Psyche

True Bugs (Heteroptera): Chemical Ecology of Invasive and Emerging Pest Species

Guest Editors: Jeffrey R. Aldrich, Jocelyn G. Millar, Antônio R. Panizzi, and Mark M. Feldlaufer

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Editorial Board

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Editorial

True Bugs (Heteroptera): Chemical Ecology of Invasive and Emerging Pest Species

Jeffrey R. Aldrich,[1,2] Jocelyn G. Millar,[3] Antônio R. Panizzi,[4] and Mark M. Feldlaufer[1]

[1] *USDA, Agricultural Research Center-West (ARS), Beltsville, MD 20705, USA*
[2] *Department of Entomology, University of California, Davis, CA 95616, USA*
[3] *Department of Entomology, University of California, Riverside, CA 92521, USA*
[4] *Laboratório de Entomologia, Embrapa Trigo, 99001-970 Passo Fundo, RS, Brazil*

Correspondence should be addressed to Jeffrey R. Aldrich, drjeffaldrich@gmail.com

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True bugs (order Hemiptera; suborder Heteroptera; about 40,000 species) are increasing in importance as pests because of international commerce, insecticide resistance, range expansion due to global warming, and immunity to genetically modified crops (GMOs). Haematophagous bugs suck blood from humans and other animals, and phytophagous species are crop pests. Damage caused by plant bugs (Miridae) and stink bugs (Pentatomidae) is escalating everywhere GMOs have been adopted, not only because bugs are unaffected by the *Bt*-endotoxins used to transform crops, but also because the advent of no-till agriculture with herbicide-resistant GMO crops leaves debris and fallen seeds favoring infield survival of bugs. Resurgences of phytophagous bugs has also occurred in crops treated for mating disruption for control of primary pests such as codling moth, with a consequent decrease in pesticides that used to incidentally control bugs. Finally, adoption of GMOs has dramatically reduced insecticide usage, facilitating establishment of invasive species such as the brown marmorated stink bug (BMSB) (*Halyomorpha halys*) that is currently wreaking havoc in many parts of the United States.

For this Special Issue we have broadened the scope of chemical ecology to include recent advances in heteropteran endocrinology, genetic research, symbionts, electronic pest detection, acoustic communication, and parasitoid host searching, as well as more mainstream investigations of semiochemicals and their use for trapping and monitoring pest species. In all, this compilation includes 17 manuscripts, with authors from nine countries.

The issue includes semiochemical-related articles on species from seven heteropteran families. T. Yasuda and H. Higuchi describe the sex pheromones of two plant bug (Miridae) species, and their use for monitoring these rice pests in Japan. A. González et al. and G. Martins et al. report original semiochemical pheromone and allomone research on a "rare" bug native to Australia, *Thaumastocoris peregrinus* (Thaumastocoridae), which is now ravaging eucalypt plantations in Brazil and Uruguay. R. A. Laumann et al. identified the female-produced attractant pheromone of a South American bean pest, *Neomegalotomus parvus* (Alydidae), and A. Khrimian et al. identified the male-produced attractant pheromone of an Australian fruit-spotting bug (*Amblypelta lutescens lutescens*) from the alydid sister family, Coreidae. G. Manrique and M. Lorenzo report on investigations of the chemical signals used between male and female triatomine bugs (Reduviidae: Triatominae), vectors of *Trypanosoma cruzi*, the protozoan etiological agent of Chagas' disease in the Americas. N. Singh et al. studied the interactions between carbon dioxide, heat, and chemical lures in attracting the bed bug, *Cimex lectularius* (Cimicidae), which is resurging as a nuisance pest worldwide. There are five manuscripts involving stink bug (Pentatomidae) communication. (1) T. C. Leskey et al. report on the impact of the invasive BMSB (*H. halys*) in mid-Atlantic fruit orchards in the United States, based upon traps baited with the aggregation pheromone

(methyl (*2E,4E,6Z*)-decatrienoate) of the Asian pentatomid (*Plautia stali*) to which BMSBs are powerfully attracted. (2) N. Endo et al. documented age-related and individual pheromone variation in males of another pentatomid exhibiting pheromonal cross-attraction in Japan, *Piezodorus hybneri*. (3) P. G. Tillman and T. E. Cottrell reported results of a 2-year on-farm experiment in the southeastern United States using a trap crop with pheromone-baited traps to suppress *Euschistus servus* in cotton. (4) C. C. A. Silva et al. studied substrate vibrations, the close-range acoustic communicative modality common to pentatomids and other bugs, that are produced by males and females of the South American stink bug, *Edessa meditabunda*. (5) D. C. Degenhardt et al. described their research on an electronic nose ("E-nose") for monitoring stink bug feeding in cotton as a guide for management decisions.

The Special Issue also includes five review papers on aspects more or less related to mainstream heteropteran chemical ecology. E. Conti and S. Colazza thoroughly review the chemical ecology of egg parasitoids associated with true bugs. I. Hurwitz et al. review the amazing paratransgenic "vaccination" of Chagas' disease vectors against transmission of *T. cruzi*; and S. S. Prado and T. D. Zucchi summarize the host-symbiont interactions of heteropteran pests, especially plant pests, and discuss the potential for managing these pests via manipulation of their symbionts. J. M. C. Ribeiro et al. provide a detailed insight into the sialomes (i.e., the salivary gland transcriptomes) of bloodsucking Heteroptera. Finally, T. Kotaki et al. present details of their discovery of a novel juvenile hormone (JH) from the pentatomid, *P. stali*, containing a second epoxide moiety (methyl (2*R*,3*S*,10*R*)-2,3;10,11-bisepoxyfarnesoate: JHSB3). Overall, we believe that this Special Issue highlights most current aspects of semiochemical-related research on true bugs.

Jeffrey R. Aldrich
Jocelyn G. Millar
Antônio R. Panizzi
Mark M. Feldlaufer

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Research Article

Impact of the Invasive Brown Marmorated Stink Bug, *Halyomorpha halys* (Stål), in Mid-Atlantic Tree Fruit Orchards in the United States: Case Studies of Commercial Management

Tracy C. Leskey,[1] Brent D. Short,[1] Bryan R. Butler,[2] and Starker E. Wright[1]

[1] *USDA-ARS, Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430-2771, USA*
[2] *University of Maryland Extension, Carroll County, 700 Agriculture Center Drive, Westminster, MD 21157, USA*

Correspondence should be addressed to Tracy C. Leskey, tracy.leskey@ars.usda.gov

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Four commercial orchards in the mid-Atlantic region of the United States were surveyed weekly in 2010 and 2011 for the presence of brown marmorated stink bug and the injury caused to both apple and peaches. Among tested sampling techniques, pyramid traps baited with the aggregation pheromone of *Plautia stali* Scott, methyl-(2*E*,4*E*,6*Z*)-decatrienoate, yielded the most brown marmorated stink bug adults and nymphs, followed by visual observations. Brown marmorated stink bugs began to feed on apples and peaches soon after fruit set and continued to feed on fruit throughout the growing season. Injury to apple was relatively inconsequential until after mid-June, whereas feeding on peaches resulted in immediate economic injury as the surface became distorted, dented, discolored, and the flesh beneath turned brown. Significantly more apples were injured and with greater severity in 2010 than in 2011. Likewise, percent injury on the exterior portion of each apple plot was significantly greater than injury reported from the interior in both years. Growers increased the number of insecticide applications nearly 4-fold from 2010 to 2011. In addition to the increased number of targeted insecticide applications, growers also reduced the interval between treatments in 2011. A metric was created to compare the relative intensity of each grower's commercial management program between seasons and amongst each other.

1. Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive stink bug native to Japan, Korea, China, and Taiwan [1], now well established throughout the mid-Atlantic region of the United States. Evidence of established populations in Switzerland [2] and Canada [3] has also been reported. Brown marmorated stink bug is an extremely polyphagous species, and a pest of many crops in Asia [4] including tree fruit, vegetables, shade trees, and leguminous crops with specific mention of apple, cherry, peach, and pear [4, 5]. Surveys conducted in the United States identified a number of tree fruit hosts for brown marmorated stink bug including apple, plum, peach, pear, and cherry [5–7]. In 2010, populations of this invasive species increased dramatically, causing widespread injury to many crops throughout the mid-Atlantic region [8]. Tree fruit, in particular, was hit hard with some growers losing entire crops of stone fruit. Among apple growers, losses were totaled in excess of 37 million dollars in the region [9].

Within the United States, native stink bugs generally have been classified as secondary pests of tree fruit orchards and have been successfully managed with broad-spectrum insecticide applications typically directed at other key pests. However, with the passage of the Food Quality Protection Act in 1996, many broad-spectrum materials have been eliminated or severely curtailed for use through regulatory measures. Subsequently, populations of native stink bugs, long considered to be secondary pests, became more prevalent in orchard agroecosystems [10, 11]. Furthermore, when brown marmorated stink bug populations increased dramatically, this led to devastating levels of fruit injury as

this invasive species quickly replaced lepidopteran pests such as codling moth, *Cydia pomonella* L., and oriental fruit moth, *Grapholita molesta* (Busck), as the key pest driving management decisions in the mid-Atlantic region of the United States.

Because brown marmorated stink bug is a newly established invasive species, management programs for this pest are still being developed. In 2010, no specific management recommendations were in place and only a single laboratory study evaluating a select number of compounds against brown marmorated stink bug had been conducted [12]. Growers were forced to rely on recommendations made for native stink bugs, which did not result in satisfactory control [8]. In general, pyrethroid insecticides, considered to be effective against native stink bugs, but are a poor fit in IPM programs because of their negative impact on beneficial arthropods [13, 14] were applied.

Recent insecticide trials against brown marmorated stink bug have revealed that numerous pyrethroid and neonicotinoid compounds at field-labeled rates are not particularly effective, with many compounds resulting in greater than 33% of the individuals recovering from a moribund or "knockdown" state [15]. This conforms to earlier laboratory [12] and field studies [16] that documented knockdown and recovery from pyrethroids specifically. On the other hand, there are a number of materials labeled for either stone or pome fruit that resulted in substantial mortality of tested individuals. Some effective materials reported in the previous study were endosulfan, a chlorinated hydrocarbon; malathion, an organophosphate; permethrin and fenpropathrin, pyrethroids; dinotefuran, a neonicotinoid; methomyl, a carbamate [15]. Prior to the establishment of brown marmorated stink bug, growers likely would not have applied many of these materials in their management programs as they were not needed to achieve acceptable levels of control of other key pests. However, management programs have rapidly evolved to meet the challenge posed by brown marmorated stink bug.

Simultaneously, development of monitoring tools that can be used to assess the presence, abundance, and seasonal activity of this invasive species is considered paramount [8]. Stink bug species are typically monitored in cropping systems using sweep nets, beating samples, pheromone-baited traps, and/or black light traps. Among native stink bugs in tree fruit, baited yellow pyramid traps [10, 11] and baited mullein plants [17] were effective at monitoring native *Euschistus* spp. while *Chinavia hilaris* (Say) was monitored in vegetable and row crops using black light traps [18]. Black light traps have been evaluated for brown marmorated stink bug in Japan [19] and in New Jersey [6]. Most recently, black pyramid traps baited with the aggregation pheromone of *Plautia stali* Scott, methyl-(2*E*,4*E*,6*Z*)-decatrienoate [20], were found to be an effective means to trap brown marmorated stink bug adults and nymphs [21, 22]. However, none of these tools have been evaluated extensively against brown marmorated stink bug in commercial tree fruit orchards.

In 2010 and 2011, we surveyed commercial fruit orchards in the mid-Atlantic to quantify the amount and severity of injury to stone and pome fruit crops. We also evaluated the efficacy of established monitoring techniques for other stink bug species to measure presence, abundance, and seasonal activity of populations of brown marmorated stink bug. Finally, we quantified the changes in management programs from 2010 to 2011 in terms of material selection, interval, and application method.

2. Materials and Methods

2.1. Commercial Site Selection. We attempted to evaluate the population density of brown marmorated stink bug and severity of injury to apple and peach fruit in two West Virginia and two Maryland orchards. In 2010, the project began during mid-season (July) and continued through November. Grower orchards were selected based on (1) the presence of brown marmorated stink bug infestations and (2) the availability of both apples and peaches as hosts. Specific apple and peach plots within orchards were chosen based on grower reports of stink bug injury and close proximity (<20 m) to wooded/wild habitats. Orchard A consisted of a 2.9 ha apple orchard ("Fuji" on M7A; Spacing: 4.9 m × 7.3 m) planted in 1995 and a 1.3 ha peach orchard ("Redhaven" on Tenn. Nat. and "Sentry" and "Bounty" on Lovell; Spacing: 4.3 m × 7.3 m) planted in 2001. Orchard B consisted of a 5.7 ha apple orchard ("Delicious" and "Golden Delicious" on M111; Spacing: 6.2 m × 8.6 m) planted in 1996 and a 5.3 ha peach orchard ("Sunbright" on Lovell; Spacing: 6.2 m × 7.4 m) planted in 1996. Orchard C consisted of 1.9 ha apple orchard ("Golden Delicious" on M111; Spacing: 4.2 m × 7.6 m) planted in 1975 and a 0.7 ha peach orchard ("Red Haven" and "Blake" on Lovell; Spacing: 4.2 m × 7.6 m) planted in 1997. Orchard D consisted of a 1.8 ha apple orchard ("Delicious" on M26, "Fuji", "Braeburn", "Mutsu", "Empire", "Jonagold" and "Magnolia Gold" on M9; Spacing: 2.7 m × 4.0 m) planted in 1992 and a 2.4 ha peach orchard (mostly "Loring", "Cresthaven", "Encore", "White Lady" and "Redhaven" on Lovell, "Beekman" on Tenn. Nat. and "John Boy" on Guardian; Spacing: 5.2 m × 6.1 m) planted in 1998. In 2011, the same four growers' orchards were monitored and evaluated for brown marmorated stink bug presence and injury to fruit throughout the entire growing season (April–November). The only exception was at orchard C where the apple plot was 1.8 ha ("Fuji" and "York" on M111/M9 interstem; Spacing: 3.0 m × 6.1 m) planted in 2001 and the peach plot was 1.9 ha (mostly "Canadian Harmony", "Bounty", and "Blake" on Lovell; Spacing: 3.7 m × 6.1 m) planted in 2003.

2.2. Aggregate Insecticide Metric (AIM). Growers selected their own spray programs for both seasons; however, growers used more targeted treatments against brown marmorated stink bug in 2011. In order to assess the insecticide treatment programs used in 2010 and 2011, a metric (AIM) was created that would compare quantitatively the differences in material and application method for each chemical used. The AIM takes into account the lethality of the active ingredient against brown marmorated stink bug [15], the number of insect Orders listed as controlled on each product label, the proportion of chemical used by the grower versus the

maximum allowed according to approved label directions, and the method of application (complete block or alternate row middle).

2.2.1. Material. Each material/active ingredient was compared by a series of three steps: (1) general insect toxicity, (2) specific brown marmorated stink bug toxicity, and (3) amount of active ingredient used. First, general insect toxicity (G_i) was assessed by counting the number of insect Orders presumed (according to the specific product label) to be controlled upon use of the chemical, then dividing that number by the number of insect Orders available for control and presented as a proportion (0 to 1). The identified Orders of insects available for control were Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, Orthoptera, and Thysanoptera [23]. Specific brown marmorated stink bug toxicity (S_i) was evaluated among chemicals by use of the lethality index reported in Leskey et al. [15]. This index was based on the results from laboratory tests on adult brown marmorated stink bugs exposed to high field-rate doses of various active ingredients, presented as dislodgeable, dry residues for a period of 4.5 h. Subsequently, all test subjects were evaluated daily over a 7-d period for their condition (alive, moribund, or dead). These data comprise the lethality index, which assigned a value 0 to 100 based on the speed and efficacy at which a chemical acted against the brown marmorated stink bug; however, in this publication the lethality index was assessed from 0 to 1 to standardize with other factors in the model. Increased efficacy yielded a higher number and vice versa. In the third examination of each material, the amount of active ingredient (A_i) used in each application was calculated as a proportion (0 to 1) of the amount of material the label permits per application. Thus the amount of material used was divided by the maximum amount that could have been used.

2.2.2. Application Method (M_i). All growers used one of two methods for applying pesticides to their fruit trees: complete block or alternate row middle sprays [24]. In the complete block spray, chemicals were applied to every tree from every drive row within a plot. In the alternate row middle spray, chemicals were applied to one half of every tree via the use of every other drive row within a plot. A complete block spray was assigned a value of 1 whereas an alternate row middle spray was assigned a value of 0.5.

2.2.3. AIM Formula. The AIM value for each insecticide application was calculated by multiplying each factor:

$$G_i \times S_i \times A_i \times M_i. \quad (1)$$

For each grower by year and fruit species, we calculated the mean AIM and total AIM. Given that grower management programs were not limited to control of brown marmorated stink bug, statistical comparisons of the mean interval between applications and the mean AIM score was computed for all growers combined. These means were compared using Student's t-test ($P < 0.05$).

