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The spittlebug *Mahanarva fimbriolata* (Stål) produces foam as a thermoregulatory and defensive strategy

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Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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1. Cigarrinha-das-raízes 2. Ecologia química 3. Termorregulação 4. Defesa química 5. Predação 6. Irritação tópica 7. Microbioma. I. Título

DEDICATION

This thesis is dedicated to my parents
Paulo and Marlene, and my sisters
Luiza and Maíra.

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EPIGRAPH

"No one after lighting a lamp puts it in a cellar or under a basket, but on a stand, so that those who enter may see the light."

Luke 11:33

SUMMARY

RESUMO

A cigarrinha *Mahanarva fimbriolata* (Stål) produz espuma como estratégia termorregulatória e defensiva

Os insetos da família Cercopidae são facilmente reconhecidos pela espuma que produzem durante o desenvolvimento ninfal para recobrir o próprio corpo. Essas ninfas, conhecidas como cigarrinhas, podem ser observadas em uma ampla gama de plantas hospedeiras. Dentre as funções atribuídas à espuma estão a proteção contra elevadas temperaturas, dessecação e inimigos naturais. Contudo, evidências experimentais para confirmar estas hipóteses são escassas. *Mahanarva fimbriolata* é uma cigarrinha de importância econômica em áreas de produção de cana-de-açúcar no Brasil, especialmente após a proibição da colheita da cana com queima prévia. As ninfas de *M. fimbriolata* se desenvolvem nas raízes de cana-de-açúcar expostas na superfície ou abaixo do solo, onde sugam a seiva do xilema, bloqueando o transporte de água e nutrientes e causando desordens fisiológicas. Nesta tese foi avaliada a importância da espuma para a termorregulação de ninfas de *M. fimbriolata*, seu microbioma bacteriano e sua ação protetora contra predadores. A tese foi dividida em quatro capítulos. O **Capítulo 1** apresenta uma introdução sobre os aspectos bioecológicos de *M. fimbriolata* e o possível papel da espuma produzida pelas ninfas, o qual foi explorado em detalhes ao longo dos demais capítulos. O **Capítulo 2** examinou a importância da espuma e de seus compostos químicos na termorregulação das ninfas. O **Capítulo 3** explorou a diversidade e composição da comunidade bacteriana presente na espuma, no intestino das ninfas, e no solo próximo à espuma. O **Capítulo 4** investigou se a espuma atua na sua proteção das ninfas contra formigas predadoras *Solenopsis invicta*, e sua irritação tópica a baratas *Periplaneta americana*. A espuma produzida por *M. fimbriolata* demonstrou: (*i*) servir como um importante microhabit térmico, mantendo a temperatura próxima da ideal para o desenvolvimento das ninfas; (*ii*) abrigar uma diversidade de bactérias previamente reportadas como simbiontes protetivos de insetos e que, provavelmente, são provenientes do intestino das ninfas; e (*iii*) ser repelente a formigas predadoras e irritante a outro artrópode (baratas). Tomados em conjunto, a espuma produzida por *M. fimbriolata* serve como uma estratégia termorregulatória e defensiva para as ninfas. Ademais, esta tese serve como base para futuras pesquisas que visam investigar a importância da espuma para o crescimento e desenvolvimento das cigarrinhas.

Palavras-chave: Cigarrinha-das-raízes; Ecologia química; Termorregulação; Defesa química; Predação; Irritação tópica; Microbioma

ABSTRACT

The spittlebug *Mahanarva fimbriolata* (Stål) produces foam as a thermoregulatory and defensive strategy

Insects of the family Cercopidae are easily identified by the spit-like foam that they produce to surround themselves during the nymphal stage. Known as spittlebugs, these insects can be observed developing in a wide range of host plants. Among the functions attributed to the foam, there are the protection of nymphs against high temperatures, desiccation and natural enemies. However, experimental evidence to confirm these hypotheses are sparse. *Mahanarva fimbriolata* is an economically important spittlebug in Brazilian sugarcane crops, especially after the harvest with burning had been prohibited. The nymphs of *M. fimbriolata* develop on the soil surface or below ground where they suck the xylem content of exposed sugarcane roots, blocking the water and nutrients transport, eventually causing physiological disorder. In this thesis, it was evaluated the importance of foam for the thermoregulation of *M. fimbriolata* nymphs, its bacterial microbiome and the protective action against predators. The thesis was divided into four chapters. The **Chapter 1** presented an introduction to the bioecological aspects of *M. fimbriolata* and the possible role of foam produced by the nymphs, which was explored in details along the other chapters. The **Chapter 2** examined the importance of foam and its chemical compounds in the thermoregulation of nymphs. The **Chapter 3** explored the diversity and composition of bacterial community present in the foam, in the gut of nymphs, and in the soil close to the foam. The **Chapter 4** investigated whether the foam acts in protecting the nymphs against the predatory ants *Solenopsis invicta*, and its topical irritancy to the cockroaches *Periplaneta americana*. It was demonstrated that the foam produced by *M. fimbriolata*: (*i*) serves as an important thermal microhabitat, maintaining the temperature close to the ideal for the nymphs development; (*ii*) harbor a diversity of bacteria previously reported as protective symbionts of insects, which are probably originated from the nymphs's gut; and (*iii*) is a repellent to predators and topical irritant to another arthropod (roaches). Taken together, the foam produced by *M. fimbriolata* is a thermoregulatory and defensive strategy to the nymphs. In addition, this thesis serves as a background for future research that aims to investigate the importance of foam for the growth and development of spittlebugs.

Keywords: Spittlebug; Chemical ecology; Thermoregulation; Chemical defense; Predation; Topical irritancy; Microbiome

1. GENERAL INTRODUCTION

Spittlebugs are insects of the family Cercopidae that produce and cover themselves with a distinctive foam during their nymphal stage (Guilbeau 1908; Kato 1958). The conspicuous white foam masses are produced by the nymphs by sucking air into the ventral cavity of their abdomen, that is then trapped in the fluid of the Malpighian tubules, resulting in the creation of bubbles at the end of the abdomen (Weaver and King 1954). The foam comprises a liquid derived from the plant in which the insect feed on, and surface-active molecules that reduce surface and interfacial tension to form emulsions (Marshall 1964; Mello et al. 1987). It has been proposed that the foam creates a protective microhabitat to the nymphs against high temperatures, desiccation and natural enemies (Whittaker 1970; del Campo et al. 2011).

The creation of microhabitats is, in fact, an important and efficient strategy used by insects to regulate their body temperature (May 1979). Eusocial hymenopterans and termites construct elaborate nests which reduce the stress caused by environmental temperature fluctuation (Korb 2003; Kadochová and Frouz 2013). Cicada nymphs are able to build below ground tunnels where they live for years under favorable thermal conditions (Williams 1995). Due to that, it is not surprising to speculate the foam produced by cercopid nymphs is a potential thermoregulation mechanism for these insects. However, experimental evidence presenting the foam as a thermoregulatory adaptation for spittlebugs is sparse (Whittaker 1970).

Reports of predation on cercopid nymphs are relatively rare, and previous works demonstrated that the nymphs are safer when inside the foam than when exposed to the environment (Whittaker 1970; del Campo et al. 2011). Indeed, insects that surround themselves with their waste products of feces have been shown to increase their protection from natural enemies' attack. For example, the beetle larva of *Hemisphaerota cyanea* constructs a thatch from long filamentous fecal strands as a physical protection against predators (Eisner and Eisner 2000). Furthermore, the use of chemical defensive secretions in insects is widespread, in which the secretions can act as a repellent or irritant to the predators (Eisner and Meinwald 1966). So far, little is known about the chemical composition of foam from spittlebugs (Mello et al. 1987; del Campo et al. 2011). Identifying the biochemical components may provide insights into the capacity of foam to protect the insect against natural enemies.

The spittlebug *Mahanarva fimbriolata* has greatly increased their population and become a serious pest in Brazilian sugarcane crops after sugarcane harvest with the use of burning have been prohibited (Dinardo-Miranda and Gil 2007). The adults of this insect feed

on and inject toxins into leaves, whereas the nymphs develop on the exposed roots on the soil surface or below ground and suck the xylem content of roots (Leite et al. 2005), blocking the water and nutrients transport and causing physiological disorder (Garcia et al. 2007; Dinardo-Miranda et al. 2014).

Since the nymphs of *M. fimbriolata* live close to the soil and the conditions in the foam are humid, there is a continuous threat of microbiological infestation, mainly because the entomopathogenic fungus *Metarhizium anisopliae* naturally occurs in areas of sugarcane where *M. fimbriolata* develops (Rezende et al. 2015). The strategy used by the nymphs to protect themselves from the attack of entomopathogenic microorganisms is poorly understood. It is widely recognized, however, that the association between insects and beneficial microorganism for a protective adaptation are common (Kaltenpoth et al. 2005; Kaltenpoth 2009; Kaltenpoth and Engl 2014). The mechanism by which the symbiont can protect their host includes the exclusion of pathogenic microbes by competition for resources, activation of the insect host immune system, and the production of secondary metabolites with antibiotic properties (Flórez et al. 2015). In this case, the Actinobacteria are the main group of bacteria that have been found to defend insects against microorganism by producing antimicrobial compounds in different groups of insects (Kaltenpoth 2009). Therefore, a promising candidate for such a protective function in nymphs of *M. fimbriolata* is the presence of beneficial microorganisms in the foam covering the nymphs, since little is known about the microbiome of this species and the bacteria present in the foam.

Based on that, the aims of this thesis were: (*i*) to examine the role that the foam produced by *M. fimbriolata* nymphs may play in their thermoregulation, and identify their principal chemical compounds; (*ii*) to assess the composition of the bacterial community present in the gut and foam produced by *M. fimbriolata* nymphs, and in the soil close to the foam in a sugarcane field; and (*iii*) to investigate the importance of foam produced by *M. fimbriolata* in protecting the nymphs against predators.

The results showed that the foam forms a thermal microhabitat and it was also discussed the importance of the compounds found in our chemical analysis as surfactants to stabilize this protective thermal layer of bubbles around the nymphs. Also, it was demonstrated that the foam produced by *M. fimbriolata* nymphs harbor a diversity of bacteria previously reported as protective symbionts of insects. Finally, this thesis provides evidence that the foam serves as a repellent to a relevant predator and topical irritant to another insect, and that the irritant propriety of foam derives, at least in part, from the fatty acids identified in the chemical analysis.

