

**UNIVERSIDADE ESTADUAL DE CAMPINAS**  
**INSTITUTO DE BIOLOGIA**



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**“Interação entre formigas, o membracídeo *Guayaquila xiphias* (Hemiptera) e sua planta hospedeira *Schefflera vinosa* (Araliaceae): o papel dos lipídios cuticulares na camuflagem química dos membracídeos”**

Este exemplar corresponde à redação final  
da tese defendida pelo(a) candidato (a)  
HENRIQUE CESAR PELICCI SILVEIRA  
e aprovada pela Comissão Julgadora.

Dissertação apresentada ao  
Instituto de Biologia para  
obtenção do Título de  
Mestre em Ecologia.

A handwritten signature in black ink, appearing to read "Paulo Moreira".

Orientador: Prof. Dr. Paulo Sérgio Moreira Carvalho de Oliveira

Co-orientador: Prof. Dr. José Roberto Trigo

**Campinas, 2008**

**FICHA CATALOGRÁFICA ELABORADA PELA  
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**Si39i**

Silveira, Henrique César Pelicci

Interação entre formigas, o membracídeo *Guayaquila xiphias* (Hemiptera: Membracidae) e sua planta hospedeira *Schefflera vinosa* (Araliaceae): o papel dos lipídios cuticulares na camuflagem química dos membracídeos / Henrique César Pelicci Silveira. – Campinas, SP: [s.n.], 2008.

Orientadores: Paulo Sérgio Carvalho de Oliveira, José Roberto Trigo.

Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. *Camponotus crassus*. 2. Camuflagem química. 3. Membracídeos. 4. Mirmecofilia. 5. *Schefflera vinosa*. I. Oliveira, Paulo Sérgio Carvalho de. II. Trigo, José Roberto. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

(rcdt/ib)

**Título em inglês:** Ants and *Guayaquila xiphias* (Hemiptera: Membracidae) on *Schefflera vinosa* (Araliaceae): the role of cuticular lipids in the chemical camouflage of treehoppers.

**Palavras-chave em inglês:** *Camponotus crassus*; Chemical camouflage; Treehoppers; Myrmecophily; *Schefflera vinosa*.

**Área de concentração:** Ecologia.

**Titulação:** Mestre em Ecologia.

**Banca examinadora:** Paulo Sérgio Carvalho de Oliveira, Alexander Vicente Christianini, Angelo Pires do Prado.

**Data da defesa:** 11/07/2008.

**Programa de Pós-Graduação:** Ecologia.

Campinas, 11 de julho de 2008.

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(Orientador)



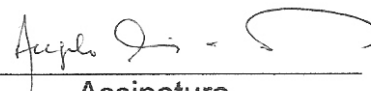
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*Para minha querida avó Idalina (1927-2008).*

## Agradecimentos

Em primeiro, agradeço à minha princesa, por me apoiar de todas as maneiras possíveis ao longo desses dois anos de mestrado. Sua ajuda foi essencial em todas as etapas desse trabalho, auxiliando-me tanto no laboratório quanto no campo (passando quase a páscoa inteira contando formigas). Além disso, com ela passei os melhores momentos de minha vida, o que certamente contribuiu para renovar minhas forças, e assim conseguir superar todos os desafios.

Agradeço aos meus pais que mesmo não conhecendo muito meu trabalho sempre me incentivaram, e continuam me incentivando até hoje. Com o carinho e apoio de vocês chego ao fim de mais uma etapa de minha vida. Aos meus irmãos, os quais sempre me acolheram bem nos fins de semana que voltava para casa. À Tia Eliana, que apesar de não ter contribuído diretamente, foi imprescindível em minha formação pessoal.

Agradeço ao meu orientador Prof. Dr. Paulo S. Oliveira, que ao longo de quatro anos foi o grande responsável por meu crescimento profissional, e também pessoal. Ao meu co-orientador Prof. Dr. José R. Trigo, por me apresentar à ecologia química, e me ajudar sempre que precisei.

Agradeço aos meus amigos da pós, os quais ajudaram imensamente no campo, no laboratório, e também com discussões valiosas nos inúmeros descansos para cafezinhos e cervejas: Alex, Buzatto, Chris, Daniel, Dri, Fião, Gabi, Lucas, Pedro, Seba. Aos meus amigos de república, Bixo, Dotô, Cunha, Daniel, Fran, Halley, Jundiaí, Jugs, Marcel, Rafa, e Tiagão, por todos os momentos divertidos que passamos no sítio. Agradeço também aos meus amigos de São Paulo por todas as viagens e longa amizade, que certamente perdurará por muitos anos mais.

Agradeço ao Dan que revisou com todo cuidado o texto em inglês, ao Lucas por tirar as fotos e o Marden por tratá-las no Photoshop.

Agradeço aos membros da pré-banca pelas críticas e sugestões valiosas.

Agradeço aos funcionários do Departamento de Zoologia, ao Instituto de Botânica que me ajudaram na logística, e parte burocrática do trabalho.

À CAPES, agradeço pela bolsa concedida.

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## Resumo

A grande abundância de formigas é um dos fatores que torna esses artrópodes os maiores predadores de insetos herbívoros, sendo que sua intensa atividade de forrageamento na vegetação resulta em um número expressivo de associações com plantas e outros artrópodes. O sistema entre formigas, *Guayaquila xiphias* (Hemiptera: Membracidae) e sua planta hospedeira *Schefflera vinosa* (Araliaceae) é um exemplo que ocorre no cerrado. As ninfas e os adultos do hemíptero se alimentam da seiva da planta, apresentando hábito gregário e produzindo exsudação ricamente energética (majoritariamente carboidratos), que é coletada dia e noite por diversas espécies de formigas, as quais protegem esses membracídeos contra predadores. Entretanto, será que somente o exsudato seria o suficiente para apaziguar as formigas fazendo com que elas não ataquem os membracídeos? Nós testamos a hipótese que a semelhança química entre o membracídeo (ninfas e adultos) e sua planta hospedeira confere a esse herbívoro uma camuflagem química contra formigas, resultando em uma defesa adicional à produção de exsudato. Ninfas liofilizadas de *G. xiphias* foram coladas em uma planta diferente da hospedeira (*Solanum tabacifolium*; Solanaceae), o que resultou em uma predação significativamente maior pela formiga predadora generalista *Camponotus crassus* (Formicinae), quando comparadas com ninfas coladas na planta hospedeira. O padrão químico dos lipídios cuticulares de ninfas e adultos dos membracídeos, e do caule jovem de *S. vinosa* foi similar, apresentando como componentes majoritários C<sub>27</sub>; octacosanol; 13-,11-MeC<sub>29</sub>; triacontanol; sitosterol e dotriacontanol. Através de bioensaios diferentes realizados com ninfas liofilizadas de *G. xiphias* e larvas de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) oferecidas em laboratório à *C. crassus*, verificamos índices de recrutamento de formigas menores nos organismos que apresentam uma grande semelhança química com o pano de fundo. Até onde temos conhecimento, essa é a primeira demonstração que outro mecanismo, além do apaziguamento por liberação de exsudato, está envolvido na proteção de membracídeos contra formigas que os atendem.

*Palavras-chave:* *C. crassus*, camuflagem química, membracídeos, mirmecofilia, *S. vinosa*.

## Abstract

Ants exert a strong predatory pressure on insect herbivores, mainly due to their great abundance in various terrestrial habitats. This intense activity of ants on vegetation results in a great number of associations with plants, and other arthropods. The system between ants, the honeydew-producing treehopper *Guayaquila xiphias* (Hemiptera: Membracidae) and its host plant *Schefflera vinosa* (Araliaceae) is an example of interaction that occurs in the cerrado savanna. Nymphs of *G. xiphias* exhibit gregarious behavior and are tended day and night by several ant species; the ants use honeydew droplets that fall on lower leaves and on the ground as cues to find the aggregations. We demonstrated through laboratory bioassays that both nymphs and adults of *G. xiphias* have similar cuticular chemical profiles to their host plant *S. vinosa*, and this similarity represents a mechanism that protects the treehoppers against attacks by tending ants. Freeze-dried nymphs of *G. xiphias* placed on a different plant (*Solanum tabacifolium*; Solanaceae) were significantly more removed by the predatory ant *C. crassus* (Hymenoptera: Formicidae) than those placed on *S. vinosa*. Gas chromatography-mass spectrometry analyses revealed that both nymphs and adults of *G. xiphias* share the same major cuticular lipid components with the young stem of *S. vinosa*: C<sub>27</sub>; octacosanol; 13-, 11-MeC<sub>29</sub>; triacontanol; sitosterol; and dotriacontanol. Based on the recruitment index, experiments with *G. xiphias* nymphs and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae supported the hypothesis that *C. crassus* foragers do not recognize as prey the organisms that have a high chemical similarity (basically cuticular lipids) with the background. To our knowledge, this is the first study showing that chemical camouflage is involved in the protection of treehoppers against tending ants.