2.3. Sampling/Monitoring. From 12 May to 20 October 2011, two commercial orchards (A & C) were monitored weekly for brown marmorated stink bug presence by the use of three sampling techniques: sweep net, limb jarring, and visual surveying. Each sampling technique was performed in both apple [12 May to 6 October (orchard A) and from 12 May to 20 October(orchard C)] and peach (12 May to 7 July) plots at each orchard.

2.3.1. Sweep Net Samples. Three areas were sampled at the border of the wooded/wild habitat proximal to each orchard plot and spaced equidistant to span the length of the plot. Fifty sweeps of the ground flora, consisting of a back-and-forth motion, were performed weekly in each area covering approximately 5 m^2. The number of nymphs and adults collected were taken to the laboratory and counted.

2.3.2. Limb Jarring Samples. Eight apple and peach trees were selected from the perimeter row of each plot that bordered a wooded/wild habitat and were then revisited each week for subsequent samples. Two limbs on opposing sides were sampled by striking each limb three times onto a 1 m^2 canvas beating sheet (BioQuip, Rancho Dominguez, CA) to collect dislodged insects. All nymphs and adults on the sheet were counted and totaled for each tree.

2.3.3. Visual Surveys. Eight additional apple and peach trees were selected from the perimeter row of each plot that bordered a wooded/wild habitat and were then revisited each week for subsequent samples. Each sample consisted of a 3-min visual inspection of all parts of the tree. All brown marmorated stink bug eggs (hatched and unhatched), nymphs, and adults were counted and any hatched eggs discovered were removed from the tree.

2.3.4. Trapping. On 4 August 2010, three black pyramid traps [21] were deployed in the perimeter tree row of apple plots at each commercial orchard. All traps were placed along the perimeter that bordered a wooded/wild habitat. In 2010, traps were baited with 50 mg of methyl-(2*E*,4*E*,6*Z*)-decatrienoate (ChemTica Intl., Atlanta, GA), an aggregation pheromone of *Plautia stali* Scott [20] and a known cross-attractant to the brown marmorated stink bug [22]. Traps were also provisioned with an insecticidal strip containing 10% 2,2-dichlorovinyl dimethyl phosphate (Vaportape II, Hercon, Emigsville, PA) to inhibit stink bug escape from the trap. The chemical attractant and insecticidal strip were replaced at 4-wk intervals. Brown marmorated stink bug adults captured in traps were sexed, and nymphs were separated by instar and then removed from the trap weekly until 10 November. In 2011, a prototype trap developed by AgBio (Westminster, CO), patterned after the 2010 trap, was used in both apple and peach blocks at the four commercial orchards described previously. The pyramid base was constructed from 2 sheets of laminated plywood joined together with glue and staples. The trap was painted with flat black exterior latex paint and was 1.1 m tall × 0.5 m wide at base × 0.64 cm thick (Figure 1(a)). A 1.9 L plastic jar

(a) (b)

Figure 1: (a) Photo of pyramid trap and (b) jar top used in commercial orchards in 2011.

top was fixed atop a yellow plastic funnel with an opening of approximately 2.5 cm, which served as the entry point at the base of the jar. The funnel was not permanently attached to the jar top because its contents were accessed by separation of the jar top from the funnel. A hole was drilled into each edge of the pyramid trap so that the funnel and jar could be held in place at the top of the pyramid by four 5.1 cm, 6-penny nails pushed into the sides of the trap. The four sides of the jar top contained 23 holes, 1 mm in diameter, spaced 1 cm apart over 14 sq·cm (Figure 1(b)). The same lure and insecticidal strips were used as in 2010, except the load was increased to 66 mg. Placement of traps and sampling protocols were similar to those used in 2010, with only sampling period duration differing. Traps were sampled weekly from 8 April (Orchards A–C) and 15 April (Orchard D) through 18 November. The number of brown marmorated stink bugs captured per week from 4 August to 12 November was compared between years with Student's *t*-test. The change in the ratio of adults: nymphs captured in apples in 2010 at each orchard was compared with a chi-square test. The same test was not performed in 2011 due to limited captures in all orchards.

2.4. Injury Assessments. In 2010, fruit were evaluated weekly from 30 July to harvest, relative to each cultivar, for the presence of stink bug damage in apples and peaches. One hundred apples and one hundred peaches were picked from both the exterior and interior at each commercial orchard. The exterior was limited to the three outermost rows of each plot and was bordered by a wooded/wild habitat; while interior fruit were selected from the middle third of each plot. The surface of each fruit was visually examined and the side of the fruit appearing to have the greatest number of injury sites was sectioned to the core. The total number of injured fruit and independent injury sites on one side of the fruit, indicated by the presence of subsurface corking (Figures 2 and 3(b)), was recorded. In 2011, fruit evaluations were conducted weekly from 18 May to harvest of each cultivar using a similar protocol to that established in 2010. Evaluations were conducted prior to 18 May as on-tree visual samples of the surface of both 100 apples and peaches, but proved too unreliable to accurately assess the level of injury and so all subsequent evaluations involved removal of fruit from the tree. Samples prior to 18 May will not be reported in this paper. Thereafter, 200 peaches and 100 apples were destructively sampled weekly from the exterior of each plot. The peach evaluation was the same as that in 2010. This level of recording persisted until 13 July, where the protocol returned to that of 2010. Due to variation in expression of injury in apple relative to fruit maturity, the entire surface of each apple was evaluated for the presence of a feeding hole or dimple until apple injury was expressed as a depression or discolored depression [25]. At this time, fruit were sectioned to the core, and the total number of injured fruit, based on the presence of corking in the flesh (Figure 3), was recorded. In 2011, only the exterior of each apple and peach plot was sampled until 5% of fruit contained at least one subsurface corky spot. Once an interior sample was triggered, only 100 fruit of each species were sampled. Thus, from August on, fruit were evaluated for the presence of corking in the flesh and the number of individual corking spots. Percent corking injury and number of injury sites per injured fruit on the exterior and interior of plots were compared using a Student's *t*-test and percentages were arcsin-square root transformed as needed.

3. Results

3.1. Aggregate Insecticide Metric

3.1.1. Apple. At all four commercial orchards, growers increased the number of brown marmorated stink bug-targeted insecticide applications and decreased the time between

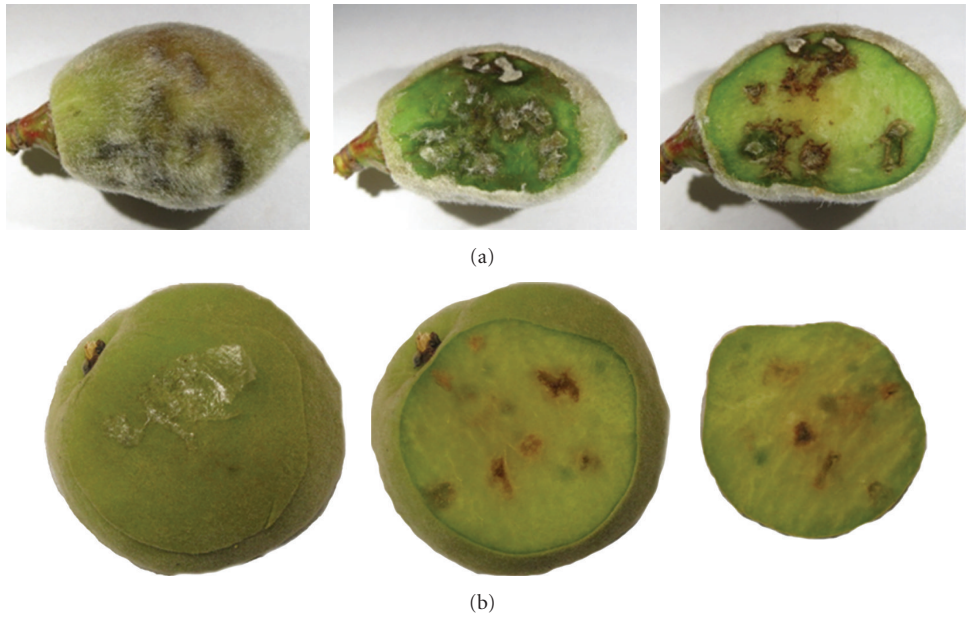

Figure 2: (a) Photos of subsurface corking injury to a 15 mm "Loring" peach in the early season and to (b) a 40 mm "Red Haven" peach ~3 weeks prior to harvest.

Figure 3: (a) Photo of subsurface feeding sheath that is the result of feeding in the early season on "Golden Delicious" apple and of (b) subsurface corking injury on "Turley Winesap" which is the result of feeding taking place later in the season (from ~6–8 weeks after petal fall until harvest).

consecutive applications from 2010 to 2011 ($t = 5.67$; df = 118; $P < 0.0001$). The total AIM score increased numerically from 2010 to 2011, but there was no statistical difference in the mean AIM score ($t = 1.078$; df = 150; $P = 0.2827$) (Table 1).

3.1.2. Peach. At all four commercial orchards, growers increased the number of brown marmorated stink bug-targeted insecticide applications and decreased the time between consecutive applications ($t = 3.45$; df = 86; $P = 0.0009$). The mean AIM score increased significantly ($t = 2.486$; df = 109; $P = 0.0144$) and total AIM score also increased from 2010 to 2011 (Table 2).

3.2. Sampling/Monitoring

3.2.1. Apple. Orchard A yielded a total of 12 brown marmorated stink bugs in limb jarring, 21 in sweep net, and 77 in visual samples season-long. At orchard C, 9 brown marmorated stink bugs were collected in limb jarring samples, 14 from sweep nets, and 49 in visual observations season long (Figure 4(a)).

3.2.2. Peach. At orchard A, a total of 3 brown marmorated stink bugs were recovered from limb jarring samples, 4 from visual observations and 0 from sweep net ground samples season-long. No brown marmorated stink bugs were

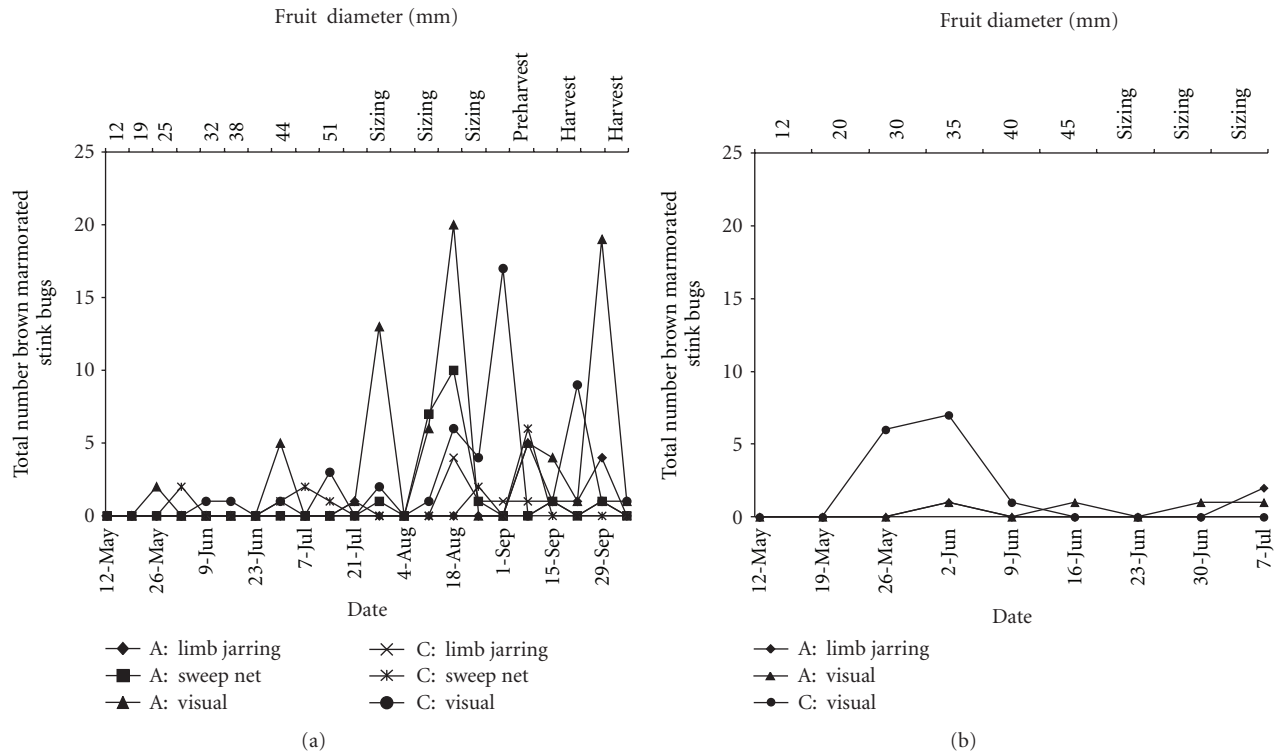

Figure 4: (a) Total number of brown marmorated stink bug eggs, nymphs, and adults recovered from limb jarring, sweep net, and visual samples in apple trees and (b) the total number recovered from limb jarring and visual samples in peach trees at orchard A and C in 2011.

Table 1: Total number of targeted brown marmorated stink bug insecticide applications, mean interval (d) ± SEM between insecticide applications, and mean A.I.M. score ± SEM and total A.I.M. score in apple plots from 2010 to 2011 at four commercial orchards.

Orchard	Number of targeted insecticide applications		Mean insecticide interval ± SEM*		A.I.M. score Mean ± SEM*		Total	
	2010	2011	2010	2011	2010	2011	2010	2011
A	3	20	10.6 ± 1.9	7.2 ± 0.4	0.06 ± 0.02	0.19 ± 0.02	0.89	5.26
B	5	7	22.2 ± 5.7	18.8 ± 2.5	0.40 ± 0.10	0.46 ± 0.10	3.63	5.47
C	4	12	18.5 ± 1.3	11.4 ± 1.4	0.18 ± 0.05	0.29 ± 0.06	1.78	5.31
D	7	42	10.6 ± 1.0	4.1 ± 0.3	0.21 ± 0.05	0.18 ± 0.02	3.28	8.16
All Orchards	19	81	14.0 ± 1.3 a	7.1 ± 0.6 b	0.20 ± 0.03 a	0.24 ± 0.02 a	9.58	24.2

* Means for all orchards combined, compared between years within a paired column, followed by a different letter are significantly different ($P < 0.05$).

collected in sweep net or limb jarring samples at orchard C and a total of 14 brown marmorated stink bugs were observed in visual samples between 25 May and 7 June with no other bugs documented for the remainder of the season (Figure 4(b)).

3.3. Trapping. In all four commercial apple plots sampled from August to November in 2010 and 2011, the number of adults ($t = 3.81$; df = 60.776; $P = 0.0003$) and nymphs ($t = 2.49$; df = 59.108; $P = 0.0155$) captured was significantly lower in 2011 (Figure 5(a)). On 8 September 2010, there was a significant shift in the ratio of adults:nymphs captured in traps at all orchards ($\chi^2 = 1762.3737$; df = 1; $P < 0.0001$). Prior to that date, significantly fewer adults were captured than nymphs at Orchard A ($\chi^2 = 21586.7131$; df = 1; $P < 0.0001$), C ($\chi^2 = 3410.2565$; df = 1; $P < 0.0001$), and D ($\chi^2 = 78.5714$; df = 1; $P < 0.0001$); however there was no difference between adult and nymph captures at orchard B ($\chi^2 = 1.5077$; df = 1; $P = 0.2195$). During the entire 2011 growing season, very few adults or nymphs were captured in traps deployed in apple and peach blocks; however, those that were captured were primarily recovered after July (Figure 5). In fact, 72% of all adult captures were recovered from traps on 29 September 2011.

