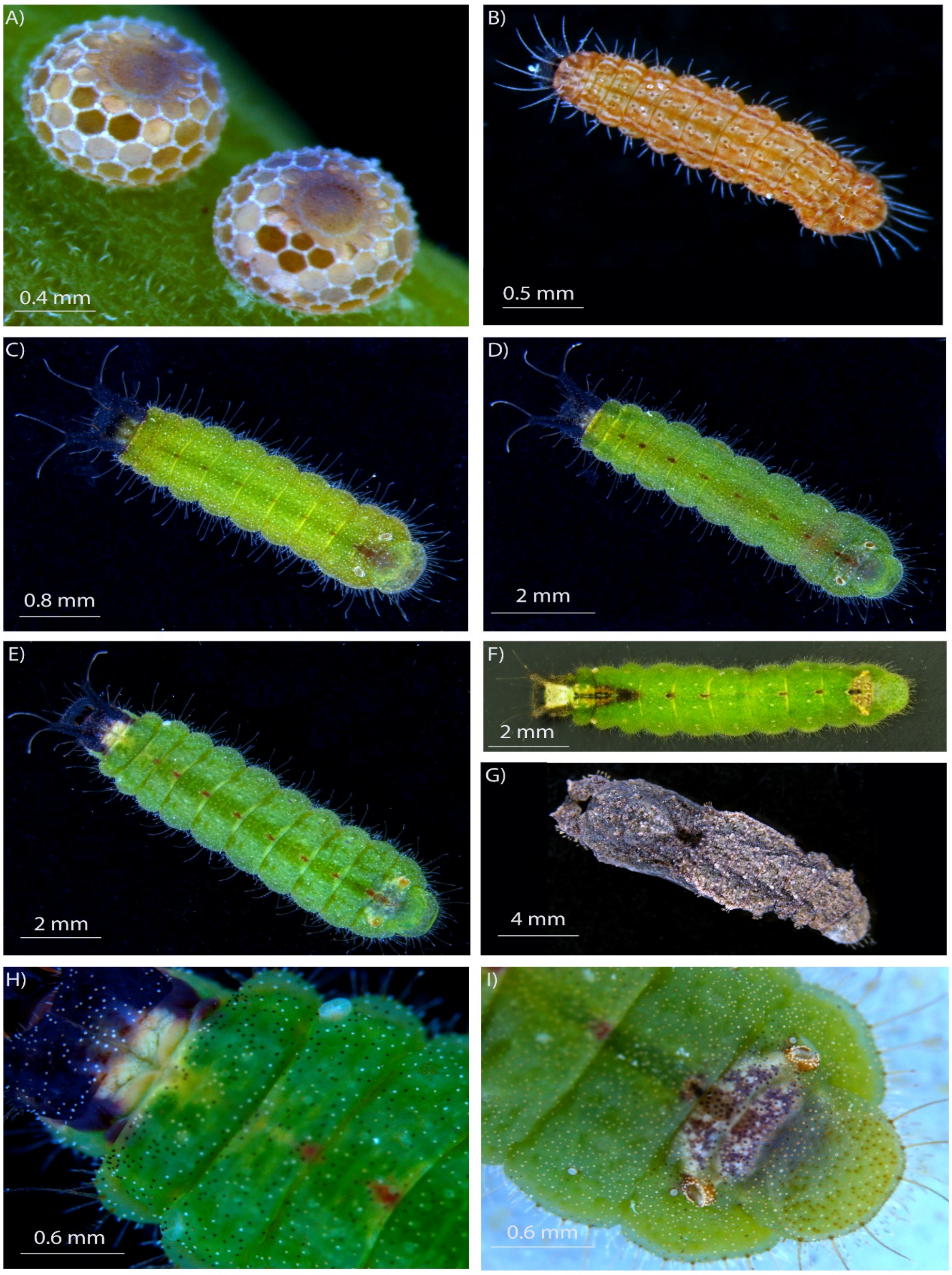


Fig 1. Life cycle of *S. calyce*. A) egg, B) first-instar caterpillar, C) second-instar caterpillar, D) third-instar caterpillar, E) fourth-instar caterpillar, F) fifth-instar caterpillar, G) pupa, H) ATOs on the third thoracic segment, dorsal view, and I) TNOs on the eighth abdominal segment, dorsal view. Photos by André Rodrigues.

From the third instar onwards, where the myrmecophilous organs ATOs and TNOs became noticeable (Fig 1D, eighth abdominal segment), ant attendance in the field became more consistent. In field observations, caterpillars were always seen being attended by ants, with *C. crassus* ants being among the most observed. The same caterpillar is attended by one ant species during the day (*C. crassus*) and another ant species during the night (*C. renggeri*) (LAK personal observations). Despite being consistently attended by ants, caterpillars are frequently attacked by parasitoids. The 40% of the caterpillars collected in the field were parasitized. In the study area, we recorded attacks by parasitoids from two wasp species, *Cotesia* sp. (Hymenoptera: Braconidae) and one species from the family Chalcididae (Hymenoptera), as well as a fly from the family Tachinidae (Diptera). Observations in the field and available records showed that caterpillars feed on at least 17 plant species, with *Inga* sp being one of the most reported. It was also reported that *S. calyce* is attended by 15 ant species, with *Camponotus* ants being the most commonly reported (Fig 2, Table1). Pupae observed in the field were ignored by attendant ants, and no attacks were observed.

Table 1. Record of host plant species and attendant ants for *Synargis calyce* and *Nymphidium lisimon.*

Species	Host plant	Attendant ant	Country	References
Synargis Calyce	*Piptadenia* sp.	*Camponotus crassus* *Camponotus blandus*	Argentina Brazil	iNaturalist
	Senna alata	*Camponotus crassus*	Brazil	iNaturalist
	Inga sp. 1	*Camponotus crassus* *Camponotus* sp2 *Camponotus blandus* *Ectatomma tuberculatum* *Camponotus atriceps*	Brazil Trinidad and Tobago	iNaturalist
	Inga laurina	*Camponotus crassus* *Pheidole* sp.	Brazil	iNaturalist
	Senegalia polyphylla	*Camponotus crassus* *Camponotus renggeri*	Brazil	iNaturalist, Lima et al.,

Species	Host plant	Attendant ant	Country	References
		Wasmannia auropunctata *Paratrechina longicornis*		2023
	Triplaris gardneriana	*Camponotus crassus*	Brazil	Guedes and Kaminski, 2023
	Piptadenia sp.	*Camponotus crassus* *Camponotus blandus*	Brazil	iNaturalist
	Cynophalla flexuosa	*Camponotus* crassus	Brazil	iNaturalist
	Canavalia rosea	*Camponotus* sp. 1	Brazil	iNaturalist
	Fridericia platyphylla	*Camponotus* crassus	Brazil	iNaturalist
	Acacia sp.	*Camponotus arboreus*	Brazil	iNaturalist
	Luehea grandiflora	*Camponotus blandus* *Camponotus melanoticus* *Cephalotes pusillus*	Brazil	iNaturalist
	Bauhinia sp.	*Camponotus renggeri*	Brazil	iNaturalist
	Ficus sp.	*Camponotus rufipes*	Brazil	iNaturalist
	Alchornea triplinervia	*Camponotus renggeri*	Brazil	iNaturalist
	Thunbergia grandiflora	*Paratrechina longicornis* *Camponotus crassus*	Brazil	iNaturalist
	Terminalia catappa	*Camponotus crassus*	Brazil	iNaturalist
	Unidentified	*Pheidole* sp.	Colombia	iNaturalist
	Banisteriopsis malifolia	*Camponotus blandus* *Ectatomma tuberculatum*	Brazil	Alves-Silva et al., 2018
	Dalbergia ecastophylla	*Camponotus crassus* *Azteca* sp.	Brazil	Callaghan, 1986
Nymphidium lisimon	*Senegalia polyphylla*	*Wasmannia auropunctata*	Brazil	iNaturalist
	Davilla sp.	*Wasmannia auropunctata*	Brazil	iNaturalist
	Piptadenia sp.	*Wasmannia auropunctata*	Brazil	iNaturalist
	Inga sp. 1	*Wasmannia auropunctata*	Brazil	Callaghan, 1988
	Triplaris gardneriana	*Wasmannia auropunctata*	Brazil	iNaturalist
	Inga laurina	*Wasmannia auropunctata*	Brazil	iNaturalist
	Coffea arabica	*Wasmannia auropunctata*	Brazil	iNaturalist
	Croton sp.	*Wasmannia auropunctata*	Colombia	iNaturalist
	Unidentified	*Wasmannia auropunctata*	Colombia	iNaturalist
	Theobroma cacao	*Wasmannia auropunctata*	Colombia	Nielsen, 2016

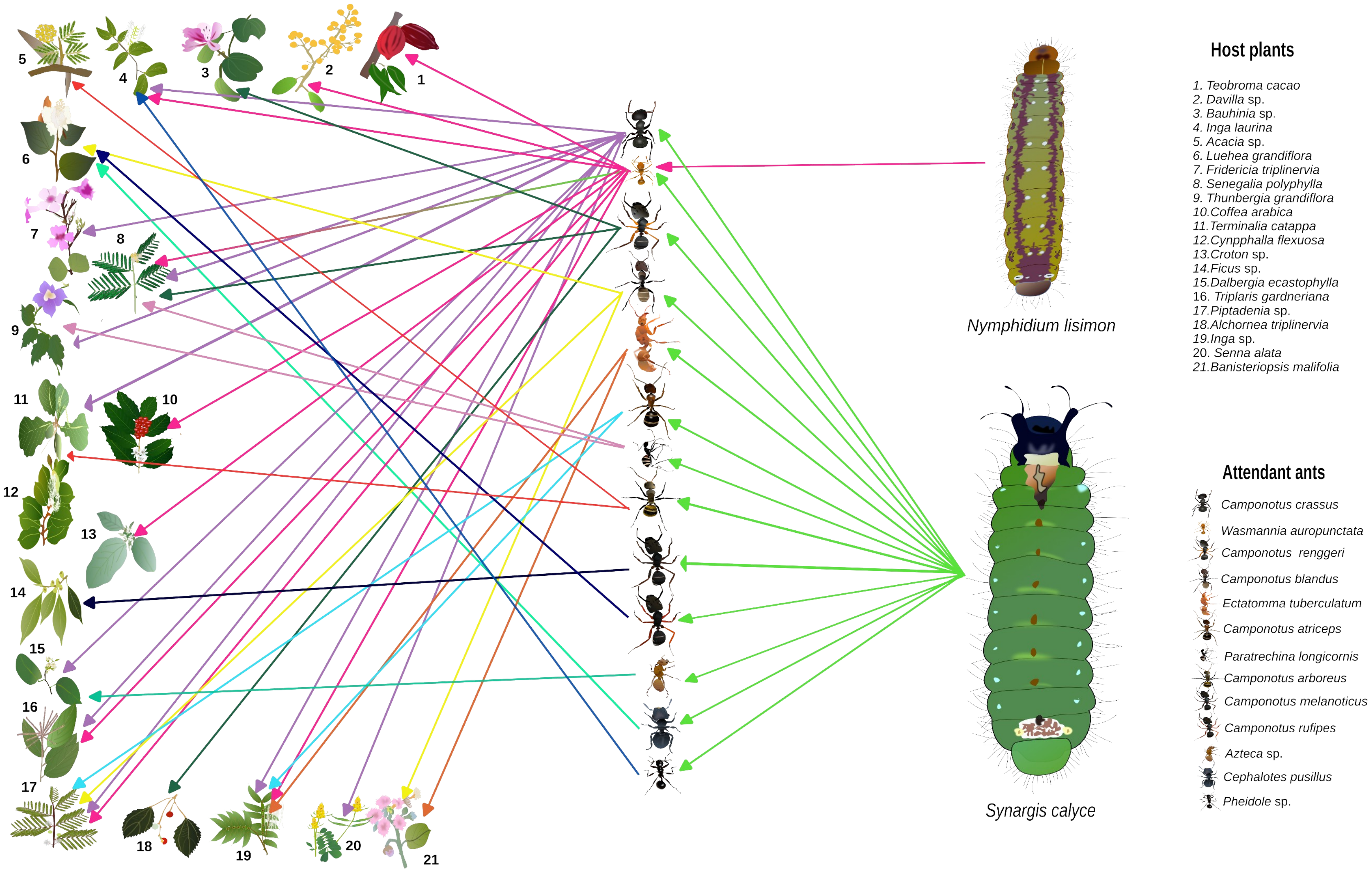


Fig 2. Network of interactions between myrmecophilous caterpillars (*S. calyce* and *N. lisimon*), attending ants, and host plants. Different colors in the arrows indicate caterpillars of different species or ants of different species.

Nymphidium lisimon- Adult females were observed laying eggs between 12:00 and 15:00 h on plants patrolled by *Wasmannia auropunctata* Roger, 1863. However, in the laboratory, females laid eggs on plants without the presence of ants. The egg masses are typically observed during oviposition.

Egg: Color bright black when laid, changing to dark lilac before hatching. It has a semi-spherical shape with a concave upper surface. The egg is covered by small cells forming hexagonal patterns (Fig 3A). The egg stage endured for five days (length: 0.5mm)

First instar: Black-brown head and red-white body. Numerous setae are distributed throughout the body, especially long setae on the thorax and the end part of the abdomen (Fig 3B). Duration four days (length of the whole body: 1mm).

Second instar: Brown head. Thorax with a brown shield. Abdomen is light red with small white lines. TNOs are already visible on the eighth abdominal segment. Many irregular orange-brown spots around the body (Fig 3C). Duration four days (length of the whole body: 2mm).

Third instar: Brown head. Thorax and abdomen are light red with irregular dark brown spots and small white lines (Fig 3D). Duration four days (length of the whole body: 3-4mm).

Fourth instar: Brown head. Thorax and abdomen are light green with irregular dark brown and white spots (Fig 3E). Duration three days (length of the whole body: 8-9mm).

Fifth instar: Brown head. Thorax and abdomen are green with irregular dark brown and white spots. The area of TNOs is well differentiated (Fig 3F, 3G). Duration six days (length of the whole body: 13mm).

Pupa: Light green and translucent before emerging as a butterfly. Areas from abdomen and wings are visible (Fig 3H). Duration eight days (length of the whole body: 10mm).

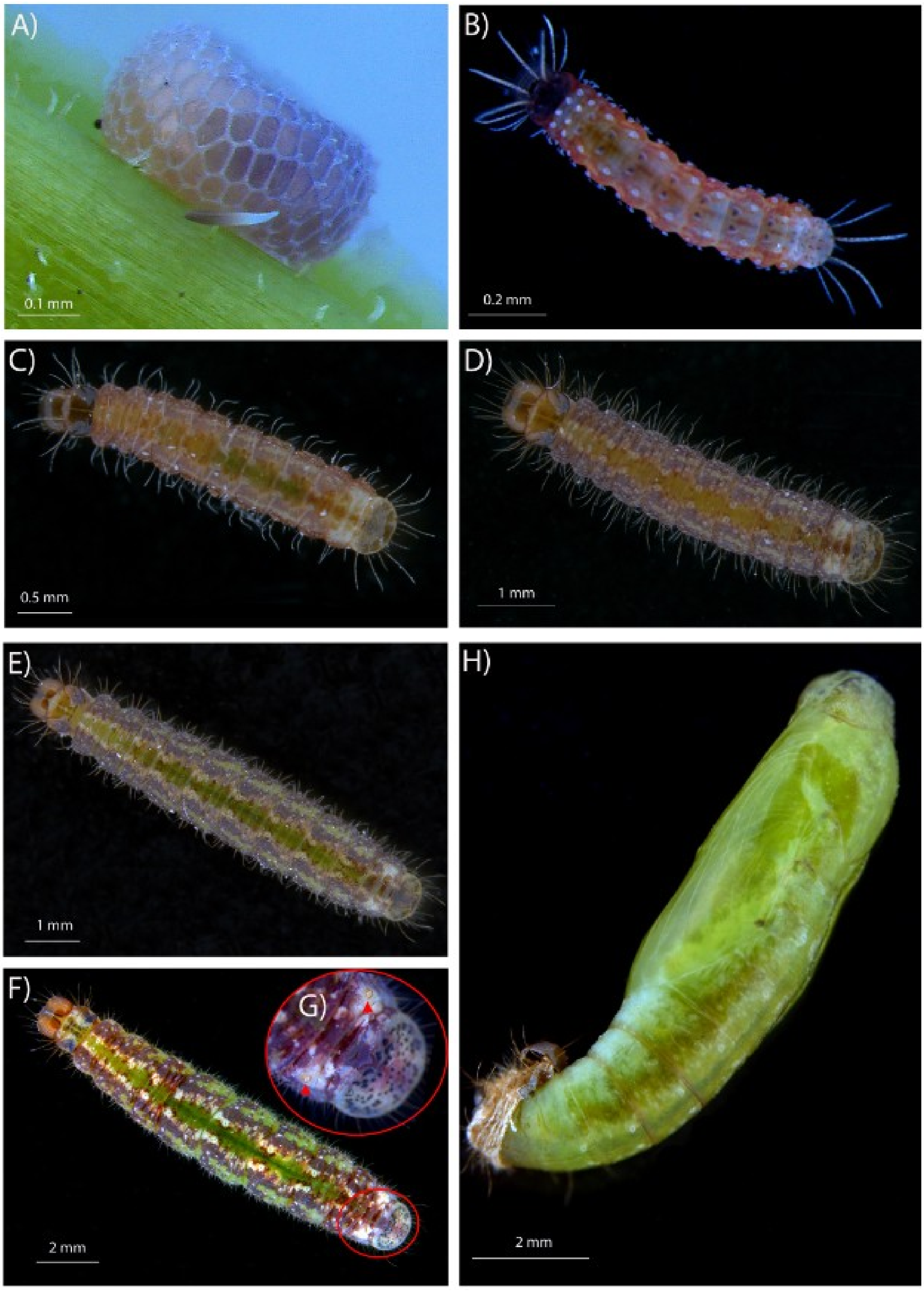


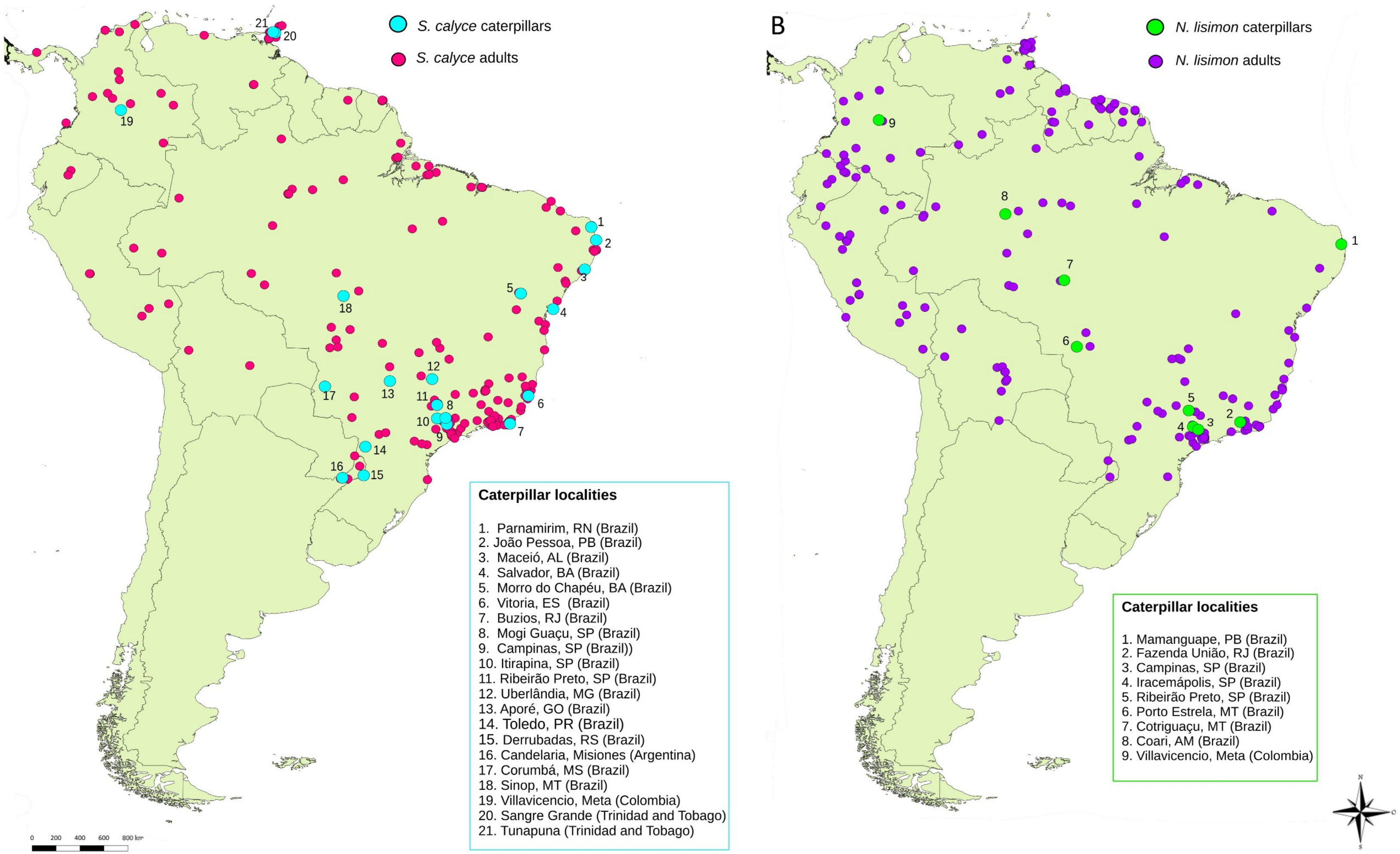
Fig 3. Life cycle of *N. lisimon*. A) egg, B) first-instar caterpillar, C) second-instar caterpillar, D) third-instar caterpillar, E) fourth-instar caterpillar, F) fifth-instar caterpillar, G) TNOs on the eighth abdominal segment, dorsal view, and H) pupa. Photos by André Rodrigues.

Caterpillars exhibit functional TNOs, but the eversion of ATOs was not observed. A large number of ants attend to the caterpillars, from the first instar to the pupal stage. While some caterpillars were observed parasitized by flies, the occurrences were minimal throughout the observations (2021-2023). Caterpillars typically conceal themselves in compartments constructed with silk and folded leaves. In the area of study, the species is abundant in localities disturbed by human intervention, where *W. auropunctata* is also numerous. Observations in the field and available records show that caterpillars feed on at least nine plant species, including some of economic importance such as *Coffea arabica* L. var. typica Cramer (Fig 2, Table1).

Distribution records

Synargis calyce is widely distributed in several countries in South America, including Argentina, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, Trinidad and Tobago, and Venezuela. In Central America, there is one report from Panama. The majority of the reports are of adults, and Brazil has the highest number of records for both adults and caterpillars. (Fig 4)

Nymphidium lisimon also has a broad distribution in South America, including Argentina, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Suriname, Trinidad and Tobago, and Venezuela. Brazil has the highest number of records for both adults and caterpillars (Fig 4.)



Caterpillar localities

1. Parnamirim, RN (Brazil)
2. João Pessoa, PB (Brazil)
3. Maceió, AL (Brazil)
4. Salvador, BA (Brazil)
5. Morro do Chapéu, BA (Brazil)
6. Vitoria, ES (Brazil)
7. Buzios, RJ (Brazil)
8. Mogi Guaçu, SP (Brazil)
9. Campinas, SP (Brazil))
10. Itirapina, SP (Brazil)
11. Ribeirão Preto, SP (Brazil)
12. Uberlândia, MG (Brazil)
13. Aporé, GO (Brazil)
14. Toledo, PR (Brazil)
15. Derrubadas, RS (Brazil)
16. Candelaria, Misiones (Argentina)
17. Corumbá, MS (Brazil)
18. Sinop, MT (Brazil)
19. Villavicencio, Meta (Colombia)
20. Sangre Grande (Trinidad and Tobago)
21. Tunapuna (Trinidad and Tobago)

Caterpillar localities

1. Mamanguape, PB (Brazil)
2. Fazenda União, RJ (Brazil)
3. Campinas, SP (Brazil)
4. Iracemápolis, SP (Brazil)
5. Ribeirão Preto, SP (Brazil)
6. Porto Estrela, MT (Brazil)
7. Cotriguaçu, MT (Brazil)
8. Coari, AM (Brazil)
9. Villavicencio, Meta (Colombia)

Fig 4. Distribution map of (A) *S. calyce* and (B) *N. lisimon*, with different colors representing records of adults and immatures. Localities where caterpillars were observed being attended by ants are indicated.

Discussion

The life cycle data for *S. calyce* are similar to those reported in other studies, including morphological traits for each instar, such as coloration, presence of setae across the whole body, and myrmecophilous organs, the number of instars, length measurements of caterpillars at each instar, and the duration of each stage (Callaghan, 1986). At the study site, *Inga laruina* (Sw.) Willd plants were also utilized by *Juditha molpe* Hübner, 1808, which has caterpillars very similar to those of *S. calyce*. However, *J. molpe* has a lighter green color and maintains an obligate relationship with *Dolichoderus bispinosus* Olivier, 1792 (Callaghan 1981, DeVries et al., 1992). For *N. lisimon*, there is only descriptive data available for the third instar of *N. lisimon attenuatum* (Callaghan, 1988). However, comparing them with our observations of the same instar, they are similar in terms the length of the whole body and coloration. The data also align with findings from Nielsen (2016), which include photos, length measurements, and characteristics of each developmental stage of the species.

The caterpillars of *S. calyce* and *N. lisimon* employ strategies to avoid natural enemies, such as feeding at night and remaining still during the day. *Synargis calyce*, in particular, exhibits coloration similar to that of its host plants (Callaghan, 1986). On the other hand, *N. lisimon* larvae construct shelters with leaves, resting inside them when not feeding (Nielsen, 2016). It is well-known that the pupal and larval stages are the most vulnerable periods for a butterfly, and developing additional strategies, such as appeasing ants for protection, can be highly effective against predators and parasitoids (Casacci et al. 2019).