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2. SPITTLEBUGS PRODUCE FOAM AS A THERMOREGULATORY ADAPTATION

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Abstract

Insects have evolved multiple mechanisms to adapt to variations in environmental temperatures, including postural control of solar input, variations in diurnal activity, external morphological structures and selecting/generating microhabitats. Foam produced by *Mahanarva fimbriolata* nymphs (also known as root spittlebugs) was found to aid in creating a constant thermal microhabitat despite environmental temperature fluctuations. The temperature within the foam was found to be similar to that of soil during the day and remained constant despite fluctuating external temperatures. In chemically analysing the composition of the foam, palmitic and stearic acids, carbohydrates and proteins were detected. These substances have previously been shown to act as a surfactant to stabilize and modulate foams. Since the immature ancestor of the spittlebug developed below ground, it is speculated that the foam may function as an 'extension' of the soil and, thus, may have enabled the spittlebug to emerge from the soil and adopt an epigean lifestyle.

Keywords: *Mahanarva fimbriolata*; Temperature; Cercopidae; Froth; Fatty acids

2.1. Introduction

As insects are ectothermic (i.e., the internal temperature of the body varies according

to the air temperature), they have evolved different mechanisms to regulate body temperature (May 1979). One common adaptation for thermoregulation is the creation of microhabitats. For example, eusocial hymenopterans and termites build elaborate nests to reduce the stress caused by environmental temperature fluctuations (Korb 2003; Kadochová and Frouz 2013). Cicada nymphs build below ground tunnels that allow them to live for years under favourable thermal conditions (Williams 1995). In contrast, nymphs of spittlebugs, a group that is phylogenetically closely related to cicadas, can be found below the soil surface (Cryan 2005; Leite et al. 2005), at ground level (Cryan and Svenson 2010) or even far above the soil surface (Thompson 1997). How they maintain a constant/suitable body temperature without the protection of ground insulation has not been previously reported.

Phylogenetic studies have shown that the first spittlebugs evolved approximately 200 million years ago from an ancestor in which nymphs developed below ground (Dietrich 2002; Rakitov 2002; Cryan and Svenson 2010). However, unlike the closely related cicadas, the front legs of spittlebug nymphs are not strong enough to burrow into the soil (Rakitov 2002). One potential mechanism that has been proposed for thermoregulation in spittlebugs is the foam that they produce and cover themselves with (commonly referred to as 'cuckoo spit') (Guilbeau 1908; Marshall 1973). The nymphs produce foam by sucking air into the ventral cavity of their abdomen, that is then trapped in the fluid of the Malpighian tubules, resulting in the creation of bubbles in the terminal part of the abdomen (Guilbeau 1908; Weaver and King 1954). The foam comprises liquid, air, and surface-active molecules that reduce surface and interfacial tension to form emulsions (Marshall 1973; Mello et al. 1987). The liquid in the foam is derived from the plant sap upon which the nymphs feed (Wilson and Dorsey 1957).

Interestingly, some amphibians, such as frogs, produce foam that protects their eggs and embryos against predation and desiccation while maintaining temperature and oxygen at appropriate levels (Seymour and Loveridge 1994; Haddad and Hödl 1997; Fleming et al. 2009). Even though a similar function has been proposed for spittlebug foam (Whittaker 1970; del Campo et al. 2011), experimental evidence of thermoregulation by their foam has not been shown.

The chemical composition allowing for a rigid bubble architecture in spittlebug foam is poorly understood (Mello et al. 1987). Identifying the biochemical components may provide insight into the capacity of froth to contribute to thermoregulation. Proteins, carbohydrates and lipids can stabilize foams (Arakawa and Timasheff 1982; Bos and van Vliet 2001; Margolles Cabrales et al. 2003; Herceg et al. 2007), however little is known about the presence and quantity of these substances in spittlebug foam.

Mahanarva fimbriolata are spittlebugs that feed on sugarcane roots (Dinardo-Miranda et al. 2007). These cercopids develop on the exposed roots on soil surface or below ground (Leite et al. 2005) and form a distinctive foam when in the nymph stage. Here we examined the role that foam may play in thermoregulation for spittlebug nymphs.

2.2. Materials and methods

Thermal microhabitat

To determine whether the foam covering *M. fimbriolata* nymphs has a thermoregulatory role, we conducted a field bioassay during the summer of 2015 in a sugarcane field in Piracicaba, São Paulo, Brazil (22°42'06"S, 47°33'50"W). The sugarcane plants were approximately 2 m tall, with 1 m between rows. We selected 25 sites inhabited by foam-covered fourth- and fifth-instar nymphs of *M. fimbriolata*, maintaining a minimum distance of 10 m between each site. Using a type K thermocouple (RDXL4SD, Omega Engineering, Stamford, CT, USA), we measured the temperature inside the foam, 0.1 m from foam and ground, 2.5 m and 1.5 m above ground, and 0.1 m below ground level at five times intervals that represented the natural variation in temperature during the day: 07h00–09h00, 09h00–11h00, 11h00–13h00, 13h00–15h00 and 15h00–17h00 (Fig. 1). We also constructed a thermogram using an infrared camera (SC640 FLIR Systems, Boston, MA, USA) and analysed the temperature of the thermographic images using the ThermaCAM Researcher 2.9 software (FLIR Systems, Boston, MA, USA).

To investigate if the thermoregulation occurs at higher environmental temperatures than those achieved in the field experiments, we evaluated the thermophysiology of the foam under controlled conditions using a growth chamber (ELETROLAB, São Paulo, SP, Brazil). Fourth- and fifth-instar nymphs of *M. fimbriolata* were collected from the same field and carefully transferred to the roots of sugarcane plants aged 25–30 days growing in pots (200 ml) containing organic substrate (Golden-Mix, Ananindeua, PA, Brazil), with one nymph per plant, for a total of 25 replicates. The plants and insects were initially equilibrated at room conditions (25 ± 2 °C, 70 ± 10% UR) for 30 min before nymphs began to produce foam. Next, they were arranged within the growth chamber at a temperature of approximately 32 ± 0.11 °C. After 30 min of acclimation and temperature stabilization, we recorded the temperatures in the chamber, 1 cm below the surface of the soil in the pots, and inside the foam using the type K thermocouple.

Fatty acid analysis

To analyse the fatty acids present in the foam, five foam samples were collected in the same sugarcane field cited above, placed in glass vials (10 ml) and stored at –30 °C until analysis. Following an extraction sequence, 1 ml of each sample was derivatised through the application of ethyl chloroformate (Qiu et al. 2007). At the end of the derivatisation process, each sample was adjusted to 0.5 ml with cyclohexane as the solvent. Each sample received 5 µl of octacosane (internal standard solution at 1000 ng μl^{-1}) (Sigma-Aldrich, St Louis, MO, USA). The derivatised samples were initially analysed by gas chromatography-flame ionization detection (GC-FID, Shimadzu GC-2010, Kyoto, Japan) using an HP-1 capillary column (Agilent Scientific, Santa Clara, CA, USA; 30 m × 0.25 mm × 0.25 µm). A 1 µl aliquot of each sample was injected in the splitless mode with an injector temperature of 240 °C using helium as the carrier gas. The column temperature was held at 60 °C for 1 min and then increased to 320 °C (15 °C min^{-1}) and held for 10 min. The extract with the best resolution was reanalysed with a gas chromatograph coupled to a mass spectrometer (GC-MS, Varian 4000, Palo Alto, CA, USA) using an HP5-MS column (JeW Scientific, Folsom, CA, USA; 30 m × 0.25 mm × 0.25 µm) and helium as the carrier gas. Injection (1 µl aliquot) was conducted in the splitless mode, and the column temperature programme was the same as that described for the GC-FID procedure above. The two major peaks were identified by comparing their mass spectra with those of the NIST 98 library and confirmed by co-injecting the authentic standards (Sigma-Aldrich, St Louis, MO, USA) with the sample. Amounts were estimated based on the peak area relative to the amount of internal standard (octacosane) and corrected according to the volume of foam used for the derivatisation.

Carbohydrate analysis

Total carbohydrate was estimated using the phenol-sulfuric acid method (Dubois et al. 1956). Briefly, five samples of foam produced by *M. fimbriolata* nymphs were collected, and an aliquot of 1 ml of each sample was vortexed with 25 µl of phenol (80% m/v) and 2.5 ml of sulfuric acid. The resulting mixture was allowed to stand for 20 min and then vortexed again. For the control, we used 1 ml of distilled water and followed the same steps as above. The absorbance of the mixed samples was measured spectrophotometrically using a UV mini 1240 Shimadzu (Shimadzu, Tokyo, Japan) at a wavelength of 490 nm. The total concentration was calculated based on a standard curve using glucose (0.01 mg l^{-1}) in the range of 100–1000 µg.

Protein analysis

Total protein present in the foam was quantified according to the Bradford technique

(Bradford 1976). Five samples of foam were collected in the same sugarcane field and 50 µl of each sample was dissolved in 3.95 ml of Milli-Q water, and 1 ml of Bradford reagent (Coomassie Brilliant Blue G with phosphoric acid and methanol) was added. The absorbance of the samples was measured using a spectrophotometer UV mini 1240 Shimadzu (Shimadzu, Tokyo, Japan) at a wavelength of 595 nm. As a control, we used 4 ml of Milli-Q water and 1 ml of protein reagent. The total protein concentration in the foam was calculated based on a standard curve for bovine serum albumin (BSA) at intervals of 0.02 to 0.3 mg.

Statistical analysis

The normality and homogeneity of the temperatures recorded in the field observations and in the laboratory assay were analysed using Kolmogorov-Smirnov and Bartlett tests. To limit the experiment-wise error rates to acceptable level in multiple comparisons with a low number of related groups, means the temperatures of the foam and those of other recorded sites were compared using one-way ANOVA followed by a Bonferroni post hoc test ($P < 0.05$) (Armstrong 2014). All analyses were performed using the SAS statistical software (SAS Institute 2011).