3.4. Injury Assessments

3.4.1. Apple: 2010. In 2010, significantly more apples were injured on the plot exterior than in the interior at orchards A ($t = 2.18$; df = 18.836; $P = 0.0421$), B ($t = 4.48$; df = 15.964;

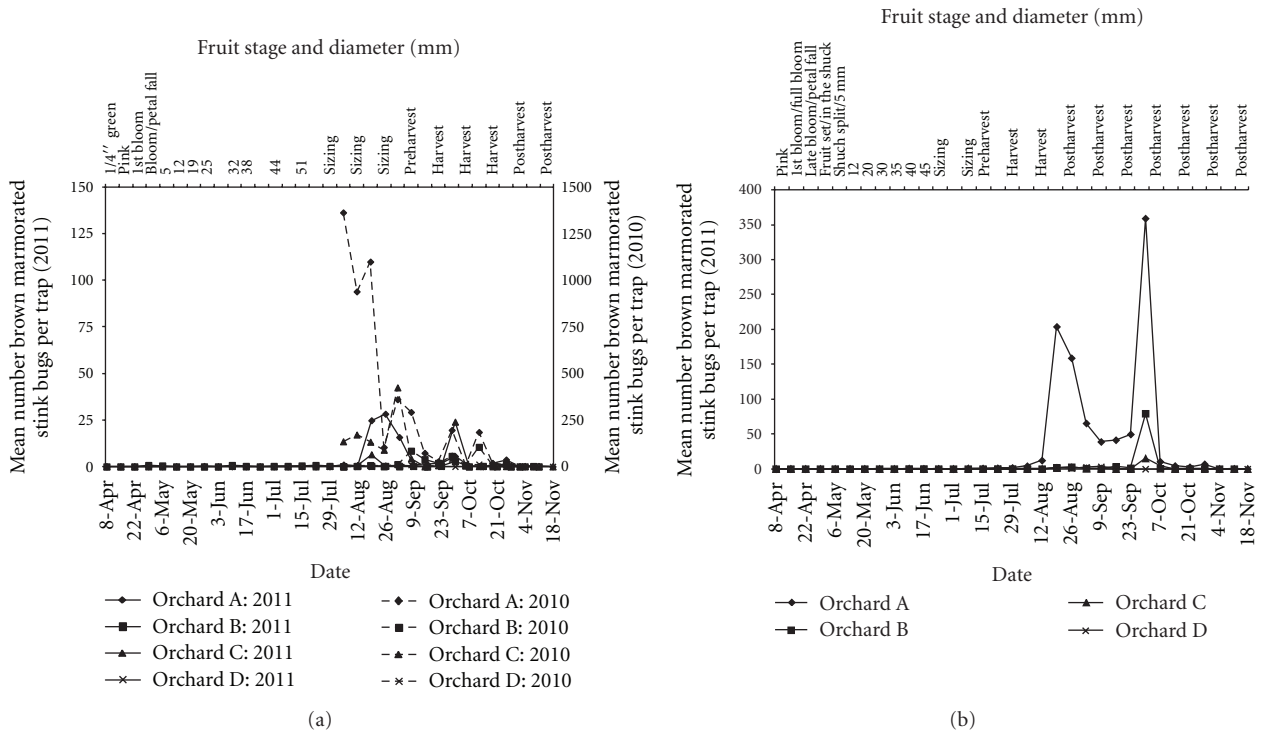

Figure 5: Mean number of brown marmrorated stink bug adults and nymphs captured per trap at orchards A-D in (a) apple in 2010 and 2011 and (b) peach plots in 2011.

Table 2: Total number of targeted brown marmorated stink bug insecticide applications, mean interval (d) ± SEM between insecticide applications, and mean A.I.M. score ± SEM and total A.I.M. score in peach plots from 2010 to 2011 at four commercial orchards.

Orchard	Number of targeted insecticide applications		Mean interval insecticide interval + SEM*		A.I.M. score Mean ± SEM*		Total	
	2010	2011	2010	2011	2010	2011	2010	2011
A	4	16	8.5 ± 1.0	6.7 ± 0.3	0.14 ± 0.03	0.30 ± 0.03	1.67	5.73
B	6	8	16.0 ± 1.9	15.7 ± 1.4	0.16 ± 0.03	0.25 ± 0.04	2.13	2.72
C	4	9	17.0 ± 3.2	14.1 ± 1.2	0.24 ± 0.06	0.30 ± 0.02	2.12	3.26
D	6	22	12.0 ± 2.6	4.5 ± 0.9	0.23 ± 0.05	0.18 ± 0.02	2.73	4.38
All Orchards	20	55	12.8 ± 1.2 a	8.1 ± 0.8 b	0.18 ± 0.02 a	0.25 ± 0.01 b	8.65	16.09

*Means for all orchards combined, compared between years within a paired column, followed by a different letter are significantly different ($P < 0.05$).

$P = 0.0004$), and C ($t = 2.64$; df = 9.4638; $P = 0.0258$); however, there was no statistical difference at orchard D ($t = 0.87$; df = 14.901; $P = 0.4007$) (Figure 6(a)). No differences in the number of injury sites were observed between apples on the exterior or interior of plots for any orchard (A: $t = 1.20$; df = 19.761; $P = 0.2452$, B: $t = 1.02$; df = 15.96; $P = 0.3211$, C: $t = 1.38$; df = 11.963; $P = 0.1923$, D: $t = -0.22$; df = 14.474; $P = .8273$) (Figure 6(b)).

3.4.2. Peach: 2010. Fruit sampling in peach started relatively close to harvest in 2010, so few samples were available for comparison and thus only data summaries were performed. The percent injury and number of injury sites recorded at harvest did not vary greatly from injury recorded at the first sample. Orchard A and B had more injured fruit on the exterior; however the reverse was true at orchard D. There was no interior peach sample at orchard C due to the size and layout of the plot. At the three orchards that had both exterior and interior peach samples, the number of injury sites per injured fruit was higher on the exterior of the plots (Figures 7(a) and 7(b)).

3.4.3. Apple: 2011. In 2011, significantly more apples were injured on the plot exterior than in the interior at orchard A ($t = 2.89$; df = 10.473; $P = 0.0153$), C ($t = 6.43$; df = 24; $P < 0.0001$), and D ($t = 2.61$; df = 18.507; $P = 0.0174$); however, there was no statistical difference at orchard B ($t = 1.75$; df = 6.8897; $P = 0.1235$) (Figure 8(a)). Only orchard C ($t = 2.53$; df = 21.895; $P = 0.0189$) had significantly more injury sites per injured apple on the plot exterior than on the interior (Figure 8(b)).

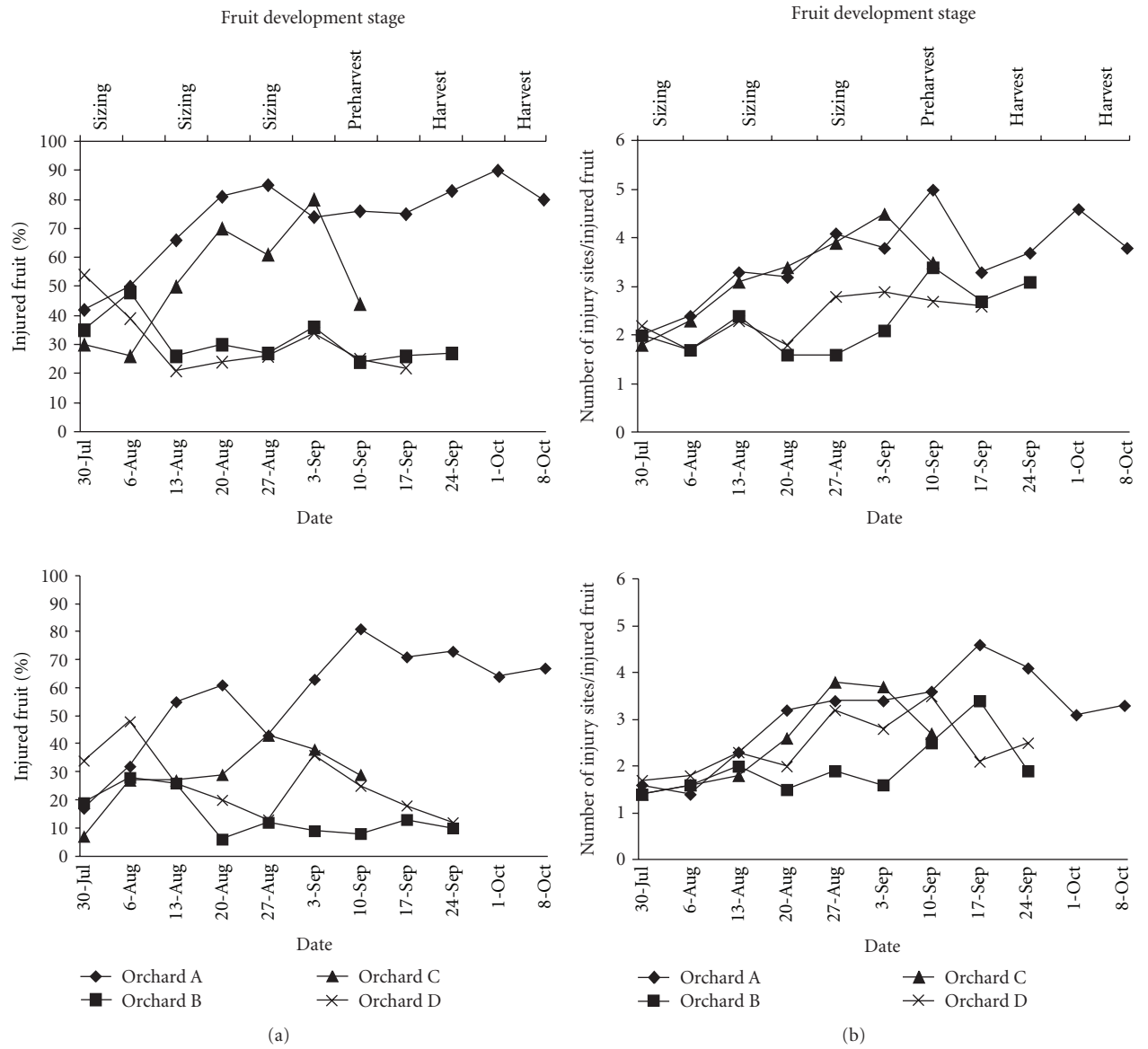

Figure 6: (a) Percent injured apples in 2010 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2010 at orchards A-D in the plot exterior (top) and interior (bottom).

3.4.4. Peach: 2011. In the peach plots, orchard B ($t = 2.13$; df = 17; $P = 0.0477$) and D ($t = 3.34$; df = 17.451; $P = 0.0038$) had significantly more injured fruit on the exterior than in the interior, whereas there was no difference at orchard A ($t = -0.50$; df = 16.598; $P = 0.6230$) or C ($t = -0.46$; df = 13.548; $P = 0.6548$) (Figure 9(a)). Only orchard B ($t = 4.14$; df = 4; $P = 0.0143$) had significantly more injury sites per injured peach on the exterior of the plot than the interior; there was no difference at the other orchards (Figure 9).

3.4.5. Apple: 2010 versus 2011. Finally, significantly more apples were injured from 30 July through harvest in total in 2010 than 2011 at each orchard (A: $t = 13.25$; df = 40; $P < 0.0001$, B: $t = 5.03$; df = 33; $P < 0.0001$, C: $t = 5.32$; df = 38; $P < 0.0001$, D: $t = 5.69$; df = 40; $P < 0.0001$). Likewise, the total number of injury sites per injured fruit was significantly greater in 2010 than 2011 at orchard A ($t = 6.51$; df = 34.78; $P < 0.0001$), C ($t = 2.45$; df = 25.765; $P = 0.0212$), and D ($t = 4.83$; df = 32.231; $P < 0.0001$), but not at orchard B ($t = 1.74$; df = 26.376; $P = 0.0940$).

3.4.6. Peach: 2010 versus 2011. No statistical comparisons were performed due to minimal peach samples collected in orchards in 2010. However, injury was generally higher in both exterior and interior samples in 2010 compared with 2011 at harvest.

4. Discussion

Brown marmorated stink bug has been documented as utilizing apple as a host in Japan [26] and the United States

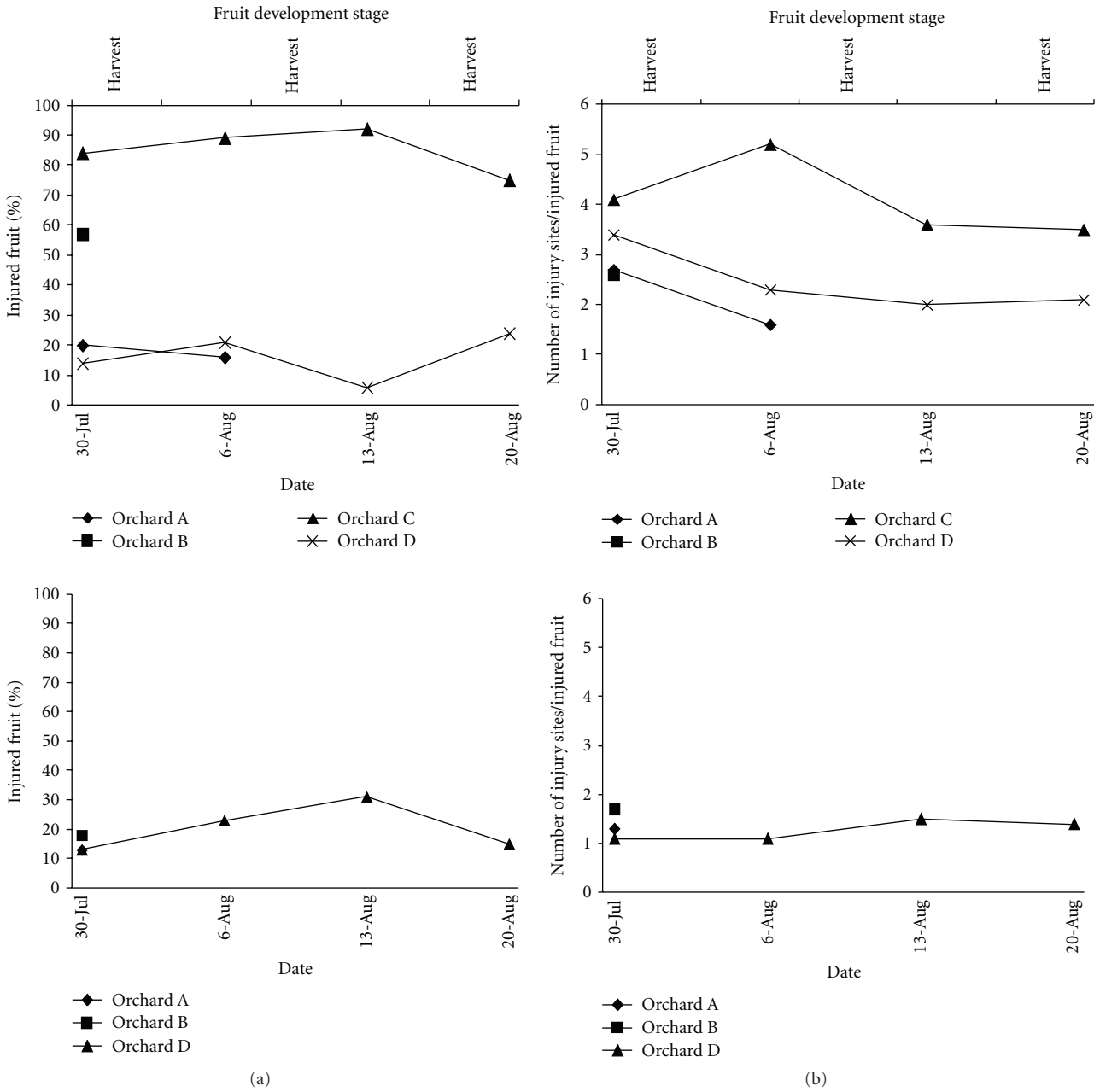

Figure 7: (a) Percent injured peaches in 2010 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2010 at orchards A–D in the plot exterior (top) and interior (bottom).