Synargis calyce and *N. lisimon* maintain associations with ants during their larval stage, but their strategies are opposite. *Synargis calyce* exhibits a facultative interaction with ants, being attended by different ant species, although its performance against natural enemies is likely better when attended by a particular species compared to others (Pierce and Dankowicz, 2022). This species displays high plasticity in its relationships with ants. For example, in periodic observations of a caterpillar feeding on *S. polyphylla*, it was initially attended to by *C. crassus*. However, when *W. auropunctata* began to dominate the plant, the caterpillar began to receive attention from this new species (AVCG personal observation). Although facultative association implies that caterpillars may occasionally be found without ants (Marquis and Koptur, 2022), this is not the case for *S. calyce* caterpillars, as no observations showed caterpillars without ant attendance in the field. On the other hand, *N. lisimon* has an obligate interaction with *W. auropunctata*. Typically, a large number of *W. auropunctata* workers attend caterpillars, completely covering its body, and even when the caterpillar walks on branches, the ants follow it. There is a record of a pupa being attended by workers inside a cavity in a *Piptadenia* sp. trunk, where *W. auropunctata* larvae were also present (AVCG personal observation). In the laboratory, caterpillars raised without ants show high mortality, especially during the first two instars, highlighting the importance of ant attendance for caterpillars to complete their life cycle (Pierce and Dankowicz, 2022). This could be related to the dependence of caterpillars on ants as a food source through regurgitation (Marquis and Koptur, 2022); however, further data are needed to test this hypothesis for this species.

Both species have Tentacle Nectary Organs (TNOs), analogous to the Dorsal Nectary Organ (DNO) in Lycaenidae, widely recognized as a source of nutritious rewards that maintain caretaking behavior in attendant ants (Cottrell, 1984; DeVries, 1988; Pierce et al., 2002; Casacci et al., 2019). In *S. calyce*, Anterior Tentacle Organs (ATOs) structural tentacle organs whose function has not been fully clarified but are similar to tentacle organs (TOs) in

Lycaenidae were also observed (DeVries, 1988, 1997; Penz and DeVries, 2006; Kaminski and Carvalho-Filho, 2012; Kaminski et al., 2016; Marquis and Koptur, 2022). In *N. lisimon*, ATOs were not observed, consistent with observations made by Callaghan (1988) and other reports in Penz and DeVries (2006).

Our field observations and records for *S. calyce* and *N. lisimon* reveal that the caterpillars have a wide variety of host plants. This pattern extends to other myrmecophilous riodinids and even lycaenids, which seem to have a broader diversity of host plants compared to non-myrmecophilous species from the same families (Pierce and Elgar, 1985; Fiedler, 1995). This could be explained by the fact that adults of myrmecophilous butterflies use the presence of ants as cues for oviposition (Pierce and Elgar, 1985; Fiedler, 1995; Pierce et al., 2002). *Synargis calyce* establishes association with at least 16 ant species, hence it is not surprising to see the wide variety of host plants used by its caterpillars. For obligate species, there is a tendency for caterpillars to associate with highly dominant ants (Kaminski, 2008; Kaminski et al., 2013). This is the case for *N. lisimon*, which only interacts with *W. auropunctata*, a species known for its generalist feeding habits and dominance in disturbed areas (Wetterer and Porter, 2003).

Many of the plant species reported for the two species studied here have extrafloral nectaries (EFNs), and evidence show that myrmecophilous caterpillars of riodinid or lycaenid butterflies prefer plants with extrafloral nectaries, which serve as attractants for ants. Some caterpillars even use these extrafloral nectaries as a food source (DeVries and Baker, 1989; DeVries, 1991b; Fiedler, 1995). This would also explain the dominance of species from Fabaceae as host plants, where many of them have EFNs. However, another aspect to consider is that plants of this family are rich in nitrogen, which has been suggested to be fundamental for myrmecophilous species, as it allows them to compensate the cost of secreting nutritious substances rich in amino acids to maintain myrmecophily (Fiedler, 1994).

Synargis calyce and *N. lisimon* are widely distributed in South America (excluding Uruguay and Chile). There is a report suggesting that *S. calyce* has reached Panama, however, the specimen in question is part of the collection at the Natural History Museum of the University of Rio de Janeiro, which recently suffered a fire. Therefore, this information requires confirmation from another source. Possible explanations for the distribution of these two species, which are restricted to South America and may also apply to all members of Riodinidae that interact with ants and are limited to the Neotropics, could include climatic conditions and the lack of suitable ant partners in other locations (Marquis, Koptur, 2022). The success of these two species in different biomes of South America can generally be explained by their association with ants, which are widely distributed in terrestrial environments, especially in the Neotropics (Hölldobler and Wilson, 1990; Brandão et al., 2012). Specifically, the association of *S. calyce* with at least 16 ant species, many of which are dominant in various environments, suggests that caterpillars may have a broad distribution range. In the case of *N. lisimon*, the wide distribution could also be related to its association with *W. auropunctata,* a cosmopolitan and dominant ant species (Wetterer and Porter, 2003; Tennant, 2021).

Despite several studies in recent years, research exploring strategies in interactions between caterpillars and ants is still limited, especially concerning Riodinidae. The situation is even more concerning regarding studies on the distribution of myrmecophilous butterfly species, the use of host plants, and the diversity of ant attendant species. Such studies are necessary because they provide a more complete understanding of how obligatore and facultative myrmecophilous mutualistic systems in butterflies’ function. Additionally, they allow comparisons between these two strategies from evolutionary and ecological perspectives, aiming to identify general patterns that may extend to other groups of myrmecophilic organisms.

References

Alves-Silva, E., Bächtold, A., & Del-Claro, K. (2018) Florivorous myrmecophilous caterpillars exploit an ant–plant mutualism and distract ants from extrafloral nectaries. Austral Ecology 43:643–650. https://doi.org/10.1111/aec.12609

Brandão, C. R., Silva, R. R., & Delabie, J. H. (2012). Neotropical ants (Hymenoptera) functional groups: nutritional and applied implications. Insect Bioecology and Nutrition for Integrated Pest Management, CRC Press pp 213-236.

Callaghan, C. J. (1981) Notes on the Immature Biology of Two Myrmecophilous Lycaenidae: *Juditha molpe* (Riodininae) and *Panthiades*. Journal of Research on the Lepidoptera, 20, 36-42.

Callaghan, S. (1986) Restinga butterflies: biology of *Synargis brennus* (Stichel)(Riodinidae). Journal of the Lepidopterists' Society, 40, 93-96.

Callaghan, C. J. (1988) Notes on the biology of three riodinine species: *Nymphidium lisimon attenuatum, Phaenochitonia sagaris satnius,* and *Metacharis ptolomaeus* (Lycaenidae: Riodininae). Journal of Research on the Lepidoptera, 27, 109-114.

Campbell, D. L., & Pierce, N. E. (2003) Phylogenetic relationships of the Riodinidae: implications for the evolution of ant association. Butterflies: Ecology and Evolution Taking Flight. University of Chicago, Chicago, IL, pp 395-408.

Casacci, L.P., Bonelli. S., Balletto, E., & Barbero, F. (2019) Multimodal signaling in myrmecophilous butterflies. Frontiers in Ecology and Evolution 7:454. https://doi.org/10.3389/fevo.2019.00454.

Cottrell, C.B. (1984) Aphytophagy in butterflies: its relationship to myrmecophily. Zoological Journal of the Linnean Society, 79, 1–57. https://doi.org/10.1111/j.1096-3642.1984.tb02318.x.

DeVries, P. J. (1988) The larval ant-organs of *Thisbe irenea* (Lepidoptera: Riodinidae) and their effects upon attending ants. Zoological Journal of the Linnean Society, 94, 379-393. https://doi.org/10.1111/j.1096-3642.1988.tb01201.x.

DeVries, P. J. (1991a). Mutualism between *Thisbe irenea* butterflies and ants, and the role of ant ecology in the evolution of larval-ant associations. Biological Journal of the Linnean Society, 43, 179-195. https://doi.org/10.1111/j.1095-8312.1991.tb00592.x.

DeVries, P. J. (1991b) Evolutionary and ecological patterns in myrmecophilous riodinid butterflies. Ant-plant interactions. Oxford University Press, Oxford, 143-156.

DeVries, P. J. (1994). Patterns of butterfly diversity and promising topics in natural history and ecology. La Selva: ecology and natural history of a Neotropical rain forest. University of Chicago Press, Chicago, 187-194.

DeVries, P. J. (1997). Riodinidae. In The butterflies of Costa Rica and their natural history, volume II, Princetown University Press, p 368

DeVries, P. J., & Baker, I. (1989) Butterfly exploitation of an ant-plant mutualism: adding insult to herbivory. Journal of the New York Entomological Society, 332-340. https://www.jstor.org/stable/25009777.

DeVries, P. J., Chacon, I. A., & Murray, D. (1992) Toward a better understanding of host use and biodiversity in riodinid butterflies(Lepidoptera). Journal of Research on the Lepidoptera, 31, 103-126.

DeVries, P. J., & Penz, C. M. (2000). Entomophagy, Behavior, and Elongated Thoracic Legs in the Myrmecophilous Neotropical Butterfly *Alesa amesis* (Riodinidae) 1. Biotropica, 32(4a), 712-721. https://doi.org/10.1111/j.1744-7429.2000.tb00519.x

Espeland, M., Hall, J. P., DeVries, P. J., Lees, D. C., Cornwall, M., Hsu, Y. F., ... & Pierce, N. E. (2015) Ancient Neotropical origin and recent recolonisation: Phylogeny, biogeography and diversification of the Riodinidae (Lepidoptera: Papilionoidea). Molecular Phylogenetics and Evolution, 93, 296-306. https://doi.org/10.1016/j.ympev.2015.08.006.

Faynel, C., Bénéluz, F., Brûlé, S., & Fernandez, S. (2017) *Dioclea guianensis* et *Dioclea virgata* (Fabaceae): plantes-hôtes de nombreux Lycaenidae et Riodinidae en Guyane. Exemples de polychromatisme larvaire cryptique (Lepidoptera). Revue de l'Association Roussillonnaise d'Entomologie, 26, 135-143.

Fiedler, K. 1991. Systematic, evolutionary, and ecological implications of myrmecophily within the Lycaenidae (Insecta: Lepidoptera: Papilionoidea). Bonner Zoologische Monographien 31, 1-210. https://eref.uni-bayreuth.de/id/eprint/30964.

Fiedler, K. (1994a) Lycaenid butterflies and plants: is myrmecophily associated with amplified hostplant diversity? Ecological Entomology 19, 79–82. https://doi.org/10.1111/j.1365-2311.1994.tb00393.x.

Fiedler, K. (1995) Lycaenid butterflies and plants: is myrmecophily associated with particular hostplant preferences?. Ethology ecology & evolution, 7, 107-132. https://doi.org/10.1080/08927014.1995.9522960

Global Biodiversity Information Facility. https://www.gbif.org Accessed Sep 2023

Guedes, T. K., & Kaminski, L. A. (2023). Behavioral observations of ant-butterfly symbioses in the Pantanal wetlands of west-central Brazil. Tropical Lepidoptera Research, 111-116.

Hall, J. P. W. (2018) A Monograph of the Nymphidiina: Phylogeny, Taxonomy, Biology and Biogeography (Lepidoptera: Riodinidae: Nymphidiini). The Entomological Society of Washington, Washington, DC.

Hall, J. P., & Harvey, D. J. (2002) Basal subtribes of the Nymphidiini (Lepidoptera: Riodinidae): phylogeny and myrmecophily. Cladistics, 18, 539-569. https://doi.org/10.1111/j.1096-0031.2002.tb00292.x.

Hill, G. M., Trager, M. D., Lucky, A., & Daniels, J. C. (2022). Protective Benefits of Tending Ants to a Critically Endangered Butterfly. Journal of Insect Science, 22(6), 9.

Hinton, H.E. (1951) Myrmecophilous Lycaenidae and other Lepidoptera—a summary. Proceedings of the London Entomological & Natural History Society 1949–1950, 111–175.

Hölldobler, B., & Wilson, E.O. (1990) The Ants. The Ants. The Belknap Press of Harvard University Press, Cambridge p 732.

Horvitz, C. C., Turnbull, C., & Harvey, D. J. (1987). Biology of immature *Eurybia elvina* (Lepidoptera: Riodinidae), a myrmecophilous metalmark butterfly. Annals of the Entomological Society of America, 80, 513-519. https://doi.org/10.1093/aesa/80.4.513.

Kaminski, L. A. (2008). Polyphagy and obligate myrmecophily in the butterfly *Hallonympha paucipuncta* (Lepidoptera: Riodinidae) in the Neotropical Cerrado savanna. Biotropica, 40(3), 390-394. https://doi.org/10.1111/j.1744-7429.2007.00379.x.

Kaminski LA (2021) Ant-butterfly interactions - Borboletas formigueiras. iNaturalist. https://www.inaturalist.org/projects/ant-butterfly-interactions-borboletas-formigueiras . Accessed 26 Apr 2022

Kaminski, L. A., & Carvalho-Filho, F. S. (2012). Life history of *Aricoris propitia* (Lepidoptera: Riodinidae)—A myrmecophilous butterfly obligately associated with fire ants. Psyche: A Journal of Entomology, 2012. https://doi.org/10.1155/2012/126876.

Kaminski, L. A., Mota, L. L., Freitas, A. V., & Moreira, G. R. (2013) Two ways to be a myrmecophilous butterfly: natural history and comparative immature-stage morphology of two species of *Theope* (Lepidoptera: Riodinidae). Biological Journal of the Linnean Society, 108, 844-870. https://doi.org/10.1111/bij.12014.

Kaminski, L. A., Iserhard, C. A., & Freitas, A. V. (2016) *Thisbe silvestr*e sp. nov.(L epidoptera: R iodinidae): a new myrmecophilous butterfly from the Brazilian Atlantic Forest. Austral Entomology, 55, 138-146. https://doi.org/10.1111/aen.12159.

Kaminski, L. A., & Lima, L. D. (2019). Larval omnivory in the myrmecophilous butterfly *Setabis lagus lagus* (Riodinidae: Nymphidiini). The Journal of the Lepidopterists' Society, 73(4), 276-279. https://doi.org/10.18473/lepi.73i4.a4.

Kaminski, L. A., Carneiro, E., Dolibaina, D. R., Casagrande, M. M., & Mielke, O. H. (2020). Oviposition of *Minstrellus grandis* (Lepidoptera: Riodinidae) in a harmful ant-plant symbiosis.Acta Amazonica,50, 256-259. https://10.1590/1809-4392202001801e.

Kaminski, L. A., Volkmann, L., Callaghan, C. J., DeVries, P. J., & Vila, R. (2021) The first known riodinid ‘cuckoo’ butterfly reveals deep-time convergence and parallelism in ant social parasites. Zoological Journal of the Linnean Society, 193, 860-879. https://doi.org/10.1093/zoolinnean/zlaa150.

Lima, L,D., Ceballos-González, A.V., Prato, A., Kaminski, L.A., & Nascimento, F.S. (2023) Plant-treehopper convergence may trick butterflies into trophic oviposition mistakes. Biotropica 55:292–298. https://doi.org/10.1111/btp.13194

Nielsen, (2016) Neotropical Riodinidae. https://riodinidae.org/2016/03/24/nymphidium-lisimon/. Accessed in 15 Aug 2023.

Marquis, R.J., & Koptur, S. (2022) Caterpillars in the middle: Tritrophic interactions in a changing world. Springer. pp. 319-391.

Mota, L. L., Kaminski, L. A., & Freitas, A. V. (2020) The tortoise caterpillar: carnivory and armoured larval morphology of the metalmark butterfly *Pachythone xanthe* (Lepidoptera: Riodinidae). Journal of Natural History, 54, 309-319. https://doi.org/10.1080/00222933.2020.1759720.

Penz, C. M., & DeVries, P. J. (2006) Systematic position of *Apodemia paucipuncta* (Riodinidae), and a critical evaluation of the nymphidiine transtilla. Zootaxa,1190, 1-50.

Pérez-Lachaud, G., Rocha, F. H., Pozo, C., Kaminski, L. A., Seraphim, N., & Lachaud, J. P. (2021) A new ant-butterfly symbiosis in the forest canopy fills an evolutionary gap. Scientific Reports, 11, 20770. https://doi.org/10.1038/s41598-021-00274-x.

Pierce, N. E., Braby, M. F., Heath, A., Lohman, D. J., Mathew, J., Rand, D. B., & Travassos, M. A. (2002). The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). Annual Review of Entomology, 47, 733-771. https://doi.org/10.1146/annurev.ento.47.091201.145257.

Pierce, N. E., Kitching, R. L., Buckley, R. C., Taylor, M. F. J., & Benbow, K. F. (1987) The costs and benefits of cooperation between the Australian lycaenid butterfly, *Jalmenus evagoras*, and its attendant ants. Behavioral Ecology and Sociobiology, 21, 237-248. https://doi.org/10.1007/BF00292505.

Pierce, N. E., & Dankowicz, E. (2022) Behavioral, ecological and evolutionary mechanisms underlying caterpillar-ant symbioses. Current Opinion in Insect Science, 52, 100898. https://doi.org/10.1016/j.cois.2022.100898.

Pierce, N. E., & Elgar, M. A. (1985) The influence of ants on host plant selection by Jalmenus evagoras, a myrmecophilous lycaenid butterfly. Behavioral Ecology and Sociobiology, 16, 209-222. https://doi.org/10.1007/BF00310983.

Torres, P. J., & Pomerantz, A. F. (2016) Butterfly kleptoparasitism and first account of immature stages, myrmecophily, and bamboo host plant of the metalmark *Adelotypa annulifera* (Riodinidae).The Journal of the Lepidopterists' Society, 70, 130-138. https://doi.org/10.18473/lepi.70i2.a8.

Ross, G. N. (1966) Life-history studies on Mexican butterflies. IV. The ecology and ethology of *Anatole rossi*, a myrmecophilous metalmark (Lepidoptera: Riodinidae). Annals of the Entomological Society of America,59(5), 985-1004. https://doi.org/10.1093/aesa/59.5.985.

Seraphim, N., Kaminski, L. A., DeVries, P. J., Penz, C., Callaghan, C., Wahlberg, N., ... & Freitas, A. V. (2018) Molecular phylogeny and higher systematics of the metalmark butterflies (Lepidoptera: Riodinidae). Systematic Entomology, 43, 407-425. https://doi.org/10.1111/syen.12282

Tennant, L. E. (2021) The ecology of *Wasmannia auropunctata* in primary tropical rainforest in Costa Rica and Panama. In Exotic ants CRC Press pp 80-90.

Wetterer, J. K., & Porter, S. D. (2003) The little fire ant, *Wasmannia auropunctata:* distribution, impact and control. Sociobiology, 41, 1-37

CHAPTER 2

INFLUENCE OF HOST PLANTS AND TENDING ANTS ON THE CUTICULAR HYDROCARBON PROFILE OF A GENERALIST MYRMECOPHILOUS CATERPILLAR

Abstract

In myrmecophilous organisms, which live in symbiosis with ants, cuticular hydrocarbons (CHCs) play a pivotal role in interspecific communication and defense against chemical-oriented predators. Although these interactions form complex information webs, little is known about the influence of biotic environmental factors on the CHC profiles of myrmecophiles. Here, we analyzed the effect of different host plants and tending ants on the larval CHC profile of *Synargis calyce* (Lepidoptera: Riodinidae), a polyphagous species with facultative myrmecophily. Groups of caterpillars were fed individually with three host plant species (without tending ants), and with two tending ant species. Through gas chromatography analysis, we compared the cuticular profiles of treatments and found a high similarity between plants and caterpillars (65-82%), but a low similarity between caterpillars and their tending ants (30-25%). Cluster analysis showed that caterpillars, ants, and plants form distinct groups, indicating that *S. calyce* caterpillars have their own chemical profile. These results are similar to those observed for Lycaenidae caterpillars indicating that there is functional convergence in the chemical strategies used by myrmecophilous caterpillar species with similar ecology. Also, the results suggest that the cuticular compounds of *S. calyce* are primarily influenced by their host plants rather than their tending ants. Thus, we propose that these caterpillars present a trade-off between camouflage and directly informing their presence to ants, maintaining their unique chemical profile, though slightly affected by biotic environmental factors.

Key Words- Chemical compounds, mutualistic interactions, myrmecophilous butterflies, Riodinidae, *Synargis calyce.*

Introduction

Ants are predominant in many terrestrial ecosystems in terms of abundance and biomass (von Beeren et al., 2012). These eusocial insects live in complex societies where communication plays a crucial role in their functioning (Hölldobler and Wilson, 1990; Yamaoka, 1990; Lenoir et al., 1999; Akino 2008). In ants and other social insects, communication and particularly the recognition of nestmates is primarily based on chemical cues and signals (Yamaoka, 1990; Lenoir et al., 1999; Akino, 2008; Blomquist and Bagnères, 2010, Nunes et al., 2014). Cuticular hydrocarbons (CHCs), the main class of cuticular compounds in ants, are colony-specific and actively participate in nestmate recognition (Howard and Blomquist, 2005; Hefetz, 2007; Ferguson et al., 2021). The ecological success, wide distribution, and social organization of ants have contributed to the evolution of diverse associations with organisms from various kingdoms, including plants, fungi, and animals, particularly other insects (Casacci et al., 2019). Organisms that interact with ants during at least part of their lifecycle are called myrmecophiles (Hölldobler and Wilson, 1990; Kronauer and Pierce, 2011; Hölldobler and Kwapich, 2022). There are about 10,000 species of myrmecophiles across various insect orders (Thomas et al., 2005; Parker, 2016; Hölldobler and Kwapich, 2022).

Myrmecophily in Lepidoptera is primarily observed in the families Lycaenidae and Riodinidae, with 75% of the species in these families having immatures stages that interact with ants (Pierce et al., 2002; Casacci et al., 2019). These families commonly exhibit facultative and unspecific relationships, involving interactions with various ant taxa. However, there are butterfly species that establish obligate and specific relationships with specific ant taxa (Fiedler, 1994, 2021; Kaminski, 2008; Pierce and Dankowicz, 2022).

Interactions between butterflies and ants can range from mutually beneficial outcomes, such as mutualism, to interactions where butterflies benefit without harming ants in commensalism, and to antagonist interactions where butterflies may be preyed by ants or where ants may be harmed by butterflies, as seen in social parasitism and competition for resources (Fiedler, 1995, 1996; Pierce and Dankowicz, 2022). Both lycaenid and riodinid species exhibit various adaptations resulting from the pressures exerted by their association with ants (Pierce et al., 2002). These adaptations include highly specialized ant-organs involved in chemical and acoustic deception (Cottrell, 1984; Fiedler et al., 1996; Pierce et al., 2002; Elmes et al., 2002; Barbero et al., 2012; Hill et al., 2022). For instance, nectary organs such as the dorsal nectary organ (DNO) in Lycaenidae and tentacle nectary organs (TNOs) in Riodinidae liquid secretions rich in sugar and amino acids (Newcomer, 1912; Malicky, 1970; DeVries, 1988). Caterpillars of these two families are also equipped with a pair tentacle organs (TOs) in Lycaenidae and anterior tentacle organs (ATOs) in Riodinidae, which emit chemical signals or vibroacoustic signals that modify the ant behavior (Henning, 1983; DeVries et al., 1986; DeVries ,1988; Gnatzy et al., 2017; Schönrogge et al., 2017).

Chemical strategies mediated by cuticular compounds enable myrmecophiles to overcome the barrier of chemical recognition employed by ants (von Beeren et al., 2012). One such strategy is chemical camouflage, where organisms resemble their background and avoid detection by chemically oriented predators (Silveira et al., 2010). In herbivorous organisms, achieving chemical camouflage with their host plants is possible through diet (Espelie et al., 1991; Barbero, 2016; Lima et al., 2024). In Lepidoptera, this strategy has already been demonstrated in both non-myrmecophilous and myrmecophilous caterpillars (Akino et al., 2004; Portugal and Trigo, 2005; Lima et al., 2021). One of the most extensively studied strategies is chemical mimicry, where organisms possess chemical compounds that close resemble those of other organisms such as ants. This strategy has been observed in social parasitic species (Akino et al., 1999; Elmes et al., 2002; Schlick-Steiner et al., 2004; Schönrogge et al., 2004; Akino, 2008). Additionally, some myrmecophilous caterpillars present low concentration of compounds on their surface, making their detection challenging - a strategy known as chemical insignificance (Inui et al., 2015; Barbero, 2016). Recently, a new strategy has been proposed in myrmecophilous caterpillars called chemical conspicuousness. In this strategy, caterpillars that do not provide caloric rewards for ants exhibit a distinct cuticular profile compared to ants or host plants. However, their profile is similar to that of other caterpillars that offer caloric rewards to ants (Lima et al., 2021).

Although ant-plant-herbivore systems embrace complex communication networks involving multiple species (e.g., Lima et al., 2021, 2023), there is still a lack of information regarding the influence of phenotypic plasticity and biotic environmental factors on the cuticle compounds of generalist myrmecophilous species (Otte et al., 2018; Sprenger and Menzel, 2020). Therefore, our aim was to investigate whether the CHC profile of a polyphagous caterpillar changes (1) according to their diet on different host plant species and (2) according to their interaction with different tending ant species. Considering that the caterpillar species used here were fed on their host plants, we hypothesized that their CHCs would be influenced primarily by their food source rather than the contact established with tending ants. Furthermore, due to the production of caloric rewards by caterpillars (trophobiosis) we predicted that caterpillars would exhibit a specific chemical profile distinct of both host plant and tending ants.

Methods and Materials

Study system

Synargis calyce C. Felder and R. Felder, 1862 (Lepidoptera: Riodinidae) (Fig. S1) is a Neotropical herbivorous and myrmecophilous butterfly whose caterpillars feed on several host plants in different families, including non-native species (Callaghan, 1986; Beccaloni et al., 2008; Alves-Silva et al., 2018; Kaminski, 2021). Female butterflies tend to lay their eggs on plants that are frequented by attendant ants and caterpillars are tended by ants during all instars (Callaghan, 1986). Although it presents facultative myrmecophily, the caterpillars are almost always found with tending ants of several genera, but mainly *Camponotus* species (Callaghan, 1986; Alves-Silva et al., 2018; Kaminski, 2021). It is common to observe temporal turnover, with attendance by different species of ants during the day and night (LAK, personal observation). Due to the high degree of ecological plasticity of this butterfly in terms of both host plants and tending ants, it is an excellent model of a generalist myrmecophile.