**Keywords:** *C. crassus*, chemical camouflage, treehoppers, myrmecophily, *S. vinosa*.



## Introdução Geral

Diversas espécies de formigas forrageiam na superfície de plantas em busca tanto de presas animais, como também outros recursos alimentares derivados de plantas (Carrol & Janzen 1973). A atividade das formigas pode ser mantida através da existência de fontes alimentares previsíveis, e imediatamente renováveis, tais como: nectários extraflorais, exsudatos de hemípteros e secreções de larvas de lepidópteros (Koptur 1992). Esses líquidos aparentemente fornecem uma alta quantidade de energia para as formigas visitantes (Davidson *et al.* 2003). Embora os recursos alimentares fornecidos na folhagem sejam explorados mais freqüentemente por espécies de formigas arbóreas, aquelas espécies que nidificam no solo amplificam suas áreas de forrageamento utilizando também a vegetação herbácea e arbórea para procurar alimento (Rico-Gray 1993).

A grande abundância de formigas nos diversos habitats terrestres torna estes artrópodes os maiores predadores de insetos herbívoros (Jeanne 1979). Assim, a intensa atividade de formigas na vegetação resulta em um número expressivo de associações entre formigas, plantas e outros artrópodes; variando de interações facultativas até obrigatórias (Beattie 1985; Hölldobler & Wilson 1990; Rico-Gray & Oliveira 2007). O resultado de muitas dessas interações são mediadas pelo modo que o comportamento das formigas pode afetar a aptidão de um herbívoro em uma determinada planta hospedeira (Oliveira & Del-Calro 2005). O comportamento agressivo e/ou predatório das formigas na região próxima aos nectários extraflorais e aos insetos que excretam açúcares (ver Way 1963) resulta em conseqüências variáveis tanto para o herbívoro quanto para a planta (Oliveira *et. al* 2002).

No caso de plantas que apresentam nectários extraflorais, as formigas visitantes afetam positivamente a reprodução da planta, através da predação de herbívoros; diminuindo o consumo de tecidos reprodutivos e vegetativos (Koptur 1992). No entanto, os resultados de sistemas constituídos por formigas e plantas podem ser muito variáveis e alguns estudos mostram que a visitaçao de formigas a nectários extraflorais pode não trazer quaisquer benefícios para planta (Beattie 1985; O'Dowd & Catchpole 1983; Heads & Lawton 1984; Rico-Gray & Oliveira 2007).

As formigas requisitam a exsudação de insetos diretamente através do toque no abdome, no caso de hemípteros; ou estimulando a glândula dorsal produtora de exsudato, no caso de lepidópteros (Way 1963). Cushman *et al.* (1994) e Stadler & Dixon (1999) sugerem que a visitação de formigas apresenta variados efeitos (tanto positivos quanto negativos) na aptidão dos insetos produtores de exsudato, através de mudanças fisiológicas e comportamentais desses artrópodes.

Um exemplo desse tipo de interação ocorre no Cerrado, envolvendo o hemíptero produtor de exsudato *Guayaquila xiphias* Fabricius (Hemiptera: Membracidae), sua planta hospedeira *Schefflera vinosa* (Cham. & Schltdl.) Frodin & Fläschi (Araliaceae) e formigas. Estes membracídeos são encontrados ao longo de todo ano em *S. vinosa* e os níveis de infestação variam de apenas uma fêmea com ovos a 200 indivíduos em uma única agregação (Del-Claro & Oliveira 1999). As ninfas completam seu ciclo de vida aproximadamente após três semanas e os adultos recém-emergidos dispersam para outras plantas hospedeiras (Del-Claro & Oliveira 1999). Del-Claro & Oliveira (2000) demonstraram que a presença de formigas atendentes reduz a abundância de inimigos naturais de *G. xiphias* na planta hospedeira, aumentando a sobrevivência dos membracídeos. Essa defesa indireta produzida pela presença de formigas é condicional, pois varia ao longo do tempo, de acordo com as mudanças bióticas e abióticas do meio (Del-Claro & Oliveira 2000). O presente estudo busca uma ampliação do entendimento do sistema composto por *G. xiphias*, *S. vinosa* e formigas, acrescentando novas informações relacionadas à defesa do membracídeo, do ponto de vista da ecologia química.

Há poucos estudos mostrando que a predação de herbívoros por formigas pode ser reduzida pela semelhança de seus hidrocarbonetos cuticulares com aqueles encontrados na planta hospedeira. Portugal & Trigo (2005) encontraram evidências desse tipo de camuflagem química em larvas do lepidóptero *Mechanitis polymnia* na planta *Solanum tabacifolium* (Solanaceae) e Nogueira-de-Sá (2004) para o coleóptero *Plagjometriona flavescens* em *Aureliana lucida* (Solanaceae).

A função primária dos lipídeos que compõem a cutícula dos insetos é diminuir a perda de água, o que representa uma adaptação importante para os

artrópodes terrestres. Alguns componentes cuticulares também estão envolvidos, das mais diversas maneiras, na comunicação química inter e intra-específicas (Howard 1993). Dentre tais componentes, os lipídeos representam um dos principais caracteres químicos relacionados com o reconhecimento em insetos sociais (Howard *et. al* 1980), atuando como componente de integração das colônias, feromônios sexuais, entre outros (Dettner & Liepert 1994).

Tanto na cutícula dos insetos quanto das plantas, pode-se encontrar uma alta variedade de componentes alifáticos de longa cadeia (Espelie *et al.* 1991). Em ambos os grupos tais componentes são estruturalmente muito semelhantes, sendo idênticos em alguns casos (Howard & Blomquist 2005).

A maioria das espécies de formigas que buscam recursos líquidos na folhagem é predadora (Kaspari 2000) e desse modo podem potencialmente atacar os insetos produtores de exsudatos dependendo das condições do meio (ver Billick *et al.* 2007). A partir destas informações, nós testamos a hipótese de que a semelhança química entre ninfas e adultos de *G. xiphias* e sua planta hospedeira confere a esse herbívoro uma camuflagem química contra ataques por formigas, resultando em uma defesa adicional à produção de exsudato.

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## **CAPÍTULO 1:**

**"Ants and *Guayaquila xiphias* (Hemiptera: Membracidae) on *Schefflera vinosa* (Araliaceae): the role of cuticular lipids in the chemical camouflage of treehoppers"**

## Introduction

The vast majority of animals are subject to predation, therefore, the interactions between predators and prey may represent a strong natural selection force (Endler 1991). A successful predation must complete five stages: detection, identification, approach, subjugation, and consumption (Endler 1986). A variety of anti-predation strategies has evolved among animals, and can interrupt at any one of the five predation stages (Brandt & Mahsberg 2002). Cryptic coloration, some morphological structures, and chemical defenses are the main early stage defenses (Schmidt 2003).

Crypsis, also termed camouflage, is the reproduction of a background of an uninteresting form or object (Vane-Wright 1976), thus diminishing the probability of detection. Even after the prey is detected as a different object than the background, cryptic animals can still prevent their recognition as a food item (Jackson & Pollard 2007). Insects can be cryptic in different ways, such as low dissemination of olfactory, auditory, or tactile cues; body coloration; and remaining motionless (Stamp & Wikens 1993). Moreover, some morphological appendages of prey, such as spines and horns, hinder the capture and processing by their predators (Galatowitsch & Mumme 2004).