[6]. Nielsen and Hamilton [7] found that based on a caging study, injury was significantly greater during the late-season compared with petal fall or mid-season. In our studies, we found that natural populations of brown marmorated stink bug in commercial apple blocks will feed on fruit throughout the season, but like native stink bug species [25], feeding injury that occurs in the early season results in a small feeding puncture in the fruit skin and nominal injury to the flesh, while injury inflicted 6-8 weeks after petal fall until harvest results in indented depressions on the surface of the fruit with corky flesh beneath [25, 27]. However, like native stink bugs [25, 27] injury symptoms may take several weeks to manifest completely (S. Joseph, personal communication). Native stink bugs found in mid-Atlantic tree fruit orchards in the United States include *Euschistus servus*, *E. tristigmus*, and *C. hilaris* predominantly [11]. These species will feed on many cultivars of apples, though higher injury rates have been recorded, in one study, on "Braeburn," "Jonica," "Jonagold," "Starkspur Dixiered," "Granny Smith" and "Stayman" [28]. However in our study, no direct comparisons of cultivar susceptibility were conducted. Injury patterns within apple blocks indicate the brown marmorated stink bug is a perimeter-driven threat. Indeed in 2010 and 2011, injury was usually significantly greater at the exterior of orchard blocks

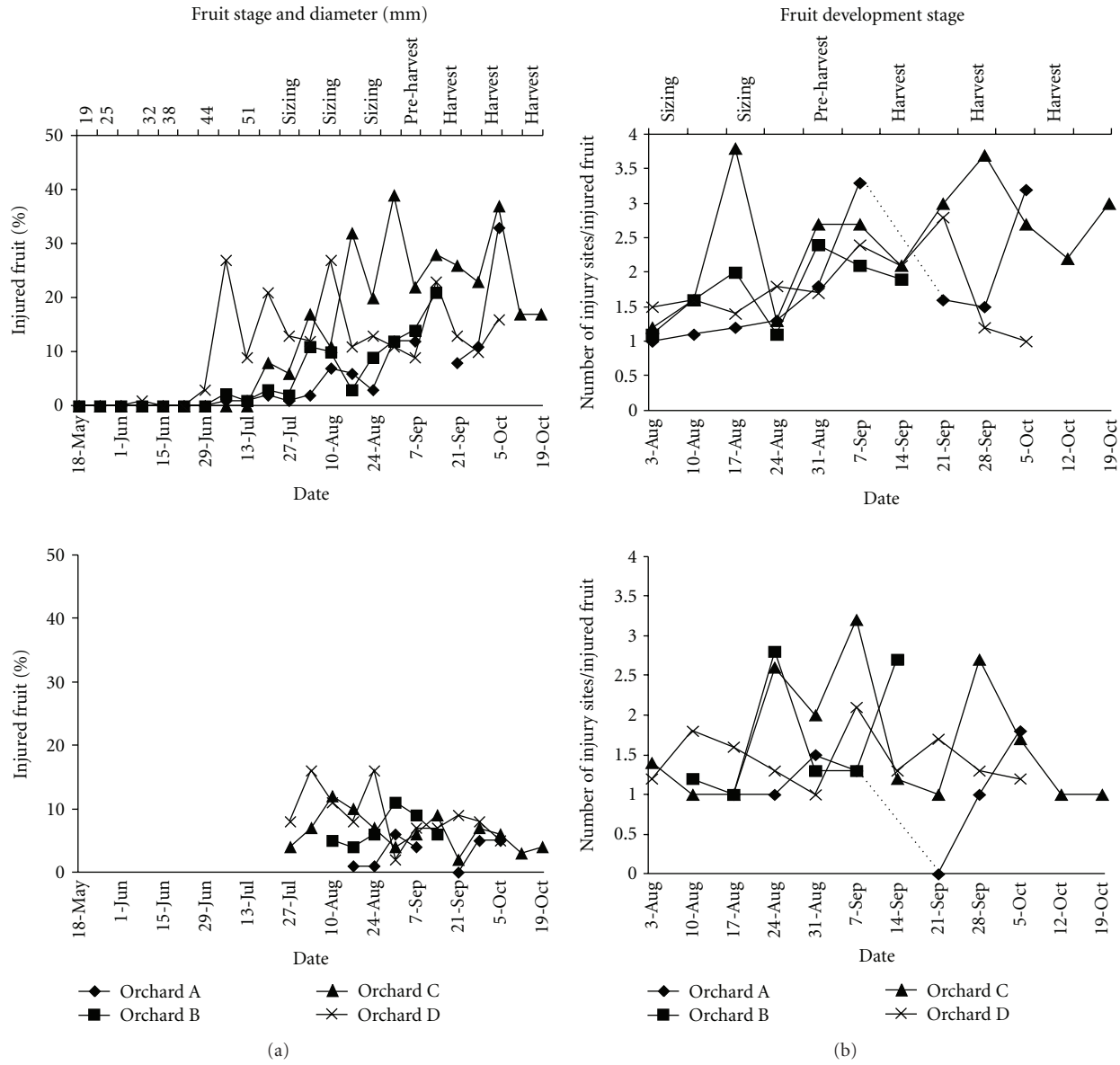

Figure 8: (a) Percent injured apples in 2011 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2011 at orchards A–D in the plot exterior (top) and interior (bottom). Note: the dotted lines in 8B represent missing data on 14 September.

relative to the interior suggesting that adults, emigrating from overwintering sites in the early season and from wood lots or cultivated hosts such as corn and soybean later in the season, constantly invade orchards. Similar patterns of movement have been observed for native stink bug species in other cropping systems [29, 30].

Peach is also an excellent host for brown marmorated stink bug. In cage studies, brown marmorated stink bug caused the greatest injury during the late season [7]. In our studies, natural populations of adults have proven to be extremely damaging in commercial peach orchards in the early season soon after fruit set. In 2011, large numbers of adults moving from overwintering sites began to target the developing peach fruit by 1 June (~30 mm diameter fruit); two orchards had already recorded over 20% damage. In 2010, early-season feeding by adults led to devastating injury to peach growers in many mid-Atlantic states [8]. Unlike apple injury, peach symptoms appeared to manifest very quickly after feeding, within several days. Typically injury inflicted by native stink bugs results in cat-facing and gummosis [31], while early season brown marmorated stink bug injury, though resulting in gummosis, often results in dead pockets of tissue deep in the flesh of the fruit that are not obvious on the surface as the fruit matures. While native stink bugs are capable of inflicting this type of injury as well, it has proven far more prevalent from brown marmorated stink bug. Damage in commercial peach blocks was significantly greater in the exterior compared with the

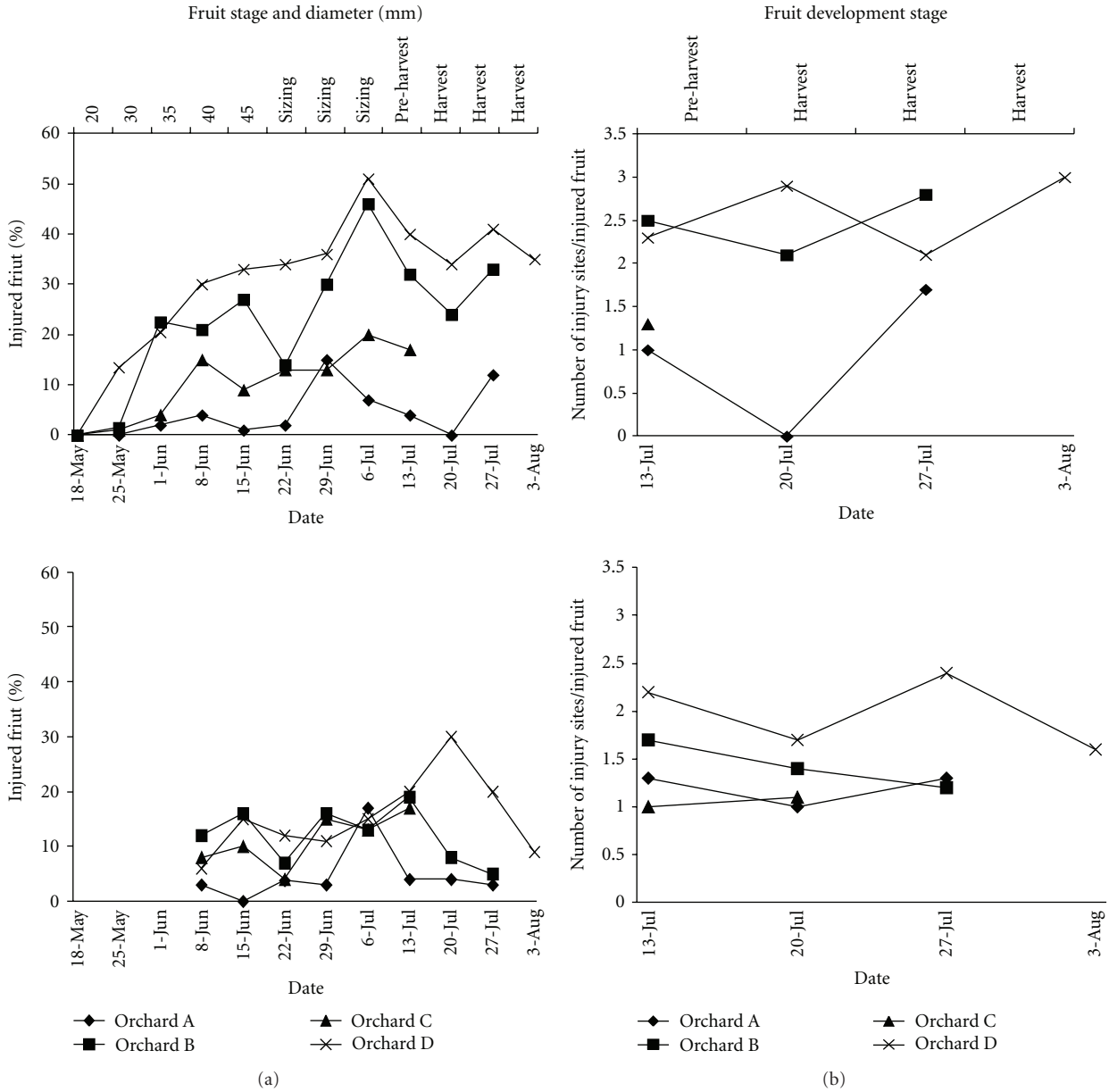

Figure 9: (a) Percent injured peaches in 2011 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2011 at orchards A–D in the plot exterior (top) and interior (bottom).

interior, indicating adults were immigrating into blocks from the outside as was found for apple.

Based on the presence of economic injury, peach fruit is vulnerable soon after fruit set and this vulnerability continues to harvest. By contrast, economic injury to apple generally begins 6–8 weeks after petal fall and can continue until harvest (Figure 8). However, because reproduction can occur in commercial orchards, mitigating treatments must be undertaken early to prevent the threat of nymphal populations contributing to overall injury.

In apple and peach orchard blocks, growers changed their management programs tremendously from 2010 to 2011. In 2010, growers either did not target brown marmorated stink bug specifically or used materials recommended for native stink bugs, particularly pyrethroids. In peach orchards in the southeastern United States, where cat-facing bugs including native stink bugs and tarnished plant bug, *Lygus lineolaris* Palisot de Beauvois, are considered key pests, control is typically achieved using pyrethroids [32]. Unfortunately, many of these same materials did not provide adequate control of brown marmorated stink bug in 2010, as documented in a field trial in which over 25% of moribund bugs exposed to cyfluthrin in treated apple canopies recovered to an actively foraging state [16]. Furthermore, in laboratory trials, treatments of beta-cyfluthrin, lambda-cyhalothrin, zeta-cypermethrin, cyfluthrin, and esfenvalerate resulted in high initial knockdown of adults, but also high levels of recovery [15].

Thus, in 2011, growers relied on materials such as endosulfan, methomyl, permethrin, fenpropathrin, dinotefuran, clothianidin, and thiamethoxam. All of these materials not only demonstrated high levels of immediate knockdown, but also very little recovery [15]. In general, the mean AIM score showed that a more intense insecticide program was implemented in 2011 in response to the threat posed by brown marmorated stink bug. Likewise, the total AIM score illustrates that growers used more of these targeted spray applications in 2011 than in 2010. In addition, growers shortened the intervals between insecticide applications from 2010 to 2011. For apple plots in 2010, growers sprayed at approximately 2-wk intervals but cut that to a 1-wk interval in 2011. Similarly in peach plots in 2010 growers, treated at approximately 13-d intervals but shortened that to 8-d intervals in 2011. These factors likely contributed to both the decrease in overall percent injury and lower trap captures. However, the sustainability of this type of program financially and ecologically is not feasible and growers will certainly need to implement a sensitive and reliable monitoring program for future seasons.

Monitoring tools can be used to effectively assess presence, abundance, and seasonal activity of a pest species, allowing growers to make informed management decisions. In 2010 and 2011, we evaluated the use of black pyramid traps baited with methyl-(2*E*,4*E*,6*Z*)-decatrienoate as a monitoring tool for brown marmorated stink bug populations. This trap and lure combination had been previously shown effective at capturing large numbers of adults and nymphs of brown marmorated stink bug [21]. Significantly more adults were captured in traps in 2010 compared with 2011. This pattern likely reflects four key considerations. First, in 2010, as stated above, growers were not specifically targeting brown marmorated stink bug with insecticide applications, allowing adults to reproduce and populations to build within the orchards. Second, weather patterns were quite different between years. In 2010, a second generation of adults had completed development by 13 September [21], whereas in 2011, development was not complete until 11 October (Leskey, unpublished data). Based on developmental rates developed by Nielsen et al. [33], degree day accumulations required for completion of the second generation were much slower in 2011, possibly leading to much smaller populations observed and subsequently trapped. Third, a different trap top was used in 2011 than in 2010. This trap top has subsequently been reported to be less effective in capturing and/or retaining brown marmorated stink bugs. Finally, differences in overall lure effectiveness may exist between 2010 and 2011 based on overall trap captures and release rates [34] contributing to lower captures in 2011.

However, more problematic is the fact that very few adults were captured in traps throughout the growing season in 2011. It is true that growers instituted season-long management programs in 2011 against brown marmorated stink bug; however, injury rates early in the season indicate that adults were surely present. Funayama [35] captured brown marmorated stink bug adults using traps baited with methyl-(2*E*,4*E*,6*Z*)-decatrieonate early in the season during outbreak years. However, our traps did not recover adults, even remotely reflective of observed populations, until mid-August in apple. Indeed, greatest trap captures actually occurred in peach orchards after the fruit was harvested. Thus, other monitoring techniques were evaluated. As evaluated for native stink bugs in the mid-Atlantic [11], we tested the use of sweep nets and limb jarring samples in commercial orchards season-long in 2011. Almost no bugs were recovered from these sampling techniques indicating that they did not adequately reflect the presence, size, or activity of populations. Timed visual counts also were conducted and did result in greater numbers of bugs being observed, but again the numbers were too low to adequately reflect population density or activity. It is likely that observed behavioral attributes of brown marmorated stink bug are not compatible with these sampling techniques. For example, brown marmorated stink bug has a tendency to be found high in the tree (Short, personal observation) lending to difficulty in obtaining individuals from limb jarring samples taken at head-height or below. Adults have not been observed feeding on many broad-leaf weeds based on host surveys conducted to date [5], which could lead to fewer captures in sweep nets. Likewise, diurnal patterns are not well understood. Certainly, adults and nymphs have been observed actively feeding and mating at night by numerous researchers.

Although cross attraction to methyl-(2*E*,4*E*,6*Z*)-decatrieonate has been observed for brown marmorated stink bug [21] as well as other species including *C. hilaris* [36], it is not attractive to brown marmorated stink bug adults season-long. Thus, identification of the aggregation pheromone of this species could provide a better tool to use with baited traps. Additionally, brown marmorated stink bug adults do respond to visible light (Leskey, unpublished data) and to ultraviolet light as they have been captured in black light traps [6]. Perhaps, creating a trap with optimized olfactory and visual stimuli including the true aggregation pheromone and specific wavelengths of light could provide a much more sensitive monitoring tool that is attractive season-long and can be used to develop treatment thresholds for this invasive species. In the interim, however, it is likely that growers still will need to continue with aggressive management programs aimed at this invasive species in order to mitigate economic injury and successfully grow tree fruit in regions where it is well established.

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Review Article

An Insight into the Sialomes of Bloodsucking Heteroptera

José M. C. Ribeiro, Teresa C. Assumpção, and Ivo M. B. Francischetti

Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Correspondence should be addressed to José M. C. Ribeiro, jribeiro@niaid.nih.gov

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Saliva of bloodsucking arthropods contains dozens or hundreds of proteins that affect their hosts' mechanisms against blood loss (hemostasis) and inflammation. Because acquisition of the hematophagous habit evolved independently in several arthropod orders and at least twice within the true bugs, there is a convergent evolutionary scenario that creates a different salivary potion for each organism evolving independently to hematophagy. Additionally, the immune pressure posed by their hosts creates additional evolutionary pressure on the genes coding for salivary proteins, including gene obsolescence, which opens the niche for coopting new genes (exaptation). In the past 10 years, several salivary transcriptomes from bloodsucking Heteroptera and one from a seed-feeding Pentatomorpha were produced, allowing insight into the salivary potion of these organisms and the evolutionary pathway to the blood-feeding mode.

1. Introduction

The order Hemiptera (bugs) comprises hemimetabolous insects having in common tubular mouthparts specialized for sucking liquid diets. The diet of Hemiptera is varied, the majority feeding on plants by either tapping the vessels conducting sap or by lacerating and flushing tissues such as leaves or seeds. Within the suborder Heteroptera (true bugs), predatory feeding (with killing of the victim) also occurs, mostly targeting other insects but also including small vertebrates such as giant water bugs and toad bugs, as well as blood or hemolymph feeding (without killing the victim) from vertebrate and invertebrate animals. The mouthparts are not only important for channeling the liquid meal but are extremely important mechanically in finding the proper spot for meal suction [1].

Saliva is produced, sometimes copiously, during the probing phase (the time between mouthpart contact with the food substrate and the commencement of the meal) and throughout the meal [31, 32]. This saliva is ejected at the tip of the maxillae by the salivary channel, which is built in between the interdigitations of the two plates that form the maxillae [33]. Saliva helps probing and feeding physically by liquefying insoluble or viscous tissues or by helping to seal the feeding site in sap suckers, were the phloem is under very high pressure [34]. Saliva has a biochemical role in aiding digestion of the meal, just as we have amylase in our own saliva; most remarkably, predacious bugs inject a highly hydrolytic cocktail into their victims that is digested while the prey is held by the predator, which can then later suck the liquefied victim and discard it as an empty shell. Saliva can also work pharmacologically by preventing the hosts' defense mechanisms against tissue loss, as occurs with the saliva of blood-feeding insects in preventing blood clotting, for example [35].