2.3. Results

Foam as a thermal microhabitat

To determine whether the internal temperature of spittlebug foam changes with fluctuations in external temperature, several local temperatures were monitored including: outside the foam, ground temperature near the foam and inside the foam. These temperature recordings were performed during summer in a sugarcane field where nymphs reside. While external temperatures ranged from 24.43 ± 0.44 °C to 29.20 ± 1.66 °C, the internal foam temperature was found to vary to a much smaller degree (Fig. 1). In the middle of the day (11h00–13h00) when the external temperature was 29.20 ± 1.66 °C, the foam temperature was observed to be significantly lower at a temperature of 25.18 ± 0.63 °C (Fig. 1, one-way ANOVA followed by a Bonferroni post hoc test, $n = 25$, $F_{(1, 100)} = 88.763$, $P < 0.0001$) (Detailed values of means ± SD corresponding to Fig.1 are shown in Supplementary Table S1). Indeed, despite fluctuating external temperatures, a uniform foam temperature was observed throughout the day (i.e. 25 ± 0.78 °C (mean ± SD)). Specifically, a previous investigation found that 25 °C resulted in the greatest nymph viability (Garcia 2006). In monitoring over 10 hours during the

day, the surface temperature difference between the foam and the soil was ≤ 0.2 °C while the maximum difference between foam and the external temperature was ≥ 4.0 °C. This indicates that the temperature of the soil and the foam are similar (Fig. 1, one-way ANOVA followed by a Bonferroni post hoc test, n = 25, $F_{(1, 100)}$ = 0.008, P = 0.928). Using thermograms obtained from an infrared camera, the difference in temperature near the foam can be visualized (Fig. 2).

To more rigorously examine the thermocapacity of the foam, nymphs were evaluated in a growth chamber in which the temperature was controlled and elevated above normal field conditions. When the growth chamber temperature was raised to 32.29 ± 0.58 °C (mean ± SD) for 30 min, foam temperature remained at 30.41 ± 1.01 °C (mean ± SD) which is approximately 2 °C below the air temperature (Fig. 3, one-way ANOVA followed by a Bonferroni post hoc test, n = 25, $F_{(1, 25)}$ = 57.220, P < 0.0001). These results indicate that the foam acts as a thermoregulator at 32 °C, which has previously been shown to be lethal for nymphs (Garcia 2006). The combined field and laboratory data indicate that nymph foam production (Supplementary Movie 1) results in a relatively constant internal temperature at a wide range of external air temperatures that creates a suitable thermal microhabitat for nymph survival.

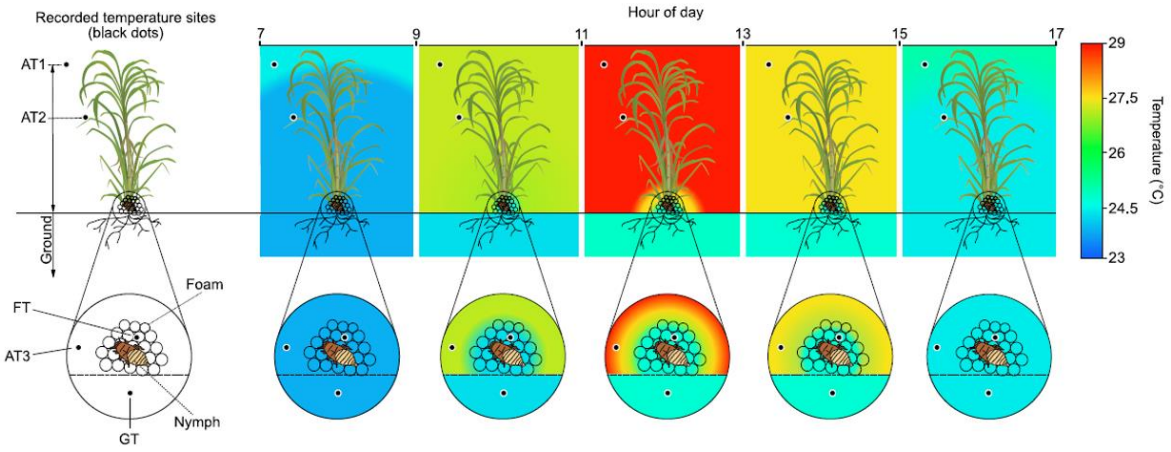

Figure 1. Thermal variation in the foam produced by *Mahanarva fimbriolata* nymphs. Temperature variation in the foam produced by *M. fimbriolata* nymphs and their environmental surroundings during a hot summer's day in a sugarcane field in Piracicaba, São Paulo, Brazil. Different colours indicate significant differences between recorded temperature sites within the same sampling time, according to one-way ANOVA followed by a Bonferroni post hoc test (P < 0.05) (n = 25). AT1 = air temperature at 2.5 m above ground; AT2 = air temperature at 1.5 m above ground; AT3 = air temperature at 0.1 m from foam and ground; FT = temperature inside foam; GT = temperature 0.1 m below ground.

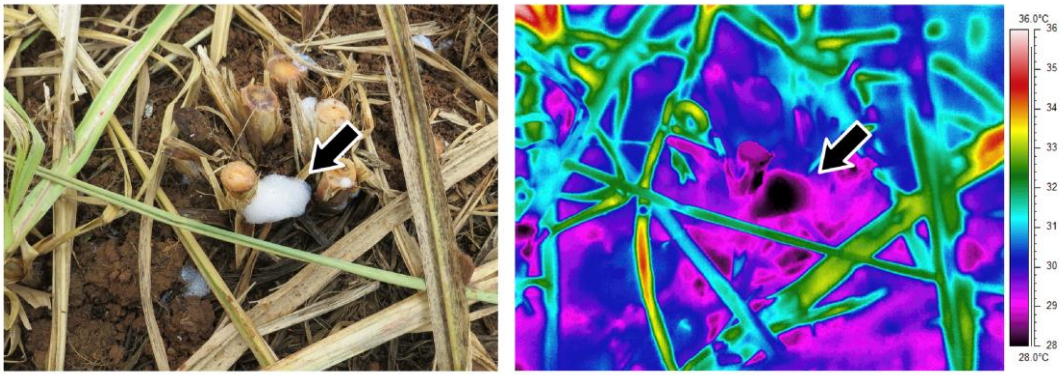

Figure 2. The foam promotes insect thermal protection. Conventional and corresponding infrared photographs of the foam produced by *Mahanarva fimbriolata* nymphs and of their surroundings in a sugarcane field in Piracicaba, São Paulo, Brazil, at 13h00 on a hot summer's day. The photographs show the importance of the foam to maintaining the microhabitat temperature lower than the surroundings temperature.

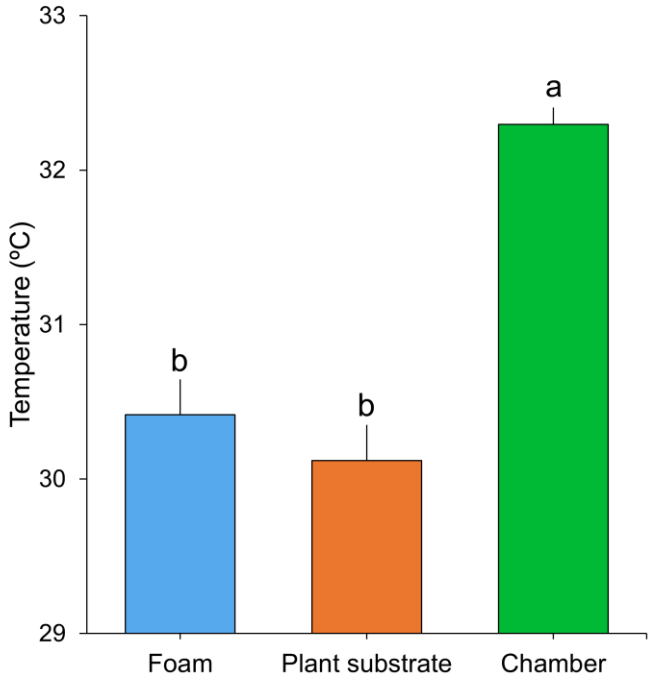

Figure 3. The temperature of spittlebug foam under controlled conditions. Comparison of the temperature of the foam produced by *Mahanarva fimbriolata*, plant substrate (soil) and surrounding air temperature in a growth chamber. Bars represent the temperature mean ± SD. Bars with different letters are significantly different according to one-way ANOVA followed by a Bonferroni post hoc test ($P < 0.05$) ($n = 25$).

Foam chemical composition

Using gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) two major peaks were identified as palmitic acid and stearic acid; co-injection with commercial standards confirmed their identity. Amounts of palmitic and stearic acid in the foam were 2.54 ± 0.88 and 2.78 ± 0.97 µg ml^{-1} of foam (means ± SE), respectively. Total carbohydrates were quantified by means of the phenol-sulfuric acid

method (Dubois et al. 1956), with 0.579 ± 0.05 µg ml^{-1} of foam (mean ± SE). Based on the Bradford method (Bradford 1976) total protein was 320 ± 50 µg ml^{-1} of foam. These components are recognized as important substances in the formation and stabilization of foam bubbles (Malysa et al. 1991; Pueyo et al. 1995; Murray and Ettelaie 2004; Mecozzi and Pietroletti 2016), and as such are likely necessary for maintaining a stable bubble layer around nymphs.

2.4. Discussion

Insects have evolved complex mechanisms to regulate their body temperature within a remarkably narrow range for successful survival and reproduction (Sanborn et al. 1992; Shi et al. 2015; Li et al. 2016). Here, we showed that *M. fimbriolata* nymphs produce and cover themselves with foam as a thermoregulatory adaptation that enable spittlebugs to maintain their body temperature within the optimal range for development (Garcia 2006). The temperature measured inside the foam was similar to the soil even though the air temperature in both the field and growth chamber varied to a much greater extent (Figs 1 and 3). Since spittlebug and some below ground insects are thought to share a common ancestor (Rakitov 2002; Cryan and Svenson 2010), insect-produced foam may serve as an 'extension' of the soil and enable immature spittlebugs to exploit food sources for above ground feeding. Without such a domestic protection, delicate cuticles would leave spittlebug nymphs vulnerable to adverse abiotic epigean environmental factors, such as high temperature and low humidity (Whittaker 1970).

Lipids, carbohydrates, and proteins were detected in *M. fimbriolata* foam similar to the foam composition of other spittlebug species (Wilson and Dorsey 1957; Kato 1958; Marshall 1973; Mello et al. 1987; Auad et al. 2012). Proteins were detected in most foams analysed (Wilson and Dorsey 1957; Marshall 1973; Mello et al. 1987; Auad et al. 2012) whereas lipids are much less common being observed only in the foam of Japanese spittlebugs (Kato 1958). While the chemical analysis has not been shown to directly provide thermal protection, lipids, carbohydrates and proteins that are present in spittlebug foam has previously been shown to function as a surfactant to stabilize the foam thereby reducing surface tension and modulating the size and distribution of bubble (Pueyo et al. 1995; Murray and Ettelaie 2004; Mecozzi and Pietroletti 2016). Lipids, including palmitic and stearic acids, are critical for the formation and stability of foams because of their elastic forces (Malysa et al. 1991). For example, palmitic, linolenic and pentadecanoic acid have been positively correlated with the height of cider-type beverage foams (Margolles Cabrales et al. 2003). Proteins are involved,

especially in the formation of film that reduce interfacial tension and increase the viscosity and elasticity of a foam (Hailing and Walstra 1981; Zayas 1997; Martin et al. 2002), which allows the foam to breathe and secure around the insect. Although carbohydrates have no direct effect on the air-water interface, they promote interactions among proteins, which create a stable film that stiffens and stabilizes the foam (Adebowale and Lawal 2003).