Chemical cues used in insect defense may be volatile, or connected to a surface, depending on the physico-chemical properties of the substances involved, and may also be composed of a single substance or a complex mixture. These chemical cues are detected by sensory organs in the receiver and generate a specific behavioral response (Dettner & Liepert 1994).

Toxic chemicals are usually associated with aposematic coloration, and can prevent predation (Pasteels 2007). Toxic and unpalatable insects utilize urticant hair or spines, and eversible glands (Bowers 1993). In addition to these morphological characteristics, other mechanisms that provide an unpleasant taste for insects include the sequestration of chemical substances from host plants, and toxic plant origin substances in their intestines (Ruxton *et al.* 2004).

Buckner (1993) suggests that chemicals can prevent predation by similarity *via* cuticular components involving crypsis or mimicry. According to Howard *et al.* (1990), chemical camouflage occurs when the mimic acquires the substance from the model, whereas chemical mimicry occurs when the



organism itself biosynthesizes the substance present in the model. However, this definition emphasizes the origin of the signal instead of the operator (predator), which is the driving force of selection in a chemical mimicry or camouflage system. Thus, in the present study a definition for chemical camouflage is used following the concepts proposed by Vane-Wright (1976): an organism uses a chemical camouflage strategy when the chemical substances present in the model, regardless of whether acquired or biosynthesized by the organism, make it uninteresting for the operator. Likewise, we used Vane-Wright's concepts to define chemical mimicry: an organism is a chemical mimic when the operator misidentifies its chemical components, and confuses it with the model.

The literature present numerous examples of chemical resemblances used in chemical mimicry (Dettner & Liepert 1994). Strategies related to chemical mimicry can involve incorporation into colonies of social insects (Howard *et al.* 1980, Moritz 1991, Akino 2002, Nash *et al.* 2008), penetration into nests of solitary insects (Tengo & Bergstrom 1977), exploitation of ant-plant mutualisms (Espelie & Hermann 1988) and ant-insect mutualisms (Völkl 1992, Gross 1993), and attraction of prey (Forsyth & Alcock 1990, Zhu & Haynes 2004). A few studies have shown that predation can be reduced by a chemical similarity between a herbivore and its host plant (chemical crypsis or camouflage). Portugal and Trigo (2005) found evidence that larvae of the ithomiine butterfly *Mechanitis polymnia* is protected against ants by cuticular lipids that are analogous to their host plant *S. tabacifolium*. Likewise, Nogueira-de-Sá (2004) recorded chemical camouflage of *Plagjometriona flavescens* (Cassidinae) on *Aureliana lucida* (Solanaceae).

The main role of cuticular lipids in plants and insects is to decrease water loss, and in both groups, the structural components are very similar (Hadley 1981, Nelson & Lee 2004). In insects and other arthropods, cuticular lipids are also involved in biochemical, physiological, and ecological interactions. They often act as substances of recognition and integration in social insect colonies, as sex and thermoregulatory pheromones, as alarm pheromones, and as barriers to microorganisms (Howard & Blomquist 2005). Espelie *et al.* (1991) argue that "the long chain aliphatic components which compose the cuticular lipids of plants and insects are well-suited to prevent dehydration, but it is not

know why plants and insects employ such a diversity of compounds to provide this protection”.

The cerrado has been cited as an environment rich in interactions between plants, herbivores, and ants (Oliveira & Freitas 2004, and references therein) which is in part due to the high frequency of liquid resources such as extrafloral nectaries, hemipteran excretion, and lepidopteran secretion on foliage (Oliveira *et al.* 2002). Treehoppers are herbivorous insects that feed on woody or herbaceous plants using stylets to extract nutrients (primarily amino acids and carbohydrates) from plant phloem (Lin 2006). Excess fluids are excreted as droplets of honeydew and may be collected by social hymenopterans such as ants, meliponine bees, and wasps (Way 1963). Such exudate-producing insects are known as trophobionts (Hölldobler & Wilson 1990). Treehoppers reduce predation with a hardened pronotum that changes coloration and hardness during development, suggesting cryptic and aposematic functions against visually-oriented predators (Wood 1993).

In the cerrado savanna, the honeydew-producing treehopper *Guayaquila xiphias* Fabricius (Hemiptera: Membracidae) exhibit gregarious behavior and are tended day and night by 21 ant species, which use honeydew droplets that fall on lower leaves and on the substrate as cues to find the aggregations (Del-Claro & Oliveira 1996; 1999). The treehoppers use *Schefflera vinosa* (Cham. & Schltld.) Frodin & Flaschi (Araliaceae) as host plant, and although this plant species does not bear extrafloral nectaries, the presence of the treehoppers increase ant visitation to the plant and reduce herbivory (Oliveira & Del-Claro 2005).

Most honeydew-gathering ants combine liquid foods with animal prey (Kaspari 2000). Thus the diet of some ant species may include both honeydew-producing insects and insect exudates (Sakata 1995; Offenberg 2001). Hölldobler & Wilson (1990) proposed that honeydew can appease tending ants, and protect the trophobionts from predation by the latter. However, to our knowledge their suggestion has never been tested. Additionally, would only honeydew be sufficient to protect *G. xiphias* against predation by ants? We hypothesized that the honeydew-producing treehoppers have similar cuticular lipid profiles to their host plant *S. vinosa*, and that this similarity may provide additional protection against tending ants. Predation and recruitment

experiments with the ant *Camponotus crassus* Mayr (Formicinae) as a model of tending ant and predator were designed to examine this hypothesis.

## Materials and Methods

### *Study area and organisms*

Branches of *S. vinosa*, containing *G. xiphias* aggregations and tending ants, were collected in the cerrado reserve of the Estação Experimental de Mogi Guaçu (Fazenda Campinha). The vegetation consists of a cerrado *sensu stricto* near Mogi Guaçu county, São Paulo state, south-eastern Brazil (22°49' S, 47° 10' W) (Oliveira-Filho & Ratter 2002). Plants and ants were used immediately for chemical extraction, while treehoppers were killed by freezing and were maintained at -15°C for the extraction of cuticular lipids, and bioassays.

Del-Claro and Oliveira (1999) reported that *C. crassus* was one of the most frequent ant species to harvest honeydew from *G. xiphias* aggregations. Therefore, this ant was used as a generalist chemically-oriented predator to test the chemical camouflage hypothesis. *C. crassus* is an arboreal species that nests in dead and decaying logs (Ribas *et al.* 2003). The colonies of *C. crassus* were collected from the area above mentioned, as well as from another cerrado *sensu stricto* area near Itirapina county, São Paulo state (22°15' S, 47° 49' W). The colonies were maintained at 25°C in test tubes (18 cm long, 2 cm in diameter) covered with red acrylic plates. Test tubes were placed in a plastic container (37 cm long x 25 cm wide x 8 cm high) connected to a foraging arena (15 cm in diameter x 7 cm high) where the experiments were carried out (Figure 1). The foraging arena was used to avoid ant aggressiveness or any other defensive behavior resulting from the proximity of the testing area to the ants' nesting chambers. Ants were prevented from escaping by applying a sticky barrier of tree Tanglefoot® (Tanglefoot Company, Grand Rapids, MI, USA) to the borders of the containers.

All colonies had similar number of individuals (30-50 workers). The ants were fed daily with 1ml of a 50% sucrose-water solution as a carbohydrate source, and three times a week with two mealworm *Tenebrio molitor*

(Coleoptera: Tenebrionidae) as a protein source. The colonies stood three days without food before the starting of each bioassay.

#### *Extraction of cuticular lipids*

Chemical extraction was performed following Portugal and Trigo (2005) and consisted in the immersion of treehopper nymphs or adults separately in 10mL of hexane (SupraSolv, Merck, Germany) for 10 minutes. Young and Schal (1997) suggest that using hexane as a solvent for a ten minute time period will sufficiently extract virtually all cuticular lipids without extracting any internal ones. Next, the hexane was treated with a small amount of sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) anhydrous for water withdrawal, then dried in a low pressure rotaevaporator at 40°C, subsequently recovered in gas chromatography-mass spectrometry vials, and finally dried in nitrogen gas ( $\text{N}_2$ ) stream. The resulting cuticular lipids extract was re-dissolved in the appropriate amount of hexane needed for analysis by gas chromatography-mass spectrometry or bioassays procedures. The cuticular lipids extraction of young stems of *S. vinosa* (where treehoppers fed upon) and *Solanum tabacifolium* Vell. (Solanaceae) was performed in the same way as described above.