Collection and Rearing of Study Species

Insects were collected at the Universidade de São Paulo (21.1637° S, 47.8592° W), Ribeirão Preto Campus, SP, Brazil, between January 2021 and April 2022. To conduct our chemical analysis, we collected ~90 eggs of *S. calyce* for rearing in the laboratory. Initially, ~20 field observations were conducted to identify plant species used by butterflies for oviposition and seven species were identified serving as host plants. Subsequently, eggs were collected from three of these host plants (*Senegalia polyphylla* (DC.) Britton and Rose (Fabaceae), *Inga laurina* (Sw.) Willd. (Fabaceae), and *Terminalia catappa* Linnaeus

(Combretaceae)) at various study locations by harvesting branches where oviposition had been observed. In the field, three ant nests of *Camponotus crassus* Mayr, 1862 (Formicidae: Formicinae) were collected and transferred to the laboratory. Each nest was placed in two connected boxes measuring 9 x 26.6 x 26.6 cm. One box served as the nesting area and contained test tubes (15 cm long) filled with water, plugged with hydrophilic cotton. The other box served as the foraging area. The ant colonies were provided with a diet of *Tenebrio molitor* Linnaeus, 1758 larvae (Coleoptera: Tenebrionidae), diluted sugar solution (10%), and water *ad libitum*. Each colony consisted of approximately 150 workers, a queen, and some brood (eggs, larvae and pupae). Additionally, approximately, 600 workers of *Paratrechina longicornis* Latreille 1802 (Formicidae: Formicinae) were collected from three established colonies near the laboratory buildings. The insects were kept under controlled conditions at a temperature of 25 °C and a photoperiod of 12h of light and 12h of darkness.

Does the Chemical Composition of Caterpillars Change According to their Food Sources?

As the caterpillars studied here are polyphagous, we selected three host plant species on which the caterpillars feed in the study area: two native, *S. polyphylla*, and *I. laurina*, and one non-native, *T. catappa*. Our aim was to investigate whether the CHCs of the caterpillar change according to its dietin the absence of tending ants. To conduct the experiment, we placed individually each egg in a plastic container (250 ml). Once the eggs hatched, we provided the caterpillars with shoots containing young leaves and extrafloral nectaries from the host plants. The shoots were replaced daily and kept in contact with moistened cotton to prevent them from drying. The *S. calyce* caterpillars were reared separately in plastic containers on three host plant species: caterpillar-*S. polyphylla* (n = 10), caterpillar-*I. laurina* (n = 10), caterpillar-*T. catappa* (n = 6). After reaching the fifth instar, we killed the caterpillars by freezing and kept them at -20 °C until CHC extractions were performed. Additionally, we collected leaves of *S. polyphylla* (n = 10), *I. laurina* (n = 10), and *T. catappa* (n = 10) for CHC extractions (See Table S1).

Does the Chemical Composition of Caterpillars Change According to their tending Ants?

In our field site, the caterpillars are attended by four different ant species: *C. crassus, P. longicornis, Camponotus renggeri* Emery, 1894, and *Wasmannia auropunctata* Roger, 1863 (AVCG, personal observation). Thus, to assess whether the caterpillar CHCs change according to their tending ants, we individually reared *S. calyce* caterpillars in a plastic container as previously described, along with a group of associated ants. Specifically, we reared the caterpillars with two experimental groups: (caterpillar-*C. crassus*) (n = 10), and (caterpillar-*P. longicornis*) (n = 10). These caterpillars were fed with the host plant *S. polyphylla*. Each caterpillar was placed together with 10 workers of *C. crassus* or 30 workers of *P. longicornis*. The number of ants was based on the average amount observed in the field. The ant workers were replaced every day until the caterpillars were frozen for chemical extraction, as previously mentioned. For chemical analysis, we also collected worker ants from colonies reared in the laboratory for *C. crassus* (n = 8 colonies; n = 20 ants for chemical analyses) and from colonies established near the laboratory for *P. longicornis* (n = 10 colonies; n = 300 ants for chemical analyses).

Chemical Analyses

To perform the chemical analyses, we placed insects or plant shoots in glass vials (1.5 ml) and covered them with *n*-hexane (Macron Fine Chemicals, 95% *n*-hexane, USA) for 1 min (Lima et al. 2023). For each sample, a fifth-instar larva of *S. calyce*, two workers of *C. crassus*, 30 workers of *P. longicornis*, and one young shoot with two leaves from each plant species were used individually. External standards were exclusively employed and the samples were not weighed. Subsequently, we left each vial at room temperature in a flow chamber to allow for drying. Once completely dried, we resuspended the contents in 5 μl of hexane, of which 2μl were manually injected. The samples were analyzed with gas chromatography coupled to a mass spectrometer (GC/MS; Shimadzu, model QP2010 Plus), using a 30 m Rxi-1ms column, with helium gas flow rate set at 1 ml/min. The oven temperature was initially set to 40 °C and then increased by 3 °C min-1 until reaching 310 °C (held for 15 min), following da Silva et al. (2021). The injector temperature was set to 250 °C. Data were analyzed by GC/MS Solutions for Windows (Shimadzu Corporation), and compounds were identified based on their mass spectra, including diagnostic and molecular ions (Carlson et al. 1998). Additionally, a retention index was calculated for each identified peak using a standard solution of different synthetic linear hydrocarbons (*n*-C_{21} to *n*-C_{40}). We also consulted the Registry of Mass Spectral Data (Wiley) and National Institute of Standards and Technology (NIST) mass spectra search program (version 2.2) Libraries database for identification (Lima et al., 2023).

Statistical Analyses

We used Morisita's Similarity Index (SI) which ranges from 0% (indicating no similarity) to 100% (representing complete similarity) (Krebs, 1999) to compare CHC profiles of different groups, following the methodology of Lima et al. (2021). This analysis was carried out using PAST software (Version 4.13) (Hammer et al., 2001). Furthermore, to assess the overall chemical similarity or dissimilarity between groups, we performed a permutation analysis (PERMANOVA). This analysis was performed using the *adonis* function from the *vegan* package (Oksanen et al., 2013) with 9999 permutations. In order to represent the multivariate chemical dataset and check for the cluster formation, we next performed a Principal Component Analysis (PCA). For this, we used the *prcomp* function of the *stats* package (R Core Team 2019). We also ran a multivariate similarity analysis (SIMPER) using the Bray-Curtis distance and adopting 999 permutations. The SIMPER analysis allowed us to determine the contribution of each chemical variable to the existing variation among samples. For this analysis, we used the *simper* function from the *vegan* package (Oksanen et al., 2013). For all tests, we determined the relative abundance percentages of each compound present in the cuticular extracts, treating the compounds as 100% and then analyzed the data. All these analyses were conducted using R version 4.0.2 (R Core Team 2019).

Results

Overall Chemical Information

A total of 78 peaks were identified in the cuticular extracts from the different groups studied (Table 1). *Senegalia polyphylla* exhibited 22 peaks, *I. laurina* had 29 peaks, *T. catappa, C. crassus* had 28 peaks each, *P. longicornis* had 22 peaks, and *S. calyce* caterpillars had 25-30 peaks. These peaks corresponded to various chemical compounds, including branched hydrocarbons (mono-, di-, and trimethylated), linear alkanes, alkenes, alcohols, and aldehydes. The carbon lengths of the identified compounds ranged from 18 to 36.

The cuticular profile of the three host plant species revealed a shared class of compounds, including linear alkanes, alcohols, and aldehydes. However, variations existed among them, particularly in the proportion and presence or absence of specific alcohols and aldehydes. For instance, 1-hexadecanol, 1-hexacosanol, and hexacosanal were exclusively present in *I. laurina*, while 1-triacontanol acetate was found only in *T. catappa* (Table 1). The SIs of the cuticular compounds of the plants varied according to the pairs of compared species. *Terminalia catappa* with *S. polyphylla* showed the highest SI of up to 77%, followed by *T. catappa* with *I. laurina*, which exhibited a SI of up to 65%, and *I. laurina* with *S. polyphyll*a, which showed a SI of up to 62%. *Senegalia polyphylla* and *I. laurina* shared 20 compounds, representing 69% and 91% of their respective cuticles. *Senegalia polyphylla* shared 15 compounds with *T. catappa*, representing 52% and 68.2% of their respective cuticles. *Terminalia catappa* shared 18 compounds with *I. laurina*, representing 60% and 62.06% of their respective cuticles. Although post hoc pairwise comparisons did not reveal a significant difference based on relative abundance or chemical composition (Table 2), the PCA revealed that the three species form separate groups, in which the first and the second principal component explained 30% and 13.77%, respectively. (Fig 1). In terms of major compounds, *S. polyphylla* had the *n*-C29, 1-triacontanol and triacontanal; *I. laurina* had the *n*-C29, Hexacosanol and *n*-C31, and *T catapp*a had the *n*-C29 and n-C31.

Table 1. Cuticular compounds found on different groups of the *S. calyce* caterpillars, attendant ants and host plants [mean relative abundance ± Standard Deviation (SD)]. Major compounds in bold. (-) = compound not detected. A total of 10 samples per group was used for the chemical analysis, with the exception of *C. crassus* (8 samples) and *S. calyce* -*Terminalia catappa* (6 samples). RT (retention time), RI (retention index), MDI (molecular diagnostic ions), Sp (*Senegalia polyphylla*), Il (*Inga laurina*), Tc *(Terminalia catappa)*,CS (caterpillars raised on *S. polyphylla*), CI (caterpillars raised on *I. laurina*), and CT (caterpillar raised on *T. catappa*). Ants were also mentioned as Cc (*Camponotus crassus*) and Pl (*Paratrechina longicornis*), along with caterpillars raised with them, like CC (caterpillars raised with *C. crassus*) and CP (caterpillars raised with *P. longicornis*).

				Host plants			**Caterpillars with host plants (without ants)**			**Ants**		**Caterpillars with ants**	
Compounds	**RT**	**RI**	***MDI***	**Sp**	**Il**	**Tc**	**CS**	**CI**	**CT**	**Cc**	**Pl**	**CC**	**CP**
4-MeC17	14.874	1756	211	-	-	-	-	-	-	-	0.76 ± 0.65	-	-
n-C18	15.159	1800	254	0.40 ± 0.72	0.34 ± 0.30	-	0.08 ± 0.05	0.11 ± 0.08	0.04 ± 0.02	-	-	-	-
1-hexadecanol	16.074	1881		-	1.59 ± 0.87	-	-	0.424 ± 0.26	-	-	-	-	-
n-C19	16.282	1900	268	0.21± 0.33	0.19 ± 0.13	-	0.10 ± 0.05	0.09 ± 0.04	0.042 ± 0.02	2.53 ± 2.17	-	0.11 ± 0.09	0.12 ± 0.04
4-meC19	16.834	1928	71/238	-	-	-	-	-	-	-	0.91 ± 0.75	-	-
n-C20	17.425	2000	282	0.42 ± 0.59	0.57 ± 0.40	-	0.25 ± 0.15	0.216 ± 0.11	0.08 ± 0.06	0.90 ± 0.61	-	0.14 ± 0.07	0.22 ±0.08
octadecanal	17.702	2024		-	-	0.15 ± 0.30	-	-	-	-	-	-	-
1-octadecanol	18.405	2084		1.02 ± 0.65	2.60 ± 1.06	0.24 ± 0.13	0.32 ± 0.37	0.68 ± 0.32	0.43 ± 0.14	-	-	0.10 ± 0.16	0.7 ± 0.66
n-C21	18.572	2100	296	0.23 ± 0.27	0.33 ± 0.21	-	0.23 ± 0.15	0.17± 0.10	0.14 ± 0.10	14.34 ± 3.54	-	1.068± 1.37	0.30 ±0.18
n-C22	19.718	2200	310	0.47 ± 0.38	0.77 ± 0.36	0.004 ± 0.01	0.33 ± 0.18	0.284 ± 0.13	0.22 ± 0.14	0.46 ± 0.32	-	0.17 ± 0.087	0.25 ± 0.10
1-eicosanol	20.733	2289		0.01 ± 0.03	1.88 ± 1.02	0.07 ± 0.14	0.14 ± 0.24	0.08 ± 0.07	0.10 ± 0.09	-	-	0.008 ± 0.01	0.06 ±0.05
C23:1	20.844	2276	322	-	-	-	-	-	-	1.00 ± 1.22	-	-	-
n-C23	20.863	2300	324	1.91 ± 0.98	1.36 ± 0.72	0.25 ± 0.35	0.86 ± 0.48	1.20 ± 0.57	0.41 ± 0.31	3.70 ± 1.05	-	0.58 ± 0.59	1.96 ± 0.83
n-C24	21.993	2400	338	0.55 ± 0.51	0.66 ± 0.34	0.18 ± 0.24	0.48 ± 0.41	0.34 ± 0.21	0.22 ± 0.23	-	-	0.19 ± 0.09	0.34 ±0.12

				Host plants			Caterpillars with host plants (without ants)			Ants		Caterpillars with ants	
Compounds	**RT**	**RI**	***MDI***	**Sp**	**Il**	**Tc**	**CS**	**CI**	**CT**	**Cc**	**Pl**	**CC**	**CP**
1-docosanol	23.060	2492		-	0.93 ± 0.59	-	-	0.27 ± 0.40	0.20 ± 0.17	-	-	-	-
ZC25	22.864	2476	352	-	-	0.08 ± 0.17	-	-	-	-	-	-	-
n-C25	23.140	2500	352	2.55 ± 1.03	1.94 ± 0.89	1.37 ± 2.78	0.83 ± 0.60	1.625 ± 1.88	0.80 ± 0.43	-	1.46 ± 0.33	0.52 ± 0.24	0.55 ±0.50
3-Mec25	24.004	2573	57/337	-	-	0.08 ± 0.13	-	-	-	-	-	-	-
n-C26	24.319	2600	366	0.59 ±0.33	1.42 ± 0.51	0.44 ± 0.44	1.04 ± 0.85	0.85 ± 0.47	0.61 ± 0.46	-	-	0.27 ±0.21	0.33 ± 0.12
ZC27	25.256	2678	380	-	-	0.41 ± 0.86	-	-	-	-	-	-	-
n-C27	25.524	2700	380	4.69 ± 1.15	6.40 ± 1.08	4.48 ± 2.28	4.83 ± 0.92	8.598 ± 3.38	5.80 ± 2.31	1.17 ± 1.19	6.43 ± 1	5.53 ± 1.97	4.87 ± 2.34
13-;11-MeC27	26.259	2732	225/197/169/253	-	-	-	-	-	-	-	0.60 ± 0.33	-	-
9-MeC27	26.305	2736	141/281	-	-	-	-	-	-	-	0.08 ± 0.03	-	-
Unknown	26.314	2766		-	-	0.11 ± 0.13	-	-	-	-	-	-	-
7-MeC27	26.369	2742	112/309	-	-	-	-	-	-	-	0.52 ± 0.12	-	-
5-MeC27	26.482	2751	85/337	-	-	-	-	-	-	-	0.17 ± 0.06	-	-
3-MeC27	26.753	2773	57/365	-	-	-	-	-	-	-	1.28 ± 0.36	-	-
n-C28	26.714	2800	394	3.56 ± 0.60	3.43 ± 0.69	1.72 ± 0.54	2.78 ± 1.39	2.57 ± 1.17	2.12 ± 0.53	4.99 ± 2.08	-	1.71 ± 0.55	1.47 ± 0.31
hexacosanal	27.202	2838		-	4.74 ± 1.58	-	-	-	-	-	-	-	-
ZC29	27.674	2876	408	-	-	0.17 ± 0.40	-	-	-	-	-	-	-
C29:1	27.806	2879	406	-	-	-	-	-	-	0.43 ± 0.39	-	-	-
1-hexacosanol	**27.964**	**2898**		**-**	**11.56 ± 3.49**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
n-C29	**27.978**	**2900**	**408**	**40.32 ± 6.33**	**20.31 ± 2.70**	**39.57 ± 3.50**	**13.35 ± 1.58**	**16.01 ± 2.12**	**16.75 ± 2.88**	**5.67 ± 1.65**	**14.16 ± 2.59**	**17.70±2.24**	**16.47 ± 1.35**
15-;13-;11-;9-MeC29	**28.759**	**2936**	**225 / 197 / 253 / 169 / 281 / 141 / 309**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**22.30 ± 1.73**	**-**	**-**
15-;13-;11-MeC29	28.670	2929	225/197/253/169/281	-	-	-	-	-	-	0.51 ± 0.40	-	-	-
7-MeC29	28.801	2940	112/337	-	-	-	-	-	-	0.053 ± 0.06	-	-	-
4-MeC29	28.671	2960	71/379	-	-	-	-	0.11 ± 0.08	0.082 ± 0.05	-	-	-	-
3-MeC29	28.809	2973	57/393	-	-	-	-	0.07 ± 0.12	0.04 ± 0.03	2.03 ± 1.20	-	0.03 ± 0.06	0.025 ± 0.04
9,17-; 9,19-	**29.118**	**2966**	**141/267/197/32**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**13.89 ± 1.86**	**-**	**-**

				Host plants			Caterpillars with host plants (without ants)			Ants		Caterpillars with ants	
Compounds	**RT**	**RI**	***MDI***	**Sp**	**Il**	**Tc**	**CS**	**CI**	**CT**	**Cc**	**Pl**	**CC**	**CP**
diMeC29			**3/169/295**										
C30:1	29.312	2982	420	-	-	-	-	-	-	3.18 ± 1.69	-	-	-
***n*-C30**	**29.124**	**3000**	**422**	**3.02 ± 0.23**	**3.35 ± 1.44**	**2.89 ± 0.46**	**4.44 ± 1.55**	**3.45 ± 1.17**	**3.84 ± 0.52**	**11.02 ± 3.99**	**-**	**3.13 ± 0.52**	**3.006 ± 0.43**
1-heptacosanol	29.286	3014		-	7.20 ± 0.98	-	-	-	-	-	-	-	-
octacosanal	29.643	3043		0.35 ± 0.14	2.93 ± 1.99	1.90 ± 0.58	0.05 ± 0.14	0.28 ± 0.34	0.20± 0.11	-	-	0.32 ± 0.24	0.52 ± 0.52
2-MeC30	29.902	3065	43/421	-	-	0.04 ± 0.05	2.91± 1.27	2.48 ± 1.85	2.38 ± 0.88	-	-	0.03 ± 0.07	0.18 ± 0.25
x.y-diMeC30	29.98	3062		-	-	-	-	-	-	0.61 ± 0.45	-	-	-
ZC31	30.078	3079	436	-	-	0.34 ± 0.10	-	-	-	-	-	-	-
C31:1	30.480	3080	434	-	-	-	-	-	-	8.64 ± 1.47	0.37 ± 0.28	-	-
n-C31	30.317	3100	436	7.78 ± 3.02	9.75 ± 6.88	32.44 ± 4.06	22.48 ± 2.56	23.23 ± 2.55	25.97 ± 2.13	5.14 ± 1.54	4.41 ± 1.11	24.34 ± 1.7	24.45 ± 1.42
1-octacosanol	30.376	3105		1.85 ± 1.12	6.30 ± 4.35	-	-	-	-	-	-	-	-
15-;13-;11-;9-MeC31	31.098	3132	225/239/197/281/169/309/141/337	-	-	-	-	-	-	-	7.58 ± 1.35	-	-
2-MeC31	31.064	3165	43/435	-	-	-	0.23 ± 0.22	0.08 ± 0.13	0.12 ± 0.09	-	-	-	-
3-MeC31	31.203	3176	57/421	-	-	-	0.08 ± 0.11	-	0.03 ± 0.03	-	-	0.98 ± 1.91	0.86 ± 1.62
C32:1-1	31.630	3177	448	-	-	-	-	-	-	1.16 ± 0.80	-	-	-
C32:1-2	31.717	3185	448	-	-	-	-	-	-	4.04 ± 1.70	-	-	-
n-C32	31.47	3200	450	0.60 ± 0.29	1.51 ± 0.60	0.90 ± 0.23	6.37 ± 0.78	4.88 ± 0.69	5.46 ± 0.91	3.97± 2.09	-	5.31 ± 0.92	5.79 ± 0.65
Unknown	31.566	3208		0.53 ± 0.28	-	-	-	-	-	-	-	-	-
1-octacosanol acetate	31.66	3216		-	3.38 ± 0.96	2.91 ± 0.95	-	-	-	-	-	-	-
X-MeC32	32.315	3235		-	-	-	-	-	-	-	2.20 ± 0.96	-	-
14,16MeC32	32.585	3258	281/253	-	-	-	-	-	-	-	2.99 ± 0.88	-	-
triacontanal	32.038	3250		9.97 ± 2.28	1.17 ± 1.11	2.11 ± 0.57	0.39 ± 0.25	0.19 ± 0.13	0.24 ± 0.14	-	-	0.267 ± 0.38	0.59 ± 0.36
2-MeC32	32.195	3262	43/448	-	-	-	0.58 ± 0.28	0.37 ± 0.51	0.58 ± 0.47	-	-	0.265 ± 0.4	0.14 ± 0.29

				Host plants			**Caterpillars with host plants (without ants)**			**Ants**		**Caterpillars with ants**	
Compounds	**RT**	**RI**	***MDI***	**Sp**	**Il**	**Tc**	**CS**	**CI**	**CT**	**Cc**	**Pl**	**CC**	**CP**
triMethyl - C32-	32.235	3266		-	-	-	-	-	-	-	2.49 ± 1.21	-	-
C33:1	**32.855**	**3283**	**462**	**-**	**-**	**-**	**-**	**-**	**-**	**13.71 ± 2.86**	**-**	**-**	**-**
***n*-C33**	**32.633**	**3300**	**464**	**-**	**1.05 ± 0.34**	**1.80 ± 0.35**	**27.79 ± 3.57**	**25.19 ± 4.89**	**26.43± 2.54**	**1.58 ± 0.56**	**-**	**29.73 ± 2.6**	**29.23 ± 3.64**
1-triacontanol	**32.741**	**3309**		**18.97 ± 7.47**	**1.66 ± 2.03**	**2.18 ± 0.89**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
17-;15-;13-Me C33	33.410	3329	253/225/281/197/30	-	-	-	-	-	-	1.75± 0.83	3.18 ± 0.53	-	-
17,21-diMeC33	33.698	3356	197/253/267/323	-	-	-	-	-	-	-	4.13 ± 0.81	-	-
C34:1-1	33.954	3380	476	-	-	-	-	-	-	1.08 ± 0.28	-	-	-
C34:1-2	34.032	3386	476	-	-	-	-	-	-	2.50 ± 1.67	-	-	-
n-C34	33.743	3400	478	-	0.7 ± 2.29	-	3.12 ± 0.53	1.69 ± 0.46	2.065 ± 0.79	0.67 ± 0.46	-	1.91 ± 0.65	2.13 ± 0.48
1-triacontanol acetate	33.935	3417		-	-	2.56 ± 0.70	-	-	-	-	-	-	-
Unknow 2	34.329	3452		-	-	0.65 ± 0.22	-	-	-	-	-	-	-
2Me-C34	34.434	3461	43/477	-	-	-	-	0.05 ± 0.08	-	-	-	-	-
C35:1	35109	3482	490	-	-	-	-	-	-	3.19 ± 0.74	-	-	-
n-C35	34.875	3500	492	-	-	-	5.61 ± 0.80	4.30 ± 1.00	4.51 ± 1.27	-	-	4.44 ± 1.82	5.15 ± 1.38
17-;15-;13-MeC35	35.627	3528	239/281/225/309/197/337	-	-	-	-	-	-	-	6.14 ± 1.25	-	-
15.19-; 15.21-diMeC35	35.627	3554	225/323/253/295	-	-	-	-	-	-	-	3.95 ± 1.1	-	-
n-C36	35.945	3600	506	-	-	-	0.37 ± 0.35	0.13± 0.14	0.15 ± 0.23	-	-	-	-

Table 2. Morisita´s similarity index (mean ± standard error), F-value, R2 and P-value of the shared cuticular hydrocarbons of pairs of groups (caterpillars, host plants and attendant ants). *S. calyce* caterpillars: C-Sp = caterpillar reared on *S. polyphylla*; C-Il = caterpillars reared on *I. laurina* C-Tc = caterpillars reared on *T. catappa*. C-Cc = caterpillars reared with *C. crassus*; C-Pl = caterpillars reared with *P. longicornis*; Plants: Sp = S. *polyphylla*; Il = I. *laurina*; Tc = *T. catappa*; Ants: Cc = *C. crassus*; Pl = *P. longicornis*.