Among the Heteroptera, the blood-feeding habit evolved at least twice in the Cimicomorpha families, once in the Cimicidae (containing the bed bug) including the small sister group Polyctenidae (bat bugs), and in the Reduviidae (kissing bugs) from possible predacious or hemolymph-sucking ancestors [1]. Within the Reduviidae, it is possible that the genus *Rhodnius* (tribe Rhodnini) is monophyletic, having evolved independently of the remaining triatomines (tribe Triatomini) [47–49]. The ancestral Cimicomorpha dates back to the Triassic/Jurassic border, over 250 MYA [1]; accordingly, the habit of blood or hemolymph feeding started

in this group well before mammals irradiated. Within these hematophagous bugs, blood is the only diet for all immature and adult stages.

To obtain blood in a fluid state, these bugs have to counteract their host's hemostasis, the physiologic process that prevents blood loss, which includes the triad of platelet aggregation, blood clotting, and vasoconstriction. Blood-circulating platelets may be triggered to aggregate by various signals, including ADP from broken cells and also released by activated platelets, collagen from subendothelial surfaces, thrombin (produced during blood clotting), and thromboxane A_2 (TXA_2—produced by activated platelets). Blood clotting may be initiated by activation of the intrinsic pathway via activation of Factor XII or by activation of the tissue factor pathway, both converging to the activation of Factor X to X_a, which activates prothrombin to thrombin which in turn cleaves fibrinogen into fibrin, forming the blood clot. Activated platelets produce the vasoconstrictor TXA_2 and also release stored serotonin and epinephrine, both powerful vasoconstrictors. A single magic bullet cannot properly destroy a redundant and complex obstacle such as this; rather, a magic potion of several antagonists is required.

Saliva of hematophagous arthropods also contains activities that interfere with the host's immune and inflammatory system in the form of immunomodulatory substances, particularly in ticks, which stay attached to their hosts for days or weeks, in contrast to minutes of host contact by bloodsucking bugs. Saliva also contains antimicrobial compounds that might help to control bacterial growth in the meal, because ejected saliva is reingested with the blood meal during blood feeding. For more detailed reviews on host hemostasis and immunity, see Francischetti et al. [56, 57].

Salivary anticlotting compounds from bloodsucking insects have been known to occur for nearly 100 years [58], while antiplatelet activity was first detected in the 1980s [59, 60] and vasodilators have only been described since the early 1990s [61]. In blood-feeding bugs, anticlotting agents from the saliva and crop of *Rhodnius* were described first by Hellman and Hawkins in 1965 [62], the first antiplatelet activity was reported in 1981 [59, 63], and *Rhodnius* salivary vasodilator was reported in 1990 [64]. Anticomplement activities have also been found [65], as well as antihistamine, antiserotonin [66], and antithromboxane [67] activities from *Rhodnius* saliva. An anesthetic was found in *Triatoma infestans* saliva in 1999 [68]. None of these earlier reports characterized the molecular nature of the compounds, most of these have been achieved in the past 20 years during the so-called "grind and find" period of discovery. Table 1 lists the molecularly characterized salivary components of blood-feeding Hemiptera.

2. On Sialomes

In the past 10 years, a new method to unveil the salivary potion of hematophagous insects has been practiced in the form of decoding their sialotranscriptomes (from the Greek, sialo = saliva), achieved by random sequencing of 500–2,000 cDNA clones originating from polyA-enriched RNA from the salivary glands of these animals. After assembly of these sequences into contigs (which represent full or near full-length mRNA), these can be compared by bioinformatic tools such as BLAST and rpsblast [69] to other proteins in public databases (such as Swissprot, Gene Ontology [70], and GenBank [71] protein data banks, and CDD, PFAM, SMART and KOG [72], which are motif databases to be explored with the rpsblast tool) to identify closely related sequences and functional motifs. Additional searches for signal sequences indicative of secretion [73], for transmembrane helices [74], and for glycosylation sites [75] are also helpful to attempt functional classification of the protein. We are now on the eve of another revolution, with the increase by thousands of fold on the number of sequences that can be economically sequenced from these libraries, which will allow identification of the lesser expressed (and possibly most potent) proteins.

So far, 12 sialotranscriptomes—all done with less than 3,000 sequenced clones per organism—have been reported from Heteroptera, 11 of which are from blood-feeding Cimicomorpha and one from the seed-feeding *Oncopeltus fasciatus*. *Oncopeltus* belongs to the Pentatomomorpha, the closest group to Cimicomorpha [76] (Table 2). Among the Cimicomorpha sialotranscriptomes, only one derives from Cimicidae (the bed bug *Cimex lectularius*); the remaining are from Triatominae, encompassing four genera (*Rhodnius, Triatoma, Dipetalogaster,* and *Panstrongylus*), although some of these transcriptomes have no proteins deposited in public databases and too few expressed sequence tags (ESTs) publicly available. A few isolated protein sequences are also available from GenBank, deriving mostly from predatory bugs. The publicly available proteins are displayed together in Additional File 1, which is a hyperlinked Excel spreadsheet where the putative secreted proteins are organized in one worksheet and the putative housekeeping proteins are displayed in another worksheet.

The secreted proteins can be classified in two major groups, those belonging to ubiquitous protein families and those of unique status among the Hemiptera family, genus, or even species level (Table 3). We will proceed to describe the protein families in the order shown on Table 3.

3. Ubiquitous Protein Families

3.1. Enzymes

3.1.1. Apyrase, 5′-Nucleotidase, and NUDIX Hydrolase. Apyrases are enzymes that can hydrolyze ATP and ADP to AMP [77–79]. Initially the existence of true apyrases was doubted, because they could originate from a mixture of enzymes such as adenylate kinase and ATPases; however, their real intracellular existence in the potato was shown later [79, 80] and its function in carbohydrate anabolism and in the promotion of glycosyltransferases was only much later discovered, as indicated [81, 82]. The role of extracellular apyrases on preventing platelet aggregation was demonstrated for the first time in *Rhodnius* saliva [63, 83, 84] and later shown in the saliva of mosquitoes [85–87] and in the vascular endothelium [88–90]. The activity from *Cimex lectularius* was purified and cloned, revealing a new type of

Table 1: Molecularly and functionally characterized salivary components of bloodfeeding Hemiptera.

Name	Family	Insect	Activity	Notes	Reference
Prolixin	Nitrophorin	*Rhodnius prolixus*	Anticlotting	FXa inhibitor	[2]
RPAI	Lipocalin	*R. prolixus*	Antiplatelet	Binds ADP	[3, 4]
Nitrophorins	Nitrophorin	*R. prolixus*	Antihistamine	Binds histamine, carrier of NO	[5, 6]
BABP	Nitrophorin	*R. prolixus*	Antiserotonin	Binds serotonin	[7]
Inositol phosphatase	Inositol phosphatase	*R. prolixus*	Inositol phosphatase	Unknown function	[8]
Lysophosphatidylcholine	Lipid	*R. prolixus*	Antihemostatic		[9]
NO	Inorganic gas	*R. prolixus, Cimex lectularius*	Vasodilatory, antiplatelet	Activates guanylate cyclase	[10, 11]
Apyrase	5′-nucleotidase	*Triatoma infestans*	Antiplatelet	Destroys ADP	[12, 13]
Triplatin	Lipocalin	*T. infestans*	Antiplatelet, vasodilator	TXA_2 binder	[14, 15]
Triafestin	Lipocalin	*T. infestans*	Anticlotting, antipain	Inhibits FXII activation	[16]
Trialysin	Trialysin	*T. infestans*	Antimicrobial	Pore forming	[17, 18]
Pallidipin	Lipocalin	*T. pallidipennis*	Antiplatelet	Collagen inhibitor (possible TXA_2 binder)	[19, 20]
Triabin	Lipocalin	*T. pallidipennis*	Anticlotting	Anti-thrombin	[21–23]
Procalin	Lipocalin	*T. protracta*	Allergen	Function unknown	[24]
Dipetalodipin	Lipocalin	*Dipetalogaster maxima*	Antiplatelet, vasodilator	TXA_2 binder	[25]
Apyrase	*Cimex* apyrase	*Cimex lectularius*	Antiplatelet	Destroys ADP	[26]
Nitrophorin	Inositol phosphatase	*C. lectularius*	Antiplatelet, vasodilator	Carrier of NO	[27–29]
Fibrinolytic enzyme	Serine proteinase	*Panstrongylus megistus*	Anticlotting		[30]

Table 2: Salivary transcriptomes of Hemiptera/Heteroptera.

Organism	Number of ESTs on DBEST	Number of derived proteins in GenBank	Reference
Rhodnius prolixus	1,439	56	[36]
R. brethesi	55	0	[37]
R. robustus	121	0	[37]
Triatoma infestans	1,738	167	[38]
Triatoma brasiliensis	2,109	28	[39]
Triatoma matogrossensis	2,230	196	[40]
Triatoma rubida	1,850	93	[41]
Triatoma dimidiata	53	53	[42]
Dipetalogaster maxima	2,671	66	[43]
Panstrongylus megistus	45	0	[44]
Cimex lectularius	1,969	102	[45]
Oncopeltus fasciatus	1,115	37	[46]

enzyme that is ubiquitous in nature [26, 91, 92]. That for *T. infestans*, though, was found to belong to a completely different family, that of the 5′-nucleotidase family of enzymes [12]. Interestingly, sand flies [93] express salivary apyrases of the *Cimex* type, while mosquito salivary apyrases belong to the 5′-nucleotidase family [87, 94], clear examples of convergent evolution.

Nudix hydrolases or bis(5′-nucleosidyl)-tetraphosphatases (EC: 3.6.1.17) are enzymes that hydrolyze nucleotides joined by their phosphate groups such as AP4A or AP5A in the case of diadenosine nucleotides, which are known agonists of platelet aggregation and inflammation [95–98]. *C. lectularius* sialotranscriptomes presents clear evidence of such enzymes, but the activity in salivary homogenates was never studied.

Lacking in these Heteroptera sialotranscriptomes are additional nucleotide-acting enzymes, such as endonucleases, found in mosquitoes and sand flies [99–101], and adenosine deaminase, found also in mosquito and some, but not all, sand flies [102–104].

Table 3: Classification of the protein families relevant to secreted products in the Hemiptera/Heteroptera.

Classification	No. of proteins	Genera found[1]	Function characterized?[2]	References
Ubiquitous protein families				
Enzymes				
Cimex apyrase	1	C, R(?)	Y	[26, 50]
5′-nucleotidase	6	T	Y	[12, 13, 51]
Cimex NUDIX hydrolase	3	C		
Secreted esterase	5	C, T		
Inositol phosphate phosphatases including *Cimex* nitrophorins	24	C, T, R	Y/N	[8, 27, 28]
Serine proteases	17	C, T	Y/N	[30]
Chitinase	1	O		
Other enzymes	4	T, O		
Protease inhibitor domains				
Kazal domain containing proteins	13	T		
Serpin	1	C		
Pacifastin-related peptide	1	O		
Cystatin	5	O		
Lipocalins	331	T, R	Y/N	[2, 4–7, 11, 14, 15, 19–21, 23, 43, 52–55]
Salivary OBP	19	C, T, R		
Salivary antigen 5 family	22	C, T, R		
Triatoma dimidiata lectin	2	T		
Immunity related				
Lysozyme	4	C		
Defensin	1	T		
Histidine-rich peptide	1	T		
Immune-related conserved insect protein	1	T		
Arthropod or insect specific families				
Cuticle-like proteins and conserved mucins	5	T		
Conserved insect secreted protein family	6	C, T, O		
Mys2 family	3	R, T		
Cimex-Triatoma family	3	C, T		
Other individual proteins of conserved insect families	4	T		
Hemiptera specific families				
Mys3/hemolysin-like family	16	T, R, O		
Triatoma-specific families				
Trialysin	8	T	Y	[17, 18]
Short trialysin	6	T		
Triatoma matogrossensis family	2	T		
Triatoma matogrossensis family 2	2	T		
Orphan *Triatoma* proteins	19	T		
Rhodnius-specific families				
Orphan *Rhodnius* proteins, include low-complexity proteins	3	R		
Cimex-specific proteins				
Cimex mucin family	2	C		
Orphan *Cimex* proteins	1	C		

Table 3: Continued.

Classification	No. of proteins	Genera found[1]	Function characterized?[2]	References
Oncopeltus-specific families				
Oncopeltus family	3	O		
Oncopeltus family 2	2	O		
Orphan *Oncopeltus* protein	12	O		
Total	559			

[1] C: *Cimex*; T: *Triatoma*/*Dipetalogaster*/*Panstrongylus*; R: *Rhodnius*; O: *Oncopeltus*.
[2] Y: yes; Y/N: characterization of a few or a single member of the family.

3.1.2. Acetylcholinesterases. Four well-expressed and closely related isoforms of a typical acetylcholinesterase enzyme were found in the sialotranscriptome of *C. lectularius* [45]. A single transcript from the same family was also found in *Triatoma matogrossensis*. Although most acetylcholinesterases are extracellular membrane-bound enzymes by virtue of a glycophosphatidyl-inositol membrane anchor in their carboxy termini, these Cimicomorpha enzymes lack this terminal region, and thus these enzymes are secreted. The role of these enzymes in blood feeding is not yet apparent.

3.1.3. Inositol Triphosphate Phosphatases (IPPase) Including Cimex Nitrophorin. This family of proteins has been found ubiquitously in the sialomes of bloodsucking Cimicomorpha, including the well-characterized enzyme from *R. prolixus* [8] and the *C. lectularius* nitrophorin [27–29], a protein found associated with a heme moiety and a carrier and stabilizer of nitric oxide (NO), a very reactive gaseous substance that is also a potent vasodilator and platelet aggregation inhibitor. While the function of *Cimex* nitrophorin is without question, the function of an extracellular inositol phosphatase is puzzling, because these inositol phosphates are intracellular and not available to an extracellular enzyme. Indeed, it appears fitting that inositol polyphosphates should be hydrolyzed, because they perform a proplatelet aggregation function as well as proinflammatory and immune-enhancing roles in leukocytes [105, 106]. Perhaps the enzyme may reach the intracellular pool by some not yet understood mechanism. On the other hand, association of heme with inositol phosphatases seen in the case of *Cimex* nitrophorins is not at all common, being unique to these proteins; investigation of the amino acids that are associated with heme binding does not reveal similarities to other IPPases from either vertebrates or invertebrates (Ribeiro, unpublished).

The phylogram of the IPPase sequences found in Additional File 1 (Figure 1) shows the *Cimex* nitrophorins contained within a strong clade with 100% bootstrap support and constituted by at least three subclades representing at least three genes expressing these NO transporters, plus alleles or other genes. *Cimex* has two additional sequences outside the nitrophorin clade and near the IPPase clade of the remaining triatomines. It is thus interesting that both *Cimex* and triatomines have a common IPPase in their sialome, even though we have no idea of their function. IPPases have not been found in any other transcriptome so far done, including those of mosquitoes, sand flies, biting midges, black flies, and ticks, being thus uniquely from Cimicomorpha blood feeders.

3.1.4. Serine Proteases. Serine proteases are commonly found in the sialotranscriptomes of insects and ticks, as well as in those of Heteroptera. An unusual serine protease activity in the saliva of *T. infestans* has been noted before, but only a partial enzyme purification of the enzyme, named triapsin, was achieved [107]. Within the bloodsucking Heteroptera, only one *Panstrongylus megistus* sequence has been molecularly characterized as a fibrinolytic enzyme [30]. Additional File 1 shows such proteins from Cimicomorpha, including plant-feeding bugs such as *Lygus lineolaris, Lygus hesperus,* and *Creontiades dilutus* [108–110]. The phylogram of these enzymes (Figure 2) shows two well-defined clades, one containing most of the *Lygus* sequences, but also two *T. matogrossensis* and one *T. brasiliensis* sequence, within a clade of 86% bootstrap support, suggesting a common ancestral salivary serine protease for plant- and blood-feeding Cimicomorpha. The fibrinolytic enzyme of *Panstrongylus* shares a strongly supported clade with two other *T. matogrossensis* sequences, which are probable orthologs of the *Panstrongylus* gene. The *Cimex* sequence appears as an outlier to the group. *Rhodnius* sialotranscriptomes have not revealed proteases, and its saliva does not hydrolyze the substrates used in the characterization of the *T. infestans* triapsin (Ribeiro, unpublished).

3.1.5. Other Enzymes. A chitinase and a lipase were found in *Oncopeltus*, while *T. matogrossensis* displayed a salivary phospholipase and a metalloprotease. The precise role of these enzymes is unknown. Salivary metalloproteases in ticks have been associated with fibrinolytic and antiangiogenic activities [111, 112], while the *Oncopeltus* enzyme may be associated with digestive or antifungal functions.