#### *Predation bioassays with different plants*

In addition to the hypothesis of ant appeasement through honeydew production (Hölldobler & Wilson 1990), treehoppers may also reduce ant attacks due to the similarity of their cuticular lipids with their host plant. We predicted that nymphs of *G. xiphias* placed on a different plant (low chemical similarity) would have a higher predation rate than treehoppers nymphs placed on *S. vinosa* (high chemical similarity).

Nymphs of the treehoppers maintained for bioassays were freeze-dried in a Labconco Freezone<sup>®</sup> 6 freeze dry system for 48 hours, and then placed ventrally on the young stem of their own host plant *S. vinosa* and on *S. tabacifolium* stem. Freeze-dried nymphs were glued to plants with a PVA-based white glue (Araúna Ind. Com. Imp. e Exp. Ltda, São Paulo, Brazil), which had no effect on ant behavior. In nature *G. xiphias* aggregations are usually found near the upper part of the principal stem of its host plant (Del-Claro &

Oliveira 1993). In contrast, *S. tabacifolium* was not been recorded as a host plant for *G. xiphias*.

The upper part of the main stem (young stem) of *S. vinosa* and *S. tabacifolium* was brought to the laboratory without leaves, and subsequently placed in different water vials (15 ml) to prevent drying. During the bioassays, 15 colonies of *C. crassus* received two vials, one with a freeze-dried treehopper nymph glued on the young stem of *S. vinosa*, and the other with a freeze-dried nymph glued on the young stem of *S. tabacifolium*. After 24 hours, the number of nymphs removed by the ants was recorded. The proportion of nymphs removed in each experimental group was compared using a binominal test for two proportions (Zar 1999).

#### *Identification and similarity analyses of cuticular lipids*

To determine the cuticular chemical similarity between organisms belonging to the study system, extracts of cuticular lipids were analyzed using electron impact gas chromatography-mass spectrometry in a Hewlett Packard 6890 gas chromatograph, equipped with an automatic injector Hewlett Packard 7683 and a silica column (HP-5MS 5% phenyl methyl siloxane capillary 95% 30m x 250 $\mu$ m x 0.25 $\mu$ m, Hewlett Packard) directly coupled to a mass selective detector Hewlett Packard 5973 (Conditions: temperature of injection: 250°C; program temperature: 70 to 300°C to 2°C/min, 20 min at 300°C, Helium 1ml/min as carrier gas; ionization energy of 70 eV). All analyses used a range of 40 to 600 uma in the mass spectrometer. The alkanes were identified according to Howard (2001), while alcohols were identified after derivatization with *N*-methyl-*N*-trimethyl-silyltrifluoroacetamide (MSTFA) (100 $\mu$ l MSTFA, 80°C, 1 h) (Attygalle 1998) in according to Menéndez *et al.* (2005) and Wang *et al.* (2007).

The cuticular lipid profile similarity of the treehoppers and its host plant was estimated according by the Morisita's index of similarity, which varies from 0 (none similarity) to 1 (full similarity) (Krebs 1999).

#### *Predations bioassays on S. vinosa, changing the cuticular lipid profile of G. xiphias and S. frugiperda*

Prey recognition bioassays were divided in two parts, both using *S. vinosa* as a background. The first part of the bioassay was done using freeze-

dried nymphs of *G. xiphias* which had approximately the same body size (0.5 cm). As described above, one treehopper nymph (treated nymph, T) was dipped in 10mL of hexane for the removal of cuticular lipids. A second nymph (control nymph, NT) did not receive the hexane treatment and its cuticular lipid profile was kept. Each nymph was glued on one side of a *S. vinosa* young stem immersed in a vial with water (Figure 2A) as in host plant alteration bioassay. Nymphs were glued in pairs at different heights to facilitate the counting of ants in contact with each nymph, and randomness regarding nymph position was decided by flipping a coin. Ten young stems of *S. vinosa* with control and treatment freeze-dried nymphs were offered to ten different *C. crassus* colonies. A recruitment index (RI) (see Portugal & Trigo 2005) was calculated as the percentage of ants in contact with T relative to the total number of ants counted on both nymphs, during a period of 30 minutes after the first ant encounter. At the end of each minute, the number of ants in contact with each nymph was recorded, totaling 60 records (30 records for each larva). If any treehopper was removed or both were not removed 30 minutes after starting observations, then the trial was terminated. The RI was calculated by the sum of the 30 records for each nymph as follows:

$$RI = \left( \frac{\text{number of ants on } T}{\text{number of ants on } T + \text{number of ants on } NT} \right) \times 100$$

A RI close or equal to 50 indicated no preference for either nymph, whereas RI lower or higher than 50 suggested a preference for the control nymph with intact cuticular lipid profile or for the hexane-treated nymph, respectively (Portugal & Trigo 2005). One sample *t*-test was used to compare the RIs of ant foragers to hexane-treated nymphs with a standard value of 50.

The second part tested if ants would recognize potential prey treated with a hexane cuticular extract from nymphs of treehoppers. Two experimental comparisons were designed in double-choice experiments using freeze-dried second instars larvae of *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae):

1. Control x Treatment: one *S. frugiperda* larva (control larva, NT) was dipped in 10mL of hexane for the removal of cuticular lipids; a second

larva (treated larva, T) had the cuticular lipids removed, being afterwards coated with cuticular extract from nymphs of *G. xiphias* in 10 $\mu$ L of hexane. Since part of the hexane extract could penetrate the body of larvae instead of remaining on the external cuticle, ten times the larval equivalent was used to compensate for this loss (Portugal & Trigo 2005). The larval equivalent was calculated based on the ratio of the dry weight of *S. frugiperda* to the dry weight of the *G. xiphias* nymph. Each larva of a pair was glued on opposite sides and different heights of a *S. vinosa* young stem (Figure 2B) and offered to a *C. crassus* colony as described above. The experiment was replicated ten times, and the RI was calculated.

2. Control x Control: both larvae (T and NT) were dipped in 10mL of hexane for the removal of cuticular lipids, were glued on opposite sides and different heights of a *S. vinosa* young stem, and were offered to a *C. crassus* colony. The experiment was replicated 10 times, and the RI was calculated.

Two independent samples *t*-test were performed to compare the RIs of each experimental comparisons.

#### *Predations bioassays on S. tabacifolium\_changing the cuticular lipid profile of S. frugiperda*

The second part of the previous bioassay with larvae of *S. frugiperda* performed on *S. vinosa* was also conducted on *S. tabacifolium* as background. As in the former bioassay, two experimental comparisons (control x control; control x treatment) were designed in double-choice experiments using freeze-dried second-instars larvae of *S. frugiperda*. The calculation and statistical analysis of recruitment indexes are similar to the previous bioassay.

## Results

*G. xiphias* freeze-dried nymphs were significantly more removed by ants when placed on non-host plant *S. tabacifolium* (73%) than on the host plant *S. vinosa* (26%) (binominal test for comparison between two proportions,  $Z = 2.556$ ,  $p = 0.011$ , Figure 3).

The analyses of cuticular extracts through gas chromatography-mass spectrometry of *G. xiphias* and the host plant *S. vinosa*, detected mainly n-alkanes and alcohols. The plant cuticle presented mainly C<sub>27</sub> (I); Octacosanol (III); 13-, 11-MeC<sub>29</sub> (IV); Triacontanol (V); Sitosterol (VI); Dotriacontanol (VII) and traces of 13-, 11-MeC<sub>29</sub> (II) (Table 1, Figure 4A). Treehopper nymphs and adults presented the same compounds, but with a higher relative abundance of 13-, 11-MeC<sub>27</sub> (II) than their host plant (Table 1, Figure 4B and C). The compositions of all three cuticular extracts were very similar (Figure 4), and were confirmed by a Morisita similarity index of 95% between nymphs and adults, 90% between nymphs and host plant, and 89% between adults and host plant. Unnumbered peaks on Figure 4 were not identified.