	Caterpillars vs plants			**Caterpillars vs ants**		**Plants vs plants**			**Caterpillars vs caterpillars**					
	C-Sp vs Sp	**C-Il vs Il**	**C-Tc vs Tc**	**C-Cc vs Cc**	**C-Pl vs Pl**	**Sp vs Il**	**Sp vs Tc**	**Tc vs Il**	**C-Sp vs C-Il**	**C-Sp vs C-Tp**	**C-Il vs C-Tc**	**C-Cc vs C-Pl**	**C-Pl vs C-Sp**	**C-Cc vs C-Sp**
Morisita's similarity index (mean ± standard error)	0.58 ± 0.05	0.63 ± 0.11	0.78 ± 0.06	0.30 ± 0.05	0.25 ± 0.05	0.62 ± 0.07	0.77 ± 0.05	0.7 ± 0.10	0.96 ± 0.02	0.95 ± 0.02	0.93 ± 0.04	0.99 ± 0.01	0.98 ± 0.02	0.99 ± 0.01
F-value	629.58	116.40	110.61	37.441	457.22	1.814	3.47	0.97	42.323	49.329	62.603	1.390	1.786	1.786
Coefficient of determination (R2)	0.9	0.89	0.86	0.675	0.962	0.091	0.167	0.051	0.70	0.89	0.68	0.071	0.090	0.090
P-value	< 0.05*	< 0.05*	< 0.05*	< 0.05*	< 0.05*	> 0.05	> 0.05	> 0.05	< 0.05*	< 0.05*	< 0.05*	> 0.05	> 0.05	> 0.05

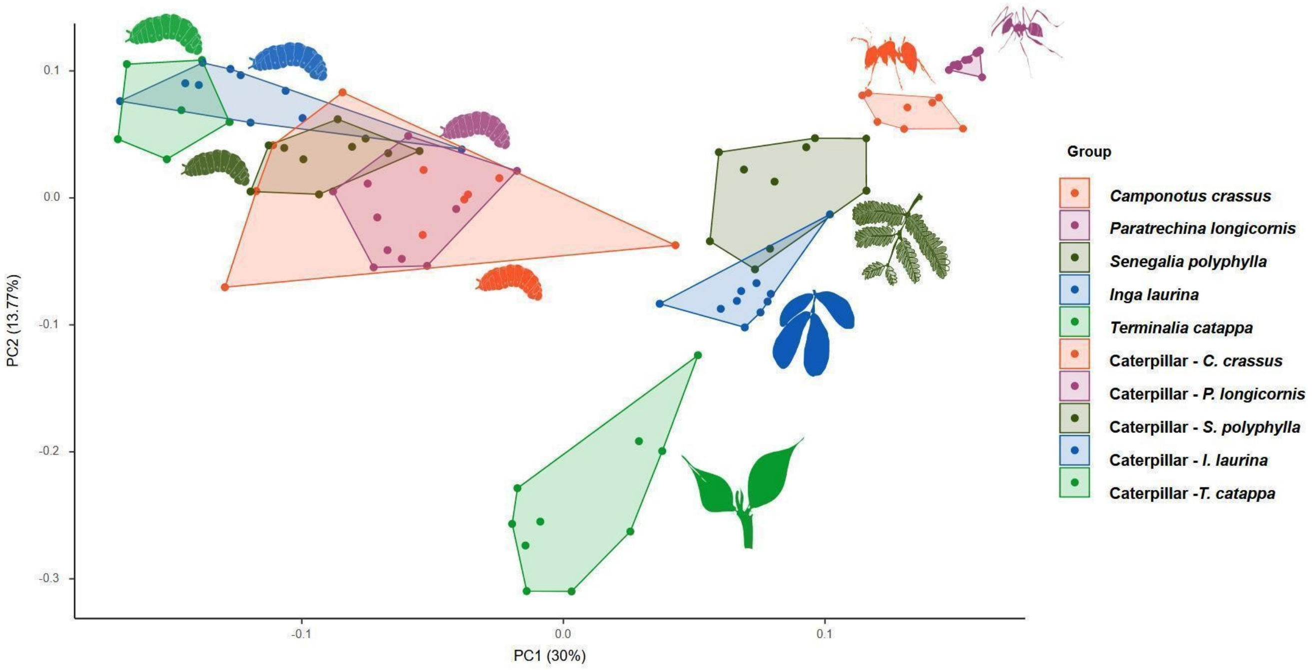


Fig. 1. Principal component analysis (PCA) of chemical compounds in *S. calyce* caterpillars (three groups reared on three plants species without ants and two groups reared with two ants species), the attendant ants (*C. crassus* and *P. longicornis*) and the host plants (*S. polyphylla, I. laurina*, and *T. catappa*)

Does the Chemical Composition of Caterpillars Change According to their Food Sources?

When comparing the compounds found in caterpillars reared on three different host plants, we found the following similarity indices: The cuticular profiles of caterpillars reared on *T. catappa* showed a SI of up to 82% with *T. catappa*, up to 74% with *I. laurina*, and up to 61% with *S. polyphylla*. The cuticular profiles of caterpillars reared on *I. laurina* showed a SI of up to 76% with *I. laurina* and *T. catappa*, and up to 60% with *S. polyphylla*. Finally, caterpillars reared on *S. polyphylla* exhibited a SI up to 65% with *S. polyphylla*, and up to 64% with *T. catappa*, and *I.*

laurina. Moreover, certain compounds were exclusively found in groups of caterpillars reared on specific plant species. For example, 1-hexadecanol was found only in the cuticular profile of caterpillars reared on *I. laurina,* and this particular compound was also identified in the chemical profile of this plant. Similarly, 1-docosanol, identified in *T. catappa*, was also detected in caterpillars reared on this plant but was absent in those reared on *S. polyphylla* (Table 1). The qualitative similarity varied according to the host plant. Specifically, caterpillars shared 19 compounds with *S. polyphylla*, representing 70.37% and 86.36% of their respective cuticles. Caterpillars and *I. laurina* shared 23 compounds, representing 76.66% and 79.31% of their respective cuticles, while caterpillars and *T. catappa* shared 17 compounds, representing 58.6% and 60.7% of their respective cuticles. However, there was a low degree of similarity in the relative abundance of compounds between caterpillars and their host plants. The post hoc pairwise comparisons revealed significant differences in relative abundance and in the chemical composition among caterpillars reared on different plants (Table 2). However, most of the compounds were shared in the three groups and we found an overlap among caterpillars in the PCA (Fig. 1). Specifically, caterpillar–*T. catappa* and caterpillar–*I. laurina* shared 27 compounds, representing 93.1% and 90% of their respective cuticles. Caterpillar-*T. catappa* and caterpillar–*S. polyphylla* shared 26 compounds, representing 89.7% and 92% of their respective cuticles. Caterpillar–*I. laurina* and caterpillar–*S. polyphylla* shared 25 compounds, representing 83.3% and 92.6% of their respective cuticles. In general, all caterpillar groups had *n*-alkanes (C29, C31, and C33) as their major compounds and they also showed a few methylated compounds, alcohols, and aldehydes, these last two also present in all plants.

Does the Chemical Composition of Caterpillars Change According to their tending Ants?

The cuticular profiles of caterpillars reared with *C. crassus*, or *P. longicornis* ants, as well as caterpillars reared without attendant ants, had a high SI (> 90%). Moreover, the cuticular profiles of all caterpillar groups were qualitatively similar, with 24 shared compounds, representing 96% of their respective cuticles. Hence, we found an overlap among samples of caterpillars in the PCA (Fig. 1). The post hoc pairwise comparisons did not show differentiation based on chemical composition among caterpillars reared with different species of attendant ants (Table 2).

In contrast, the cuticular profiles of caterpillars and attendant ants showed low similarity indexes and varied according to the attendant ant (SI < 30% for *C. crassus* and SI < 25% for *P. longicornis*). Caterpillars and *C. crassus* shared 14 compounds, representing 54% and 50% of their respective cuticles. Caterpillars and *P. longicornis* shared four compounds, representing 16% and 18.2% of their respective cuticles. The post hoc pairwise comparisons showed a significant differentiation based on chemical composition between all groups of caterpillars and their respective attendant ants (Table 2). Moreover, the PCA revealed that ants and caterpillars form separate groups.In terms of major compounds, *C. crassus* had the *n*-C21, *n*-C30 and C33:1, and *P. longicornis* had the *n*-C29, 15-;13-;11-;9-MeC29, 15-;13-;11-MeC29 and 9.17-; 9.19-diMeC29 (Table 1).

In general, caterpillars, host plants, and attendant ants shared the *n*-C27, *n*-C29, and *n*-C31 compounds (Fig. 3). Only caterpillars and plants showed alcohols and aldehydes in their chemical profiles (hexadecanol, octadecanol, eicosanol, octacosanal, docosanol and triacontanal). The most significant differentiating compounds among the groups, according to SIMPER analysis, were the n-C33, n-C31 and *n*-C29, which were major compounds in caterpillars (Fig. 2). Overall, we observed that all groups of caterpillars presented high similarity in composition and proportion of their cuticular compounds. Host plants and caterpillars had a higher number of compounds in common, with *n*-alkanes (*n*-C29 and *n*-C31) as their major compounds. Host plants also had various alcohols and aldehydes, which are also present in caterpillars, but in smaller proportions. The three plants exhibited qualitative similarities in their linear alkanes, with some variations in proportion. Additionally, there were qualitative differences in the alcohols and aldehydes among the three species. Thus, the cuticular profile of caterpillars was not influenced by attendant ants. Ant species showed a greater diversity of compounds compared to caterpillars, including various branched alkanes, alkanes, and alkenes.

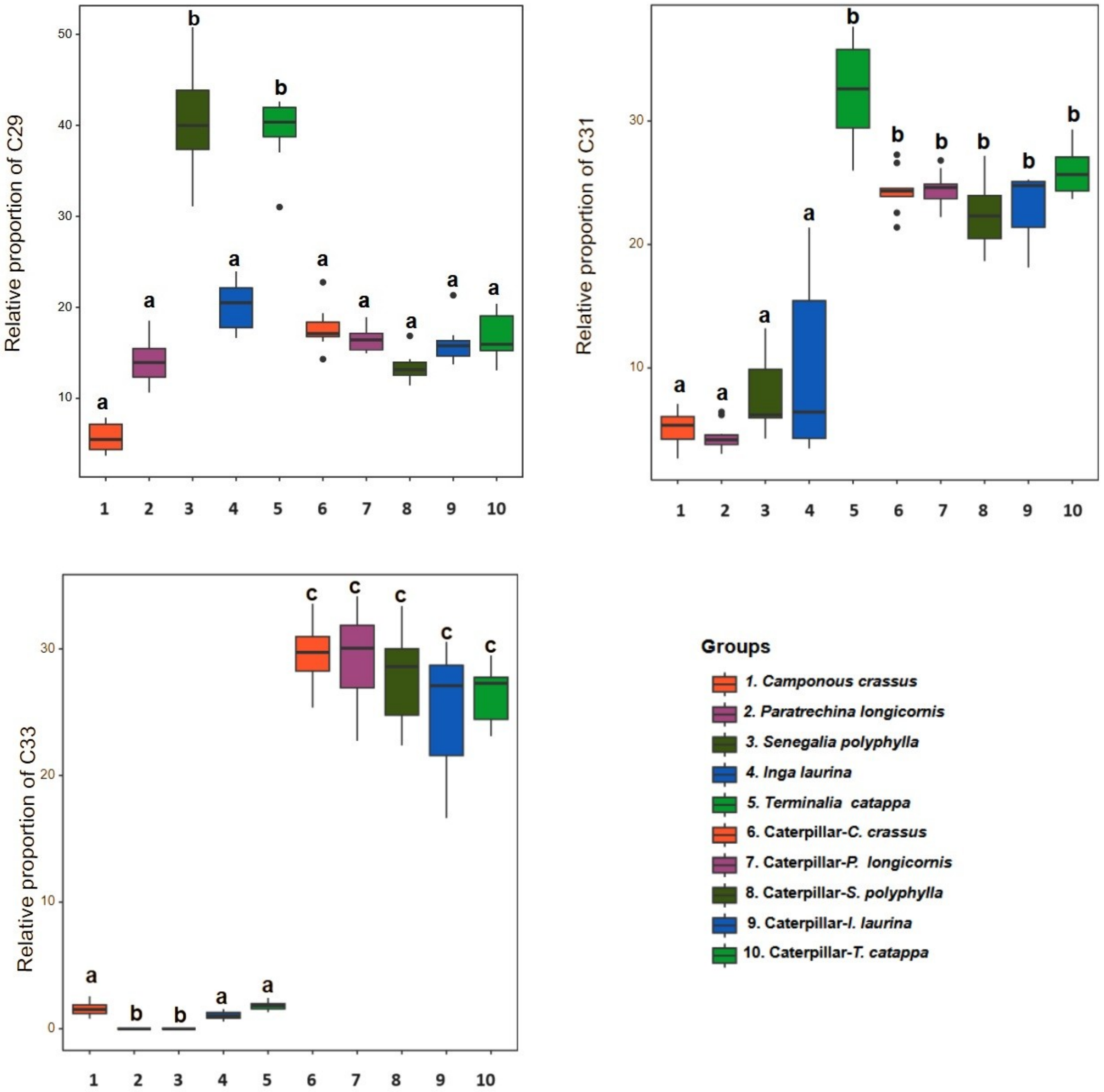


Fig. 2. Box plots of the relative abundance of the most important chemical compounds (*n*-C29, *n*-C31 and *n*-C33) that contributed to differentiating the groups according to SIMPER. Groups 1 and 2 include attendant ants; 3 – 5 include host plants and 6 – 10 include caterpillars exposed to different conditions. Different letters represent $p < 0.05$

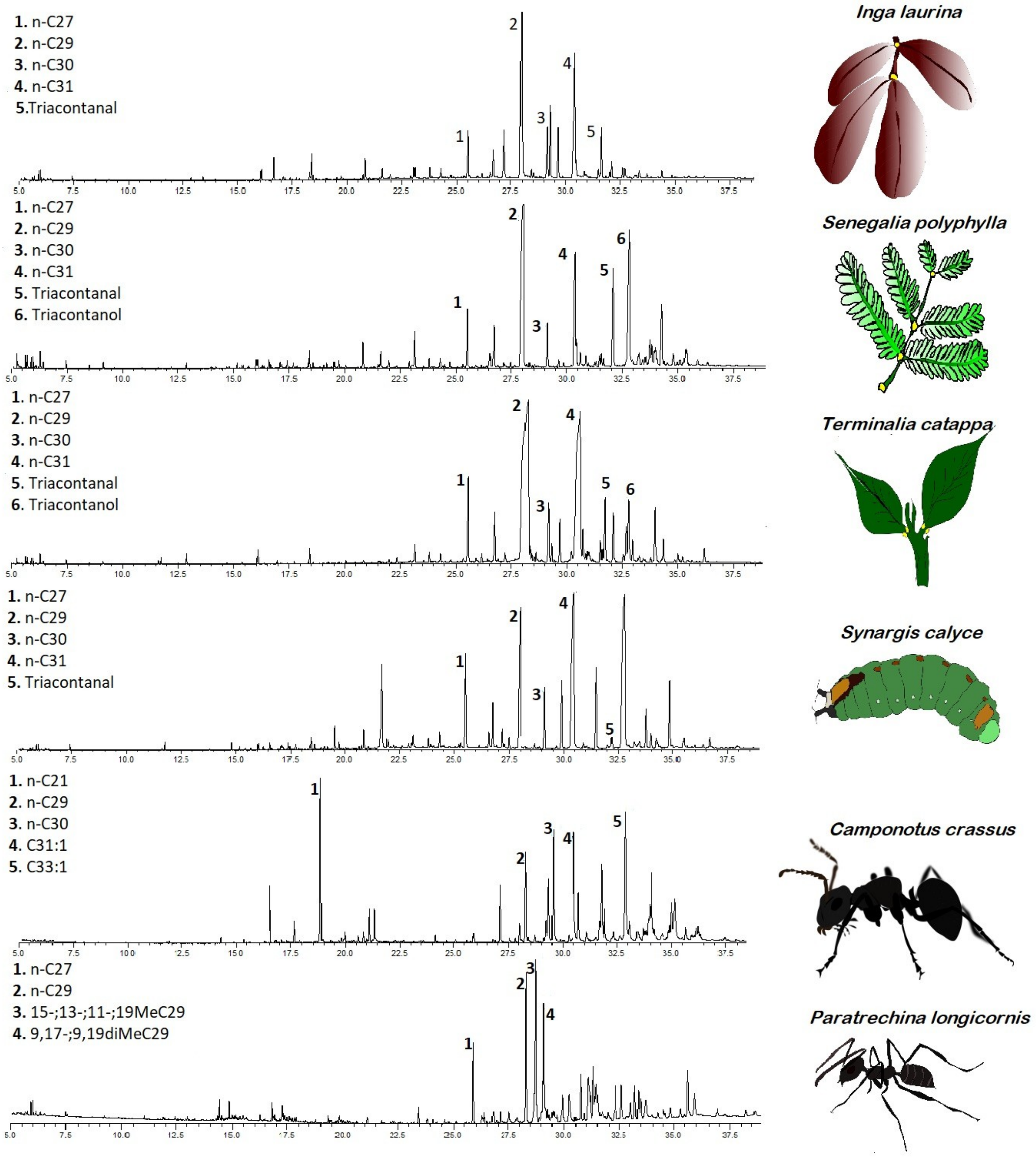


Fig. 3. Comparison of CHC profiles among host plant, myrmecophilous caterpillar, and attendant ants. All compound identities can be found in Table 1.

Discussion

We found that the cuticular profile of caterpillars are more similar to those of the host plants rather than to their tending ants. Moreover, caterpillars reared on different species of plants, without ants or in close contact with ants also had similar cuticular composition and proportion, meaning that in an overall perspective caterpillar cuticular composition is weakly affected by exogenous factors. However, some of their compounds varied depending on the host plants where they were reared, which suggests that they acquired part of these compounds through their diet. Thus, *S. calyce* caterpillars have a chemical profile that it is slightly altered by their food. Consequently, our hypothesis that the caterpillar cuticular composition is influenced by their food source was partially corroborated. Even though the caterpillars are not chemically identical to their host plants, there is a higher similarity between caterpillar-plant when compared to caterpillar-ant species. Given that some compounds, such as 1-hectacontanol, are present in one of the host plants and in caterpillars reared on that plant, but not in the other groups of caterpillars, this may suggest that at least part of the caterpillar chemical composition should derive from their food source. In this way, we suggest that the chemical composition of caterpillars is mainly genetically derived and slightly influenced by the environment. Acquiring compounds from host plants through diet and the usage of them when interacting with ants stands out as a promising strategy and it has also been observed in other plant-herbivorous insect systems (e.g., Silveira et al., 2010; Lima et al., 2021, 2024).

The three plant species used to feed the caterpillars exhibit qualitative similarities in their linear alkanes, as well as in certain alcohols and aldehydes . This suggests that *S. calyce* females likely tend to lay eggs on plants with similar chemical profiles, a similar pattern was observed in lycaenids (Lima et al., 2021). There is evidence that alkanes and alcohols can serve as signals for host plant selection (Li and Ishikawa, 2006; Barbero, 2016; Bertea et al., 2020). In a recent study involving *S. calyce*, it was observed that butterflies sometimes mistakenly lay eggs directly on treehoppers because the treehoppers have a cuticular profile similar to that of the host plant (Lima et al., 2023). The compounds found on the surface of leaves have been described to play a role in the chemical defense of plants (aliphatic hydrocarbons, fatty and phenolic acids derivatives) (Martemyanov et al., 2015: Bertea et al., 2020). Hence, there is a possibility that an evolutionary arms race between caterpillars and plants has driven caterpillars to develop mechanisms countering the chemical defenses of plants. This may involve detoxification of compounds through enzymes or the sequestration of such compounds. Consequently, caterpillars may exhibit a preference for specific chemical compounds present in various host plants (Zu et al., 2020).

Through chemical analyses, we found that the chemical profile of caterpillars was not influenced by their attendant ants thus confirming our second hypothesis that *S. calyce* CHCs are not affected by the interactions that they establish with different ant species. The two ant species studied showed distinct chemical profiles, while the groups of *S. calyce* caterpillars reared with different ant species or without ants had similar cuticular profiles composition and proportion. This indicates that *S. calyce* caterpillars have cuticular profiles that are independent of their attendant ants, indicating that they do not use chemical mimicry as a strategy when interacting with them. This finding is supported by the dissimilarity in chemical composition between caterpillars and ants, compared to the similarity between caterpillars and plants. Additionally, *S. calyce* caterpillars have facultative association with several species of ants, and they do not exploit ant nests (Callaghan, 1986; Alves-Silva et al., 2018; Kaminski, 2021). Previous studies demonstrating chemical mimicry between caterpillars and ants have typically involved obligate interactions with a few specific ant species, where the CHCs of caterpillars closely resemble those of the ants (Henning, 1983; Elmes et al., 1991; Dettner and Liepert, 1994; Akino et al., 1999; Elmes et al., 2002; Schönrogge et al., 2004; Hojo et al., 2009; Thomas et al., 2013; Witek et al., 2013; Hojo et al., 2014; Barbero, 2016; Casacci et al., 2019). Chemical mimicry with attendant ants is an effective strategy for social parasitic caterpillars, as they typically inhabit ant nests and benefit from being perceived as members of the colony, allowing them to exploit valuable resources within the nest such as ant larvae or trophallaxis (Fiedler, 1991; Barbero, 2016; Casacci et al., 2019). We also ruled out the possibility of a chemical insignificance strategy in *S. calyce* caterpillars as in previous studies where caterpillars and pupae used chemical insignificance, their cuticular profile consisted of only a few hydrocarbons in very small proportions (Lohman et al., 2006; Inui et al., 2015). In contrast, *S. calyce* caterpillars showed 27-30 cuticular compounds, with some of them in high proportions.

Thus, it seems that this species maintains its own chemical profile in a chemical strategy conspicuousness (*sensu* Lima et al., 2021). This strategy is the most likely since the chemical profiles of caterpillars exposed to various conditions remained mainly unchanged, with all groups exhibiting a high degree of similarity. Chemical conspicuousness becomes advantageous when interacting with attendant ants, increasing the likelihood that ants will associate the reward with specific cuticular profiles (Hojo et al., 2014). Studies conducted with Neotropical Lycaenidae species have demonstrated that these species possess conspicuous chemical profiles (Lima et al., 2021). Additionally, there are studies showing that caterpillars or pupae of facultative lycaenid butterflies from other regions have unique chemical profiles recognized by ants, which helps maintain their attending behavior (Ômura et al., 2009, 2012; Hojo et al., 2014; Mizuno et al., 2018). However, to our knowledge, this is the first study to explore cuticular hydrocarbons (CHCs) and investigate chemical strategies in a facultative species of the Riodinidae family.

In this study, caterpillars and plants shared several compounds when compared to ants. Notably, linear alkanes such as C29 and C31 are present in significant proportions across all groups, along with some alcohols and aldehydes. Therefore, we cannot rule out the possibility that in certain instances, caterpillars may employ chemical camouflage. Moreover, caterpillars reared on one host plant species exhibited a similarity exceeding 80%, which has been previously demonstrated as sufficient to serve as chemical camouflage strategy in other insect groups (Silveira et al., 2010). Host plants play a crucial role in providing an effective background for herbivorous organisms, allowing them to avoid detection by visually or chemically resembling their surroundings. This strategy is observed in various organisms that have close relationships with ants, enabling them to interact with ants without being attacked (von Beeren et al., 2012; Barbero, 2016; Lima et al., 2024). Consequently, we propose that these caterpillars may employ a trade-off strategy between camouflage and informing their presence to ants, which could vary depending on the presence of predators or mutualist ants. Given the presence of numerous non-attendant ant species that visit the host plants, many of which have extrafloral nectaries, it is highly likely that chemical camouflage has been selected as an efficient strategy for *S. calyce* caterpillars to avoid attacks from different ants (Akino et al., 2004). On the other hand, informing their presence to attendant ants also is efficient for the caterpillars. The complexity of CHCs profiles is well-known, and it is likely that each chemical trait serves a distinct function (Sprenger and Menzel, 2020). There may even be conflicts or trade-offs among the various functions of the chemical profile (Steiger and Stökl, 2014; Ingleby, 2015). Camouflage might be the protagonist during encounters with non-attendant ants, while directly informing their presence becomes more prominent with attendant ants, as it aims to induce ants to associate caterpillars' chemical profile with the chemical reward, consequently securing ant protection (Hojo et al., 2014).

We suggest that specific compounds such as some aldehydes present in both plants and caterpillars or methylated alkanes present in caterpillars, play a role in making the caterpillars blend with the background or informing their presence to attendant ants respectively. For instance, in studies conducted on *Lycaeides argyrognomon* (Bergsträsser, 1779) (Lepidoptera: Lycaenidae), pupal cuticular lipids were found to contain various long-chain aliphatics aldehydes, including 1-octacosanal and 1-triacontanal, which were found to suppress ant aggression (Mizuno et al., 2018). Interestingly, these two compounds were found in all groups of *S. calyce* caterpillars and host plants, suggesting their potential importance in the interaction between *S. calyce* caterpillars and ants.

Studying tri-trophic relationships can present challenges when analyzing the results. For instance, in the PCA results, we observed a low percentage for PC1 and PC2. We suspect that these low percentages values may be linked to the number of groups included in the analysis, comprising five caterpillars, three plants, and two ant groups. The low existent variation within each main group (e.g. ant, caterpillar, and plant) may have contributed to an overall lower dissimilarity percentage when comparing all the groups at once. Thus, to elucidate our findings, we employed more than one type of analysis. Using multiple approaches to analyze the data stands out as a useful strategy when working with complex systems.

Ants exert strong selection pressure on myrmecophilous caterpillars, leading to the development of multimodal adaptations (Pierce and Dankowicz, 2022; Marquis and Koptur, 2022). These adaptations include morphophysiological, behavioral, chemical, and acoustic traits that caterpillars utilize to deceive, attract, alarm, or appease attending ants (Fiedler et al., 1996; Casacci et al., 2019). Synargis calyce caterpillars possess functional TNOs, which have been shown to contribute to the association with ants in other riodinids (DeVries, 1988; Kaminski and Carvalho-Filho, 2012; Kaminski et al., 2013; Mota et al., 2020; Kaminski et al., 2021). To further enhance our understanding of the multimodal signaling in myrmecophile systems, future studies should conduct behavioral assays to experimentally confirm the chemical strategy employed and investigate the products and effects of caterpillars' ant-organs on attending ants, as well as compare them with the products of extrafloral nectaries from plants. This research will contribute to unraveling the specific role of these chemical strategies and organs in the complex interactions between myrmecophilous caterpillars and ants.

References

Alves-Silva, E., Bächtold, A, & Del-Claro, K. (2018) Florivorous myrmecophilous caterpillars exploit an ant–plant mutualism and distract ants from extrafloral nectaries. Austral Ecology 43:643–650. https://doi.org/10.1111/aec.12609

Akino, T. (2008) Chemical strategies to deal with ants: a review of mimicry, camouflage, propaganda, and phytomimesis by ants (Hymenoptera: Formicidae) and other arthropods. Myrmecological News 11:173–181.

Akino. T., Knapp, J. J, Thomas, J. A., & Elmes, G.W. (1999) Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. Proceedings of the Royal Society of London. Series B: Biological Sciences 266:1419–1426. https://doi.org/10.1098/rspb.1999.0796

Akino, T., Nakamura, K. I., & Wakamura, S. (2004) Diet-induced chemical phytomimesis by twig-like caterpillars of *Biston robustum* Butler (Lepidoptera: Geometridae). Chemoecology 14*:*165–174. https://doi.org/10.1007/s00049-004-0274-4

Barbero, F. (2016) Cuticular lipids as a cross-talk among ants, plants and butterflies. International Journal of Molecular Sciences 17:1966. https://doi.org/10.3390/ijms17121966

Barbero, F., Patricelli, D., Witek, M., Balletto, E., Casacci, L. P., Sala, M., & Bonelli S (2012). *Myrmica* ants and their butterfly parasites with special focus on the acoustic communication. Psyche 2012:1-11. https://doi.org/10.1155/2012/725237

Beccaloni. G. W., Viloria. A, L., Hall, S. K., & Robinson, G, S. (2008) Catalogue of the hostplants of the Neotropical butterflies/Catálogo de las plantas huésped de las mariposas neotropicales. Monografias Tercer Milenio, Saragosa.