While the mechanism behind the observed thermal protection is proposed to be due to thermal insulation the extent in which evaporative cooling may play a role in controlled temperature conditions still needs to be investigated. Evaporative cooling has been demonstrated for hemipterans that feed on xylem and do not produce foam, such as the cicada *Okanagodes gracilis*, that regulate their body temperature by water loss through pores in the dorsal thorax and abdomen (Sanborn et al. 1992). Interestingly, for foam-producing *Aphrophora saratoga* nymphs, water evaporation from foam was demonstrated be lower than from free-water surface, making the foam an uncertain protection against desiccation for this species (Turner 1994). In future studies, by measuring temperature of dried cercopid foam differentiating these two mechanisms experimentally should be possible.

In summary, nymph-produced foam forms a microhabitat for the thermoregulation of *M. fimbriolata* nymphs. Future investigations on physical properties of the foam, especially optical reflection and heat dissipation will provide further insights into the phenomenon reported here.

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Supplementary information

Supplementary Table S1: Temperature (°C) variation (mean ± SD) in the foam produced by *Mahanarva fimbriolata* nymphs and the environmental surroundings during a summer day in Piracicaba, São Paulo, Brazil. Different letters indicate significant difference between sampled sites within each time interval, based on a one-way ANOVA followed by a Bonferroni post hoc test ($P < 0.05$) ($n = 25$).

Recorded temperature sites	Hour of day				
	07h00–09h00	**09h00–11h00**	**11h00–13h00**	**13h00–15h00**	**15h00–17h00**
AT1	24.43 ± 0.44 a	27.13 ± 1.04 a	29.20 ± 1.66 a	27.54 ± 0.82 a	25.28 ± 0.68 a
AT2	24.09 ± 0.35 b	26.87 ± 1.00 a	28.67 ± 1.55 a	27.86 ± 0.69 a	25.23 ± 0.59 a
AT3	23.94 ± 0.24 b	26.47 ± 0.92 a	27.67 ± 0.94 b	27.61 ± 0.59 a	25.38 ± 0.52 a
FT	23.56 ± 0.29 b	24.64 ± 0.66 b	25.18 ± 0.63 c	25.24 ± 0.47 b	24.57 ± 0.36 b
GT	23.57 ± 0.24 b	24.51 ± 0.76 b	25.15 ± 0.61 c	25.13 ± 0.49 b	24.86 ± 0.44 b

AT1 = air temperature at 2.5 m above ground; **AT2** = air temperature at 1.5 m above ground; **AT3** = air temperature at 0.1 m from foam and ground; **FT** = temperature inside foam; **GT** = temperature 0.1 m below ground.

3. FOAM BACTERIAL COMMUNITY IS LINKED WITH THE GUT OF NYMPHS OF THE SPITTLEBUG *Mahanarva fimbriolata*

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Abstract

The development of insects is strongly influenced by their resident microorganisms. Symbionts play key roles in insect nutrition, reproduction and defense. Bacteria are important partners due to the wide diversity of their biochemical pathways that aid in the host development. Here, we present evidence that the foam produced by nymphs of the spittlebug *Mahanarva fimbriolata* harbors a diversity of bacteria, including some that were previously reported as defensive symbionts of insects. Analysis of the microbiomes in the nymph gut and the soil close to the foam showed that the microorganisms in the foam were more closely related to those in the gut than in the soil, suggesting that the bacteria are actively introduced into the foam by the insect. Proteobacteria, Actinobacteria and Acidobacteria were the predominant groups found in the foam. Since members of Actinobacteria have been found to protect different species of insects by producing secondary metabolites with antibiotic properties, we speculate that the froth produced by *M. fimbriolata* may aid in defending the nymphs against entomopathogenic microorganisms.

Keywords: Hemiptera; Symbiosis; Actinobacteria; Microbiome; Bacterial diversity

3.1. Introduction

Spittlebugs are cercopid insects that in the nymph stage produce a distinctive foam while they feed on the host plants (Guilbeau 1908; Kato 1958). The foam is produced by

sucking air into the ventral cavity of the abdomen, which is then trapped in the fluid of the Malpighian tubules, resulting in the formation of bubbles in the terminal part of the abdomen (Guilbeau 1908; Weaver and King 1954). The foam comprises liquid, air and surface-active molecules that reduce surface and interfacial tension to form emulsions (Marshall 1973; Mello et al. 1987; Tonelli et al. 2018).

Several functions have been attributed to the foam, such as the protection of nymphs against high temperatures (Tonelli et al. 2018), desiccation (Whittaker 1970) and natural enemies (Whittaker 1970; del Campo et al. 2011). The spittlebug *Mahanarva fimbriolata* feeds on sugarcane roots exposed on the soil surface or belowground (Leite et al. 2005; Dinardo-Miranda et al. 2007). Because of the humid nature of the foam and its proximity to the soil, there is a continuous threat of fungal and bacterial infestation, such as by the entomopathogenic fungus *Metarhizium anisopliae*, which occurs naturally in soils of sugarcane fields where *M. fimbriolata* develops (Rezende et al. 2015). The means by which the nymphs are protected from microbial attack during this stage are little understood. Associations between insects and beneficial microorganisms as a protective adaptation are common (Ferrari et al. 2004; Oh et al. 2009; Douglas 2015; Flórez et al. 2015). The mechanisms by which symbionts can protect their hosts from natural enemies are diverse and not mutually exclusive, and include competition from nutrients or space; activation of the insect immune system, which may be more deleterious to the invader than to the resident microbiome; and the production of secondary metabolites with antibiotic properties (Douglas 2015; Flórez et al. 2015). An example of this last is the females of the European beewolf (*Philantus triangulum*), which cultivate *Streptomyces* spp. in specialized antennal glands and apply them to the brood cell prior to oviposition in sandy soil, where the bacteria protect against the attack of entomopathogenic microorganisms. Survival of the nymphs is reduced from 80% to 10% if the *Streptomyces* cells are removed (Kaltenpoth et al. 2005).

Actinobacteria is the main group of bacteria that have been found to defend insects against microorganisms, by producing antimicrobial compounds (Kaltenpoth 2009). Proteobacteria, including Gammaproteobacteria and Alphaproteobacteria, are also involved in protecting some hemipterans against natural enemies (Ferrari et al. 2004; Scarborough et al. 2005; Mahadav et al. 2008). Therefore, one possible means for such a protective function in *M. fimbriolata* would be the presence of beneficial microorganisms in the foam covering the nymphs.

It has long been recognized that the gut microbiome is involved in the growth, development and adaptation of the insect host (Dillon and Dillon 2004; Krishnan et al. 2014;

Lewis and Lizé 2015). The insect gut can be considered a portion of the external environment in which the conditions and resources are controlled by the insect (Douglas 2011). A community of Actinobacteria has been reported in the gut of some hemipterans, such as firebugs and pentatomids (Zucchi et al. 2012; Salem et al. 2013). Spittlebugs are widely recognized as dependent on symbiosis with bacteria for nutritional reasons (Moran et al. 2005; Koga et al. 2013; Koga and Moran 2014). However, little is known about the microbiome in the gut of *M. fimbriolata* nymphs.

The overall aim of this study was to assess the diversity and composition of the bacterial community present in the foam produced by nymphs of *M. fimbriolata*, comparing it to communities found in their gut and in the soil close to the foam. We also discuss the likely protective role of bacteria in the foam, based on their taxonomic group.

3.2. Materials and methods

Foam, soil and gut sampling

Foam produced by fourth- and fifth-instar nymphs of *M. fimbriolata* was collected in a sugarcane field in Piracicaba, Brazil (22°42′06″S, 47°33′50″W). For comparison, the soil close to the foam was also collected. For the analysis of gut contents, fourth- and fifth-instar nymphs were collected and carefully transferred to the roots of sugarcane plants for transport to the laboratory and subsequent dissection. Insects were surface-sterilized using 0.5% sodium hypochlorite in 70% ethanol (v/v), followed by washes in sterile deionized water under aseptic conditions. Surface-sterilized nymphs were dissected in a laminar-flow hood in sterile 0.85% NaCl saline solution to obtain the whole guts. Pools of guts, each from five insects, were placed in tubes (1.5 ml) containing 1 ml of absolute ethanol (Scopel and Cônsoli 2018). A total of 15 samples were analyzed (five replicates from each environment, i.e., foam, gut and soil). All samples were maintained at –20 °C prior to DNA extraction.

DNA extraction of total bacterial community

DNA from the samples of foam, gut and soil was extracted using the Power Soil DNA Isolation kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. For gut samples, the material was removed from the freezer, triturated, and the triturated material was used in the extraction procedure. In all cases, aliquots of 0.5 g were used for DNA extraction. DNA preparations were visualized by electrophoresis in 1% agarose gel in 1×TAE (Tris-

Acetate-EDTA buffer) to assess yield and integrity. The samples were then stored at –20 °C until the following analysis.

Quantification of total bacterial community

The abundance of the total bacterial community was estimated by using the 16S rRNA partial gene as a proxy. Each sample was quantified twice, using the StepOne Real Time System (Applied Biosystems) with SYBR Green I. The reaction was performed in 25 µl of the reaction mixture from a Power SYBR Green PCR Master Mix (Applied Biosystems, Frankfurt, Germany), 0.5 µl of each primer (100 µm), and 1 µl of target DNA (≈ 10 ng). The primers used were P1/P2 (Muyzer et al. 1993) and the thermal cycling conditions were 35 cycles at 94 °C for 30 s of denaturation, followed by 55 °C for 30 s of annealing and 72 °C for 30 s in a final extension. The standard curve was constructed using serial dilutions (108 to 101) of the PCR product of the environmental soil samples. The amplification efficiency was 92.60% and r^2 = 0.995. Data from the DNA amplification was interpolated on a standard curve and the number of copies of the target gene was calculated in relation to ng of DNA in each sample. Specificity of the amplification products was confirmed by melting-curve analyses, and the expected sizes of the amplified fragments were checked in 1% agarose gel.