The recruitment index (RI) of ants to hexane-treated *G. xiphias* nymphs placed on *S. vinosa* supported the hypothesis that omnivorous ant foragers do not recognize treehoppers as prey when their cuticular chemical profile is kept intact. The RI towards hexane-treated nymphs was  $80.50 \pm 3.69$  ( $\bar{X} \pm SE$ ; N=10). This value was significantly higher than the standard value of 50 (one sample t-test,  $t = 8.26$ ,  $df = 9$ ,  $p < 0.001$ ,  $N = 10$ , Figure 5). The prey recognition bioassays performed on *S. vinosa* with *S. frugiperda* larvae, also support chemical camouflage hypothesis (Figure 6). The RI for the control x treatment experimental comparison ( $17.43 \pm 2.79$ ;  $\bar{X} \pm SE$ ) was significantly lower than the control x control comparison ( $43.55 \pm 3.14$ ) (t-test for two independent samples,  $t = 6.62$ ,  $df = 18$ ,  $p < 0.001$ , Figure 7).

Double-choice experiments with freeze-dried second-instar larvae of *S. frugiperda* on *S. tabacifolium* showed that ant recruitment did not vary with treatment. The recruitment index was  $45.62 \pm 4.29$  ( $\bar{X} \pm SE$ ) for the control x treatment experimental comparison, and  $44.72 \pm 4.29$  for the control x control comparison (t-test for two independent samples,  $t = 0.15$ ,  $df = 17$ ,  $p = 0.88$ , Figure 8).

## Discussion

The treehoppers *G. xiphias* are attacked in the cerrado vegetation mostly by three enemies: salticid spiders that prey on both nymphs and adults;



predatory larvae of *Ocyrtamus arx* (Diptera: Syrphidae) that suck the body contents of treehoppers; and *Gonatocerus* (Myrmaridae) wasps that parasitize treehopper egg masses (Oliveira & Del-Claro 2005). Honeydew-gathering ant species that forage on *G. xiphias* aggregations can attack these potential enemies, reducing their abundance and increasing treehopper survival and fecundity (Del-Claro & Oliveira 2000).

The majority of ant species that tend insect-derived liquid foods generally combine this source of food with animal prey (Kaspari 2000). Thus, ants presenting this opportunistic diet can potentially consume honeydew-producing insects while collecting their exudates. On chestnut trees (*Castanea crenata*), Sakata (1995) observed the ant *Lasius niger* collecting honeydew and preying on two aphid species – *Lachnus tropicalis* and *Myzocallis kuricola*. However, there is no record of ants attacking nymphs or adults of *G. xiphias*. This suggests that in addition to a possible role of appeasement played by honeydew production (Hölldobler & Wilson 1990), chemical camouflage may be an alternative defense mechanism providing protection to treehoppers against attacks from tending ants.

It has been proposed that honeydew composition is an important factor in mediating interactions between ants and honeydew-producing hemipterans (Kiss 1981; Del-Claro & Oliveira 1993; Völkl et al. 1999; Yao & Akimoto 2001; Blüthgen & Fiedler 2004; Woodring et al. 2004). Fischer & Shingleton (2001) demonstrated that exudates of aphid species containing high proportions of the trisaccharide melezitose are more attractive to ants and that the biosynthesis of this carbohydrate may incur metabolic costs on aphids. This ability to make the sugar composition of honeydew more suitable (Stadler & Dixon 2005) may represent a primary protection against tending ants. We did not analyze the honeydew composition of *G. xiphias*. However freeze-dried nymphs of *G. xiphias*, therefore without reward, placed on their own host plant suffered lower predation by *C. crassus* ants in laboratory than those placed on a non-host plant. This finding led us to hypothesize that chemical camouflage might be an additional defense strategy used by *G. xiphias*.

Among ants nest mate recognition is related to the ability to distinguish differences in cuticular substances (Hölldobler & Wilson 1990). Members of the same colony generally attack alien organisms (those presenting differences in

cuticular chemical profile) near the nest territory, but there are many records of non-nestmate individuals living within ant colonies (Lenoir *et al.* 2001). A combination of chemical, morphological and behavioral mimicry, defensive chemicals, and appeasement secretions enable these organisms to survive among ants (review in Dettner and Liepert 1994).

Chemical signaling represents an important factor mediating predator and prey dynamics and other ecological interactions (Cohen *et al.* 2008). It is possible that prey recognition by *C. crassus* is also based on chemical perception. Boulay and co-workers (2000) demonstrated that individuals of *C. fellah* had their cuticular lipids profile modified when isolated, and were attacked by nest mates when returned to their own colonies. Espelie *et al.* (1991) proposed that the similar cuticular lipids between herbivores and their host plants may provide an effective defense strategy against chemically-oriented predators.

Gas chromatography-mass spectrometry analyses revealed that both nymphs and adults of *G. xiphias* share the same major cuticular lipid components with the young stem of *S. vinosa*. This convergence of lipid profiles is confirmed by a high Morisita similarity index. We reported a 90% similarity between treehopper nymphs and the young stem of *S. vinosa*. Although Portugal (2001) documented a lower similarity index (59%) between larvae of *Mechanitis polymnia* and leaves of its host plant *S. tabacifolium*, he showed that this chemical similarity was sufficient to predatory ants not perceiving the herbivore.

According to Allan *et al.* (2002), “simply revealing similarities between species in their cuticular hydrocarbons is not sufficient to demonstrate that the cuticular hydrocarbons function as camouflage”. Thus, we designed prey recognition bioassays to support our chemical camouflage hypothesis.

Laboratory bioassays, with freeze-dried nymphs of *G. xiphias* indicated that *C. crassus* ants perceived treehopper nymphs when cuticular lipids were extracted, and consequently recruited more to these individuals. We suggest that the modified lipid profile of *G. xiphias* on *S. vinosa* gave treehoppers a low chemical similarity condition that enabled the ants to recognize them as potential prey.

In addition to the similarity of the cuticular lipid profile with its host plant, other morphological characteristics may be involved in the defense and recognition of treehoppers by tending ants. Size, hardness, and shape of cuticle are some characteristics that could be used by ants to recognize the treehoppers. The bioassays with *S. frugiperda*, however, confirmed the role of chemical camouflage in protecting treehoppers. *C. crassus* did not recognize as prey the larvae of the moth coated with treehopper cuticular extract on *S. vinosa* (high chemical similarity). Larvae of the *S. frugiperda* are palatable, and generally are recognized and consumed by predatory ants such as *C. crassus* (Fuller *et al.* 1997). Larvae of the moth coated with treehopper cuticular extract placed on *S. tabacifolium* (low chemical similarity) were normally recognized as prey, indicating that the treehopper cuticle does not have unpalatable substances. Moreover, Portugal (2001) demonstrated that the *S. tabacifolium* cuticle presents mainly alkanes, which is different from the *S. vinosa* chemical composition of the cuticle.

We demonstrated that nymphs and adults *G. xiphias* have similar cuticular chemical profiles to their host plant *S. vinosa*, and this similarity represents a mechanism that makes the treehoppers free from attacks by to tending ants. Although it is not clear what degree of similarity between the cuticular lipid profiles is necessary to deceive the ants, our results support the prediction that chemical camouflage acts as a defense mechanism against ant predation.

Ants exert a strong predatory pressure on herbivorous insects, mainly due to their great abundance in various terrestrial habitats (Jeanne 1979). Therefore, natural selection should favor the evolution of different kinds of strategies to escape or diminish ant attacks on foliage (Oliveira & Freitas 2004; Stadler & Dixon 2005). Interactions between organisms can range from negative to positive outcomes depending on the size, age, or abundance of participants; depending on the abiotic and biotic setting in which the interaction takes place; and depending on the availability of resources (Bronstein 1994). Offenberg (2001) demonstrated that ants not only tended their aphid partners but also consumed them according to the availability of alternative food resources. The effects of ants on *G. xiphias* survival depend on the tending ant specie and can vary with time (Del-Claro & Oliveira 2000). Thus natural

selection possibly favored treehoppers with similar cuticular lipid profiles to its host plant, reducing predation regardless of variants that occur during the interaction with tending ants. Additional bioassays with other chemically-oriented extrafloral nectaries visitors, which can act as predators, are needed to test the effectiveness of chemical camouflage as a defensive mechanism used by *G. xiphias*. This is the first study to demonstrate that chemical camouflage is involved in the protection of treehoppers against tending ants.