3.2. Protease Inhibitor Domains

3.2.1. Kazal Domain-Containing Peptides. The Kazal domain occurs in many protease inhibitors, and its structure was first determined for the proteinase inhibitor IIA from bull seminal plasma [113]. The sialotranscriptome of members of South American *Triatoma* (*T. infestans, T. matogrossensis,* and *T. brasiliensis*) but not North American *T. dimidiata* or *T. rubida,* nor any other sialotranscriptome of Cimicomorpha, abounds with transcripts coding for proteins containing this

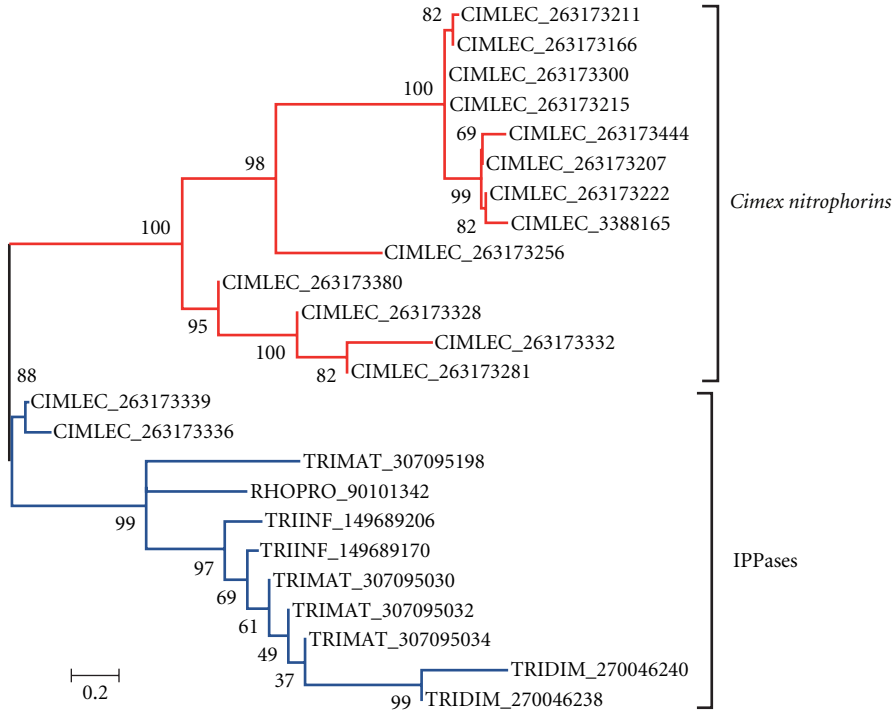

Figure 1: Phylogram of the inositol triphosphate phosphatase family of Cimicomorpha. The sequences are named with the first 3 letters of the genus name, followed by the first 3 letters of the species name, followed by their GenBank GI accession number. The sequences were aligned by Clustal, and the neighbor-joining bootstrapped phylogram was obtained with the MEGA package with 10,000 iterations, Poisson model of amino acid substitution and pairwise amino acid comparisons using the gamma rate of amino acid substitution (gamma parameter = 1). The numbers at the nodes are the percent bootstrap support. The line at the base indicates the rate of amino acid substitution per site.

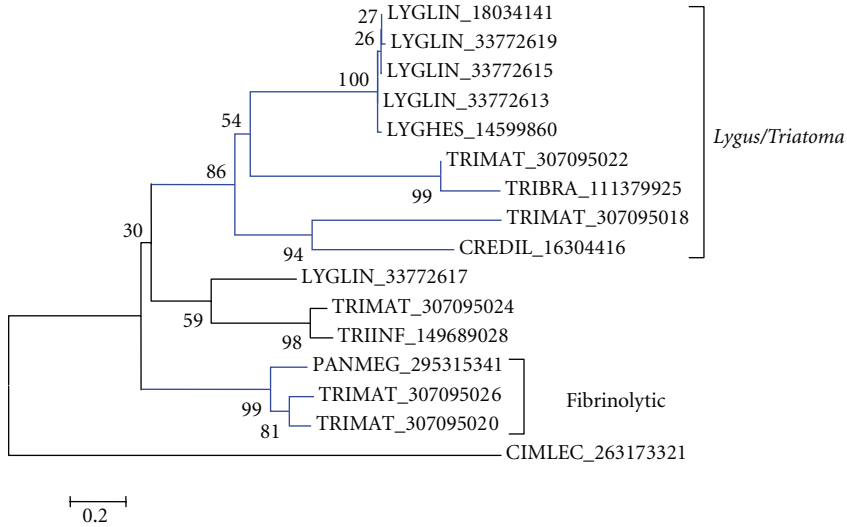

Figure 2: Phylogram of the salivary serine proteases of Cimicomorpha. See the legend of Figure 1 for more details.

domain; however, none have been so far characterized functionally. In *Rhodnius, Triatoma,* and *Dipetalogaster*, the crop antithrombin has been characterized as a protein containing two such domains [114–117], but salivary anticlotting of *Rhodnius* and *Triatoma* has been shown to be different lipocalins named prolixin S and triabin [2, 21, 118]. Kazal-type peptides can function as antimicrobials by inhibiting microbial exoproteases essential for their survival [119, 120] and can also work as vasodilators, as in the case of a tabanid salivary protein named vasotab, which is suspected to modify

ion channels [121]. These functions should be taken into consideration in functional assays of the recombinant Kazal peptides.

3.2.2. Serpin. The serine protease inhibitor (serpin) family is ubiquitous in nature, functioning mostly as endogenous regulators of proteolytic cascades such as inhibiting thrombin in vertebrates (plasmatic antithrombin 3) or regulating phenol oxidase activation cascades in invertebrates [122, 123]. The salivary anticlotting proteins of *Aedes* mosquitoes (but not those of anopheline mosquitoes) are members of this family [124, 125]. A single sequence of this family, derived from four ESTs, was found in the sialotranscriptome of *C. lectularius*. Its target is still unknown.

3.2.3. Pacifastin and Cystatin. Proteins containing these domains were only found in the sialotranscriptome of *Oncopeltus*. Pacifastins are typical serine protease inhibitors of insects and crustaceans [126], while cystatins are ubiquitous proteins typically inhibiting cysteine proteases [127]. Although a single EST was found coding for the pacifastin peptide, five well-expressed cystatins were identified in *Oncopeltus*. The targets of these peptides are unknown, but it was suggested that the salivary cystatins may prevent plant apoptosis induced by cysteine proteases [46, 128, 129]. Tick sialomes have revealed cystatins that were shown to inhibit inflammation and maturation of dendritic cells in their hosts [130].

3.3. Lipocalins. The term lipocalin literally means a cup of lipid, as these proteins form a barrel with a hydrophobic interior cavity that is suitable to transport lipids and other hydrophobic compounds in an aqueous milieu [131–133]. There is virtually no sequence conservation in the family, which is recognized by its typical 3D structure composed of a repeated +1 topology β-barrel. This protein family is by far the most abundant in sialotranscriptomes of triatomine bugs (see review [132]) but remarkably absent in *Cimex* and *Oncopeltus*; however, it was also abundantly recruited in tick sialomes [56], another case of convergent evolution. Additional File 1 provides for 331 lipocalins, which is more than half of all putative secreted proteins listed in this work. Several of these proteins may be alleles of the same gene. The sheer size of the family in individual species is indicative of gene duplication events that might have had an impact during the evolution of blood-feeding [134–136]. Following gene duplication—by retrotransposition or more commonly by forming tandem repeats due to transposable element recombination—the new genes can lead to an increased transcript load in a particular organ or tissue. If this augmented expression increases fitness (e.g., helps the bug to feed), the gene will persist; otherwise, it will evolve to be a pseudogene [137]. Once genes are duplicated and fitness is increased by the duplication, these are free to evolve independently and to diverge from each other by acquisition of novel functions. Salivary genes of bloodsucking arthropods are under selection by two different processes. First, the gene can evolve in the direction of fine tuning its function in relationship to its target. For example, a bug feeding on a bird may have "ideal" anticlotting, but if ecologic changes appear and the bug shifts to another host, this anticlotting may still work but have some room for improvement (e.g., by increasing its affinity to the specific thrombin). Second, any protein injected into the skin of a vertebrate is capable of eliciting an immune reaction, which may lead to defensive host behavior following mast cell degranulation or complement-mediated local inflammation, leading to interruption of the meal or killing of the insect. This may lead to a scenario of balanced polymorphism, with the least common epitope being the best one to have, thus multiplying the number of different alleles in a population that are selected to have the same optimal function but the least common antigenicity. Host immune pressure can also lead to gene obsolescence, creating a niche for cooption (exaptation) of new genes, including horizontal transfer [138], which may substitute for the lost function and thus may explain the appearance of novel salivary genes in related organisms [139].

Lipocalin functions in triatomines are multiple and linked to their unique barrel when working as kratagonists (from the Greek kratos = seize) [140], which are binders of relatively small agonists such as biogenic amines, TXA_2, leukotrienes, or ADP, or carrying the heme that carries NO in *Rhodnius* nitrophorins, or functions linked to their side chains when they work as anticlotting agents such as triabin (for references for these functions, see Table 1). Uniquely, the protein nitrophorin 2 from *R. prolixus* has three functions: (i) it carries NO, (ii) it binds histamine, and (iii) it is an inhibitor of the activation of Factor X [5, 141]. Notice that contrary to their names as "lipid cups," many of these lipocalin ligands are well charged and not hydrophobic, such as biogenic amines and ADP. The functions of the salivary lipocalins in ticks are similarly associated with their kratagonist activity toward biogenic amines or arachidonic acid derivatives, or as inhibitors of complement activation [142–148].

A phylogram of the triatomine lipocalins, although a bit overwhelming in size, presents a bird's-eye view of the several distinct families arranged mostly in robust clades (Figure 3 and Additional File 2). Most clades have not a single member that has been analyzed functionally (marked with Roman numerals in Figure 3), including the clade containing the *Triatoma protracta* antigen procalin; accordingly there are eight clades that have no known function. Additional File 2 is provided for high-resolution display of the sequences, which have their NCBI accession numbers for sequence retrieval. A few details deserve some comments with respect to the phylogram. (i) The clade named Pal-Tri-Dip contains the *Triatoma* proteins pallidipin, triplatin, and the *Dipetalogaster* protein dipetalodipin, which are platelet inhibitors possibly all due to being TXA_2 kratagonists as demonstrated for triplatin and dipetalodipin [25], thus indicating the conservation of this function among two different genera. (ii) Most *Rhodnius* lipocalins cluster in two clades, one containing all the known NO carriers, named nitrophorins (NP) and the other containing the adenosine nucleotide kratagonists named RPAI (*Rhodnius* platelet aggregation inhibitor). (iii) The *Rhodnius* biogenic amine-binding protein (BABP)

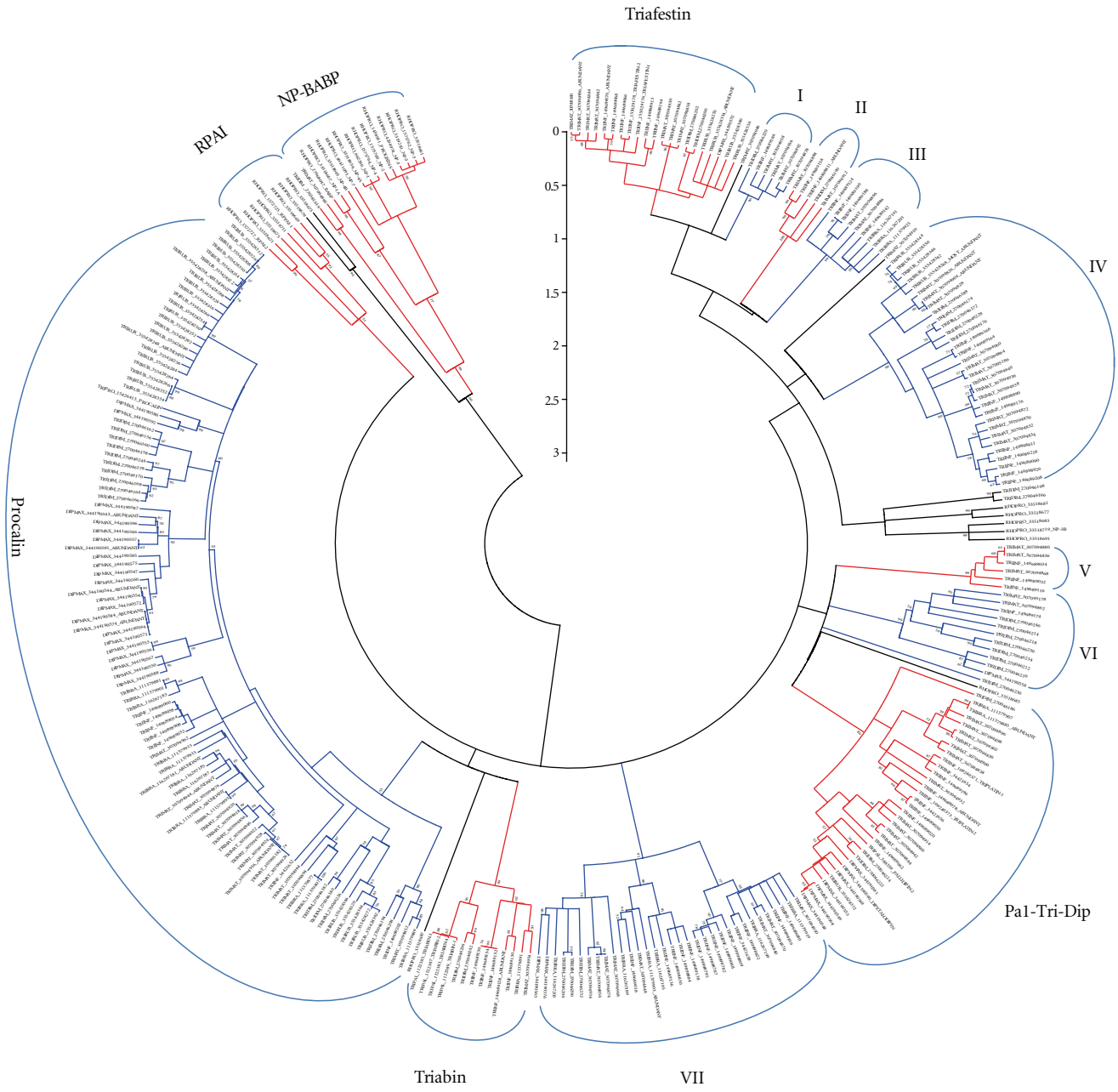

Figure 3: Phylogram of the salivary lipocalin family of triatomines. Clades containing members that have been functionally characterized are named according to these proteins. Other clades are named with Roman numerals. Except for the triafestin clade, other clades have >70% bootstrap support. The sequences are named with the first 3 letters of the genus name, followed by the first 3 letters of the species name, followed by their GenBank gi| accession number. When the protein has been functionally characterized, its name is also included after the NCBI number. Abundantly expressed proteins are also marked to indicate this fact. The numbers in the bar indicate the rate of amino acid substitution per site. For other details, see Figure 1 legend.

somewhat surprisingly clusters with the nitrophorins, but BABP does not have a heme group and has higher affinity for serotonin and norepinephrine, constituting a good example of gene duplication and divergence of function [7]. (iv) Exceptionally, one *T. matogrossensis* and one *T. dimidiata* protein sequence group with the NP-BABP clade with 99% bootstrap support. The function of these proteins could lead to the original function of the *Rhodnius* nitrophorins, which are exclusive of the genus *Rhodnius*. Indeed the abundance of these heme proteins in *Rhodnius* salivary glands makes these glands distinctively bright cherry red in color, as first pointed out by Wigglesworth nearly 70 years ago [149]. *Triatoma* and *Dipetalogaster* glands are clear or of a very pale yellow color [150]. (v) *Rhodnius* lipocalins not belonging to the NP-BABP and RPAI clades are scattered in the phylogram, including one sequence between the procalin and triabin

clades, one between the triabin and VI clades, and a group of four proteins between clades IV and V. None of these *Rhodnius* proteins group within strong bootstrap support to any of the *Dipetalogaster*- or *Triatoma*-containing clades. (vi) Finally, the procalin clade is very extensive and contains many robust subclades, many of which are of single species, indicating possible recent events of gene duplication or extensive polymorphism.

3.4. Odorant/Pheromone-Binding Family (OBP). The OBP family, like the lipocalins, is specialized in carrying small hydrophobic ligands in aqueous media [151, 152]. A modified version of the odorant-binding family of proteins is very abundant in the sialotranscriptomes of hematophagous Nematocera [138] and named as the D7 protein family. A few mosquito proteins have been crystallized and functionally characterized, showing kratagonist activity toward biogenic amines, TXA_2, and leukotrienes, in addition to anticlotting activity [153–156].

In hematophagous Cimicomorpha, members of the OBP family are found in *Rhodnius, Triatoma,* and *Cimex* but are particularly abundant in *Cimex*, with two OBP proteins having over 250 ESTs in a total of ~2,000 ESTs, suggesting the OBP family has been recruited by *Cimex* to function as the lipocalins in triatomines. No salivary member of this family in Cimicomorpha has been so far functionally characterized.