Bertea, C., Casacci, P., Bonelli, S., Zampollo, A., & Barbero, F. (2020) Chemical, physiological, and molecular responses of host plants to lepidopteran egg-laying. Frontiers in Plant Science 10: 1768. https://doi.org/10.3389/fpls.2019.01768

von Beeren. C., Pohl. S., & Witte. V. (2012) On the use of adaptive resemblance terms in chemical ecology. Psyche 2012. https://doi.org/10.1155/2012/635761

Blomquist, G. J., & Bagnères, A. G., (2010) Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press

Callaghan, C. J. (1986) Restinga butterflies: biology of *Synargis brennus* (Stichel) (Riodinidae). Journal of the Lepidopterists' Society 40:93–96

Carlson, D. A, Bernier, U. R., & Sutton, B. D. (1998) Elution patterns from capillary GC for methyl-branched alkanes. Journal of Chemical Ecology 24:1845–1865.

Casacci, L. P., Bonelli, S., Balletto, E., & Barbero, F. (2019) Multimodal Signaling in myrmecophilous Butterflies. Frontiers in Ecology and Evolution 7:454. https://doi.org/10.3389/fevo.2019.00454

Cottrell, C. B. (1984) Aphytophagy in butterflies: its relationship to myrmecophily. Zoological Journal of the Linnean Society 80:1–57. https://doi.org/10.1111/j.1096-3642.1984.tb02318.x da Silva RC., Brown, R. L., do Nascimento. F. S., Wenseelers. T., & Oi, C. A. (2021) Cuticular hydrocarbons as cues of caste and sex in the German wasp *Vespula germanica*. Insect Sociaux 68:261–276. https://doi.org/10.1007/s00040-021-00817-5

Dettner, K. & Liepert, C. (1994) Chemical mimicry and camouflage. Annual Review Entomology 39:129–154. https://doi.org/10.1146/annurev.en.39.010194.001021

DeVries, P. J. (1988) The larval ant-organs of *Thisbe irenea* (Lepidoptera: Riodinidae) and their effects upon attending ants. Zoological Journal of the Linnean Society 94:379–393. https://doi.org/10.1111/j.1096-3642.1988.tb01201.x

DeVries, P. J., Harvey, D. J., & Kitching. I. J. (1986) The ant associated epidermal organs on the larva of the lycaenid butterfly C*uretis regul*a Evans. Journal of Natural History 20*:*621–633. https://doi.org/10.1080/00222938600770421

Elmes, G., Thomas, J. A. & Wardlaw, J. C. (1991). Larvae of *Maculinea rebeli*, a large-blue butterfly, and their *Myrmica* host ants: wild adoption and behaviour in ant-nests. Journal of Zoology 223:447–460. https://doi.org/10.1111/j.1469-7998.1991.tb04775.x

Elmes, G., Akino, T., Thomas, J., Clarke, R., & Knapp, J. (2002) Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. Oecologia 130:525–535. https://doi.org/10.1007/s00442-001-0857-5

Espelie, K. E., Bernays, E. A, & Brown, J. J. (1991) Plant and insect cuticular lipids serve as behavioral cues for insects. Arch. Insect Biochemistry Physiology 17:223–233.

Ferguson, S. T., Bakis, I., & Zwiebel, L.J. (2021) Advances in the study of olfaction in eusocial ants. Insects 12:252. https://doi.org/10.3390/insects12030252

Fiedler, K. (1991) European and North West African Lycaenidae (Lepidoptera) and their associations with ants. Journal of Research on the Lepidoptera 28:239–257.

Fiedler, K. (1994) Lycaenid butterflies and plants: is myrmecophily associated with amplified hostplant diversity? Ecological Entomology 19, 79–82. https://doi.org/10.1111/j.1365-2311.1994.tb00393.x

Fiedler, K. (1995) Associations of lycaenid butterflies with ants in Turkey. Die tagfalter der türkei unter Berücksichtigung der angrenzenden Länder 1:437–50.

Fiedler, K. (1996) Host-plant relationships of lycaenid butterflies: large-scale patterns, interactions with plant chemistry, and mutualism with ants. Entomologia Experimentalis et Applicata 80: 259– 267.

Fiedler, K. (2021) The ant associates of Lycaenidae butterfly caterpillars–revisited. Nota Lepidopterologica 44: 159-174. https://doi.org/10.3897/nl.44.68993

Fiedler, K., B Hölldobler., & Seufert, P. (1996) Butterflies and ants: the communicative domain, Experientia 52: 14–24. https://doi.org/10.1007/BF01922410

Gnatzy, W., Jatho, M., Kleinteich, T., Gorb, S., & Hustert, R. (2017) The eversible tentacle organs of *Polyommatus* caterpillars (Lepidoptera, Lycaenidae): morphology, fine structure, sensory supply and functional aspects. Arthropod Structure and Development 46:788–804. https://doi.org/10.1016/j.asd.2017.10.003

Hammer, Ø., Harper, D. A.T., & Ryan, P. D (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica, 4:1–9.

Hefetz, A. (2007) The evolution of hydrocarbon pheromone parsimony in ants (Hymenoptera: Formicidae)-interplay of colony odor uniformity and odor idiosyncrasy. Myrmecological News 10:59–68.

Henning, S. F. (1983) Chemical communication between Iycaenid larvae (Lepidoptera: Lycaenidae) and ants (Hymenoptera: Formicidae). Journal of the Entomological Society Southern Africa 46:341–366

Hill, G. M., Trager, M. D., Lucky, A., & Daniels, J. C (2022) Protective Benefits of Tending Ants to a Critically Endangered Butterfly. Journal of Insect Science 22.:9. https://doi.org/10.1093/jisesa/ieac068

Hojo, M. K., Wada-Katsumata, A., Akino, T., Yamaguchi, S., Ozaki, M., & Yamaoka, R. (2009) Chemical disguise as particular caste of host ants in the ant inquiline parasite *Niphanda fusca* (Lepidoptera: Lycaenidae). Proceedings of the Royal Society B: Biological Sciences276:551–558. https://doi.org/10.1098/rspb.2008.1064

Hojo, M. K., Yamamoto, A., Akino, T., Tsuji, K., & Yamaoka, R. (2014) Ants use partner specific odors to learn to recognize a mutualistic partner. PLoS One 9:e86054. https://doi.org/10.1371/journal.pone.0086054

Hölldobler, B., Wilson, E. O. (1990) The Ants. The Ants. The Belknap Press of Harvard University Press, Cambridge p 732.

Hölldobler, B., Kwapich, C. L. (2022) The Lycaenidae: mutualists, predators, and parasites In: The guests of ants: how myrmecophiles interact with their hosts. Harvard University Press, Massachusetts, pp 149-219

Howard, R. W., Blomquist, G. J. (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annual Review Entomoloy 50:371–393. https://doi.org/10.1146/annurev.ento.50.071803.130359

Ingleby, F. C. (2015) Insect cuticular hydrocarbons as dynamic traits in sexual communication. Insects 6:732–742. https://doi.org/10.3390/insects6030732

Inui, Y., Shimizu-kaya, U., Okubo, T., Yamsaki, E., & Itioka, T. (2015) Various chemical strategies to deceive ants in three *Arhopala* species (Lepidoptera: Lycaenidae) exploiting Macaranga. PLoS One 10: e0120652. https://doi.org/10.1371/journal.pone.0120652

Kaminski, L. A. (2008) Polyphagy and obligate myrmecophily in the butterfly *Hallonympha paucipuncta* (Lepidoptera: Riodinidae) in the Neotropical Cerrado savanna. Biotropica 40: 390–394. https://doi.org/10.1111/j.1744-7429.2007.00379.x

Kaminski, L. A. (2021) Ant-butterfly interactions - Borboletas formigueiras. iNaturalist. https://www.inaturalist.org/projects/ant-butterfly-interactions-borboletas-formigueiras. Accessed 26 April 2023

Kaminski, L.A., & Carvalho-Filho, F. S. (2012) Life history of *Aricoris propitia* (Lepidoptera: Riodinidae)—A myrmecophilous butterfly obligately associated with fire ants. Psyche 2012. https://doi.org/10.1155/2012/126876

Kaminski, L.A., Mota, L. L., Freitas, A. V., & Moreira, G. R. (2013) Two ways to be a myrmecophilous butterfly: natural history and comparative immature-stage morphology of two species of *Theope* (Lepidoptera: Riodinidae). Biological Journal of the Linnean Society 108:844–870. https://doi.org/10.1111/bij.12014

Kaminski, L. A., Volkmann, L., Callaghan, C. J., DeVries, P. J, & Vila, R. (2021) The first known riodinid 'cuckoo'butterfly reveals deep-time convergence and parallelism in ant social parasites. Zool J Linn Soc, 193:860-879. https://doi.org/10.1093/zoolinnean/zlaa150

Krebs, C. J. (1999) Ecological Methodology. Addison Wesley Longman, Menlo Park, USA.

Kronauer, D. J, & Pierce, N. E. (2011) Myrmecophiles. Current Biology 21:R208–R209.

Lenoir, A., Fresneau, D., Errard, C., & Hefetz, A. (1999) The individuality and the colonial identity in ants: the emergence of the social representation concept. In: Information Processing in Social Insects, Basel, pp. 219–37.

Li, G. Q., & Ishikawa Y (2006) Leaf epicuticular wax chemicals of the Japanese knotweed *Fallopia japonica* as oviposition stimulants for *Ostrinia latipennis*. Journ of Chemical Ecoogy 32:595–604. https://doi.org/10.1007/s10886-005-9022-7

Lima, L. D., Trigo, J. R., & Kaminski, L. A. (2021) Chemical convergence between a guild of facultative myrmecophilous caterpillars and host plants. Ecological Entomology 46:66–75. https://doi.org/10.1111/een.12941

Lima, L. D., Ceballos-González, A. V., Prato, A., Kaminski, L.A., & Nascimento, F. S (2023) Plant-treehopper convergence may trick butterflies into trophic oviposition mistakes. Biotropica 55:292–298. https://doi.org/10.1111/btp.13194

Lima, L. D., Ceballos-González, A. V., Prato, A., Cavalleri, A., Trigo, J. R., & Nascimento, F. S (2024) Chemical camouflage induced by diet in a pest treehopper on host plants. Plants 13: 216. https://doi.org/10.3390/plants13020216

Lohman, D. J., Liao, Q., & Pierce, N. E. (2006) Convergence of chemical mimicry in a guild of aphid predators. Ecological Entomology 31:41–51. https://doi.org/10.1111/j.0307-6946.2006.00758.x

Malicky, H. (1970) New aspects of the association between lycaenid larvae (Lycaenidae) and ants (Formicidae, Hymenoptera). Journal of the Lepidopterists´Society 24:190–202.

Marquis, R. J., & Koptur, S. (2022) Caterpillars in the middle: Tritrophic interactions in a changing world. Springer. pp. 319-391.

Martemyanov, V. V., Pavlushin, S. V., Dubovskiy, I. M., Belousova, I. A., Yushkova, Y. V., Morosov, S. V., & Glupov, V. V. (2015) Leaf surface lipophilic compounds as one of the factors of silver birch chemical defense against larvae of gypsy moth. PLoS One 10: e0121917. https://doi.org/10.1371/journal.pone.0121917

Mizuno, T., Hagiwara, Y., & Akino, T. (2018) Chemical tactic of facultative myrmecophilous lycaenid pupa to suppress ant aggression. Chemoecology 28:173–182. https://doi.org/10.1007/s00049-018-0270-8

Mota, L. L., Kaminski, L. A., & Freitas, A. V. (2020) The tortoise caterpillar: carnivory and armoured larval morphology of the metalmark butterfly *Pachythone xanthe* (Lepidoptera: Riodinidae). Journal of Natural History 54: 309–319. https://doi.org/10.1080/00222933.2020.1759720

Newcomer, E. J (1912) Some observations on the relations of ants and lycaenid caterpillars, and a description of the relational organs of the latter. Journal of the New York Entomological Society 20:31–36.

Nunes, T. M., Mateus, S., Favaris, A. P., Amaral, M. F., von Zuben, L. G., Clososki, G. C., & Lopes, N. P. (2014). Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds. Scientific reports 4:7449. https://doi.org/10.1038/srep07449

Ômura, H., Watanabe, M. & Honda, K. (2009). Cuticular hydrocarbons of larva and pupa of Reverdin's blue, *Lycaeides argyrognomon* (Lycaenidae) and its tending ants. Lepid Sci, 60:203–210. https://doi.org/10.18984/lepid.60.3_203

Ômura, H., Watanabe, M. & Honda, K. (2012) Cuticular hydrocarbon profiles of *Lycaeides subsolanus* larvae and their attendant ants. Lepid Sci, 63:186–190. https://doi.org/10.18984/lepid.63.4_186

Otte, T., Hilker, M., & Geiselhardt, S. (2018). Phenotypic plasticity of cuticular hydrocarbon profiles in insects. Journal of Chemical Ecology, 44, 235–247. https://doi.org/10.1007/s10886-018-0934-4

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R, O'hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., & Szoecs, E. (2013) Package 'vegan'. Community ecology package. 1-295 version 2.

Parker, J. (2016). Myrmecophily in beetles (Coleoptera): evolutionary patterns and biological mechanisms. Myrmecological news 22:65–108.

Pierce, N. E., Braby, M. F, Heath, A., Lohman, D. J, Mathew, J., Rand, D. B., & Travassos, M. A. (2002) The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). Annual Review Entomology 47:733–771. https://doi.org/10.1146/annurev.ento.47.091201.145257

Pierce, N. E., Dankowicz, E. (2022) Behavioral, ecological and evolutionary mechanisms underlying caterpillar-ant symbioses. Current Opinion in Insect Science 52:100898. https://doi.org/10.1016/j.cois.2022.100898

Portugal, A. H. A., & Trigo, J. R. (2005) Similarity of cuticular lipids between a caterpillar and its host plant: a way to make prey undetectable for predatory ants?. Journal of Chemical Ecology 31:2551–2561. https://doi.org/10.1007/s10886-005-7613-y

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna https://www.R-project.org/. Accessed 16 Feb 2023.

Schlick-Steiner, B. C., Steiner, F. M., Höttinger, H., Nikiforov, A., Mistrik, R., Schafellner, C., Baier, P., & Christian, E. (2004) A butterfly's chemical key to various ant forts: Intersection-odour or aggregate-odour multi-host mimicry? Naturwissenschaften *91:*209–214. https://doi.org/10.1007/s00114-004-0518-8

Schönrogge, K., Wardlaw, J., Peters, A., Everett, S., Thomas, J., & Elmes, G. (2004) Changes in chemical signature and host specificity from larval retrieval to full social integration in the myrmecophilous butterfly *Maculinea rebeli*. Journal of Chemical Ecology 30:91–107. https://doi.org/10.1023/B:JOEC.0000013184.18176.a9

Schönrogge, K., Barbero, F., Casacci, L. P., Settele, J., & Thomas, J. A. (2017) Acoustic communication within ant societies and its mimicry by mutualistic and socially parasitic myrmecophiles. Animal Behavior 134:249–256. https://doi.org/10.1016/j.anbehav.2016.10.031

Silveira, H. C., Oliveira, P. S., & Trigo, J. R. (2010) Attracting predators without falling prey: chemical camouflage protects honeydew-producing treehoppers from ant predation. The American Naturalist, 175:261–268 https://doi.org/10.1086/649580

Sprenger, P. P., & Menzel, F. (2020) Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: how and why they differ among individuals, colonies, and species. Myrmecological news, 30:1–6. https://doi.org/10.25849/myrmecol.news_030:013

Steiger, S., & Stökl, J. (2014) The role of sexual selection in the evolution of chemical signals in insects. Insects, 5:423–438. https://doi.org/10.3390/insects5020423

Thomas, J. A., Schönrogge, K., & Elmes, G. W. (2005) Specialisations and host associations of social parasites of ants. Evolutionary ecology (ed. J. Rolff, M. Fellowes and G. Holloway). Symposium of the Royal Entomological Society XXI, pp. 475–514. https://doi.org/10.1079/9780851998121.047

Thomas, J. A., Elmes, G., Sielezniew, M., Stankiewicz-Fiedurek, A., Simcox, D. J., Settele, J. & Schönrogge, K. (2013). Mimetic host shifts in an endangered social parasite of ants. Proceedings of the Royal Society Lond B: Biological Science 280: 20122336. https://doi.org/10.1098/rspb.2012.2336.

Witek, M., Casacci, L. P., Barbero, F., Patricelli, D., Sala, M., Bossi, S., & Bonelli, S. (2013) Interspecific relationships in co-occurring populations of social parasites and their host ants. Biological Journal of the Linnean Society 109:699–709. https://doi.org/10.1111/bij.12074

Yamaoka, R. (1990) Chemical approach to understanding interactions among organisms. Physioloy and Ecology Japan 27:31–52.

Zu, P., Boege, K., Del-Val, E., Schuman, M. C., Stevenson, P. C., Zaldivar-Riverón, A., & Saavedra, S. (2020) Information arms race explains plant-herbivore chemical communication in ecological communities. Science 368:1377–1381. https://10.1126/science.aba2965.

CHAPTER 3

THE ROLE OF ANT-ORGANS OF MYRMECOPHILOUS RIODINID CATERPILLARS AND THEIR IMPACT ON ANT ATTENDANT BEHAVIOR

Abstract

Selection pressures exerted by ants over myrmecophilous butterflies have led to the evolution of specialized organs, namely ant-organs, that mediate interactions with ants. Although some studies have focused on these ant-organs, such as the dorsal nectary organ (DNO) and tentacular organs (TOs) in Lycaenidae, and the tentacular nectary organs (TNOs) and anterior tentacular organs (ATOs) in Riodinidae, there is no consensus on its functions, and very little is known about their roles in mediating ant-caterpillar interaction in the majority of the systems. Here, we first built an ethogram of the behavioral interactions between a facultative myrmecophilous caterpillars Synargis calyce (C. Felder and R. Felder, 1862) (Lepidoptera: Riodinidae) and its attendant ants Camponotus crassus Mayr, 1862 (Hymenoptera: Formicidae). Secondly, we manipulated ATOs and TNOs to determine their role in maintaining ant attendant behavior separately. We quantified the time and number of ants interacting with caterpillars in the different treatments involving occlusion of TNOs and ATOs We found that the time and number of ants interacting with caterpillars were higher in the control group and the group with TNOs not occluded, followed by the group with functional ATOs. Caterpillars with both organs occluded received less attention. Our results indicate that the TNOs sustain ant-caterpillar interactions. We propose that interactions between S. calyce caterpillars and C. crassus ants are mainly mediated by rewards offered from the TNOs ant-organs and, to a lesser extent, by the eversion of ATOs.

Key Words- Anterior tentacle organs (ATOs), *Camponotus crassus*, tentacle nectary organs (TNOs), mutualism, myrmecophilous butterflies, Riodinidae, *Synargis calyce*.

Introduction

Organisms of different species can interact in antagonistic, neutral, or cooperative ways (Bronstein, 1994). Among these interactions, cooperative relationships of mutualism are conspicuous, especially where competition for food, and pressure from predators or herbivores is intense (Leigh, 2010; Bascompte, 2019). In these cooperative interactions, the two parties involved benefit by exchanging services such as nutrition or defense (Hojo, 2022).

Many organisms establish relationships with ants, which are abundant organisms and important predators (Hölldobler and Wilson, 1990; Floren et al., 2002). Within the order Lepidoptera, a significant proportion of species in the families Lycaenidae and Riodinidae interact with ants and the selection pressures exerted by interactions with ants contributed for species from these families to develop strategies and specialized 'ant-organs' that sustain these interactions (Fiedler, 1991; DeVries, 1991; Pierce et al., 2002; Pierce and Dankowicz, 2022). The interactions with ants occur typically in the larval stage (Pierce et al., 2002) which represents a state of high vulnerability to predators and parasitoids (Casacci et al., 2019). Under these circumstances, being actively protected by ants may be less metabolically costly for caterpillars than using other defensive strategies (Wagner, 1993; Mizuno et al., 2018; Marquis and Koptur, 2022). Lycaenid and Riodinid caterpillars usually establish facultative interactions with ants, however, there are some cases of obligate interactions with a specific ant species or genus where the survival of caterpillars depends on ants (Fiedler, 1991; Bronstein, 1994; Pierce et al., 2002; Pierce and Dankowicz, 2022).

A mechanism that is often deployed by caterpillars and maintains ants' attendant behavior is releasing rewarding exudates (Casacci et al., 2019). The primary constituents of these secretions include sugars and certain amino acids (DeVries, 1988; Daniels et al., 2005;

Marquis and Koptur, 2022). These secretions are released by analogous organs in Lycaenidae and Riodinidae (DeVries, 1988; Pierce and Dankowicz, 2022). Lycaenids release secretions from the dorsal nectar organ (DNO) located in the seventh abdominal segment, and riodinids do so from a pair of tentacle nectar organs (TNOs) located in the eighth abdominal segment (Newcomer, 1912; DeVries, 1988; Fiedler and Maschwitz, 1989; Leimar and Axén, 1993; Pierce et al., 2002; Casacci et al., 2019). Although these secretions have been mainly associated with nutritional rewards, evidence shows that they may contain other compounds that alter or manipulate the behavior of attending ants (Hojo et al., 2015).

Both Lycaenidae and Riodinidae caterpillars have a second pair of organs that mediate interactions with ants (Pierce et al., 2002; Casacci et al., 2019). These analogous organs in the two families are called tentacle organs (TOs) in Lycaenidae and anterior tentacle organs (ATOs) in Riodinidae, respectively (Cottrell, 1984; Kitching and Luke, 1985; DeVries, 1988, 1991; Kaminski and Carvalho-Filho, 2012). Various debates have focused on clarifying the function of these structures; however, so far, a consensus has not been reached (Casacci et al., 2019; Marquis and Koptur, 2022). One of the hypotheses is that the organs can act by conferring visual or tactile cues (Murray, 1935; Malicky, 1970; Gnatzy et al., 2017). The second hypothesis proposes that both TOs and ATOs can induce alarm behavior in ants, since they release volatiles that mimic ant alarm pheromones, and therefore, they would convey chemical messages (Henning, 1983; DeVries, 1984, 1988; Kitching and Luke, 1985; Pierce and Dankowicz, 2022).

Only a few studies have explored the role of caterpillar ant-organs despite the life histories of almost a thousand ant-associated species have been documented (Pierce and Dankowicz, 2022). In Lycaenidae, experiments on the facultative myrmecophilous *Arhopala* *japonica* (Murray, 1875) demonstrated that occluding DNOs did not deter ant attendance on caterpillars, suggesting effects beyond nutrition, such as altering dopamine levels in ants' brains and influencing behavior (Hojo et al., 2014, 2015). In Riodinidae, experiments occluding TNOs and ATOs of *Lemonias rossi* (Clench, 1964) returned inconclusive results, as ants still attended caterpillars, possibly due to the obligate nature of their interaction (Ross, 1966). However, in the facultative myrmecophilous species *Thisbe irenea* (Stoll, 1780); experiments with occluded ATOs showed reduced ant-caterpillar contact, though limited sample size warrants cautious interpretation (DeVries, 1988).

Given that myrmecophilous caterpillars have a set of specialized ant-organs and adaptations that enable close interactions with ants, gaining a better understanding of the function and role of these organs in maintaining ants' attendant behavior can shed light on the evolutionary and ecological processes involved in these complex interactions. In many myrmecophilous butterfly species, several of these organs have received little attention and remain unexplored. For instance, the function of ATOs in Riodinidae caterpillars is still debatable, and the function of TNOs has been explored in a few species.. Thus, this study aimed at comprehending the potential role of TNOs and ATOs in maintaining ants' attendance via manipulative experiments using the caterpillar *Synargis calyce* (C. Felder and R. Felder, 1862) (Lepidoptera: Riodinidae) as a model. We hypothesized that TNOs and ATOs help sustaining ant attending behavior. We predicted that (1) caterpillars with occluded TNOs and ATOs would be either ignored by ants or would have significantly shorter attendance time, (2) caterpillars with occluded TNOs but active ATOs receive more attendant behaviors than caterpillars with occluded ATOs but less attendant behavior than caterpillars without occlusion, (3) caterpillars with occluded ATOs but active TNOs receive more attendant behaviors than caterpillars with occluded TNOs but less attendant behaviors than non-occluded caterpillars, and (4) caterpillars with active ATOs and TNOs receive more attendant behavior and for longer periods than all other combinations. This study represents one of the first investigations with sufficient and conclusive data that aims to independently understand how different tentacle organs in facultative riodinids maintain interactions with attendant ants.

Material and Methods

Collection and rearing of study species

We conducted this study between August 2021 and August 2022 at the Universidade de São Paulo (21.1637° S, 47.8592° W), Ribeirão Preto Campus, Brazil. We selected *S. calyce* as our study model because it is a facultative and polyphagous myrmecophilous species widely distributed in South America (Callaghan, 1986; Kaminski, 2021). After reaching the third instar, these caterpillars possess functional TNOs and ATOs (Callaghan, 1986). Through field observations, they were consistently observed being attended by ants (AVCG, personal observations). Despite interacting with various ant species, *Camponotus crassus* Mayr, 1862 (Formicinae), a representative and highly abundant species in the Brazilian Savanna (Lange et al., 2019), stood out as the most frequently observed ant species interacting with these caterpillars in the study area.

For our experiments, we collected approximately 100 *S. calyce* eggs in the field and we reared them in the laboratory. Upon hatching, each caterpillar was placed in a 250 ml plastic container. We fed them with shoots containing young leaves and extrafloral nectaries (EFNs) from the host plant *Senegalia pollyphyla* (DC.) Britton & Rose (Fabaceae) and changed the leaves daily. We used this plant because it was the most prevalent species in the study site and where we discovered the highest number of eggs. We collected eight ant nests of *C. crassus* in the field and we transferred them to the laboratory. We placed the nests in two interconnected plastic boxes (9 x 26.6 x 26.6 cm) where one box served as the nesting area and contained test tubes (15 cm long) filled with water plugged with hydrophilic cotton and the other box served as the foraging area (Fig. 1). We provided the ant colonies with a diet of mealworm *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) larvae, diluted sugar solution (10%), and water *ad libitum*. Each colony consisted of approximately 150 workers, a queen, and brood (including eggs, larvae and pupae). We maintained all the insects at a controlled temperature of 25 °C and a photoperiod of 12 hours of light and 12 hours of darkness.