## Conclusões gerais

1. A exsudação produzida por hemípteros resulta na formação de associações mutualísticas com formigas. Tais interações podem ser condicionais dependendo de diversos fatores bióticos e abióticos. O principal benefício oferecido pelas formigas atendentes aos hemípteros é a proteção contra inimigos naturais. Entretanto, a capacidade fisiológica de tornar a composição do exsudato mais adequado para as formigas, pode também representar uma defesa primária de hemípteros contra ataques pelas formigas que os atendem.
2. Existe uma alta similaridade química entre o padrão de lipídios cuticulares de ninfas e adultos de *G. xiphias*, com o padrão químico do meristema apical de *S. vinosa*, a planta hospedeira utilizada no cerrado por esses membracídeos. Os principais componentes químicos encontrados tanto na cutícula dos membracídeos, quanto na planta hospedeira são: C<sub>27</sub>; Octacosanol; 13-, 11-MeC<sub>29</sub>; Triacontanol; Sitosterol; and Dotriacontanol.
3. Neste trabalho demonstramos que a semelhança química entre ninfas e adultos de *Guayquila xiphias*, e sua planta hospedeira resulta em uma menor predação dos membracídeos por formigas da espécie *C. crassus* (modelo de predador utilizado nos bio-ensaios).
4. O mecanismo de defesa utilizado pelos membracídeos contra formigas coletoras de exsudatos pode ser definido como camuflagem química, e possivelmente protege *G. xiphias* contra predação de outros predadores.

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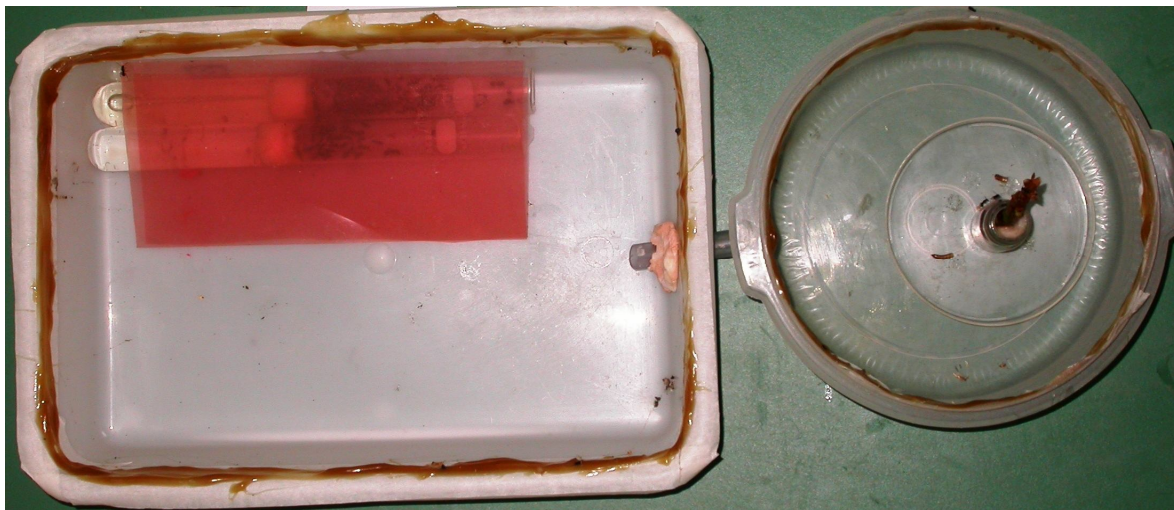


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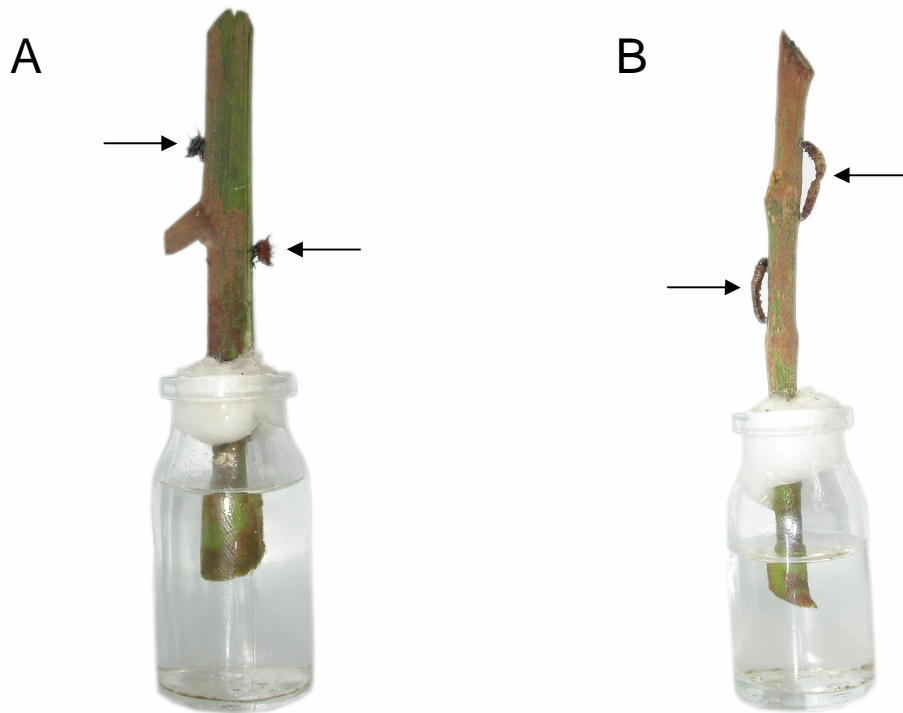
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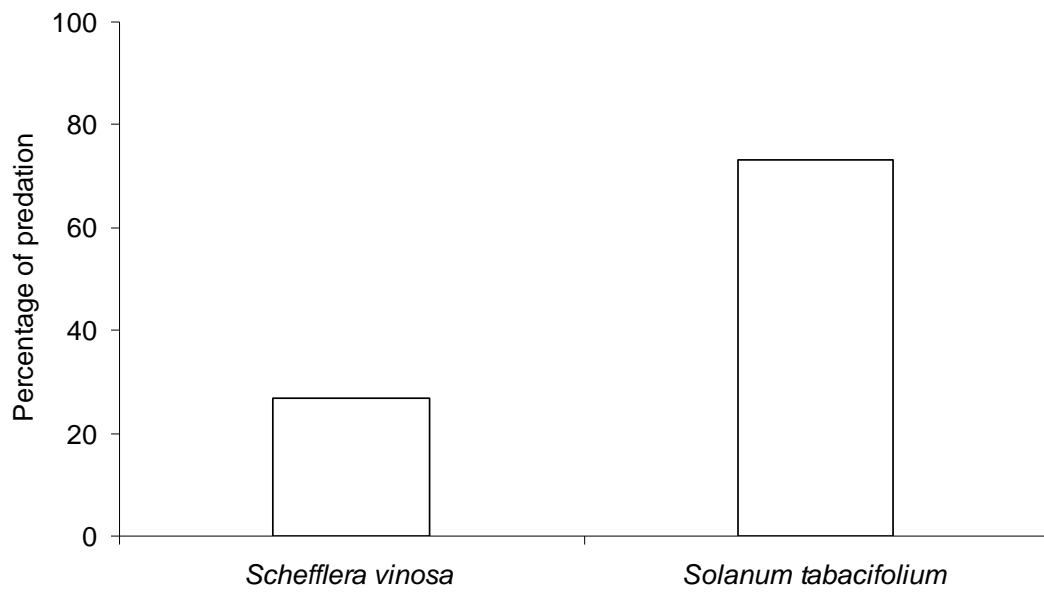
## Figures