3.5. Antigen-5 Family. This is a ubiquitous protein family found in plants and animals, including expression in the venom glands of vespids, where it was recognized as an antigen, thus the name antigen 5 for this family. They are members of the CAP superfamily, most with unknown function [157]. In snakes and lizards, they have been associated with venom toxins [158–160]. In stable flies, one salivary antigen 5 protein binds immunoglobulins and may function as an inhibitor of the classical pathway of complement activation [161]. In horse flies, one protein has acquired a disintegrin motif and is a strong inhibitor of platelet aggregation [162–164]. All triatomine sialotranscriptomes have revealed this class of proteins, which is particularly abundant in *Dipetalogaster.* The function of these proteins in triatomine blood feeding is still unknown.

3.6. Lectin. Triatoma dimidiata exclusively presents two partial sequences containing a galactose-binding domain. While lectins—mainly C-type lectins—have been described in the sialotranscriptome of mosquitoes (none with known function), this is so far a unique finding in triatomine sialotranscriptomes.

3.7. Immunity-Related, Ubiquitous Families. Immunity-related proteins and peptides are commonly found in the saliva of bloodsucking arthropods and may help to control microbial growth in the ingested meal and perhaps also avoid microbial infection of the bite site. Lysozyme, while common in mosquito sialomes, is found exclusively so far in *Cimex* sialomes, with four quite different proteins being reported. *D. maxima* presents a histidine-rich peptide that could function as an antimicrobial peptide, and a defensin is reported from *T. infestans.* The absence of commonly found salivary antimicrobial peptides in triatomines suggests that if this salivary function is present within these organisms, it may be encoded by lineage-specific gene families, one of which (trialysin) will be reported further below.

4. Arthropod-Specific Families

Several insect-specific families are further identified, none functionally characterized, and most without domains providing a clue for their function. These include proteins with chitin-binding domains and cuticle-like homologs, which may be associated with salivary ducts rather than a function in the injected saliva. One conserved secreted insect protein family of basic peptides having ~100 amino acids after signal peptide cleavage occurs in *Cimex, Oncopeltus,* and *Triatoma* sialotranscriptomes. Homologs are found by blastp to the nonredundant (NR) protein database including a venom protein from the wasp parasitoid *Nasonia vitripennis* identified in a proteomic study [165]. Exceptionally, there are also homologs to proteins from the soil bacteria *Streptomyces clavuligerus*, having 52% identity to the insect proteins. Similarly, the protein originally described in *R. prolixus* as MYS2 has homologs found in the sialotranscriptomes of *T. brasiliensis* and *T. matogrossensis* and is similar to many other insect proteins in the NR, including protein sequences deducted from the sialotranscriptome of the tsetse *Glossina morsitans* [166]. Three sequences, one each from *C. lectularius*, *T. infestans,* and *T. matogrossensis,* have 25% amino acid sequence identity but 52% similarity and little similarity to other proteins on the NR database. These sequences are grouped in Additional File 1 as the *Cimex-Triatoma* family. PSI-blast initiated by the *T. matogrossensis* sequence against the NR database initially retrieves only the two other sequences, but on first iteration it retrieves dozens of insect proteins (Additional File 3), and in the third iteration it retrieves *Daphnia* and tick proteins, suggesting this is an arthropod family of high divergence. Finally, the sialotranscriptome of *T. matogrossensis* identified four additional nonrelated proteins that have insect homologs but were not found in other reported sialotranscriptomes of Hemiptera but are similar to proteins reported from *G. morsitans* and from *Aedes aegypti* sialotranscriptomes. It is possible that these families function as antimicrobial peptides, but so far none has been characterized.

5. Hemiptera-Specific Families

5.1. Mys3/Hemolysin Family. When the *R. prolixus* sialotranscriptome was reported [36], an additional mysterious protein was named Mys3. Later, with additional sialotranscriptome reports, another protein family emerged, named as hemolysin-like because some members had weak similarity to bacterial proteins annotated as hemolysins. PSI-blast later revealed that these proteins all belong to a single family that is quite divergent, including a truncated protein from the sialome of *Oncopeltus*, suggesting a non-blood-feeding role, perhaps antimicrobial, for its members.

5.2. Triatoma-Specific Families. Sialotranscriptomes of several species of the *Triatoma* genus reveal several unique protein families, among which are the trialysin and short trialysin families. The trialysins are basic proteins of mature MW near 26 kDa that can be further processed to peptides that have lytic properties [17, 18] and may function as antimicrobials. Short trialysins have mature MW of ~6.1 and acidic pI and are so named because they match the amino terminal region of the mature trialysins. Both forms are abundantly expressed but only found in *T. infestans* and *T. matogrossensis,* which are from southern South America, and are not found in the sialotranscriptomes of *T. brasiliensis,* found in northeastern Brazil, or on those of the North American *T. dimidiata* or *T. rubida*. Additional File 1 reports 19 protein sequences from *Triatoma* that are not similar to anything deposited in the NR database and two pairs of sequences from *T. matogrossensis* that only match its pair members. None has been functionally characterized. It is interesting that of these 23 sequences only one derives from *T. rubida* and the remaining derive from *T. infestans* and *T. matogrossensis*, although the number of clones sequenced for the *T. brasiliensis*, *T. dimidiata,* and *T. rubida* was similar to those of *T. infestans* and *T. matogrossensis*, suggesting a greater sialome diversity in these bugs from southern South America.

5.3. Rhodnius-, Cimex-, and Oncopeltus-Specific Families. Additional File 1 presents 16 proteins from the bugs named above that have no significant matches to the NR database except in some cases for some proteins of low complexity. None of these proteins has been functionally characterized. This includes *Rhodnius* MY1 protein, one of three mysterious proteins revealed in the first bug sialotranscriptome [36]. As seen above, MYS2 and MYS3 were later found to be members of larger families. It is expected that, with a larger number of genomes and transcriptomes sequenced, MYS1—as well as the other orphan proteins in this group—will also be deorphanized.

6. Housekeeping Proteins

Mostly from the sialotranscriptomes shown in Table 2, many housekeeping protein sequences were also deduced, including many associated with energy metabolism, protein synthesis, modification, and export, among other classes (see worksheet named "Housekeeping" of Additional File 1). Interestingly, the sialotranscriptome of *Triatoma rubida* shows abundant expression of members of the cytochrome P450 as well as of the 15-hydroxyprostaglandin dehydrogenase, suggesting either that the salivary gland may have an active endogenous prostaglandin signaling or that prostaglandins may be secreted in the saliva of these bugs. Cyt P450 transcripts were also detected in *Rhodnius* and *T. matogrossensis,* and the prostaglandin dehydrogenase was also found in *T. infestans*. Increased depth of sequencing of these sialotranscriptomes may certainly reveal these two classes of proteins to be expressed in all triatomines.

7. Concluding Remarks

Blood-feeding Cimicomorpha have developed a sophisticated and divergent array of salivary pharmacologically active compounds that disarm their hosts' reaction against blood loss. In a few transcriptomes encompassing members of the Reduviidae and Cimicidae, the convergent evolution scenario in the sialomes of these two families is apparent. Both have apyrase activity, but from different gene families; *Cimex* and *Rhodnius* (but not any Triatomini member) use NO as a vasodilator but co-opted completely different heme proteins to carry this unstable gas. The anticlotting compounds are different at the Reduviidae tribe level and so on. The lipocalin expansion is remarkable among the triatomines and nonexistent in *Cimex*. These proteins can play many different functions as binders of small agonists (kratagonists), NO carriers, or protease inhibitors. In *Cimex*, the expanded odorant binding family may have taken this role, but none thus far has been characterized.

Notice that the sialome of *Oncopeltus*, a member of the Pentatomomorpha—the most closely related suborder to the Cimicomorpha (see http://tolweb.org/Heteroptera/10805) [76, 167]—revealed virtually nothing in common with the Cimicomorpha, and the Cimicidae sialome also revealed little in common with the Reduviidae, perhaps as expected by the divergence of these families (see http://tolweb.org/Cimicomorpha/10817). Zooming-in on the Triatomine group, it will be interesting in the future to describe the sialomes of additional tribes of the Triatomine, such as the Bolboderini, which includes bugs that feed on insect hemolymph, the Cavernicolini that are associated with bats, and members of the Linshcosteus genus that are found in India [168] and could be divergent members. Zooming a little out and as indicated by Schofield and Galvão [49], facultative blood feeding is found in non-Triatominae members of the Reduviidae, including the Emesinae, Harpactorinae, Peiratinae, Physoderinae, and Reduviinae. Sialomes of these subfamilies could be more indicative of the prevalent "preadaptations" available as stepping stones and promoted by the blood-feeding habit. On the other hand, the *Cimicidae* are closely related to the bat bugs (Polyctenidae), which is a sister group, and to the Anthocoridae (flower bugs; http://tolweb.org/Cimicomorpha/10817), which feed on small insects. These non-blood-feeding closer relatives may reveal insights into the Cimicidae evolution to hematophagy.

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Research Article

Pheromone of the Banana-Spotting Bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae): Identification, Synthesis, and Field Bioassay

Ashot Khrimian,[1] Harry A. C. Fay,[2] Filadelfo Guzman,[1] Kamlesh Chauhan,[1] Chris Moore,[3] and Jeffrey R. Aldrich[1]

[1] *Beltsville Agricultural Research Center, Agricultural Research Service, USDA, Building 007, Room 326, BARC-West, 10300 Baltimore Avenue, Beltsville, MD 20705, USA*
[2] *Department of Employment, Economic Development and Innovation, Horticulture and Forestry Science, 28 Peters Street, P.O. Box 1054, Mareeba, QLD 4880, Australia*
[3] *School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia*

Correspondence should be addressed to Ashot Khrimian, ashot.khrimian@ars.usda.gov

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The banana-spotting bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae), is one of the principal pests of tree fruits and nuts across northern and eastern Australia. Apart from visual damage assessment, there are currently no reliable methods for monitoring bug activity to aid management decisions. An attractant pheromone for this species that could be used as a trap lure could potentially fill this void. Earlier, two male-specific compounds were identified in airborne extracts from *A. lutescens lutescens*, (*E,E*)-α-farnesene and (*R,E*)-nerolidol; an unknown compound with a molecular weight 220 was also detected. We now report the identification of this hitherto unknown compound as (*R,E,E*)-α-farnesene-10,11-oxide. Synthesis of this epoxide was conducted using a regioselective asymmetric dihydroxylation of a sulfolene. A blend mimicking the natural proportions of (*E,E*)-α-farnesene, (*R,E*)-nerolidol, and (*R,E,E*)-α-farnesene-10,11-oxide attracted male and female *A. lutescens lutescens* as well as nymphs in the field, verifying that the aggregation pheromone comprises or is contained within this group of compounds.

1. Introduction

The fruit-spotting bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae), commonly known as the banana-spotting bug, is one of the most important insect pests of fruit and nut crops in tropical and subtropical Australia [1–4]. Nymphs and adults feed on shoots and developing fruits of a wide range of commercial tree crops, causing significant production losses if pesticides are not regularly applied. Monitoring banana-spotting bug activity in crops is extremely difficult and currently relies on scouts assessing damage at weekly or fortnightly intervals throughout the season [5]. This approach is reactive and not always effective, as well as being time consuming and expensive since a large proportion of a crop must be monitored because damage can be unevenly distributed throughout an orchard. Alternative monitoring tools based on semiochemicals, such as host plant volatiles and/or pheromones, are highly desirable because they could potentially be more reliable and easier to standardize. Studying male-specific compounds from Nearctic and Australasian true bugs, Aldrich et al. identified (*E,E*)-α-farnesene and (*R,E*)-nerolidol from *A. lutescens lutescens*, whereas a third compound with molecular weight 220 remained unknown [6]. Blends of these chemicals could serve as an attractant pheromone for *A. lutescens lutescens* but their biological importance has not been demonstrated, nor has the structure of the unknown compound been elucidated. The aims of this paper were to address both of these issues.

2. Material and Methods

2.1. Insects. *Amblypelta lutescens lutescens* males were hand-collected from a row of *Murraya paniculata* (orange jessamine) bushes on the northwest outskirts of Mareeba, Australia (16°59′S, 145°25′E), at the end of March 2009. Males were separated from females by the squarer shape of the tip of the abdomen when viewed laterally. The collected males were held in a 30 × 30 × 30 cm gauze-covered cage with *M. paniculata* fruit, green beans, and water at approximately 26°C and 60–70% RH under natural light until aeration experiments.

2.2. Collection of Airborne Volatiles from Males. Two samples of airborne extracts were collected in Mareeba from 14 male *A. lutescens lutescens* over a 4- and then a 3-day period in early April 2009. During aerations, the males were held in a 500 mL triple-neck vacuum flask with 3 green beans as food. The flask inlet was connected by silicone tubing (8 mm i.d.) to a modified Pasteur pipette (5 mm i.d.) containing 50 mm of acid-cleansed activated charcoal to filter the incoming air. The flask outlet was also connected by silicone tubing to an 18 cm long glass tube (5 mm i.d.) containing 50 mm of Super Q absorbent (Alltech, USA) held in place on each end by glass wool. Silicone tubing connected the glass tube to an Airchek 224-PCXR8 Sampler pump (SKC Inc., Eight-Four PA), which operated at 250–500 mL/min during the aerations. All connections in the inlet and outlet lines were sealed with Teflon tape. Sampling was conducted at approximately 26°C and 60–70% RH. No bugs died during either sampling period. Once collected, the aeration samples in their glass tubes were sealed in individual airtight containers and sent to USDA-ARS, Beltsville, where they were eluted with 600 μL of hexane. The extracts were concentrated to 50 μL under a gentle stream of N_2 and analyzed by gas chromatography-mass spectrometry (GC-MS) on an Rtx-5MS and by GC on Chiraldex B-DM columns (see below).

2.3. Analytical Methods. Electron-impact (EI) mass spectra were obtained at 70 eV with an Agilent Technologies 5973 mass selective detector interfaced with a 6890 N GC equipped with a 30 m × 0.25 mm i.d. × 0.25 μm film Rtx-5MS (Restek Corporation, Bellefonte, PA, USA) column. Column temperature was maintained at 50°C for 5 min and then raised to 260°C at 10°C/min. Helium was used as a carrier gas at 1 mL/min. Enantioselective GC analyses of nerolidol and racemic and optically active (*E*,*E*)-α-farnesene oxide were performed on an Agilent Technologies 6890 N GC equipped with an FID detector and 30 m × 0.25 mm i.d. × 0.12 μm film Chiraldex B-DM column (Astec, Whippany, NJ, USA). Column temperature was maintained at 100°C for 2 min and then raised to 180°C at 2°C/min. Hydrogen was used as a carrier gas at 1 mL/min. ^{1}H and ^{13}C NMR spectra of compounds **6** and **8** were obtained on a Bruker AVIII-600 MHz spectrometer. ^{1}H NMR spectra of compounds **4** and **10** and ^{1}H-^{1}H COSY of **6** were obtained on a Bruker AV-400 MHz spectrometer. The ^{1}H NMR spectrum of compound **5** was obtained on an Anasazi 90 MHz spectrometer (Indianapolis, IN). Chemical shifts are reported in δ units and referenced to the residual $CDCl_3$ solvent signal. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter with a 1.0 mL cell. Thin layer chromatography analyses were conducted on Whatman AL SIL G/UV plates using 20% ethanol solution of phosphomolybdic acid and/or UV for visualization of spots. Flash chromatography was carried out with 230–400 mesh silica gel (Fisher Scientific, Fair Lawn, NJ).

2.4. Chemicals. All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise specified. Hexamethylphosphoramide (HMPA, highly toxic) was distilled from P_2O_5, and tetrahydrofuran (THF) was dried by distillation from sodium benzophenone-ketyl. (*R*,*E*)-Nerolidol was synthesized from (*E*,*E*)-farnesol using a four-step procedure described by Kigoshi et al. [7] that involved a Sharpless-Katsuki asymmetric epoxidation, conversion of the resulting (2*R*,3*R*)-epoxyfarnesol to a tosylate and further to (2*R*,3*R*)-epoxyfarnesyl iodide, and, finally, a stereoselective zinc reduction of the latter. The enantiomeric purity of (*R*,*E*)-nerolidol, as judged from GC analysis on a Chiraldex B-DM column, was 93% (see Section 2.3 for GC conditions). Racemic (*E*,*E*)-α-farnesene oxide (*rac*-**4**) was prepared by epoxidation of (*E*,*E*)-α-farnesene with *m*-chloroperbenzoic acid (MCPBA) as described in Spicer et al. [8].