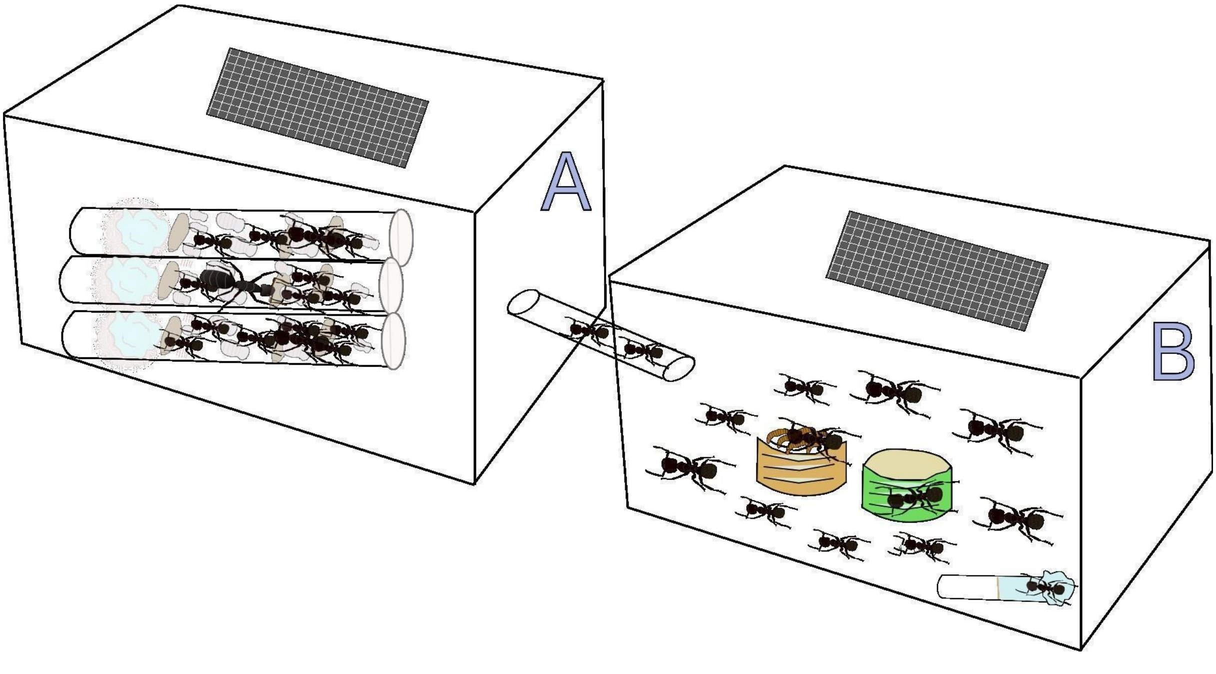


Fig. 1. Scheme illustrating the ant nests of *Camponotus crassus* maintained in the laboratory. Box A has been designated as the nesting area, while Box B served as the foraging area.

Ethogram of the interaction between caterpillars and ants

To create an ethogram of the interaction between *S. calyce* myrmecophilous caterpillars and *C. crassus* ants, we employed ants from three nests and fifth-instar caterpillars. The ethogram was established based on 10-min recordings of interactions between a single caterpillar and a single ant (n = 15) using a video camera (Handycam Hdr-CX405 HD, Sony, Japan). This approach enabled a detailed determination and description of the behaviors occurring during the interaction between the caterpillars and ants. For the ethogram, each caterpillar was introduced into the ants' foraging area along with a branch of the host plant to simulate natural conditions. Observations were recorded between 8:00- 12:00 and the focal animal sampling method (Altmann, 1974) was employed for this purpose. We used different caterpillars and ants in each replicate.

Organ occlusion experimental design

We collected 15 workers of *C. crassus* from the foraging area of each of the eight nests collected and placed them in a petri dish (150 x 25 mm) along with a single fifth-instar caterpillar of *S. calyce*. Insects were given a 15 min-interval of adaptation, which was followed by a 5-min period of interactions recording with a video camera.

We subjected the caterpillars to the following treatments:

1. Control: Caterpillars with intact TNOs and ATOs (no occlusion);
2. Caterpillars with both their TNOs and ATOs occluded;
3. Caterpillars with their TNOs occluded;
4. Caterpillars with their ATOs occluded.

We repeated each treatment 15 times, resulting in a total of 60 assays. To occlude the TNOs and ATOs in the respective treatments, we applied a small amount of clear nail polish in and around the organs. For the caterpillars without occlusion, we added a small amount of clear nail polish to the caterpillar but without occluding the organs. Previous studies have demonstrated that the nail polish does not disturb neither caterpillar nor ant behaviors (DeVries, 1988; Hojo, 2014).

We measured different variables during the video analysis of this experiment: (1) the total time in seconds that ants attended the caterpillars, (2) the latency time until the ants started interacting with caterpillars and, (3) the number of ants attending the caterpillars at time 0, at 2.5 min, and at 5 min. We used different caterpillars and ants in each replicate.

Statistical analysis

To analyze the recorded interactions, we used the BORIS software (Friard and Gamba, 2016) and defined behavioral actions, quantifying them in terms of their frequency and their duration.

Data on the interaction time of ants with caterpillars, the latency time of ants until their first contact with caterpillars, and the number of ants interacting with caterpillars during the experiment were analyzed using a linear mixed model (LMM). For the first case, a model was constructed with the interaction time of ants with caterpillars as the response variable. Nest number, date, and time of day were treated as random effects (random intercept), and treatment groups (control, ATOs occluded, TNOs occluded, and both occluded) were considered as fixed effects. In the second case, a model was created using the latency time of ants until their first contact with caterpillars as the response variable. Nest number, date, and time of day were included as random effects (random intercept), and treatment groups (control, ATOs occluded, TNOs occluded, and both occluded) were considered as fixed effects. For the third case, another model was developed, taking the number of ants interacting with caterpillars during the experiment as the response variable. Nest number, date, and time of day were treated as random effects (random intercept), and treatment groups (control, ATOs occluded, TNOs occluded, and both occluded) were considered as fixed effects. All analyses were performed using R version 2023.06.1 (R Core Team, 2021).

Results

Ethogram of the interaction between caterpillars and ants

In total, we conducted 150 min of observations and recorded a total of 1386 behavioral events during this time. These observations were then categorized into 10 distinct and consistent behavioral events exhibited by both ants and caterpillars during their interactions (see Table 1). Among ants, antennation over the caterpillar thorax and ants walking on caterpillars were the most frequent behaviors, both accounting for a relative frequency of 18.1 %. Additionally, the latter, walking behavior over the caterpillar, had the longest duration. For caterpillars, everting TNOs was both the most frequent and longest-lasting behavior. The total duration of ant-caterpillar interactions was approximately eight minutes within the total of 10 min observation.

In general, caterpillars and ants exhibited a series of behavioral responses to each other's stimuli (see Fig. 2). For instance, caterpillars responded to ant antennation over the thorax by everting their ATOs, and to ant antennation over their abdomen by everting their

TNOs. After the TNOs were everted, ants fed on the droplets released by these organs. On the other hand, after the eversion of the ATOs, ants exhibited a characteristic behavior by jumping over the caterpillars close to the ATOs and decreasing their locomotion. Defensive display behavior in caterpillars, which is characterized by raising their heads and moving backward, was observed in only 3% of instances after being touched by ants. The full list of interactions between ants and caterpillars is depicted in Table 1 and Fig. 2.

Table 1. Behavioral repertoire of the interaction between *Sinargis calyce* caterpillars and *Camponotus crassus* attendant ants, presenting the frequency, duration range, and occurrence range of each behavior in a 10-min recording interval. The total observed time was 150 min, during which 1386 observations were recorded.

Behavior	**Code**	**Relative frequency (%)**	**Duration range (sec)**	**Occurrence range**
Ant antennation over caterpillar thorax	AAT	18.1	44.2-121.9	9-31
Ants walking on caterpillar	AWC	18.1	43-136.4	8-56
Ant patrolling in foraging area	APFA	15.7	35.7-118.5	5-26
Ant antennation over caterpillar abdomen	AAA	13.0	24.2-112.4	5-26
Caterpillar everting TNOs	CET	9.4	16.2-71.0	2-20
Ants feeding on TNOs	AFT	8.7	22.9-118.5	2-18
Caterpillar everting ATOs	CEA	5.8	8.2-24.8	2-17
Ant jumping over caterpillars	AJC	5.5	7.9-23.6	1-16
Ant selfgrooming antenna + 1rst pair of legs	AAPL	5.4	7.5-60.2	2-14
Caterpillar showing aggressive display	CSAD	0.3	0.9-6.9	0-3
TOTAL		100		

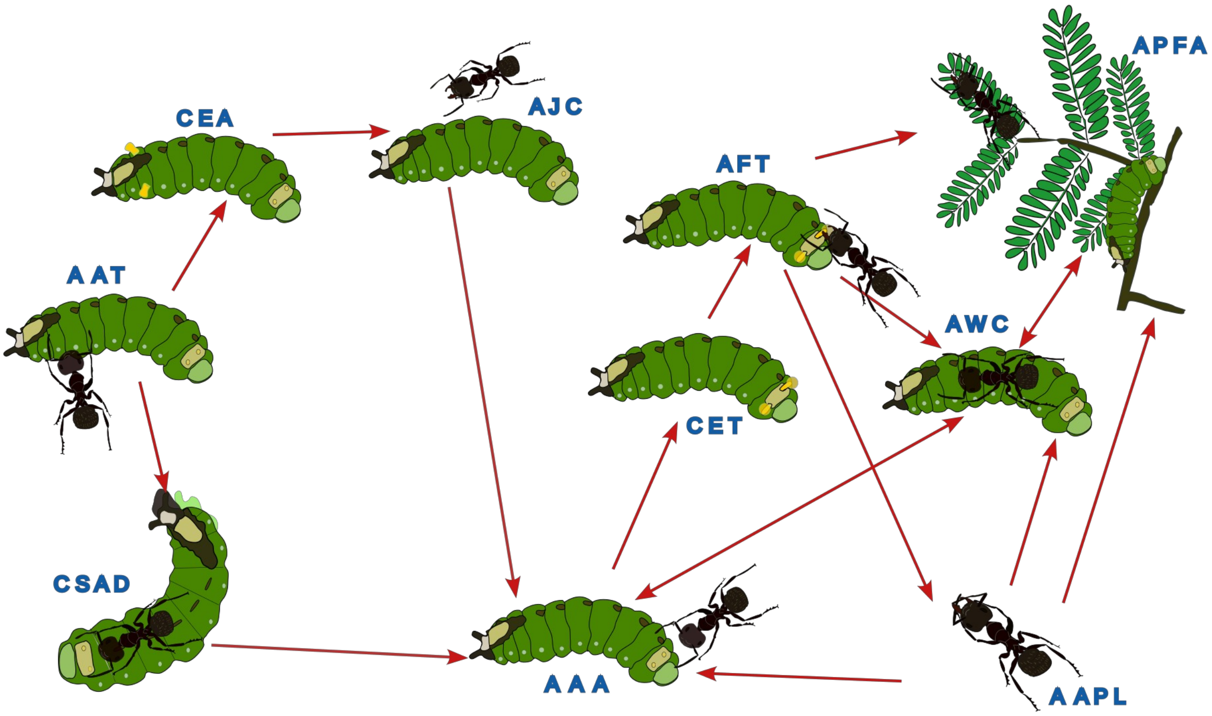


Fig. 2. Ethogram sequence of behaviors exhibited by *Synargis calyce* caterpillars and *Camponotus crassus* attendant ants. The codes correspond to: **AAT**: ant antennation over caterpillar thorax (duration range: 44.2-121.9; occurrence range: 9-31); **AWC**: ants walking on caterpillar (duration range: 43-136.4; occurrence range: 8-56); **APFA**: ant patrolling in foraging area (duration range: 35.7-118.5; occurrence range: 5-26); **AAA**: ant antennation over caterpillar abdomen (duration range: 24.2-112.4; occurrence range: 5-26); **CET**: caterpillar everting **TNOs** (duration range: 16.2-71.0; occurrence range: 2-20); **AFT**: ants feeding on **TNOs** (duration range: 22.9-118.5; occurrence range: 2-18); **CEA**: caterpillar everting **ATOs** (duration range: 8.2-24.8; occurrence range: 2-17); **AJC**: ant jumping over caterpillars (duration range: 7.9-23.6; occurrence range: 1-16); **AAPL**: Ant selfgrooming antenna + 1rst pair of legs (duration range: 7.5-60.2; occurrence range: 2-14); **CSAD**: caterpillar showing aggressive display (duration range: 0.9-6.9; occurrence range: 0-3).

Organ occlusion assays

When comparing the duration of ant interactions, we observed a significant difference between the group of caterpillars with both TNOs and ATOs occluded and the other groups. Specifically, the attendance time was significantly shorter for the group that had both organs occluded compared to all other groups of caterpillars (Control vs ATOs occluded $p=0.667$, Control vs both occluded $p < 0.05$, ATOS occluded vs both occluded $p < 0.05$, ATOs occluded vs TNOs occluded $p < 0.05$, Both occluded vs TNOs occluded $p < 0.05$) (Fig. 3; Table 2). Caterpillars with occluded TNOs also exhibited a significant difference in the interaction time with the control group and the group of caterpillars with occluded ATOs (control vs TNOs occluded $p < 0.05$, ATOs occluded vs TNOs occluded $p < 0.05$). On the other hand, there was no significant difference in interaction time between caterpillars with occluded ATOs and the control group caterpillars with functional TNOs and ATOs). Moreover, the interaction time between these two caterpillar groups and ants was longer in comparison with the other two groups (Fig. 3; Table 2).

Regarding the latency time before the first interaction with the caterpillars, we did not find a significant difference across the four treatments. However, there was a trend for ants interacting with caterpillars deprived from functional organs to take more time to start interacting with them (Fig. 4; Table 2). The number of ants interacting with ants over time differed significantly among the groups. The number of ants interacting with caterpillars over time increased significantly in both control and caterpillars with ATOs occluded in comparison to caterpillars from the other two groups (Control vs both organs occluded < 0.05, ATOS occluded vs both occluded $p < 0.05$, ATOs occluded vs TNOs occluded $p < 0.05$, control vs TNOs occluded $p < 0.05$). Caterpillars with TNOs occluded represented the following group with the highest number of ants tending across the 5 min observation. The number of ants interacting with caterpillars with both organs occluded was the lowest of the four groups (Fig. 5; Table 2).

Overall, the interaction time of ants with caterpillars was the longest for the control group and the group with ATOs occluded, followed by the group with TNOs occluded, and the group with both organs occluded. The latency time for ants making the first contact with caterpillars was similar across the four treatments, although there was a trend for ants taking more time to start the first interactions with caterpillars with both organs occluded. On the other hand, the number of ants attending the caterpillars during the experiment was higher for the control group and the group with ATOs occluded, followed by the group with TNOs occluded, and then the group with both organs occluded.

Table 2. Linear Mixed Model (LMM) showing standard error (SE), degrees of freedom (df), t.ratios, and p-values to assess ants' interaction time with caterpillars, the latency time until the first contact with caterpillars, and the number of ants during the experiment. This analysis considered different treatments (control, caterpillars with occluded ATOs, caterpillars with occluded TNOs, and caterpillars with both organs occluded). P values below 0.05 are shown in italics.

Time of ant interactions	**SE**	**df**	**t.ratio**	**p.value**
Control vs ATOs occluded	17.8	17.7	0.433	0.6699
Control vs both organs occluded	17.7	40.9	13.482	*<.0001*
Control vs TNOs occluded	17.2	16.1	5.003	*0.0002*
ATOS occluded vs both organs occluded	18.0	19.0	12.837	*<.0001*
ATOs occluded vs TNOs occluded	17.5	40.8	4.480	*0.0001*
Both organs occluded vs TNOs occluded	17.5	17.5	-8.756	*<.0001*
Latency time	**SE**	**df**	**t.ratio**	**p.value**
Control vs ATOs occluded	0.960	17.0	0.453	0.8275
Control vs both organs occluded	0.897	42.3	-1.967	0.1380
Control vs TNOs occluded	0.934	15.5	0.085	0.9335
ATOS occluded vs both organs occluded	0.972	17.9	2.261	0.1380
ATOs occluded vs TNOs occluded	0.883	42.4	0.402	0.8275
both organs occluded vs TNOs occluded	0.947	16.4	1.946	0.1380
Number of ants across time	**SE**	**df**	**t.ratio**	**p.value**
Control vs ATOs occluded	0.203	21.1	-0.330	0.7446
Control vs both organs occluded	0.203	171.7	-5.914	*<.0001*
Control vs TNOs occluded	0.200	20.6	-2.518	*0.0242*
ATOS occluded vs both organs occluded	0.206	22.9	5.497	*<.0001*
ATOs occluded vs TNOs occluded	0.200	171.6	3.148	*0.0033*
both organs occluded vs TNOs occluded	0.197	18.9	3.539	*0.0033*

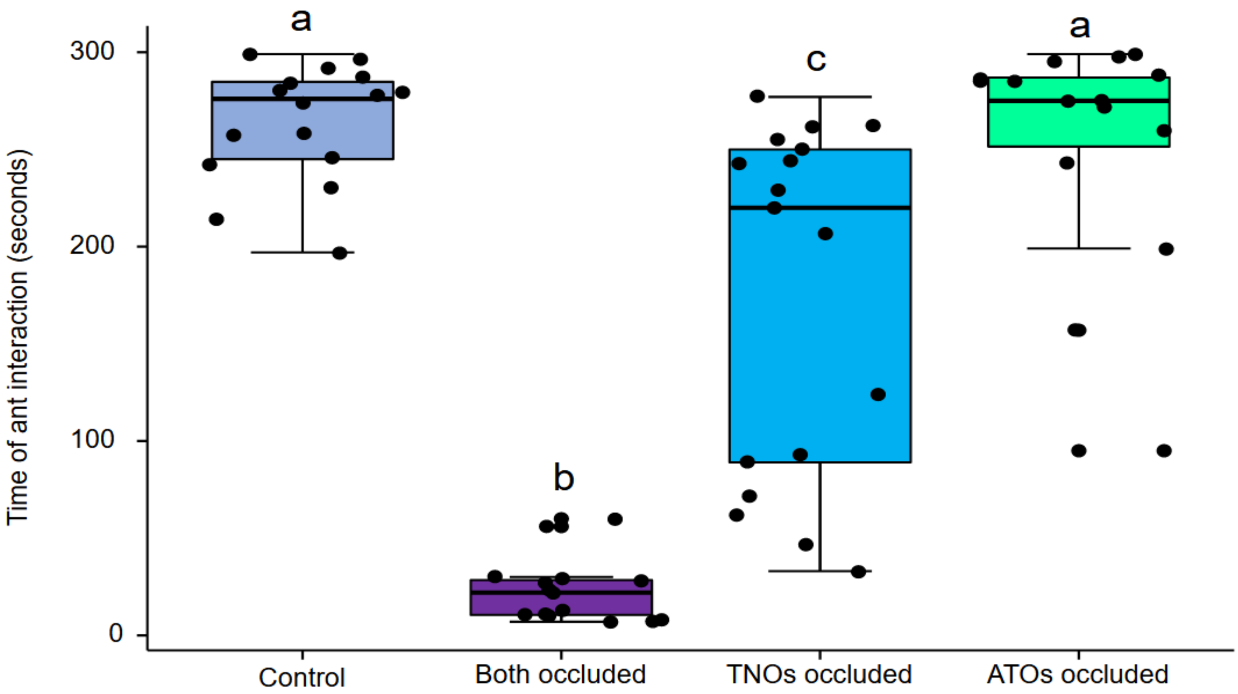


Fig. 3 Interaction time of ants according to each treatment (control, caterpillars with occluded ATOs, caterpillars with occluded TNOs, and caterpillars with both occluded organs). Letters represent $p < 0.05$.

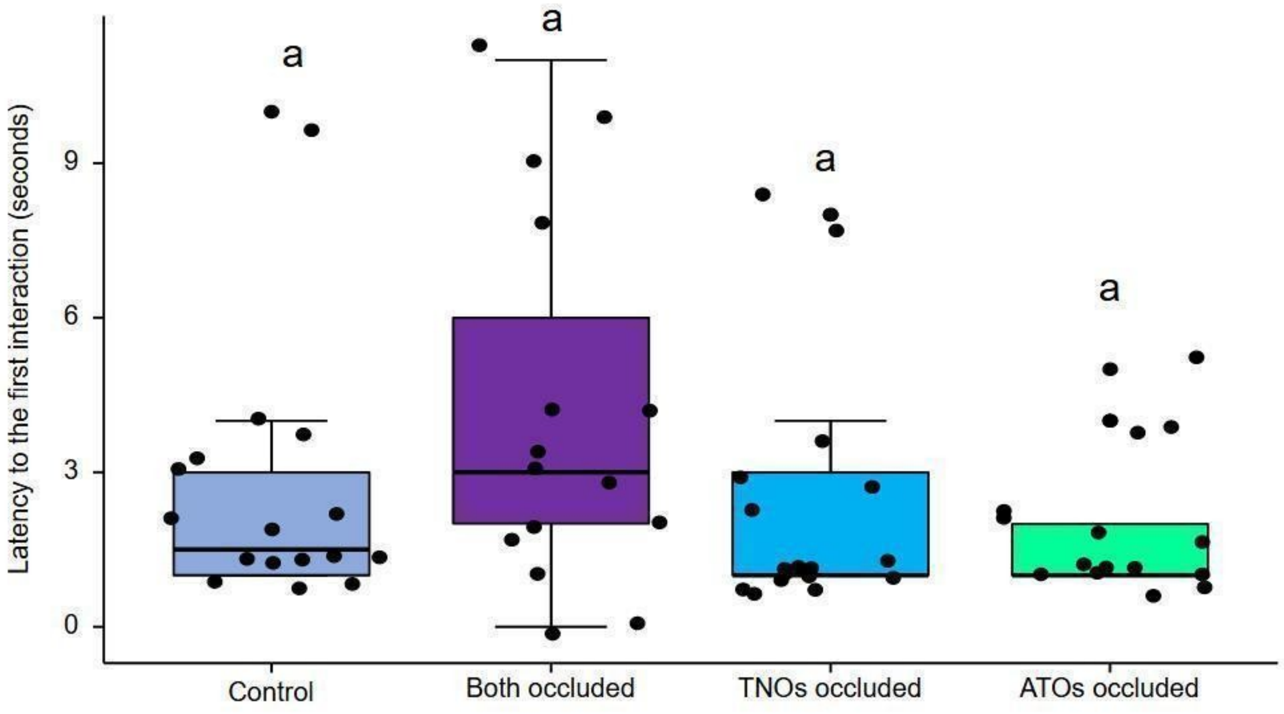


Fig 4 Latency time of ants before first contact with caterpillars, according to each treatment (control, caterpillars with occluded ATOs, caterpillars with occluded TNOs, and caterpillars with both occluded organs). Different letters represent $p < 0.05$.

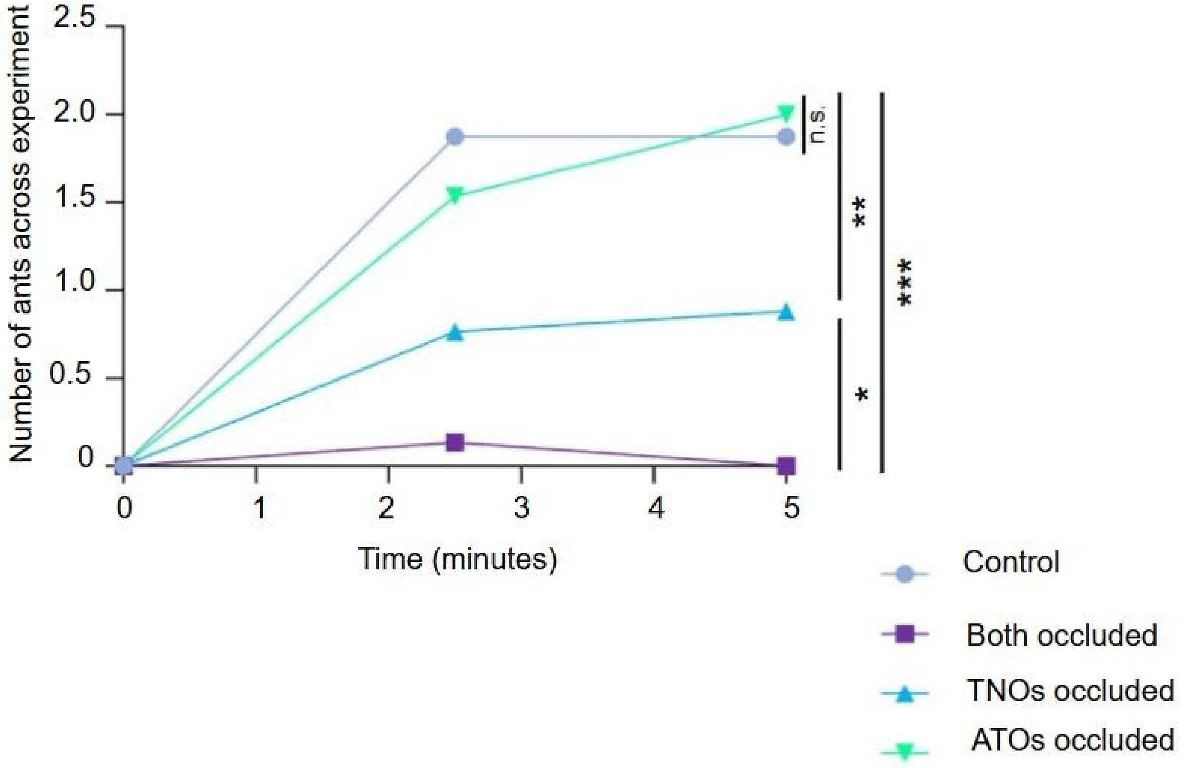


Fig 5. Mean number of ants interacting with caterpillars throughout the experiment according to each treatment (control, caterpillars with occluded ATOs, caterpillars with occluded TNOs, and caterpillars with both occluded organs). Asterisks indicate (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

Discussion

The ethogram revealed that caterpillars primarily spent more time everting their TNOs, while ants were more engaged in antennation behavior and walking on the caterpillars. Consequently, these ant behaviors consistently triggered the eversion of TNOs or ATOs in caterpillars. The eversion of both organs at the same time was also observed. This behavioral sequence was also noted in the riodinids *L. rossi* and *T. irenea* (Ross, 1966; DeVries, 1988). After their initial feeding on TNO secretion, ants maintained close proximity to the caterpillars, even without constantly receiving a nutritional reward. Similar observations were documented for *T. irenea,* where attending ants stayed within a few centimeters far from the caterpillars after consuming the TNO secretion (DeVries, 1988).