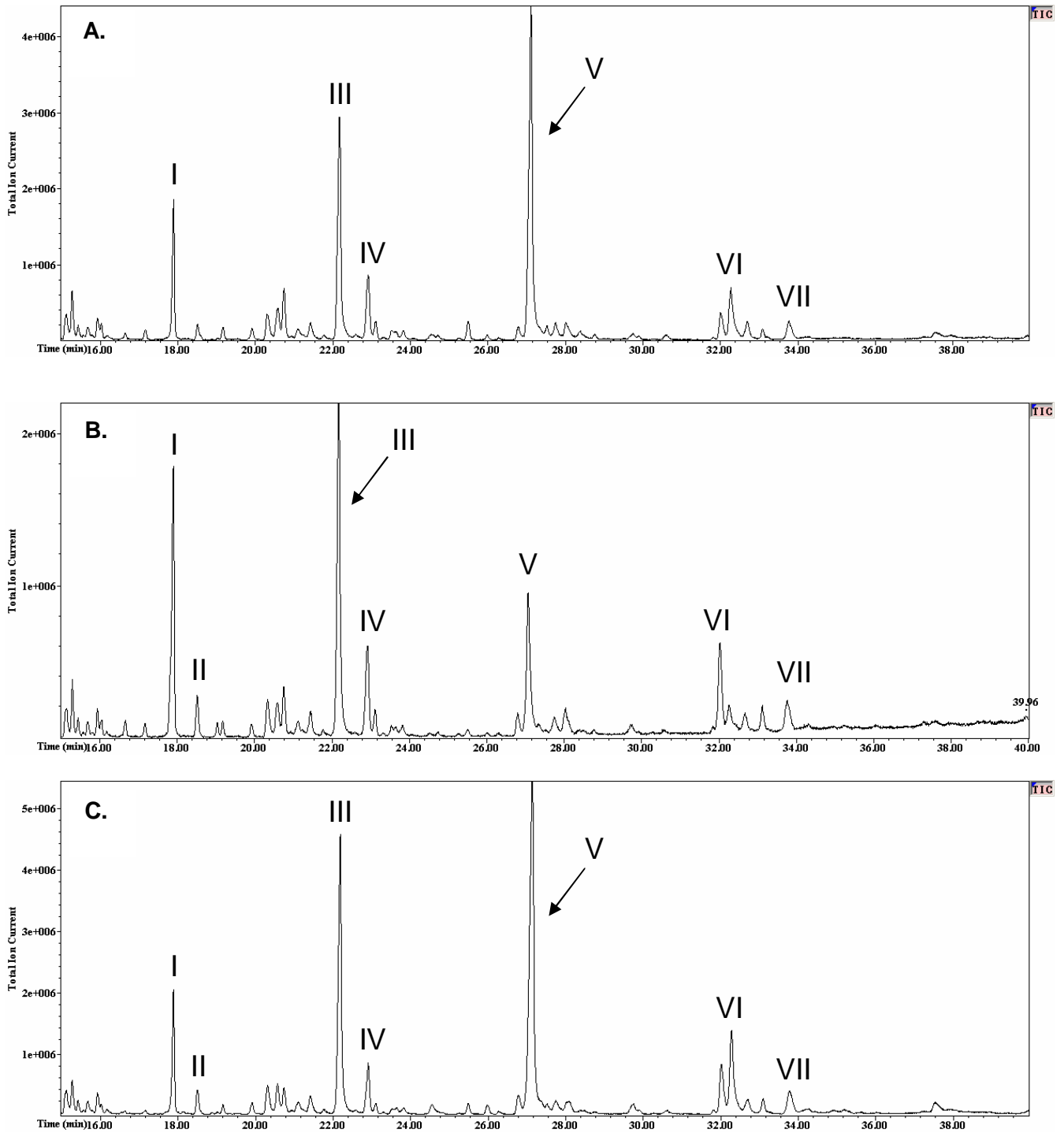
**Figure 1.** Artificial nest (37 cm long x 25 cm wide x 8 cm high) and foraging arena where *C. crassus* colonies were maintained. Ants were reared in test tubes (18 cm long, 2 cm in diameter) covered with a red acrylic plate.



**Figure 2.** Bioassay with *G. xiphias* freeze-dried nymphs (A), and *S. frugiperda* freeze-dried larvae (B). The insects (arrows) were glued on the upper part (young stem) of the principal stem of *S. vinosa*.



**Figure 3.** Percentage of *G. xiphias* freeze-dried nymphs removed by *C. crassus* ants after 24 hours. Fifteen replications were done for each plant. Percentages are significantly different (binominal test for two proportions,  $Z= 2.56$ ,  $p= 0.011$ ).

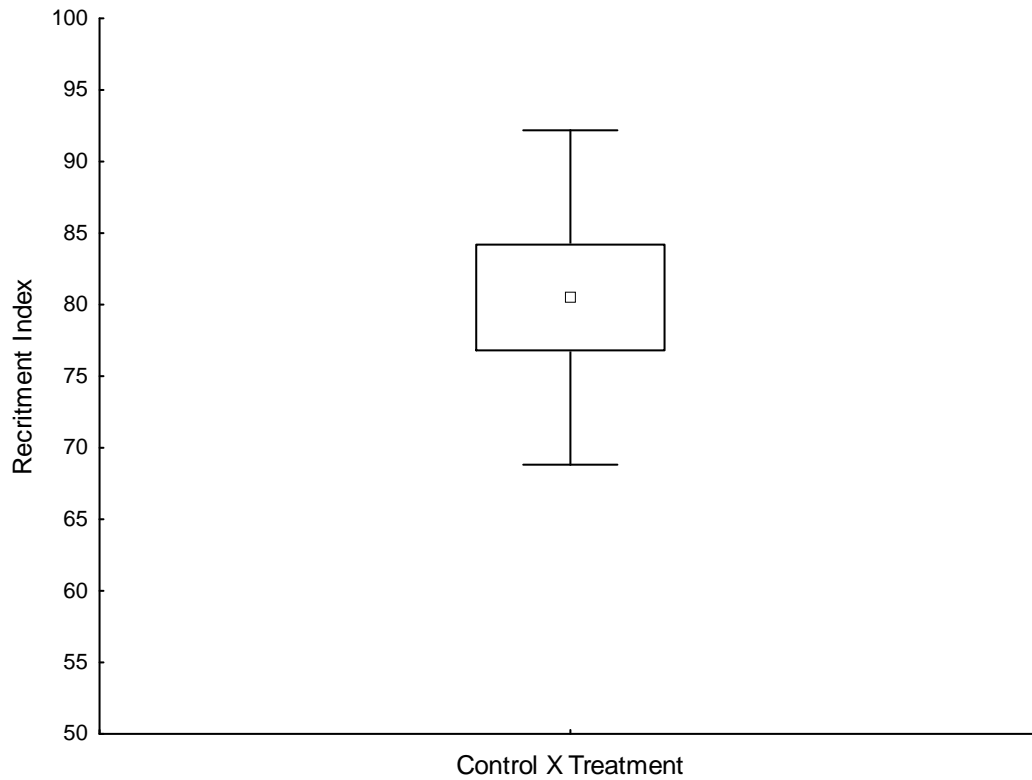


**Figure 4.** Chromatograms of cuticular extract from *S. vinosa* (A), *G. xiphias* nymphs (B), and adults (B). Numbered peaks, which were identified by their mass spectra, are listed in table 1.