Sequencing of the 16S rRNA gene

The DNA extracted from the foam, gut and soil samples (3 replicates of each) was used for PCR amplification, using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al. 2013) (coupled with Illumina adapters), which cover the hypervariable regions V3-V4 of the 16S rRNA gene. Amplification, pooling and purification were performed at the University of São Paulo, Brazil (http://genfis40.esalq.usp.br/multi/). All samples were sequenced using a MiSeq Platform and the Nextera XT index kit for library preparation (Illumina, USA).

Next-generation sequence analysis

The sequenced paired-end reads were separated by sample and analyzed using the QIIME software and BPM pipeline (Caporaso et al. 2010; Pylro et al. 2014). Quality-control analyses were performed to eliminate low-quality reads, short reads and chimeric sequences, and to trim the low-quality 3’ region of individual reads in order to achieve a minimum quality of Q28 (Phred scale). The remaining sequences were clustered in operational taxonomic units (OTUs) at 97% sequence identity using VSEARCH 6.1 (v6.1.544), followed by selection of a

representative sequence for each OTU (Edgar 2010). The reads were then aligned with the Greengenes Core Set (DeSantis et al. 2006), using the PyNAST algorithm, and filtered. An OTU table was generated, singletons were excluded, and the OTU table was rarefied (50,000 sequences per sample) to prevent bias related to the different number of reads in the samples. All sequences have been deposited to the MG-RAST under accession number mgm4796924.3, mgm4796929.3, mgm4796930.3, mgm4796931.3, mgm4796932.3, mgm4796933.3, mgm4796934.3, mgm4796935.3, mgm4796936.3.

Statistical analyses

Statistical analyses were performed using the software R (R Core Team 2017). Significant differences in gene abundance were identified by analysis of variance (ANOVA) followed by Tukey's post-hoc test, using the "ExpDes" package (Ferreira et al. 2014). The differential abundance of bacterial groups was analyzed using the "edgeR" package (McMurdie and Holmes 2014). A Venn diagram was constructed to determine the proportion of groups that were exclusive and shared between samples, using the webtool developed by Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/). Principal Coordinates Analysis (PCoA) ordination was performed based on the Bray-Curtis ecological distance. The matrix originated by the PCoA was used to calculate the Permutational Multivariate Analysis of Variance (PERMANOVA). These analyses were performed based on OTU-generated matrices exported into Microbiome Analyst (Dhariwal et al. 2017).

3.3. Results

Abundance of total bacterial community

The abundance of the total bacterial community at the sampled sites ranged from 8.610 to 9.938 log of copies of 16S rRNA gene / g of sample (Table 1). Bacteria were most abundant in the soil samples (log values of 9.938 copies of 16S rRNA / g of soil). Bacterial abundance was similar in the foam and gut samples (log values of 8.703 and 8.610 copies of 16S rRNA, respectively), with no statistical difference according to the ANOVA + Tukey test.

Table 1. Abundance of the total bacterial community present in soil close to the foam in a sugarcane field, in the foam produced by *Mahanarva fimbriolata* nymphs, and in the gut of nymphs (n = 5 per sampled site).

Samples	Log of copies of 16S rRNA / g of sample*
Soil	9.938 ± 0.01^{a}
Foam	8.703 ± 0.03^{b}
Gut	8.610 ± 0.02^{b}

*Different letters correspond to statistical differences between means detected by ANOVA + Tukey test ($P < 0.05$).

Bacterial communities in foam, gut, and soil samples

After quality filtering, a total of 50,000 high-quality sequences per sample were obtained. A mean Good's coverage of 99% was determined, indicating that the dataset was representative of the bacterial community analyzed (Supplementary Table S1).

Evaluating the differences in microbial community structure, the relative abundance of OTUs in different samples was used to compute a Bray-Curtis dissimilarity matrix coordinated with PCoA. The contrasting patterns observed indicated that the microbiome was composed differently in the three environments sampled (Fig. 1). The main difference was between the foam samples from gut and from soil (observed on the first axis), while the difference between gut and soil samples was observed when two axes were used to plot samples (Fig. 1). The replicates of soil samples showed higher variability than the replicates from the other sites (gut and foam), indicating a stricter selection of the microbial communities composing the foam and gut microbiomes. The differences among the communities were further confirmed by PERMANOVA (F-value = 3.363; $r^2 = 0.528$; $P < 0.006$).

The Venn diagram showed that the majority of OTUs were found in only one environment. Twenty-eight OTUs were shared among the foam, gut and soil samples. Forty-eight OTUs were shared between samples of foam and gut, while foam and soil samples shared twenty-four OTUs, and soil and gut shared only eight OTUs. Foam samples hosted 257 specific OTUs, while gut samples hosted 291 specific OTUs and soil hosted 288 specific OTUs (Fig. 2).

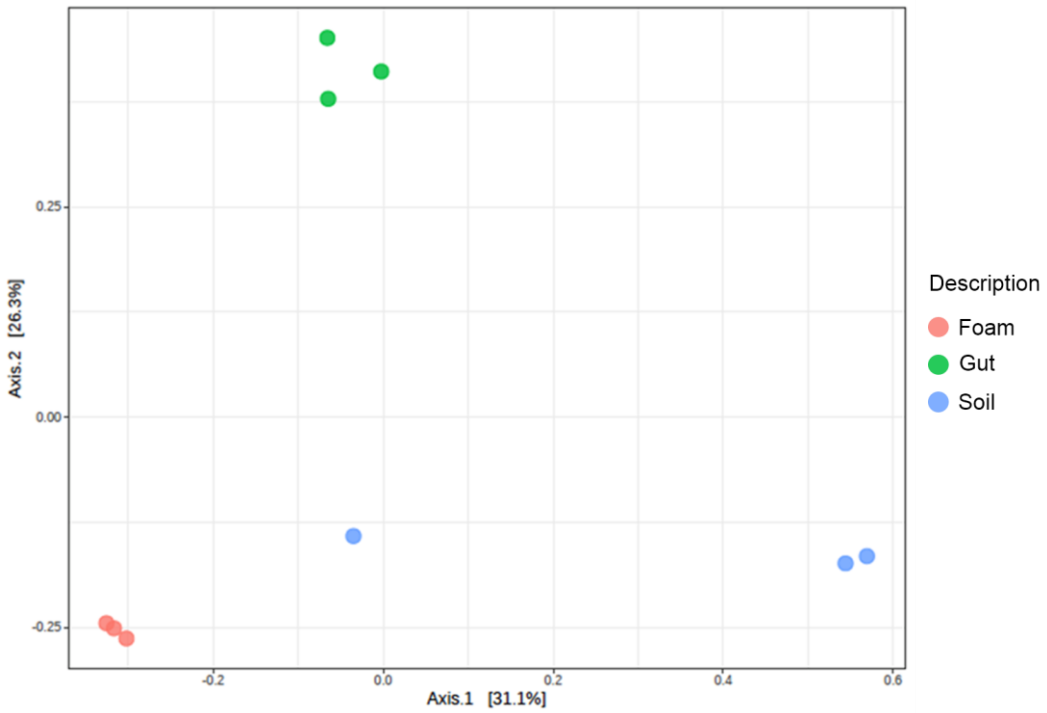

Figure 1. Principal coordinates analysis (PCoA) based on the differences in microbial community structures in the foam produced by *Mahanarva fimbriolata* nymphs, in the gut of nymphs, and in the soil close to the foam in a sugarcane field. The values on the axes indicate the percentage of the variance represented on each axis. Distinctions among communities were confirmed by PERMANOVA (F-value = 3.363; $r^2 = 0.528$; $P < 0.006$).

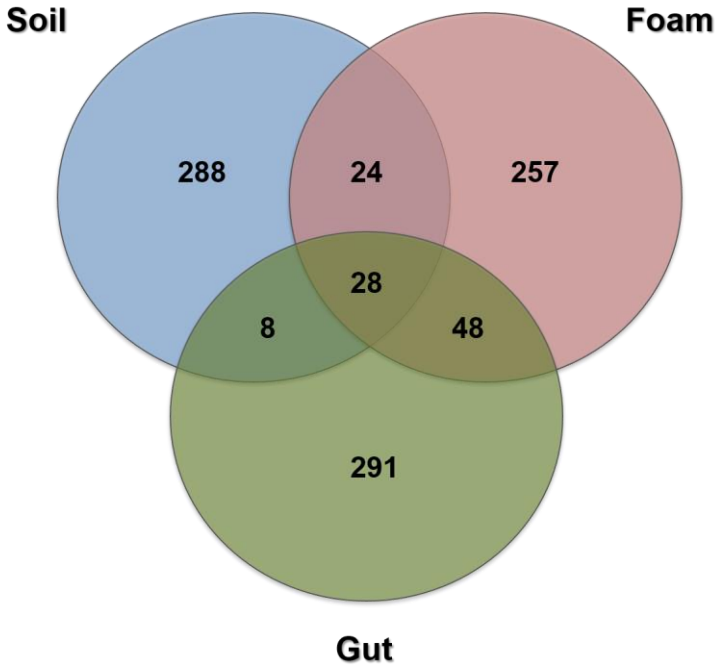

Figure 2. Venn diagram constructed to determine the proportion of operational taxonomic groups (OTUs) of bacteria exclusive to and shared in the foam produced by *Mahanarva fimbriolata* nymphs, in the gut of nymphs, and in the soil close to the foam in a sugarcane field. The data were analyzed using the webtool developed by Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Metrics of alpha-diversity

Both the foam and the gut samples were more diverse than the soil samples (Supplementary Table S1). The highest mean values of the Shannon index were observed for foam samples (8.69), followed by gut (8.08) and soil samples (2.26) ($P < 0.05$) (Supplementary Table S1). Analysis of the sample richness using the Chao1 index showed that the spittlebug-derived samples showed higher mean richness than soil samples. Samples from foam showed Chao1 values of 172,333, gut samples showed 132,993, and soil samples were estimated to harbor 698 species (Supplementary Table S1) ($P < 0.05$).

Identification of bacterial taxa in samples

Considering the bacterial phyla represented by more than 3% of the sequences, the major bacterial groups found in soil were Proteobacteria (38.1%), Bacteroidetes (37.3%) and Firmicutes (11.1%). Samples from foam were composed mainly of Proteobacteria (40.33%), Actinobacteria (19.5%) and Acidobacteria (17.0%). in gut samples, the most prevalent phyla were Actinobacteria (33.7%), Proteobacteria (16.6%) and Acidobacteria (10.1%) (Fig. 3a).