In this study, in every instance, the eversion of ATOs elicited behavioral reactions in attending ants. When caterpillars everted their ATOs, ants jumped towards the caterpillars, remained motionless for a few seconds, and adopted an alert posture. A comparable behavior was previously observed in *T. irenea*, where, after 15 or more reversals of ATOs, attending ants would open their mandibles, bend their abdomens, and jump toward the ATOs (DeVries, 1988). DeVries (1988) also noted that, when exposed to external stimuli, these ants maintained a state of alertness and attempted to attack any small object moving close by. In the case of *L. rossi*, when the caterpillars evert their ATOs, the attending ants exhibit heightened locomotion and agitation (Ross, 1966). Thus, it is likely that both behaviors—the eversion of TNOs and ATOs—have an effective action in these species, maintaining the attention of the ants and leading them to spend most of their time with the caterpillars in a 'enticement and binding' process (*sensu* DeVries, 1988).

We corroborated our hypothesis that TNOs and ATOs help sustaining ant attending behavior as we found that caterpillars with unmanipulated TNOs and ATOs, as well as caterpillars with unmanipulated TNOs, had the longest attendant time coming from ants. Additionally, the number of ants present throughout the experiment was greater for these two groups. These findings suggest that TNOs are more effective at maintaining ant attendant behavior compared to ATOs. Some studies focusing on lycaenids caterpillars have highlighted the crucial role of secretions released by the DNO (mainly sugars and amino acids). This ant organ is analogous to TNOs in riodinids and plays a significant role in sustaining the attendant behavior of associated ants (see examples in Pierce et al., 2002; Cassaci et al., 2019). One of the main effects of TNOs in the occlusion experiments of this study is the amount of time that ants spend next to caterpillars. This is advantageous for the caterpillars, as a longer interaction time with ants implies a more extended period of protection against parasitoids and predators (Pierce & Dankowicz, 2022). This is the case for several species of hemipterans and butterflies that maintain interactions with ants (Pierce and Mead, 1981; DeVries, 1991; Weeks, 2003; Cushman et al., 1994; Camacho and Avilés, 2021; Nelson and Mooney, 2022). On the other hand, ants also benefit, as TNO secretions represent a profitable source of carbohydrates that have already been shown to increase colony growth (Helms and Vinson, 2008; Wilder et al., 2011).

The same does not occur with ATOs, which in this study have been shown to have an intermediate effect in maintaining the ants' attention towards caterpillars. Caterpillars continue to benefit from receiving attention from ants even without constantly offering a reward that is costly to produce (Hojo, 2022). However, for the ants, this dynamic is costly, as they attend the caterpillars without receiving a reward and engage time that could be used for acquiring food resources. There is likely synergy between TNOs and ATOs, along with other ant-organs (Casacci et al., 2019), that maintains ants associated with caterpillars, even without receiving constant rewards. Moreover, the ants may recognize the caterpillars despite the absence of an attractant (Ross, 1966). This may pose disadvantages for ants, for example, plants can offer a more reliable and constant resource through

EFNs. However, there is evidence that ants prefer to invest more time in attending to caterpillars than in patrolling plant EFNs (DeVries, 1988).

Research involving some lycaenid and riodinid species has indicated that the contents released by DNO and TNOs primarily consist of sugars and amino acids (DeVries, 1988; Cushman et al., 1994; Pierce and Nash, 1999; Wada et al., 2001; Pierce et al., 2002; Daniels et al., 2005; Cassaci et al., 2019; Marquis and Koptur, 2022). Unlike hemipteran honeydew, the nutritional reward provided by specialized glands is costly for caterpillars (Daniels et al., 2005; Kaminski and Rodrigues, 2011; Lima et al., 2021). In our observations, ants continued to attend caterpillars with active TNOs and inactive ATOs, even when they did not receive constant rewards. Therefore, it is likely that certain compounds, such as amino acids, have a long-term impact on ants, leading them to sustain their attendant behavior (Cassaci et al., 2019). In studies conducted with the species *Niphanda fusca* (Bremer & Grey, 1853) (Lepidoptera: Lycaenidae), it was reported that the nutritional rewards released by the caterpillar contained significant amounts of a specific amino acid, glycine. The presence of this amino acid, in conjunction with trehalose, increased the attractiveness to attending ants (Wada et al., 2001; Hojo et al., 2009). Recent research indicates that the substances referred to as nutritional rewards produced by these organs may be more than simple nourishment (Cassaci et al., 2019). Experiments involving *A. japonica* (Lepidoptera: Lycaenidae) have shown that secretions from the DNO reduce the locomotor activity of *Pristomyrmex punctatus* (Smith, 1860) (Myrmicinae) ants (Hojo et al., 2015). Analysis of the ant brain function suggests that these secretions can influence ant behavior by modifying dopamine levels (Hojo et al., 2015).

However, as the ATOs were not as effective as TNOs in maintaining the attendant behavior and number of ants in this study, our results suggest that they have a medium-term effect. Caterpillars with occluded TNOs but functional ATOs received more attendant behaviors and were visited by a higher number of ants compared to caterpillars with both organs occluded. Some studies involving TOs in Lycaenidae and ATOs in Riodinidae, are not clear about the role played by these organs. The possibilities include them conveying tactile or visual cues (Murray, 1935; Gnatzy, 2017) or chemical cues, acting like a source of ant alarm pheromones (Henning, 1983; DeVries, 1984; Kitching and Luke, 1985; Pierce and Dankowicz, 2022).

Both the ethogram bioassays and occlusion experiments demonstrated that *C. crassus* ants exhibit characteristic behavior after *S. calyce* caterpillars evert their ATOs. When *S. calyce* caterpillars interacted with *Camponotus renggeri* Emery, 1894, this behavior was not observed (AVCG, personal observations). This phenomenon has been reported for other Lycaenid species that interact with various ant species (Fiedler, 1991). Moreover, in bioassays involving volatile compounds from *C. crassus* and *C. renggeri*, we observed that different compounds can act as alarm pheromones (Ceballos-González et al., unpublished data). Considering that ants are primarily oriented via chemical cues (Akino, 2008; Blomquist and Bagnères, 2010; Blomquist and Ginzel, 2021), we suggest that ATOs act as sources of chemical cues in this species and hence affect ants' behavior. Although TOs and ATOs functions are largely unknown (Pierce and Nash, 1999), some research supports the hypothesis of these organs conveying chemical cues. For instance, in a study with the lycaenid *Aloeides dentatis* (Swierstra, 1909), extracts obtained from areas close to the TOs were consistent with compounds acting as alarm pheromones in attendant ants (Henning, 1983). When an ant is in danger, the release of an alarm pheromone can alert or recruit more ants and stimulate aggressive reactions against the threat (Blum, 1985; Verheggen et al., 2010). Thus, it is likely that in caterpillars, the mechanism is the same. In nature, caterpillars face various enemies such as predators and parasitoids. The release of substances that mimic the ant alarm pheromone can recruit more ants and intensify the attack against possible threats (Vander Meer Robert and Alonso, 2019)

We demonstrate that TNOs alone have a greater effect on attending ants, and although to a lesser extent, the ATOs also have a positive effect in ant attendant behavior. Given the social organizational complexity present in ant colonies, it has been suggested that ants can use various signals, including chemical, tactile, and even visual cues, both inside and outside the colony (Hölldobler and Wilson, 1990). Thus, it is also likely that myrmecophilous butterflies have developed the ability to use multimodal signals to facilitate interaction with ants (Casacci et al., 2019). For example, in species of the lycaenid genus *Phengaris*, cuticular hydrocarbons (CHCs) that mimic the odor of attendant ants may play an essential role in the initial contact with ants, and when caterpillars are inside the colony; vibroacoustic signals seem to take center stage (Sala et al., 2014; Casacci et al., 2019). In the case of *S. calyce*, a facultative species that does not enter the ant colony (Callaghan, 1986; Alves-Silva et al., 2018; Lima et al., 2023), the use of multimodal signals is probably reinforced by the nutritional reward of TNOs and by chemical cues such as CHCs, ATOs, and pore cupola organs (PCOs), and to a lesser extent, by vibroacoustic signals (Casacci et al., 2019). Recent studies on the cuticular profile show that this species has its own distinctive profile, potentially enabling ants to learn to associate this profile with the rewards of the tentacle nectar organs and reinforce their attention (Ceballos-González et al., unpublished data).

In our study, TNOs seem to be the primary resource utilized by caterpillars to prolong interactions with assistant ants. While ATOs, though with a slightly diminished impact, also demonstrate effectiveness in maintaining attention. This highlights a positive impact, particularly for caterpillars, enhancing their likelihood of survival and success in a world full of parasitoids and predators. Although there are studies showing that ant protection can outweigh costs in reward production (Oliveira, 1997), some species may even reduce costs in this production by having multiple organs involved in interactions with ants (Casacci et al., 2019; Marquis and Koptur, 2022). *Synargis calyce* is a polyphagous caterpillar that feeds on a wide variety of plants. However, the majority of plants it uses as food have EFNs, which make them attractive to several ant species. Caterpillars may benefit if the compounds released by their TNOs resemble those found on the plants, potentially deterring ants from attacking them by recognizing them as being part of the plant. Nonetheless, it would also be advantageous for caterpillars to offer richer rewards than EFNs, enabling them to compete against and be preferred over EFNs as variability in these secretions may play a key role in ant preferences and nutrition over honeydew (Blüthgen et al., 2004). Future research identifying the compounds in TNOs and EFNs, as well as their effects on the behavior of attending ants, may contribute to increasing our understanding about this intricate ant-caterpillar relationship. The primary challenge for this aim is to isolate the volatile compounds of the ATOs (if they exist), as the caterpillars do not evert their ATOs without the presence of ants. Additionally, in the case of TNOs, overcoming the obstacle of collecting sufficient quantities of the released secretion may also be difficult to address.

References

Alves-Silva, E., Bächtold, A., & Del-Claro, K. (2018) Florivorous myrmecophilous caterpillars exploit an ant–plant mutualism and distract ants from extrafloral nectaries. Austral Ecology 43,643-650. https://doi.org/10.1111/aec.12609

Altmann, J. (1974) Observational study of behavior: sampling methods. Behaviour, 49, 227-266.

Akino, T, Knapp, J.J., Thomas, JA., & Elmes, G.W. (1999) Chemical mimicry and host specificity in the butterfly *Maculinea rebeli,* a social parasite of *Myrmica* ant colonies. Proceedings of the Royal Society B: Biological Sciences, 266, 1419–1426. https://doi.org/10.1098/rspb.1999.0796.

Akino, T. (2008). Chemical strategies to deal with ants: a review of mimicry, camouflage, propaganda, and phytomimesis by ants (Hymenoptera: Formicidae) and other arthropods. Myrmecological News, 11, 173-181.

Barbero, F. (2016) Cuticular lipids as a cross-talk among ants, plants and butterflies. International Journal of Molecular Sciences, 17,1966. https://doi.org/10.3390/ijms17121966

Bascompte, J. (2019). Mutualism and biodiversity. Current Biology, 29, R467-R470.

Blomquist, G. J., & Bagnères, A. G. (Eds.). (2010). Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, p. 492

Blomquist, G. J., & Ginzel, M. D. (2021) Chemical ecology, biochemistry, and molecular biology of insect hydrocarbons. Annual Review of Entomology, 66, 45-60. https://doi.org/10.1146/annurev-ento-031620-071754.

Blum, M. S. (1985). Alarm pheromones. In "Comprehensive Insect physiology, Biochemistry and Pharmacology," (G. A. Kerkut and L. I. Gilbert, Eds.) Pergamon Press, pp. 193–224.

Bluthgen, N., Gottsberger, G., & Fiedler, K. (2004) Sugar and amino acid composition of ant-attended nectar and honeydew sources from an Australian rainforest. Austral Ecology, 29, 418-429. https://doi.org/10.1111/j.1442-9993.2004.01380.x

Bronstein, J. L. (1994). Our current understanding of mutualism. The Quarterly Review of Biology, *69*, 31-51. https://doi.org/10.1086/418432.

Callaghan, C.J. (1986) Restinga butterflies: biology of *Synargis brennus* (Stichel) (Riodinidae). Journal of the Lepidopterists' Society, 40,93–96.

Camacho, L. F., & Avilés, L. (2021) Resource exchange and partner recognition mediate mutualistic interactions between prey and their would-be predators. Biology Letters, 17(8), 20210316. https://doi.org/10.1098/rsbl.2021.0316.

Casacci, L.P, Bonelli, S, Balletto E., & Barbero, F. (2019) Multimodal Signaling in Myrmecophilous Butterflies. Frontiers in Ecology and Evolution, 7,454. https://doi.org/10.3389/fevo.2019.00454.

Cottrell, C.B. (1984) Aphytophagy in butterflies: its relationship to myrmecophily. Zoological Journal of the Linnean Society, 79, 1–57. https://doi.org/10.1111/j.1096-3642.1984.tb02318.x.

Cushman, J. H., Rashbrook, V. K., & Beattie, A. J. (1994) Assessing benefits to both participants in a lycaenid-ant association. Ecology 75, 1031–1041. https://doi.org/10.2307/1939427.

Daniels, H., Gottsberger, G., & Fiedler, K. (2005). Nutrient composition of larval nectar secretions from three species of myrmecophilous butterflies. Journal of Chemical Ecology, 31, 2805-2821. https://doi.org/10.1007/s10886-005-8395-y.

DeVries, P. J. (1984). Of crazy-ants and Curetinae: are *Curetis* butterflies tended by ants?. Zoological journal of the Linnean Society, 80, 59-66. https://doi.org/10.1111/j.1096-3642.1984.tb02319.x.

DeVries, P. J. (1988). The larval ant-organs of *Thisbe irenea* (Lepidoptera: Riodinidae) and their effects upon attending ants. Zoological Journal of the Linnean Society, *94*, 379-393. https://doi.org/10.1111/j.1096-3642.1988.tb01201.x.

DeVries, P. J. (1991). Mutualism between *Thisbe irenea* butterflies and ants, and the role of ant ecology in the evolution of larval-ant associations. Biological Journal of the Linnean Society, 43, 179-195. https://doi.org/10.1111/j.1095-8312.1991.tb00592.x.

Fiedler, K. (1991) European and North West African Lycaenidae (Lepidoptera) and their associations with ants. The Journal the Research on the Lepidoptera 28,239–257.

Fiedler, K., & Maschwitz, U. (1988) Functional analysis of the myrmecophilous relationships between ants (Hymenoptera: Formicidae) and lycaenids (Lepidoptera: Lycaenidae) II. Lycaenid larvae as trophobiotic partners of ants—a quantitative approach. Oecologia, 75, 204-206. https://doi.org/10.1007/BF00378598.

Floren, A., Biun, A., & Linsenmair, E. K. (2002). Arboreal ants as key predators in tropical lowland rainforest trees. Oecologia, 131, 137-144. https://doi.org/10.1007/s00442-002-0874-z.

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods in ecology and evolution, 7, 1325-1330. https://doi.org/10.1111/2041-210X.12584

Gnatzy, W., Jatho, M., Kleinteich, T., Gorb, S. N., & Hustert, R. (2017) The eversible tentacle organs of *Polyommatus* caterpillars (Lepidoptera, Lycaenidae): Morphology, fine structure, sensory supply and functional aspects. Arthropod Structure & Development, 46, 788-804. https://doi.org/10.1016/j.asd.2017.10.003.

Helms, K. R., & Vinson, S. B. (2008). Plant resources and colony growth in an invasive ant: the importance of honeydew-producing hemiptera in carbohydrate transfer across trophic levels. Environmental entomology, 37, 487-493. https://doi.org/10.1093/ee/37.2.487.

Henning, S. F. (1983) Chemical communication between Iycaenid larvae (Lepidoptera: Lycaenidae) and ants (Hymenoptera: Formicidae). Journal of the Entomological Society of Southern Africa, 46, 341-366. https://hdl.handle.net/10520/AJA00128789_3496.

Hojo, M. K. (2022) Evolution of chemical interactions between ants and their mutualist partners. Current Opinion in Insect Science, 52, 100943. https://doi.org/10.1016/j.cois.2022.100943.

Hojo, M. K., Pierce, N. E., & Tsuji, K. (2015). Lycaenid caterpillar secretions manipulate attendant ant behavior. Current Biology, 25, 2260-2264.

Hojo, M.K., Yamamoto, A., Akino, T., Tsuji, K., & Yamaoka, R. (2014) Ants use partner specific odors to learn to recognize a mutualistic partner. PLoS One 9,e86054. https://doi.org/10.1371/journal.pone.0086054

Hojo, M. K., Wada-Katsumata, A., Akino, T., Yamaguchi, S., Ozaki, M., & Yamaoka, R. (2009). Chemical disguise as particular caste of host ants in the ant inquiline parasite *Niphanda fusca* (Lepidoptera: Lycaenidae). Proceedings of the Royal Society B: Biological Sciences, 276, 551-558. https://doi.org/10.1098/rspb.2008.1064.

Hölldobler, B., & Wilson, E.O. (1990) The Ants. The Ants. The Belknap Press of Harvard University Press, Cambridge p 732.

Kaminski, L. A., & Rodrigues, D. (2011). Species-specific levels of ant attendance mediate performance costs in a facultative myrmecophilous butterfly. Physiological Entomology, 36, 208-214. https://doi.org/10.1111/j.1365-3032.2011.00785.x.

Kaminski, L. A., & Carvalho-Filho, F. S. (2012). Life history of *Aricoris propitia* (Lepidoptera: Riodinidae)—A myrmecophilous butterfly obligately associated with fire ants. Psyche: A Journal of Entomology, 2012. https://doi.org/10.1155/2012/126876.

Kaminski LA (2021) Ant-butterfly interactions - Borboletas formigueiras. iNaturalist. https://www.inaturalist.org/projects/ant-butterfly-interactions-borboletas-formigueiras. Accessed 15 Oct 2023.

Kitching, R. L., & Luke, B. (1985). The myrmecophilous organs of the larvae of some British Lycaenidae (Lepidoptera): a comparative study. Journal of Natural History, 19, 259-276. https://doi.org/10.1080/00222938500770211.

Lange, D., Calixto, E. S., Rosa, B. B., Sales, T. A., & Del-Claro, K. (2019). Natural history and ecology of foraging of the *Camponotus crassus* Mayr, 1862 (Hymenoptera: Formicidae). Journal of Natural History, 53(27-28), 1737-1749. https://doi.org/10.1080/00222933.2019.1660430.

Leigh Jr, E. G. (2010) The evolution of mutualism. Journal of Evolutionary Biology, 23, 2507-2528. https://doi.org/10.1111/j.1420-9101.2010.02114.x.

Leimar, O., & Axén, A. H. (1993). Strategic behaviour in an interspecific mutualism: interactions between lycaenid larvae and ants. Animal Behaviour, 46, 1177-1182. https://doi.org/10.1006/anbe.1993.1307

Lenoir, A., d'Ettorre, P., Errard, C., & Hefetz, A. (2001) Chemical ecology and social parasitism in ants. Annual Review of Entomology, 46, 573-599. https://doi.org/10.1146/annurev.ento.46.1.573.

Lima, L.D, Trigo, J.R, Kaminski, L.A (2021) Chemical convergence between a guild of facultative myrmecophilous caterpillars and host plants. Ecological Entomology 46,66–75. https://doi.org/10.1111/een.12941.

Lima, L. D., Ceballos-González, A. V., Prato, A., Kaminski, L. A., & do Nascimento, F. S. (2023) Plant–treehopper convergence may trick butterflies into trophic oviposition mistakes. Biotropica, 55, 292-298. https://doi.org/10.1111/btp.13194.

Lorenzi, M. C., & d'Ettorre, P. (2020) Nestmate recognition in social insects: what does it mean to be chemically insignificant?. Frontiers in Ecology and Evolution, 7, 488. https://doi.org/10.3389/fevo.2019.00488.

Malicky, H. (1970) New aspects of the association between lycaenid larvae (Lycaenidae) and ants (Formicidae, Hymenoptera). Journal of the Lepidopterists´ Society, 24, 190-202.

Marquis, R.J., & Koptur, S. (2022) Caterpillars in the middle: Tritrophic interactions in a changing world. Springer. pp. 319-391

Mizuno, T., Hagiwara, Y., & Akino, T. (2018) Chemical tactic of facultative myrmecophilous lycaenid pupa to suppress ant aggression. Chemoecology 28,173–182. https://doi.org/10.1007/s00049-018-0270-8

Murray, F. V. (1935). The metamorphosis of Calandra oryzae. Journal of Cell Science, 2, 405-495. https://doi.org/10.1242/jcs.s2-77.307.405.

Nelson, A. S., & Mooney, K. A. (2022). The evolution and ecology of interactions between ants and honeydew-producing hemipteran insects. Annual Review of Ecology, Evolution, and Systematics, 53, 379-402. https://doi.org/10.1146/annurev-ecolsys-102220-014840

Newcomer, E. J. (1912) Some observations on the relations of ants and lycaenid caterpillars, and a description of the relational organs of the latter. Journal of the New York Entomological Society, 20, 31-36. https://www.jstor.org/stable/25003494

Mizuno, T., Hagiwara, Y., & Akino, T. (2018) Chemical tactic of facultative myrmecophilous lycaenid pupa to suppress ant aggression. Chemoecology, 28, 173-182. https://doi.org/10.1007/s00049-018-0270-8.

Oliveira, P. S. (1997) The ecological function of extrafloral nectaries: herbivore deterrence by visiting ants and reproductive output in Caryocar brasiliense (Caryocaraceae). Functional ecology, 11, 323-330. https://doi.org/10.1046/j.1365-2435.1997.00087.x.

Pierce, N. E., & Mead, P. S. (1981). Parasitoids as selective agents in the symbiosis between lycaenid butterfly larvae and ants. Science, 211, 1185-1187. https://doi.org/10.1126/science.211.4487.1185.

Pierce, N. E., & Nash, D. R. (1999). The imperial blue, *Jalmenus evagoras* (Lycaenidae). Monographs on Australian Lepidoptera, 6, 279–315.

Pierce, N.E., Braby, M.F., Heath, A., Lohman, D.J., Mathew, J., Rand, D.B., & Travassos, M.A. (2002) The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). Annual Review of Entomology, 47,733–771. https://doi.org/10.1146/annurev.ento.47.091201.145257.

Pierce, N. E., & Dankowicz, E. (2022). Behavioral, ecological and evolutionary mechanisms underlying caterpillar-ant symbioses. Current Opinion in Insect Science, 52, 100898. https://doi.org/10.1016/j.cois.2022.100898.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. Accessed in Aug 2023.

Ross, G. N. (1966) Life-history studies on Mexican butterflies. IV. The ecology and ethology of *Anatole rossi*, a myrmecophilous metalmark (Lepidoptera: Riodinidae). Annals of the Entomological Society of America,59(5), 985-1004. https://doi.org/10.1093/aesa/59.5.985.

Sala, M., Casacci, L. P., Balletto, E., Bonelli, S., & Barbero, F. (2014) Variation in butterfly larval acoustics as a strategy to infiltrate and exploit host ant colony resources. PLOS one, 9, e94341. https://doi.org/10.1371/journal.pone.0094341.

Silveira, H. C., Oliveira, P. S., & Trigo, J. R. (2010). Attracting predators without falling prey: chemical camouflage protects honeydew-producing treehoppers from ant predation. The American Naturalist, 175, 261-268. https://doi.org/10.1086/649580.

Vander Meer Robert, K., & Alonso, L. E. (2019) Pheromone directed behavior in ants. In Pheromone Communication in Social Insects. CRC Press., pp. 159-192.

Verheggen, F. J., Haubruge, E., & Mescher, M. C. (2010). Alarm pheromones—chemical signaling in response to danger. Vitamins & Hormones, 83, 215-239. https://doi.org/10.1016/S0083-6729(10)83009-2.

Wada, A., Isobe, Y., Yamaguchi, S., Yamaoka, R., & Ozaki, M. (2001) Taste-enhancing effects of glycine on the sweetness of glucose: a gustatory aspect of symbiosis between the ant, *Camponotus japonicus,* and the larvae of the lycaenid butterfly, *Niphanda fusca*. Chemical Senses, 26, 983-992. https://doi.org/10.1093/chemse/26.8.983

Wagner, D. (1993). Species-specific effects of tending ants on the development of lycaenid butterfly larvae. Oecologia, 96, 276-281. https://doi.org/10.1007/BF00317742.

Weeks, J. A. (2003). Parasitism and ant protection alter the survival of the lycaenid Hemiargus isola. Ecological Entomology, 28(2), 228-232. https://doi.org/10.1046/j.1365-2311.2003.00489.x.

Wilder, S. M., Holway, D. A., Suarez, A. V., LeBrun, E. G., & Eubanks, M. D. (2011). Intercontinental differences in resource use reveal the importance of mutualisms in fire ant invasions. Proceedings of the National Academy of Sciences, 108(51), 20639-20644. https://doi.org/10.1073/pnas.1115263108

General Discussion

Chemical communication plays a pivotal role in the interactions between caterpillars and ants (Pierce et al., 2002; Marquis and Koptur, 2022). Thus, the present work focused on some aspects of chemical communication in the myrmecophilous relationships of two riodinid caterpillars, examining the cuticular hydrocarbons of caterpillars, host plants, and attendant ants, as well the specialized organs Anterior Tentacle Organs (ATOs) and Tentacle Nectary Organs (TNOs).