**Table 1.** Relative abundance of compounds from cuticular lipids of *G. xiphias* nymphs, adults, and their host plant *Schefflera vinosa* analysed by GC-MS.

| Compounds                      | Relative Abundance |               |              |
|--------------------------------|--------------------|---------------|--------------|
|                                | Nymphs             | Adults        | Host plant   |
| Unknown                        | 1.29 ± 1.03        | 1.40 ± 1.14   | 0.60 ± 0.79  |
| Unknown                        | 2.49 ± 1.94        | 1.70 ± 1.01   | 2.78 ± 4.42  |
| Unknown                        | 0.48 ± 0.47        | 0.21 ± 0.43   | 0.18 ± 0.26  |
| Unknown                        | 1.17 ± 1.34        | 0.58 ± 0.80   | 0.33 ± 0.47  |
| C <sub>27</sub> (I)            | 12.94 ± 9.24       | 10.62 ± 6.92  | 4.11 ± 2.27  |
| 13-, 11-MeC <sub>27</sub> (II) | 0.98 ± 1.23        | 2.51 ± 1.56   | -            |
| Unknown                        | 1.68 ± 1.33        | 1.31 ± 1.32   | 0.95 ± 1.10  |
| Unknown                        | 1.52 ± 1.12        | 1.13 ± 1.30   | 2.42 ± 2.34  |
| Unknown                        | 1.76 ± 1.25        | 1.15 ± 1.30   | 2.25 ± 0.79  |
| Octacosanol (III)              | 23.55 ± 12.51      | 15.05 ± 4.74  | 20.31 ± 9.00 |
| 13-, 11-MeC <sub>29</sub> (IV) | 4.30 ± 3.39        | 8.55 ± 6.95   | 1.09 ± 1.58  |
| Unknown                        | 1.33 ± 0.94        | 0.84 ± 0.89   | 0.38 ± 0.62  |
| Triacotanol (V)                | 15.89 ± 15.89      | 18.60 ± 10.38 | 25.83 ± 8.46 |
| Sitosterol (VI)                | 2.58 ± 2.31        | 3.47 ± 2.54   | 2.32 ± 1.70  |
| Dotriacontanol (VII)           | 3.36 ± 3.49        | 3.93 ± 2.66   | 4.00 ± 1.73  |

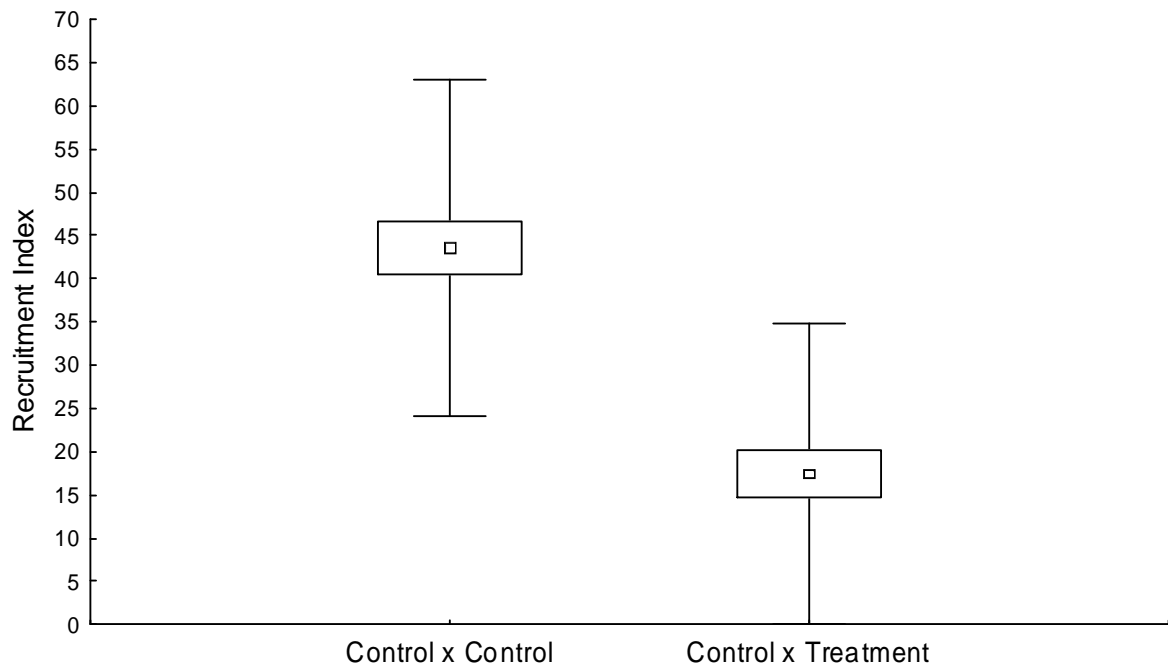




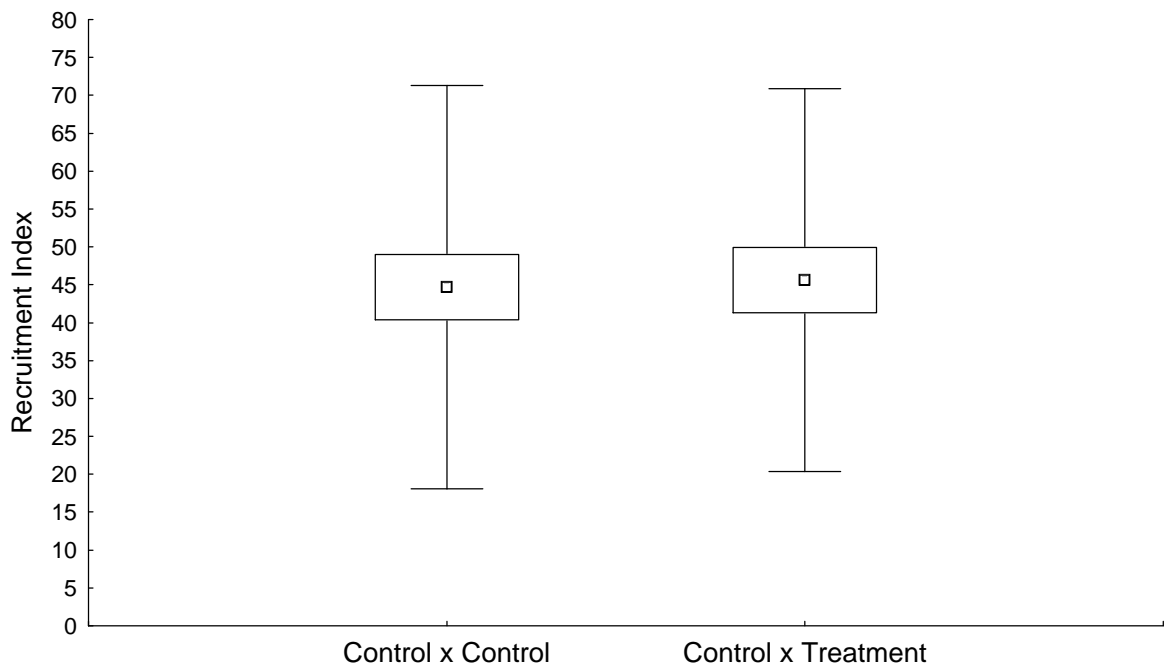
**Figure 5.** Recruitment index (RI) calculated as the percentage of ants in contact with hexane-treated nymph (treatment) relative to the sum of ants recruited to non-treated nymphs (control) and hexane-treated ones. Treated and control nymphs were each placed on opposite sides of the same young stem of *S. vinosa*. The RI mean is represented by the square in the center of the box, which represents the standard error, and whiskers represent the standard deviation. RI mean was statistically tested against a standard value of 50 (no preference for either nymph – see Material and Methods) (one sample *t*-test,  $t=8.26$ ,  $df=9$ ,  $p<0.001$ ,  $N=10$  replications).



**Figure 6.** Bioassay with freeze-dried *S. frugiperda* larvae glued on the young stem of *S. vinosa* with *C. crassus* ants. The larva on the left was treated with *G. xiphias* cuticular extract, and the other one was treated only with hexane.



**Figure 7.** Recruitment index (RI) calculated for two experimental comparisons (control x control and control x treatment). Control represents a *S. frugiperda* treated with hexane, while treatment represents a *S. frugiperda* larvae treated with the cuticular extract of *G. xiphias*. One larva of each experimental group was placed on the opposite side of the same young stem of *S. vinosa*. The RI mean is represented by the square in the center of the boxes, which represent standard errors, and whiskers represent standard deviations. Comparisons were performed with the *t*-test for two independent samples ( $t= 6.62$ ,  $df= 18$ ,  $p< 0.001$ ,  $N= 10$ ).



**Figure 8.** Recruitment index (RI) calculated for two experimental comparisons (control x control and control x treatment). Control represents a *S. frugiperda* treated with hexane, while treatment represents a *S. frugiperda* larvae treated with the cuticular extract of *G. xiphias*. One larva of each experimental group was placed on the opposite side of the same young stem of *S. tabacifolium*. The RI mean is represented by the square in the center of the boxes, which represent standard errors, and whiskers represent standard deviations. Comparisons were performed with the *t*-test for two independent samples ( $t = -0.15$ ,  $df = 17$ ,  $p = 0.88$ ,  $N = 10$ ).