Considering bacterial classes represented by more than 2% of the sequences, Betaproteobacteria (20.5%), Alphaproteobacteria (15.1%) and Flavobacteriia (14.7%) were the most prevalent classes in soil samples, while Alphaproteobacteria (29.9%), Actinobacteria (14.0%) and Chloroacidobacteria (6.37%) were the most prevalent in foam samples. Actinobacteria (18.37%), Thermoleophilia (7.70%) and Betaproteobacteria (6.40%) were the most prevalent in gut samples (Fig. 3b).

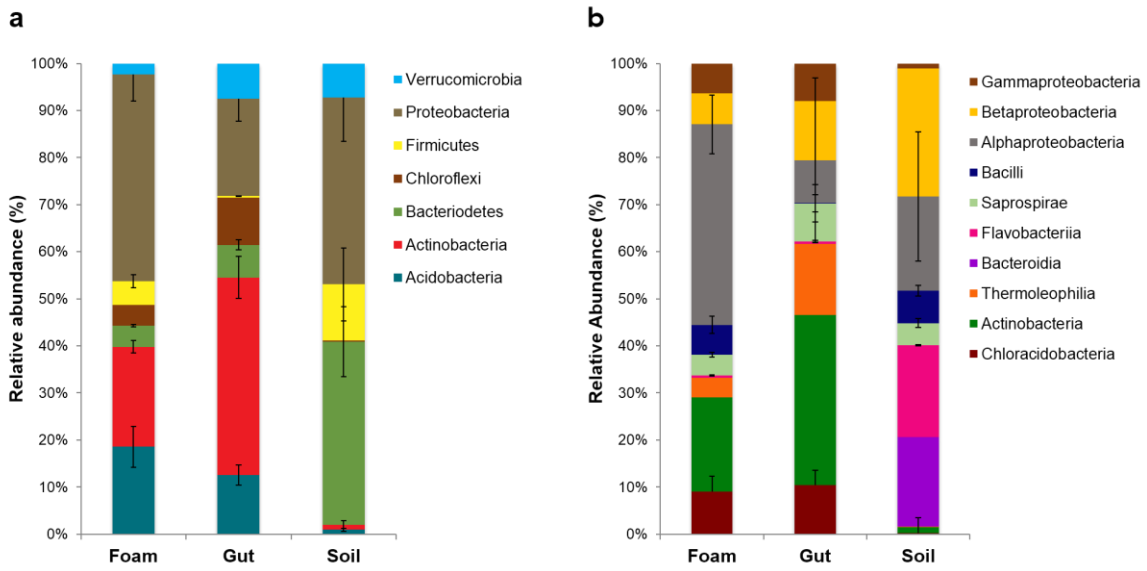

Figure 3. Relative abundance (%) of bacterial groups found in the foam produced by *Mahanarva fimbriolata* nymphs, in the gut of nymphs, and soil close to the foam in a sugarcane field. (a) Bacterial phyla represented by more than 3% of the sequences. (b) Bacterial classes represented by more than 2% of the sequences.

Regarding bacterial genera in the spittlebug-derived samples, *Bacillus*, *Streptomyces*, *Nocardia* and *Sphingomonas* were the most prevalent genera in foam, while *Nitrospira* was the most prevalent in gut samples ($P < 0.05$) (Fig. 4).

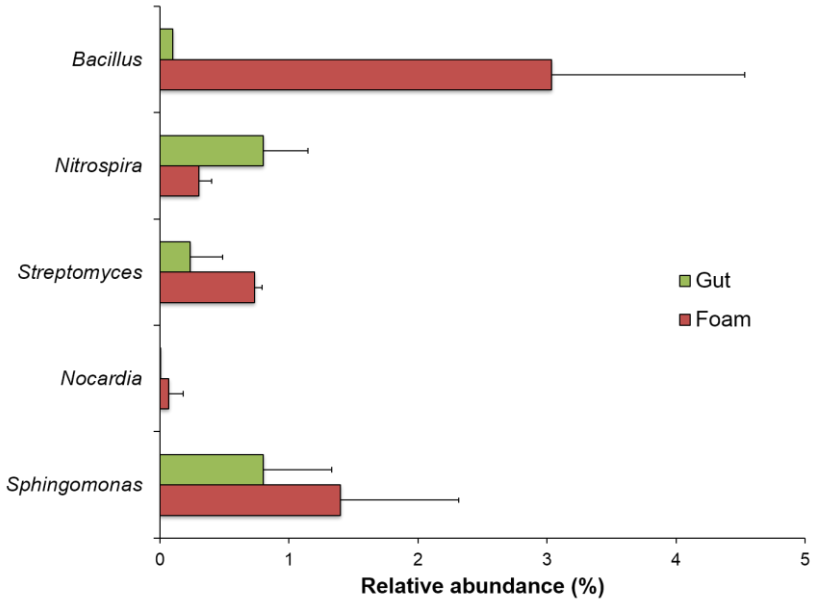

Figure 4. Differential relative abundance (%) of bacterial genera in the foam produced by *Mahanarva fimbriolata* nymphs and in the gut of nymphs. All genera differed statistically in abundance among the sites sampled, according to analyses using the "edgeR" software package ($P < 0.05$).

3.4. Discussion

The successful dissemination of insects worldwide has been strongly influenced by their associations with microorganisms (Douglas 2015). While symbiotic microorganisms are well known to play a key role in providing nutrients in many insects, several studies have shown that they can also be important in protecting the host against pathogen attack (Scarborough et al. 2005; Kaltenpoth 2009; Kroiss et al. 2010). Here, we examined the bacterial community present in the foam of *M. fimbriolata* nymphs, by comparing its abundance and composition with the bacterial communities in soil and in the spittlebug gut.

Our results revealed the presence of a large number of bacteria in the foam of *M. fimbriolata*, as previously reported for other spittlebugs (Wilson and Dorsey 1957). The complexity of the bacterial community present in the foam and its similarity to that in the insect gut suggests that these symbionts are incorporated actively into the spittle. The presence of lipids, carbohydrates and proteins can make the foam a favorable and beneficial environment with satisfactory nutritional requirements for bacterial growth (Wilson and Dorsey 1957; Mello et al. 1987; Tonelli et al. 2018).

Analysis of the bacterial community structure revealed a clear differentiation between foam and soil samples. Also, the similarity between the bacterial communities from the foam and the nymph gut reinforces the supposition that most microorganisms in the foam are not contaminants from the air or soil. The access to food-associated microbial cells, the availability of nutrients, and protection from various stresses of the external environment are some attributes that make the insect gut favorable for colonization by microorganisms (Douglas 2015).

The means by which insects transmit their symbionts from one generation to another vary widely across different taxonomic groups and individual behavior. In Hemiptera, post-hatch transmission is the most common mechanism of transfer, with symbiont acquisition resulting from ingestion of adult fecal droplets (Beard et al. 2002), probing capsules containing symbionts deposited close to the eggs (Hosokawa et al. 2006), or by egg-surface contamination and further probing of symbionts by the nymphs (Prado et al. 2006).

Our results showed that the foam produced by the nymphs of *M. fimbriolata* harbors a complex community of microorganisms. We identified 257 specific OTUs in the spittle, including some that were previously reported as defensive symbionts of insects (Figs. 2 and 3). The diversity in the foam was similar to the other microenvironments examined, such as the 288 OTUs in the soil and the 291 OTUs in the insect gut. Actinobacteria was present predominantly in the foam and nymph gut, whereas Proteobacteria was found in all environments, but with higher frequency in the foam than in the gut and soil (Fig. 3a).

The presence of Actinobacteria in the foam and gut of nymphs may be connected to the capacity of these bacteria to exploit a wide range of nutrient sources and their ability to inhabit the intestinal tract of several insects (Park et al. 2007; Lefebvre et al. 2009; Zucchi et al. 2012). The ubiquity of this phylum in the environment and their capacity to produce substances with antimicrobial activity has probably predisposed them to be involved in defensive symbioses with soil-dwelling insects (Kaltenpoth 2009). For instance, *Streptomyces* spp. were found to produce streptochlorin and a complex of eight piericidin derivates that provide a potent antimicrobial defense for the larval cocoon of *P. triangulum* wasps in the presence of noxious pathogens (Kroiss et al. 2010). There is also evidence that Proteobacteria promote resistance of insects to certain natural enemies. The Alphaproteobacteria *Wolbachia* protects *Drosophila melanogaster* against various viruses (Hedges et al. 2008), and the Betaproteobacteria *Regiella insecticola* reduces the mortality of pea aphids infected with entomopathogenic fungi (Scarborough et al. 2005). In both cases, the underlying mechanisms are not known.

Since the members of Actinobacteria seem to be rarely involved in nutritional symbiosis with insects (Kaltenpoth 2009; Zucchi et al. 2012; Salem et al. 2013), we hypothesized that the presence of these bacteria in the foam may aid in protecting against entomopathogenic microorganisms of spittlebug nymphs. However, our current findings do not address this hypothesis, since we did not evaluate the antimicrobial compounds in the foam.

Elucidating the contribution of the resident symbiotic community in the foam to the development of *M. fimbriolata* nymphs expands our understanding of the ecology of this spittlebug, which is a serious agricultural pest in Brazilian sugarcane crops and suggests possibilities for biological control by manipulation of the host's microbiome. Here, we demonstrated that the foam produced by nymphs of *M. fimbriolata* harbors a diversity of bacteria that were previously reported as protective symbionts of insects. Further investigations on the isolation and identification of substances in the foam can help to understand the possible protective mechanism(s) involved and the presence of antibiotics in the foam.

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Supplementary information

Supplementary Table S1: Sequencing information for foam produced by *Mahanarva fimbriolata* nymphs, the gut of nymphs, and soil close to the foam in a sugarcane field (n = 3).

Samples	No. of reads	Observed OTUs	Shannon	Chao1	Good's Coverage
Foam_R1	93,517	1,537[a]	8.81[a]	175,110[a]	0.99
Foam_R2	57,294	1,572[a]	8.94[a]	173,700[a]	0.99
Foam_R3	97,687	1,395[a]	8.33[a]	168,190[a]	0.99
Gut_R1	60,731	1,052[a]	8.16[a]	121,240[a]	0.99
Gut_R2	84,435	1,155[a]	7.94[a]	139,320[a]	0.99
Gut_R3	80,213	1,155[a]	8.16[a]	138,420[a]	0.99
Soil_R1	53,823	998[b]	1.66[b]	1259[b]	0.99
Soil_R2	96,654	233[b]	1.46[b]	495[b]	0.99
Soil_R3	108,867	86[b]	3.66[b]	340[b]	0.99

Different letters correspond to statistical differences in means detected by metrics of alpha-diversity ($P < 0.05$).