In the first chapter, I present data on the natural history, distribution, host plant diversity, and attendant ants for *S. calyce* and *N. lisimon*. These findings complement existing literature on both species, particularly addressing gaps in knowledge, such as incomplete life cycle data for *N. lisimon* in previous studies (Callaghan, 1986; Callaghan, 1988). Despite being undervalued, descriptive and natural history studies serve as the foundation for understanding broad evolutionary and ecological concepts (Greeney et al., 2012). Furthermore, many species with fascinating behaviors may remain undiscovered due to the ongoing loss of biodiversity resulting from human activities (Vitousek, 1994). While there are numerous studies on the natural history of myrmecophilous caterpillars, progress in research on tropical species remains limited (DeVries, 1997; Greeney et al., 2012; Seraphim et al. 2018). Another aspect to consider is that myrmecophilous species cannot be studied in isolation, as their evolutionary history, behavior, and ecology are profoundly influenced by their close associations with ants (Casacci et al., 2019; Marquis and Koptur, 2022; Pierce and Dankowicz, 2022). Therefore, the attendant ant records presented here are valuable not only for elucidating specific biogeographic aspects of the Riodinidae family but also for understanding the impact of attendant ants on host plant selection (Fiedler, 1994).

The field observations and collection of records available in the literature show that the two species studied here have contrasting ant-interaction strategies, with *S. calyce* interacting with several ant species and *N. lisimon* interacting with a single ant species, having facultative and obligate relationships, respectively (Casacci et al. 2019; Marquis and Koptur, 2022). As it is presented here, these differences possibly impact the chemical strategies employed by each species when interacting with ants. For example, regarding strategies involving cuticular hydrocarbons (CHCs), it has been shown by various authors that obligate caterpillars generally employ chemical mimicry, while facultative species can employ camouflage or chemical conspicuousness (Akino et al., 1999; Elmes et al., 2002; Schlick-Steiner et al., 2004; Schönrogge et al., 2004; Akino, 2008; Lima et al., 2021).

In our second chapter, it was shown that *S. calyce,* a facultative species, exhibits a chemical profile that is more influenced by the host plant than by its attendant ants. However, PCA analyses indicate that overall, the chemical profile of caterpillars in all treatment groups remains relatively the same, suggesting that these caterpillars maintain their own profile, employing the strategy of chemical conspicuousness (Lima et al., 2021). Thus, we propose that this species displays a trade-off between camouflage and chemical conspicuousness, depending on the species of ants present on the host plant. It is known that each compound or group of compounds within the same profile likely has a different function, with conflicts existing between these various functions (Steiger and Stökl, 2014; Ingleby, 2015; Sprenger and Menzel, 2020). Thus, specific compounds in the cuticular profile of *S. calyce*, such as certain alcohols and aldehydes found also in host plants, may play a greater role in camouflage, while others, like branched alkanes, may contribute to conspicuousness for attendant ants.

The opposite applies to *N. lisimon*, an obligate species that showed cuticular profiles more influenced by attendant ants than by host plants (Appendix 1). When comparing groups of caterpillars raised without ants to groups raised with ants, it was observed that caterpillars raised with ants exhibited more CHCs, especially alkenes, in their cuticular profile, which were also present in both workers and queens of *W. auropunctata*. Although experiments are needed, this could suggest that this species may be using chemical mimicry. It is known that chemical mimicry is the predominant chemical strategy in parasitic species living within ant nests, and several examples have been reported within Lycaenidae (see Casacci et al, 2019; Marquis and Koptur, 2022). However, this would be the first report for an obligate species of Riodinidae, that to our knowledge, it is not parasitic.

Strategies involving CHCs can be effective in avoiding ant attacks or even attracting their attention when caterpillars mimic the cuticular compounds of attending ants (Akino et al., 1999; Hojo et al., 2009; Barbero, 2016; Mizuno et al., 2018; Casacci et al., 2019). However, this may not be sufficient, especially for facultative species like *S. calyce*, thus caterpillars of various Lycaenidae and Riodinidae species possess highly specialized organs to interact with ants (Fiedler, 1991). In our third chapter, we explore the role of TNOs and ATOs in maintaining the attending behavior of *C. crassus* with *S. calyce*. We found that TNOs have a greater impact on maintaining ant attendance, although ATOs also showed a positive effect, albeit relatively minor. In both cases, there was a longer interaction time with ants compared to caterpillars with both organs occluded. This has positive effects on caterpillars in nature, as the time spent in interaction can be extrapolated to increased protection against predators and parasitoids (Pierce and Mead, 1981; DeVries, 1991; Cushman et al., 1994; Weeks, 2003; Camacho and Avilés, 2021; Nelson and Mooney, 2022; Pierce and Dankowicz, 2022). However, the constant production of carbohydrates and sugars by TNOs can be highly costly for caterpillars and is conditioned by the quality of the consumed food or the host plant (Fiedler, 1994). In the case of ATOs, the advantage is greater because they offer protection without representing a high cost for caterpillars. Therefore, there is likely a synergy between TNOs, ATOs, and other specialized organs of caterpillars under natural conditions, which resulted from selection pressures exerted by ants during the evolutionary process (Pierce and Dankowicz, 2022).

This study compiles life cycle, field notes, diversity, and distribution data for two myrmecophilous riodinids, emphasizing their importance as baseline information for further studies on behavior, chemical ecology, and myrmecophily. Additionally, it presents the first published study that explores the influence of host plants and attending ants on caterpillars of one riodinid species. While studying the strategies employed by myrmecophilous caterpillars to interact with ants is fundamental to assessing the function of each, we know that in nature they do not act separately. For example, even though *S. calyce* may use cuticular hydrocarbons in the initial contact, the TNOs and ATOs can reinforce the signals and effectively maintain the association. This has been suggested for obligate species of the genus *Phengaris*, where various types of signals can play different roles and be decisive even in different phases of the life cycle (Casacci et al., 2019).

This study represents a significant advancement, expanding the studies on chemical strategies, effects of the TNOs and ATOs on attendant ants, and the natural history of riodinids. However, there are still many aspects to be explored in the future, especially about myrmecophilous riodinids (Kaminski, 2008). For instance, ant-tending organs (ATOs), especially regarding their composition and function (Casacci et al., 2019; Marquis and Koptur, 2022), remain largely unexplored for most species. In facultative species like *S. calyce*, it is possible that attendant ants learn to recognize chemical signals from caterpillars, so future studies should also be conducted on this aspect. Moreover, more field experiments are necessary to evaluate the cuticular hydrocarbons of myrmecophilous riodinid caterpillars, as they could provide greater clarity and consistency regarding their role in maintaining mutualistic relationships with ants. Studies comparing the products of caterpillar TNOs with EFNs of plants and assessing the preferences of attending ants could be useful in establishing costs and benefits for both plants, caterpillars, and ants. Another interesting aspect is the parasitoids as *S. calyce* caterpillars suffer high levels of parasitism, and it has been observed that parasitized caterpillars continue to receive ant attention (AVCG, personal observation). Evaluating the CHCs of parasitized caterpillars, the quality of nutritive rewards from their TNOs after parasitism, and the effectiveness in protection against parasitoids depending on the attendant ant species could add another layer to expand our knowledge in these types of interactions.

References

Akino, T. (2008). Chemical strategies to deal with ants: a review of mimicry, camouflage, propaganda, and phytomimesis by ants (Hymenoptera: Formicidae) and other arthropods. Myrmecological News, 11, 173-181.

Akino, T., Knapp, J. J., Thomas, J. A., & Elmes, G. W. (1999). Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. Proceedings of the Royal Society of London. Series B: Biological Sciences, 266, 1419-1426. https://doi.org/10.1098/rspb.1999.0796.

Barbero, F. (2016) Cuticular lipids as a cross-talk among ants, plants and butterflies. International Journal of Molecular Sciences, 17,1966. https://doi.org/10.3390/ijms17121966.

Callaghan, S. (1986) Restinga butterflies: biology of *Synargis brennus* (Stichel)(Riodinidae). Journal of the Lepidopterists' Society, 40, 93-96.

Callaghan, C. J. (1988) Notes on the biology of three riodinine species: *Nymphidium lisimon attenuatum, Phaenochitonia sagaris satnius,* and *Metacharis ptolomaeus* (Lycaenidae: Riodininae). Journal of Research on the Lepidoptera, 27, 109-114.

Camacho, L. F., & Avilés, L. (2021) Resource exchange and partner recognition mediate mutualistic interactions between prey and their would-be predators. Biology Letters, 17(8), 20210316. https://doi.org/10.1098/rsbl.2021.0316.

Casacci, L.P, Bonelli, S, Balletto E., & Barbero, F. (2019) Multimodal Signaling in Myrmecophilous Butterflies. Frontiers in Ecology and Evolution, 7,454. https://doi.org/10.3389/fevo.2019.00454.

Cushman, J. H., Rashbrook, V. K., & Beattie, A. J. (1994) Assessing benefits to both participants in a lycaenid-ant association. Ecology 75, 1031–1041. https://doi.org/10.2307/1939427.

Daniels, H., Gottsberger, G., & Fiedler, K. (2005). Nutrient composition of larval nectar secretions

Elmes, G., Akino, T., Thomas, J., Clarke, R., & Knapp, J. (2002) Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. Oecologia, 130, 525-535. https://doi.org/10.1007/s00442-001-0857-5.

Greeney, H. F., Dyer, L. A., & Smilanich, A. M. (2012) Feeding by lepidopteran larvae is dangerous: a review of caterpillars' chemical, physiological, morphological, and behavioral defenses against natural enemies. Invertebrate Survival Journal, 9, 7-34.

DeVries, P. J. (1991). Mutualism between *Thisbe irenea* butterflies and ants, and the role of ant ecology in the evolution of larval-ant associations. Biological Journal of the Linnean Society, 43, 179-195. https://doi.org/10.1111/j.1095-8312.1991.tb00592.x.

DeVries, P. J. (1997). Riodinidae. In The butterflies of Costa Rica and their natural history, volume II, Princetown University Press, p 368.

Fiedler, K. (1994) Lycaenid butterflies and plants: is myrmecophily associated with amplified hostplant diversity? Ecological Entomology 19, 79–82. https://doi.org/10.1111/j.1365-2311.1994.tb00393.x.

Hojo, M. K., Wada-Katsumata, A., Akino, T., Yamaguchi, S., Ozaki, M., & Yamaoka, R. (2009). Chemical disguise as particular caste of host ants in the ant inquiline parasite Niphanda fusca (Lepidoptera: Lycaenidae). Proceedings of the Royal Society B: Biological Sciences, 276, 551-558. https://doi.org/10.1098/rspb.2008.1064.

Ingleby, F. C. (2015) Insect cuticular hydrocarbons as dynamic traits in sexual communication. Insects, 6, 732-742. https://doi.org/10.3390/insects6030732.

Kaminski, L. A. (2008) Polyphagy and obligate myrmecophily in the butterfly *Hallonympha paucipuncta* (Lepidoptera: Riodinidae) in the Neotropical Cerrado savanna. Biotropica, 40, 390-394. https://doi.org/10.1111/j.1744-7429.2007.00379.x.

Lima, L.D, Trigo, J.R, Kaminski, L.A (2021) Chemical convergence between a guild of facultative myrmecophilous caterpillars and host plants. Ecological Entomology 46,66–75. https://doi.org/10.1111/een.12941.

Pierce, N. E., & Mead, P. S. (1981). Parasitoids as selective agents in the symbiosis between lycaenid butterfly larvae and ants. Science, 211, 1185-1187. https://doi.org/10.1126/science.211.4487.1185.

Marquis, R.J., & Koptur, S. (2022) Caterpillars in the middle: Tritrophic interactions in a changing world. Springer. pp. 319-391.

Mizuno, T., Hagiwara, Y., & Akino, T. (2018) Chemical tactic of facultative myrmecophilous lycaenid pupa to suppress ant aggression. Chemoecology 28,173–182. https://doi.org/10.1007/s00049-018-0270-8

Nelson, A. S., & Mooney, K. A. (2022). The evolution and ecology of interactions between ants and honeydew-producing hemipteran insects. Annual Review of Ecology, Evolution, and Systematics, 53, 379-402. https://doi.org/10.1146/annurev-ecolsys-102220-014840

Pierce, N. E., Braby, M. F., Heath, A., Lohman, D. J., Mathew, J., Rand, D. B., & Travassos, M. A. (2002). The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). Annual Review of Entomology, 47, 733-771. https://doi.org/10.1146/annurev.ento.47.091201.145257.

Pierce, N. E., & Dankowicz, E. (2022). Behavioral, ecological and evolutionary mechanisms underlying caterpillar-ant symbioses. Current Opinion in Insect Science, 52, 100898. https://doi.org/10.1016/j.cois.2022.100898.

Seraphim, N., Kaminski, L. A., DeVries, P. J., Penz, C., Callaghan, C., Wahlberg, N., ... & Freitas, A. V. (2018) Molecular phylogeny and higher systematics of the metalmark butterflies (Lepidoptera: Riodinidae). Systematic Entomology, 43, 407-425. https://doi.org/10.1111/syen.12282.

Schlick-Steiner, B. C., Steiner, F. M., Höttinger, H., Nikiforov, A., Mistrik, R., Schafellner, C., ... & Christian, E. (2004). A butterfly's chemical key to various ant forts: intersection-odour or aggregate-odour multi-host mimicry?. Naturwissenschaften, 91, 209-214. https://doi.org/10.1007/s00114-004-0518-8.

Schönrogge, K., Wardlaw, J. C., Peters, A. J., Everett, S., Thomas, J. A., & Elmes, G. W. (2004) Changes in chemical signature and host specificity from larval retrieval to full social integration in the myrmecophilous butterfly *Maculinea rebeli*. Journal of Chemical Ecology, 30, 91-107. https://doi.org/10.1023/B:JOEC.0000013184.18176.a9.

Sprenger, P. P., & Menzel, F. (2020) Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: how and why they differ among individuals, colonies, and species. Myrmecological News, 30. https://doi.org/10.25849/myrmecol.news_030:013.

Steiger, S., & Stökl, J. (2014).The role of sexual selection in the evolution of chemical signals in insects. Insects, 5, 423-438. https://doi.org/10.3390/insects5020423.

Vitousek, P. M. (1994) Beyond global warming: ecology and global change. Ecology, 75, 1861-1876. https://doi.org/10.2307/1941591.

Weeks, J. A. (2003). Parasitism and ant protection alter the survival of the lycaenid Hemiargus isola. Ecological Entomology, 28(2), 228-232. https://doi.org/10.1046/j.1365-2311.2003.00489.x.

Appendix 1. Preliminary results on the influence of attending ants and host plant on the cuticular profile of the obligate caterpillar *N. lisimon.*

A total of 92 peaks of cuticular extracts from *N. lisimon* caterpillars, attending ants *W. auropunctata* (workers and queens), and host plants were identified. These peaks were identified as branched hydrocarbons (mono-, di-, and trimethylated), linear alkanes, alkadienes, alkenes, alcohols, and aldehydes (Fig 1). The carbon chain length of the compounds ranged from 18 to 37. Ant and caterpillar species showed greater compound diversity compared to plant samples, including several branched alkanes, alkanes, and alkenes. Only plants exhibited alcohols and aldehydes in their chemical profile (1-octadecanol, octacosanal, 1-triacontanol, and triacontanal). Caterpillars raised in the absence of ants (workers and queens) shared 60% of cuticular compounds, whereas caterpillars raised with ants shared 66-78% of cuticular compounds. Alkenes (C31:1, C33:1, and C35:1) were present in both ants and caterpillars raised with ants but absent in caterpillars raised in the absence of ants (Fig. 2). Both caterpillars raised with ants and those raised without ants shared less than 45% of compounds with the host plant. These preliminary results suggest that *N. lisimon* caterpillars, unlike another studied myrmecophilous butterfly species (*S. calyce*), have a cuticular profile influenced by their attendant ants (Fig 3).

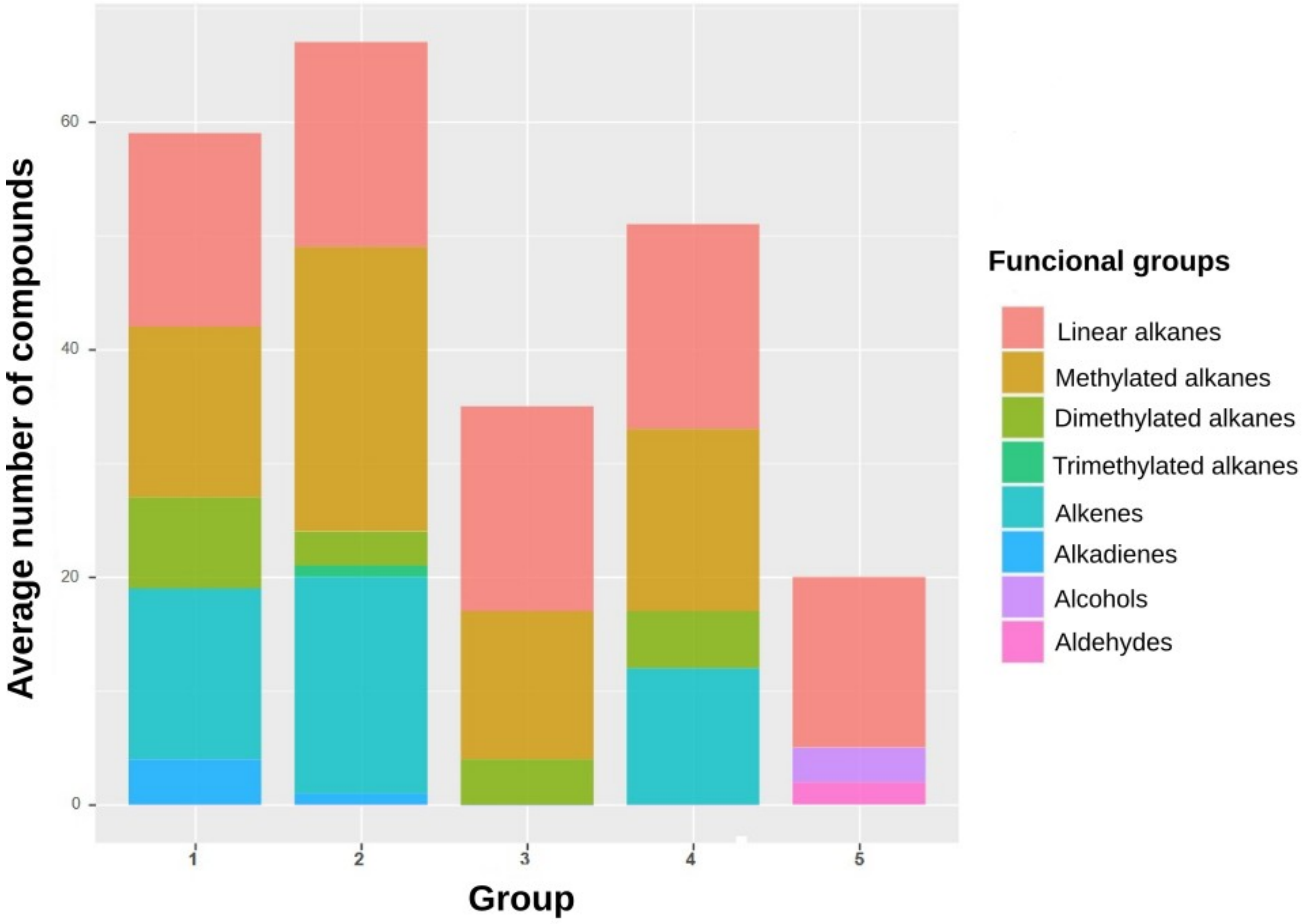


Fig 1. Number of compounds by functional groups for: 1. *W. auropunctata* (workers), 2. *W. auropunctata* (queen) 3. *N. lisimon* without ants, 4. *N. lisimon* with ants, and 5. *S. polyphylla* (host plant).

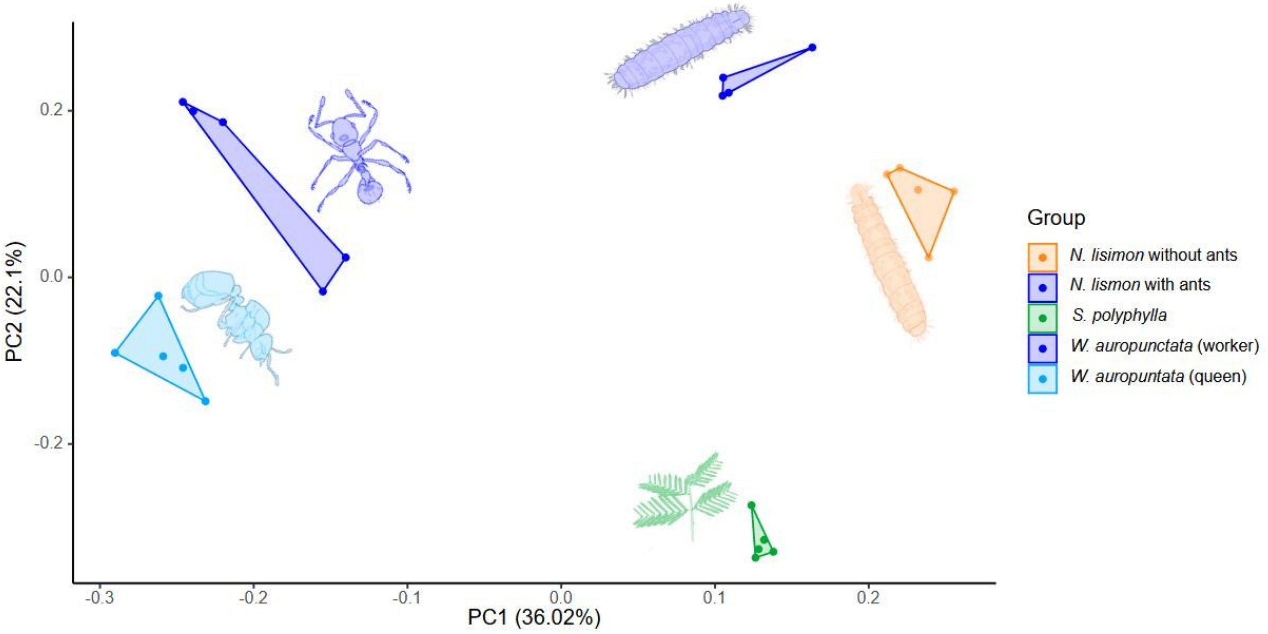


Fig 2. Principal Component Analysis (PCA) of the cuticular profile of *W. auropunctata* (workers), *W. auropunctata* (queens), host plant (*S. polyphylla*), and *N. lisimon* caterpillars raised with ants and without ants.

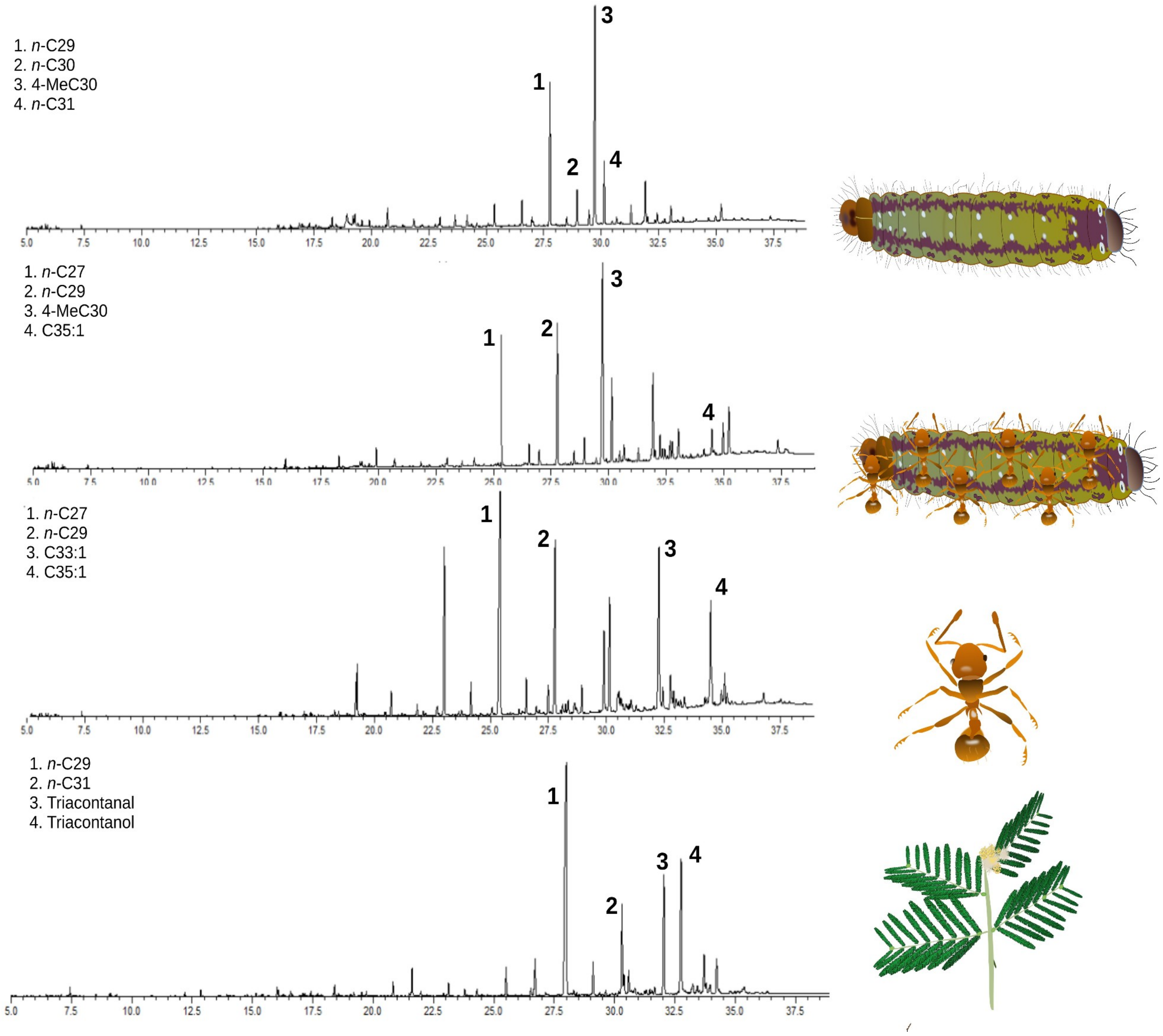


Fig 3. Chromatograms of the cuticular profile of *N. lisimon* caterpillars raised with and without ants, workers of *W. auropunctata*, and host plant *S. polyphylla.*