4. THE SPITTLEBUG *Mahanarva fimbriolata* PRODUCES FOAM AS A CHEMICAL DEFENSIVE STRATEGY

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Abstract

The use of defensive secretions is widespread among arthropods. The secretions are essentially chemical signals that represent the means by which predators and other potential enemies are warned to desist of attack or withdraw. Nymphs of the spittlebug *Mahanarva fimbriolata* produce and cover themselves with foam while feeding on the exposed roots of sugarcane. In chemically analyzing the composition of the foam produced by *M. fimbriolata* nymphs, palmitic and stearic acids were detected. Bioassays showed that the natural foam was repellent and topical irritant to the predatory ant *Solenopsis invicta* and the cockroaches *Periplaneta americana*, respectively. Testing the synthetic mixture of compounds found in our chemical analysis, we observed that the topical irritancy derives, at least partially, from the fatty acids present in the foam. The repellent behavior of *S. invicta* was stereotyped after touching the foam, suggesting that the defensive action of froth was not based on volatiles compounds. The defensive role of foam of *M. fimbriolata* nymphs reinforce the importance of the protective layer of bubbles around these insects and open new perspectives in the biological significance of fatty acids for insect's development.

Keywords: Repellency; Fatty acids; *Solenopsis invicta*; Cercopidae; Topical irritancy

4.1. Introduction

Spittlebug nymphs are insects easily identified by the foam that they produce to surround themselves while feeding on the host plants (Guilbeau 1908; Kato 1958). These insects can be observed developing on a large diversity of host plant in different environments

around the world (Carvalho and Webb 2005; Dinardo-Miranda et al. 2008; del Campo et al. 2011). The nymphs produce foam by sucking air into the ventral cavity of the abdomen, that is then trapped in the fluid of the Malpighian tubules, resulting in the creation of bubbles at the end of the abdomen (Guilbeau 1908; Weaver and King 1954).

It has been suggested that the foam has several functions, including protection against high temperatures (Tonelli et al. 2018), desiccation (Whittaker 1970) and natural enemies (Whittaker 1970; del Campo et al. 2011). Indeed, reports of predation on cercopid nymphs are relatively rare, and previous works demonstrated that the nymphs are less likely to be carried away or be attacked by predators while being inside the foam than when exposed (Whittaker 1970; del Campo et al. 2011).

Interestingly, insects that surround themselves with their waste products or feces has been shown to increase their protection from natural enemy's attack (Weiss 2006). An example is the beetle larva of *Hemisphaerota cyanea* that construct a thatch from long filamentous fecal strands as a protection from the predators *Cycloneda sanguinea* and *Stiretrus anchorago* (Eisner and Eisner 2000). Besides, the use of the chemical defense among arthropods is widespread (Eisner and Meinwald 1966; Pasteels et al. 1983; Eisner et al. 2000b), in which the defensive secretions may act as a repellent or topical irritant, causing the predators to desist from the attack or to perform vigorous cleaning activities (Eisner and Meinwald 1966).

Mahanarva fimbriolata is a spittlebug extremely conspicuous when feeding on the preferred host, the sugarcane roots. The nymphs develop on soil surface or below ground and form a distinctive white foam mass (Leite et al. 2005). The list of enemies potentially damaging the nymphs is sparse. Among arthropods, only the larvae of the syrphid *Salpingogaster nigra* has been reported preying on *M. fimbriolata* nymphs (Koller 1984). The predatory ant *Solenopsis invicta* is common in Brazilian sugarcane fields where *M. fimbriolata* develops (Rossi and Fowler 2000), however, the reason why the nymphs are not regularly attacked by the ants is unknown.

The chemical composition of spittlebugs foam is poorly understood (Marshall 1973; Mello et al. 1987; del Campo et al. 2011). Proteins, carbohydrates, lipids and alcohols are the main groups of components found so far (Kato 1958; Marshall 1973; Mello et al. 1987; del Campo et al. 2011). Nevertheless, little is known about the small-molecule of cercopid foam. Further identification of the chemical components may provide insight into the capacity of foam to contribute to the protection of nymphs against predators.

Here, we investigated the role that the foam produced by nymphs of *M. fimbriolata* may play in defending the insect against the predatory ant *S. invicta* and also evaluated the

topical irritancy of foam and the synthetic mixture of fatty acids found in our chemical analysis when applied to the cockroaches *Periplaneta americana*.

4.2. Materials and methods

Spittlebugs

Nymphs of *M. fimbriolata* were collected in a sugarcane field in Piracicaba, SP, Brazil (22º 42' 06"S, 47º33'50"W) and reared on sugarcane plants under controlled conditions (25 ± 0.5 ºC, 70% RH, 14L:10D) (Garcia et al. 2007). Briefly, adults of *M. fimbriolata* were kept in plastic cages containing plants with wet cotton covering the soil to serve as a substrate for oviposition. The collected eggs were maintained in Petri dishes on wet filter paper until hatching. Newly hatched nymphs were transferred to sugarcane roots and fed for ≈ 40 days until becoming adults. Only fourth- and fifth-instar nymphs were used in the bioassays

Predation tests with ants

The repellence to predation of the natural foam was evaluated using the predatory ant *S. invicta*. Ants were from laboratory colonies established from nests collected at the Campus of the Universidade Estadual Paulista, Rio Claro, SP, Brazil. To test the foam, a tray housing a nest of ants was connected with a sterile, clean wire (40 cm length × 20 cm height) to a second tray containing two plastic disks (10.5 cm diameter). In one of the disks 5 nymphs of *M. fimbriolata* covered with 10 ml of foam were placed and on the other disc there were placed 5 nymphs without foam, obtained through a brief submersion in distilled water followed by gentle drying with absorbent paper. The ants had free access between the trays, and the behavior was evaluated with a camera (Sony DSC-HX30V®) for two hours, taking a photo every 10 minutes of each disk, and then counting the number of ants in each treatment. Three nests of *S. invicta* were used and each nest was used 4 times, respecting a minimum interval of one week for its reuse (n = 12). The nymphs and their foam were used only once in the bioassays and then discarded.

Foam chemical analyses

Five foam samples from *M. fimbriolata* of fourth- and fifth-instar were collected in the same sugarcane field cited above, placed in glass vials (10 ml) and stored at –30 ºC until analysis. Following the extraction sequence, 1 ml of each sample was derivatized using ethyl

chloroformate (Qiu et al. 2007). After derivatization, each sample was adjusted to 0.5 ml with cyclohexane as solvent. Each sample received 5 µl of octacosane (internal standard solution at 1000 ng μl^{-1}) (Sigma-Aldrich, St Louis, MO, USA). The samples were initially analyzed by gas chromatography-flame ionization detection (GC-FID, Shimadzu GC-2010, Kyoto, Japan) using a HP-1 capillary column (Agilent Scientific, Santa Clara, CA, USA; 30 m × 0.25 mm × 0.25 µm). A 1 µl aliquot of each sample was injected in the splitless mode with an injector temperature of 240 ºC using helium as the carrier gas. The column temperature was held at 60 ºC for 1 min and then increased to 320 ºC (15 ºC min^{-1}) and held for 10 min. The extract with the best resolution was reanalyzed with gas chromatography coupled to a mass spectrometer (GC-MS, Varian 4000, Palo Alto, CA, USA) using HP5-MS column (JeW Scientific, Folsom, CA, USA, 30 m × 0.25 mm × 0.25 µm) and helium as the carrier gas. Injection (1 µl aliquot) was conducted in splitless mode and the column temperature programme was the same as described for the GC-FID above. The major peaks were identified by comparing their mass spectra with those of NIST 98 library and confirmed by co-injection with the authentic standards (Sigma-Aldrich, St Louis, MO, USA). Amounts were estimated based on the peak area relative to the amount of internal standard (octacosane).

Preparation of a synthetic mixture of foam compounds

A mixture containing the compounds identified in the chemical analysis of foam from *M. fimbriolata* nymphs was prepared for use in the bioassays. The mixture was prepared so that the amount of each component was the same as found in the native samples (i.e., 2.54 ± 0.88 µg of palmitic acid and 2.78 ± 0.97 µg of stearic acid per ml of solvent (means ± SE)). Glycerol was chosen as the solvent because it is a non-irritating medium (del Campo et al. 2011). All chemicals were purchased from Sigma-Aldrich.

Topical irritancy to cockroaches

The topical irritancy of foam and the synthetic mixture were tested through topical application to the dorsal abdominal tergites of last instar nymphs of *P. americana* (Eisner et al. 2000a; del Campo et al. 2011). For the natural foam, a microcapillary was used to apply 0.25 ± 0.3 mg (mean ± SD) of foam to the cockroaches. Caprylic acid and distilled water (0.25 µl) were tested as positive and negative controls, respectively. The synthetic mixture was evaluated similarly. Aliquots of 0.25 µl of the synthetic mixture in glycerol were used and glycerol alone was added as an additional control category. If the cockroach scratched ipsilaterally within 45 s of application, it was considered as irritated. Twenty cockroaches were used per tested

substance and the insects were used only once per application and then discarded.

Statistical analysis

A generalized linear model (glm) and quasi-Poisson distribution was used to analyse the data of predation test with ants. The goodness-of-fit was evaluated using half normal plots (hnp) with simulated envelops (Demétrio et al. 2014). An analysis of deviance was performed to assess the significance of interaction between the factors.

To analyse the topical irritancy data, we adopted a glm and binomial distribution. The goodness-of-fit was also evaluated using hnp with a simulated envelops (Demétrio et al. 2014), and an analysis of deviance was performed to assess the significance of the interaction between the factors. The means were then compared (Tukey, $P < 0.05$) using the *glht* function multicomp packet in R (R Development Core Team 2012).

4.3. Results

Repellency of foam to the predatory ants

The foam produced by *M. fimbriolata* nymphs effectively repelled the predatory ants *S. invicta* (Fig. 1). Nymphs covered by foam were less visited than nymphs uncovered from the 20 min until the end of 2 hours of experiments (Fig. 1, glm, $n = 12$, $P < 0.05$). Only in the time of 10 min there was no statistical difference between the treatments (Fig. 1, glm, $n = 12$, $P = 1.00$), probably due to the low number of ants that had left the tray of nest and directed to the tray containing the spittlebug nymphs.

It was clear that after finding the uncovered nymphs, the ants started to recruit conspecifics to kill and carry the spittlebugs to the tray housing the nest. The same behavior was not observed when the ants found the covered nymphs. In this case, soon after ants contacting the foam, they engaged in conspicuous cleaning behaviour and desist to attack the cercopids. We also observed that in all replicates ($n = 12$), at the end of 2 hours of the experiments the uncovered nymphs were killed by the ants.

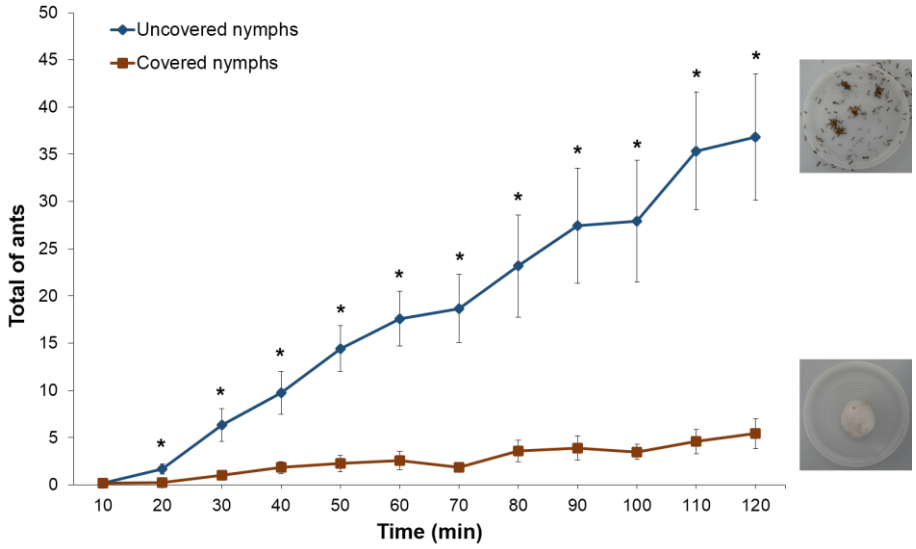

Figure 1. Results of predation tests in which nymphs of *Mahanarva fimbriolata* covered and uncovered with its foam were tested against the predatory ant *Solenopsis invicta*. The points represent the total of ants ± SE (n = 12). Asterisks indicate significant differences between treatments within the same sampling time, according to generalized linear model (glm) and quasi-Poisson distribution (*P < 0.05).

Foam chemical composition

Using GC-FID and GC-MS, two major peaks were identified as palmitic and stearic acids. Co-injection with commercial standards confirmed their identity (Fig. 2). Amounts of palmitic and stearic acids in the foam were 2.54 ± 0.88 and 2.78 ± 0.97 µg ml^{-1} of foam (means ± SE), respectively.

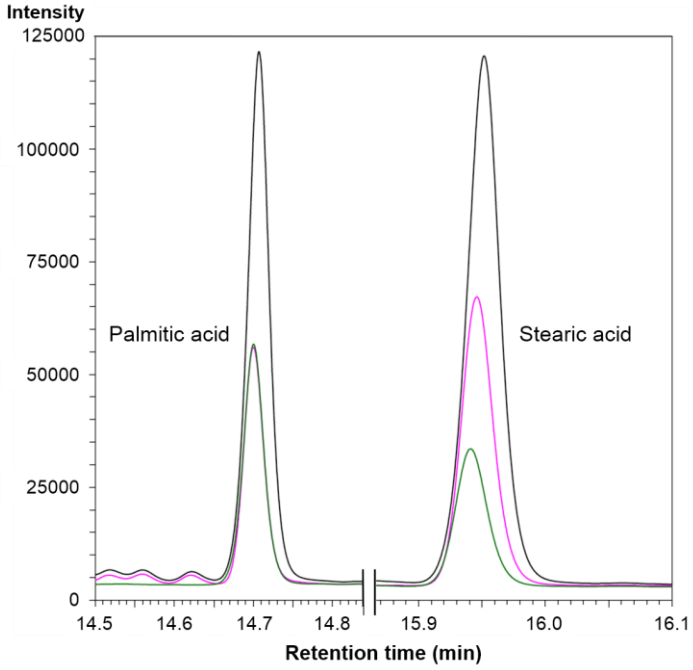

Figure 2. Representative gas chromatograms of co-injection of derivatized foam produced by nymphs of *Mahanarva fimbriolata* and the authentic standards of palmitic and stearic acids. The lines indicate: pink: natural foam extract; green: authentic standards of the fatty acids; black: coelution of foam with the authentic standards of the fatty acids.

Topical irritancy assays

The cockroaches receiving a topical application of *M. fimbriolata* foam showed a significantly higher incidence of scratching than cockroaches receiving only a droplet of distilled water or glycerol (Fig. 3, Tukey test, n = 20 per substance, $P < 0.05$). Besides, we found no significant difference of scratching between roaches that received a droplet of foam than those receiving the caprylic acid or the synthetic mixture of fatty acids in the same proportion found in the chemical analysis (Fig. 3, Tukey test, n = 20 per substance, $P > 0.05$).

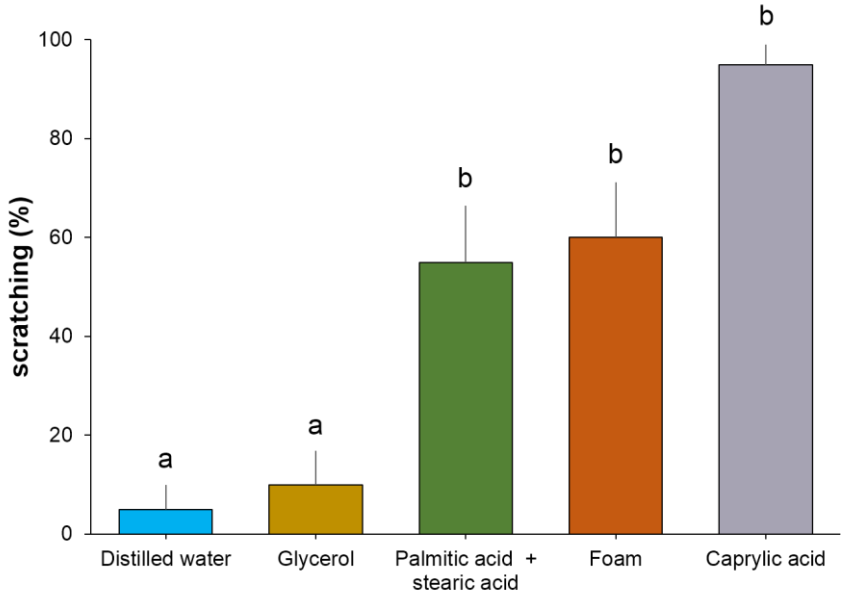

Figure 3. Incidence of scratching in *Periplaneta americana* nymphs in response to topical application of foam from *Mahanarva fimbriolata* nymphs, distilled water, glycerol, caprylic acid, and the synthetic mixture of palmitic and stearic acids (n = 20 per tested substance). Bars represent the % of scratching ± SE. Bars with different letters are significantly different (Tukey, $P < 0.05$). Means were compared using the *glht* function multicomp packet in R.

4.4. Discussion

The waste products left by an insect can affect its interactions with other organisms, including predators or prey (Weiss 2006). Spittlebugs are insects that produce and cover themselves with foam masses during the nymph stage (Guilbeau 1908). We demonstrated that the foam produced by the cercopid nymphs of *M. fimbriolata* was repellent to the predatory ant *S. invicta*, and topical irritant to cockroaches *P. americana* (Figs. 1 and 3).

Nymphs covered with the foam were suitable to repel the ants, while uncovered nymphs were attacked and killed by the predators. It was clear that the repellent effect of foam was not based on its odor, since the ants walked randomly on the trays and plastic disks, and only after touching the foam they engaged in a conspicuous cleaning behavior, which is a strong

indicator that a repellent and deterrent substance is involved (Schroeder et al. 2006).

The bioassays to test the topical irritancy to cockroaches reinforce the protective role of foam and the synthetic mixture of fatty acids. Even though the cockroaches are not natural enemies of *M. fimbriolata*, these results suggest that the foam may be a generalist defensive strategy against other arthropods. In this case, nymphs can protect themselves against a greater range of natural enemies, since the predator expend time in self-cleaning instead attacking the nymphs (Eisner and Meinwald 1966). Taken together, these results may help to explain the reason why reports of predation on *M. fimbriolata* nymphs in nature are rare (Koller 1984).

The chemical composition of foam from various cercopid species has shown components from multiple classes, including lipids, proteins, carbohydrates, alcohols, lactones, polyol pinitol and polyhydroxylakanoates (Wilson and Dorsey 1957; Marshall 1973; Mello et al. 1987; del Campo et al. 2011). In the present study we have only identified the fatty acids detected in our chemical analysis after a derivatization with ethyl chloroformate. The results of tests with synthetic mixture containing the palmitic and stearic acids allowed us to infer that the irritant effect to *P. americana* can be, at least partially, due the presence of this lipids.

Fatty acids are common in nature and have a recognized biological significance for insect's development, involvement in metabolic energy storage, cell and biomembranes structure, regulatory physiology, and as important cuticular components protecting the insects from water loss in terrestrial environments (Lockey 1988; Stanley-Samuelson et al. 1988). A wide range of fatty acids are also found in the defensive secretions of insects. The more common fatty acids in defensive secretions are C12 to C18 saturated and unsaturated components (Stanley-Samuelson et al. 1988). β-Hydroxydecanoic acid is present in the secretion of *Atta sexdens* (Schildknecht and Koob 1971), while 3-Dodecenoic acid is the major component in the defensive secretion of beetle *Deleaster dichrous* (Dettner et al. 1985). Recently, some fatty acids have been shown as repelling to insects (Hwang et al. 1982; Mullens et al. 2009) and the caprylic acid is a well-known wax solvent that acts by disrupting the epicuticle of insects, thereby facilitating the penetration of noxious compound or the water loss (Eisner and Meinwald 1966). However, the mechanism of repellency and irritancy of palmitic and stearic acid to arthropods is unknow so far.

In conclusion, our findings provide evidence that the foam produced by *M. fimbriolata* nymphs is repellent to a relevant predator and topically irritant to another insect. We also showed that the irritant propriety of foam derives, at least in part, from the fatty acids identified in our chemical analysis. Further studies to clarify the mode of action of these compounds will expand our understanding and shed new light in this defensive strategy used by *M. fimbriolata*.

The results presented here consist with previous speculations that the cercopid foam plays a key role in protecting the nymphs against predation.

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