2.4.1. (E)- 2-(3′,7′-Dimethylocta-2′,6′-dienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (5). Procedures of Yamada et al. [9] and Desai et al. [10] were followed. 3-Methyl-2,5-dihydrothiophene-1,1-dioxide (3-methyl-3-sulfolene, 10.95 g, 82.84 mmol, Alfa Aesar, Ward Hill, MA; recrystallized from methanol before use) was placed under N_2 into a four-neck round bottomed flask equipped with a thermometer, mechanical stirrer, dropping funnel, N_2 inlet, and a septum. Dry THF (300 mL) was added; the solution was cooled to −72°C and then charged sequentially with geranyl bromide (9.0 g, 41.44 mmol) and anhydrous hexamethylphosphoramide (HMPA, 29 mL). Lithium bis(trimethylsilyl)amide (41.4 mmol; 41.4 mL of 1.0 M in THF) was added slowly under vigorous stirring, maintaining the temperature below −68°C. The reaction mixture was stirred for 30 min at −70°C, then slowly warmed to −20°C, and quenched with ethyl acetate (150 mL). The mixture was concentrated on a rotary evaporator, and the residue was dissolved in ether (500 mL), washed with water and brine, and then dried over anhydrous sodium sulfate. Evaporation of the solvent and flash chromatography of the residue with hexane/ethyl acetate (3 : 1) yielded compound **5** (9.30 g, 84% from geranyl bromide). GC-MS analysis of **5** proceeded with complete extrusion of SO_2 as described in Chou et al. [11] resulting in (*E*,*E*)-α-farnesene of 95% purity. GC-MS (*m/z*, %): 204 (M^+, 1), 189 (2), 161 (5), 133 (5), 123 (33), 119 (38), 107 (46), 105 (24), 93 (100), 91 (38), 81 923), 80 (24), 79 (45), 77 (28), 69 (58), 67 (12), 55 (50), 53 (17), 43 (16), 41 (69). The mass spectral data matched those reported in NIST 08 MS Library for (*E*,*E*)-α-farnesene. ^{1}H NMR (90 MHz, $CDCl_3$): 1.58 (br s, 3H), 1.62 (br s, 6H), 1.82 (m, 3H), 1.95–2.06 (m, 4H), 2.57 (br t, J = 7.0 Hz, 2H), 3.38–3.70 (m, 3H), 5.06 (m, 1H), 5.20 (br t, J = 7.0 Hz, 1H), 5.62 (m, 1H). Data are in good agreement with those reported in [8].

2.4.2. (3E,6E)-3,7,11-Trimethyl-1,3,6,10-dodecatetraene ((E,E)-α-Farnesene 2). (*E,E*)-α-Farnesene was prepared by thermal decomposition of **5** (100 mg, 0.37 mmol) in degassed refluxing *n*-octane (5 mL) bubbled with N_2 for 1.5 h [12]. The solution was cooled, concentrated, and purified by flash chromatography with pentane to give **2** (61 mg, 81%) of 94% purity.

2.4.3. (2R/S,6′S,2′E)- 2-(3′,7′-Dimethyl-6′,7′-dihydroxy-2′-octenyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (6). A procedure by Sharpless et al. [13] was followed. AD-mix α (26.0 g) was added to a stirred mixture of *t*-BuOH (83 mL) and water (93 mL) at 0–5°C. Methanesulfonamide (1.70 g, 17.90 mmol) was added, followed by sulfolene **5** (5.0 g, 18.62 mmol). The mixture was stirred for 8 h, after which TLC analysis (hexane/ethyl acetate, 1 : 3) showed very little remaining starting material. Sodium sulfite (2.35 g) was added, and the mixture was allowed to warm to room temperature. The mixture was diluted with water (40 mL) and extracted with dichloromethane (6 × 50 mL). The organic extracts were combined, dried with Na_2SO_4, and concentrated. Flash chromatography with hexane/ethyl acetate (1 : 3) provided diol **6** (4.96 g, 88%) as a mixture of two diastereomers in about equal quantity. GC-MS (*m/z*, %): 179 (3), 159 (3), 134 (28), 121 (24), 119 (55), 107 (28), 93 (66), 91 (40), 81 (67), 80 (58), 79 (47), 59 (100), 43 (47), 41 (42). $[\alpha]_D^{25} = -9.26°$ (*c* 1.75, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): 1.149 and 1.154 (s, 3H, C7′-CH_3 A), 1.19 and 1.20 (s, 3H, C7′-CH_3 B), 1.44 (m, 1H, H-5′), 1.62 (m, 1H, H-5′), 1.68 and 1.69 (s, 3H, C3′-CH_3), 1.86 (br s, 3H, C3-CH_3), 2.13–2.28 (m, 4H, H-4′, 2OH), 2.57 (m, 2H, H-1′), 3.35 (t, J = 10.2 Hz, 1H, H-6′), 3.55–3.75 (m, 3H, H-2, H-5), 5.29 and 5.33 (br t, J = 6.5 Hz, 1H, H-2′), 5.69 (br s, 3H, H-4). ^{13}C NMR (151 MHz, $CDCl_3$): 15.97, 16.12, 17.95, 18.02, 23.36, 23.39, 25.98, 26.00, 26.27, 26.30, 28.61, 29.17, 36.52, 36.67, 43.40, 55.73, 67.25, 67.44, 72.96, 77.52, 117.16, 117.18, 119.04, 119.27, 138.26, 138.33, 138.69, 138.80.

2.4.4. (2R/S,6′R,2′E)- 2-(3′,7′-Dimethyl-6′,7′-dihydroxy-2′-octenyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (7). Analogous to the procedure described above, sulfolene **5** (1.498 g, 5.58 mmol) was dihydroxylated with AD-mix β (7.79 g) in the presence of methanesulfonamide (0.53 g, 5.58 mmol) in a mixture of *t*-BuOH (27 mL) and water (27 mL) to give diol **7** (1.200 g, 71%). $[\alpha]_D^{25} = +10.49°$ (*c* 2.64, $CHCl_3$).

2.4.5. (3S,6E,9E)-2,6,10-trimethyl-6,9,11-dodecatriene-2,3-diol (8). A two-neck conical flask equipped with a reflux condenser and N_2 inlet extending to the bottom of the flask was loaded with a mixture of sulfolenediol **6** (491 mg, 1.62 mmol) and pyridine (10 mL). The solution was degassed with a gentle steam of N_2 for 10 min and then heated in an oil bath at 122°C while maintaining the N_2 flow. After 2 h of heating, TLC (hexane/ethyl acetate, 1 : 3) showed that the reaction was almost complete. The solution was concentrated by rotary evaporation, and the remainder was purified by flash chromatography with (hexane/ethyl acetate, 1 : 1) to provide diol **8** (287 mg, 74%). GC-MS (*m/z*, %): 205 (1), 179 (5), 159 (5), 143 (7), 134 (40), 121 (32), 119 (71), 107 (33), 105 (37), 93 (72), 91 (43), 81 (74), 80 (66), 79 (48), 59 (100), 43 (31), 41 (26). $[\alpha]_D^{25} = -23.9°$ (*c* 2.70, $CHCl_3$). Lit. [14] $[\alpha]_D = -20.8°$ (*c* 0.03, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): 1.16 (s, H-1), 1.21 (s, 3H, C2-CH_3), 1.60 (m, 2H, H-4), 1.66 (s, 3H), 1.77 (s, 3H), 2.09 (m, 1H, H-5A), 2.27 (m, 1H, H-5B), 2.86 (t, J = 7.2 Hz, 2H, H-8), 3.36 (m, 1H, H-3), 4.94 (d, J = 10.8 Hz, 1H, H-12 *cis*), 5.10 (d, J = 17.4 Hz, 1H, H-12 *trans*), 5.20 (t, J = 6.0 Hz, 1H, H-7), 5.45 (t, J = 7.5 Hz, 1H, H-9), 6.36 (dd, J = 17.4, 10.8 Hz, 1H, H-11). ^{13}C NMR (151 MHz, $CDCl_3$): 11.9, 16.3, 23.31, 26.7, 27.4, 29.9, 36.9, 73.3, 78.5, 110.9, 122.9, 131.6, 134.1, 135.9, 141.7. ^{1}H NMR data are in close agreement with reported values [14].

2.4.6. (3R,6E,9E)-2,6,10-Trimethyl-6,9,11-dodecatriene-2,3-diol (9). Analogously to the preparation of diol **8**, sulfolenediol **7** (1.250 g, 4.13 mmol) was heated in the presence of pyridine (50 mL) to provide diol **9** (546 mg, 55%, 98% pure). The mass spectrum of **9** was identical to that of **8**. $[\alpha]_D^{25} = +25.0°$ (*c* 2.58, $CHCl_3$). Lit. [14] $[\alpha]_D = +21.2°$ (*c* 0.05, $CHCl_3$).

2.4.7. (10R,3E,6E)-3,7,11-Trimethyl-10,11-epoxy-1,3,6-dodecatriene ((R,E,E)-α-farnesene-10,11-oxide, 4). Methanesulfonyl chloride (138 μL, 1.73 mmol) was added to a solution of diol **8** (378 mg, 1.59 mmol) in dry pyridine (2 mL) at room temperature. After stirring for 1 h, TLC (hexane/ethyl acetate, 1 : 1) confirmed a complete conversion of the diol to the secondary mesylate. The mixture was cooled in an ice bath and quenched with a mixture of water (3 mL) and methyl *t*-butyl ether (3 mL). The organic layer was separated, and the aqueous layer was extracted with methyl *t*-butyl ether (5 × 3 mL). The organic extracts were combined, dried over Na_2SO_4, and concentrated. The crude mesylate (still containing pyridine) was dissolved in methanol (5 mL) and treated with a solution of KOH (162 mg, 2.89 mmol) in MeOH (1.5 mL) at 20°C. A precipitate formed almost instantaneously, and an additional 3 mL of MeOH was added to enable stirring for another 30 min. The methanol then was removed *in vacuo*, and the residue was partitioned between ice water (3 mL) and ether (5 mL). The organic layer was separated, and the aqueous layer was extracted with hexane/ether (1 : 1). The combined organic extracts were washed with ammonium chloride solution, dried, and concentrated. The remainder was purified by flash chromatography (hexane/ethyl acetate, 1 : 1) to provide epoxide **4** (223 mg, 64%) of 93% chemical and 95% enantiomeric purities. The latter was determined by chiral GC analysis on the Chiraldex B-DM column (see Section 2.3 for conditions). $[\alpha]_D^{25} = +0.2°$ (*c* 1.45, $CHCl_3$). GC-MS (*m/z*, %): 205 (1), 187 (2), 159 (4), 134 (40), 119 (100), 107 (26), 105 (45), 93 (83), 91 (63), 81 (57), 80 (75), 79 (70), 77 (39), 59 (43), 55 (44), 43 (56), 41 (61). The mass spectral data matched those of the natural epoxide **4** and synthetic racemic epoxide [15]. ^{1}H NMR (400 MHz, $CDCl_3$): 1.26 (s, 3H, H-12/C11-CH_3), 1.30 (s, 3H, H-12/C11-CH_3), 1.67 (br s, 3H), 1.58–1.75 (m, 2H), 1.77 (br s, 3H), 2.07–2.22 (m, 2H), 2.71 (t, J = 6.0 Hz, 1H, H-10), 2.85 (br t, J = 7.2 Hz, 2H, H-5),

4.94 (d, J = 10.8 Hz, 1H, H-1 *cis*), 5.10 (d, J = 17.6 Hz, 1H, H-1 *trans*), 5.18 (dq, J = 7.2, 1.2 Hz, 1H), 5.46 (br t, J = 7.2, 1H), 6.37 (dd, J = 17.6, 10.8 Hz, 1H, H-2). These data are in good agreement with those previously reported for the racemic epoxide [8, 15].

2.4.8. (10S,3E,6E)-3,7,11-Trimethyl-10,11-epoxy-1,3,6-dodecatriene ((S,E,E)-α-farnesene oxide, 10). Diol **9** (490 mg, 2.06 mmol) was mesylated with methanesulfonyl chloride (179 μL, 2.25 mmol) in dry pyridine (2.5 mL) as described above, and the crude mesylate was treated with KOH (233 mg) in 10 mL MeOH to yield epoxide **10** (222 mg, 50%) of 92% of chemical and 94% enantiomeric purities. $[\alpha]_D{}^{25} = -0.1^\circ$ (c 2.50, $CHCl_3$). The mass spectral and NMR data of **10** were identical to those of **4**.

2.5. Field Trapping. Two treatments were tested in a mango orchard on Southedge Research Station, approximately 50 km west of Cairns in northeast Queensland, Australia: no.1, 0.2 mg (*E*,*E*)-α-farnesene (**2**), 1.2 mg (*R*,*E*)-nerolidol (**3**), and 0.6 mg (*R*,*E*,*E*)-α-farnesene oxide (**4**); no. 2, 0.2 mg (*E*,*E*)-α-farnesene, 1.2 mg (*R*,*E*)-nerolidol, 0.6 mg (*R*,*E*,*E*)-α-farnesene oxide, and 0.6 mg hexyl hexanoate (**1**). Relative proportions of components in these blends were based on relative areas of peaks in the aeration extract. The pheromone components plus 5% BHT (butylated hydroxytoluene, or 2,6-di-*tert*-butyl-4-methylphenol) stabilizer were applied to rubber septa in hexane solutions [16] at Beltsville and shipped to Australia. The test site was located at 16°58′S, 145°20′E at an elevation 450 m, and has a mean annual rainfall of 1,110 mm. The station has several thousand mango trees, including an extensive gene pool block, mixed varietal block, a large number of hybrids, and pure blocks containing Kensington Pride, R2E2, and Keitt varieties. The traps were set out in two rows of mature trees (16–20 years old) of mixed varieties at one trap per tree with several trees without traps between them. All trees contained maturing fruit at the time of trap deployment in October. Traps consisted of panels of green-colored twin wall polypropylene sheeting (Corflute BFS Plastic Pty. Ltd., Salisbury, QLD, Australia, 0.3 × 25 × 30 cm), placed 2–2.5 m above ground and fixed to branches with wire so that they remained rigid even in windy conditions. Traps were covered on each side with double-sided cloth tape coated with emulsion acrylic adhesive (Henkel Aust. Pty. Ltd., Kilsyth, VIC, Australia). Each trap had a 3 cm diameter hole in the center in which the rubber septum containing the pheromone was fastened. There were five replicates of each pheromone treatment and five untreated control traps. Traps were assigned sequentially for treatments, repeated for each subsequent replicate down the rows. Pheromone lures were stored, sealed, and refrigerated until use, and traps were baited the morning of deployment. Traps were inspected 4 days after their initial deployment, when adult males, females, and nymphs were removed and recorded.

2.6. Analysis of Trapping Data. The data are presented as number of *A. lutescens lutescens* adult males, adult females, and nymphs per trap per day. Data were $\sqrt{(x+1)}$-transformed to compare treatments by analysis of variance using GenStat 11th Edition [17]. Significantly different means were compared by the least significant difference method at P = 0.05.

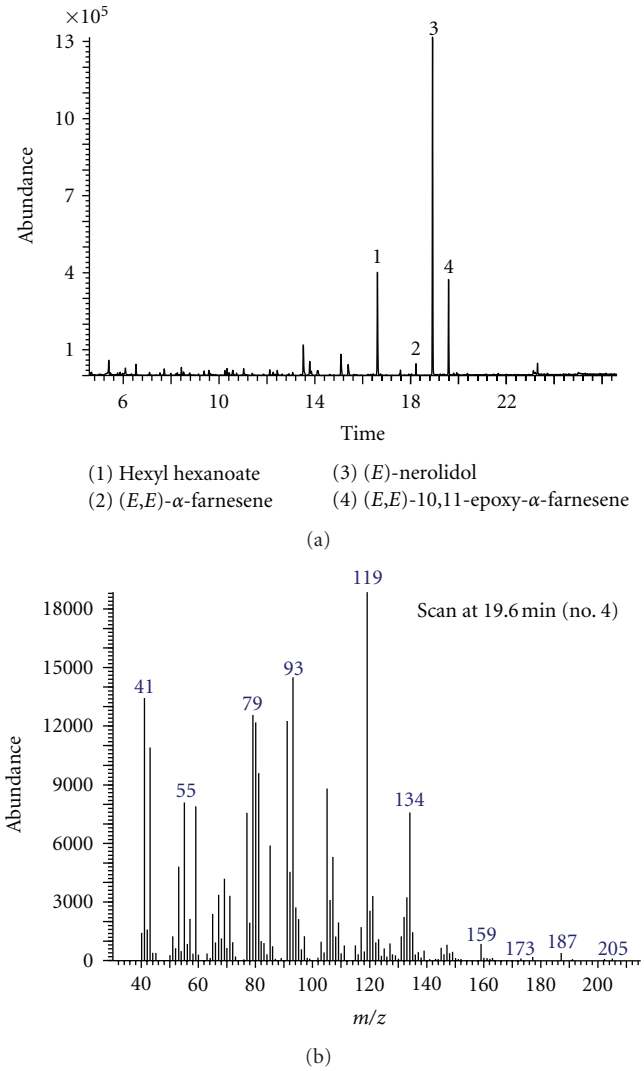


Figure 1: Total ion chromatogram of an aeration extract of male *Amblypelta lutescens lutescens* and EI mass spectrum of compound **4**. Column: Rtx-5MS (30 m × 0.25 mm i.d. × 0.25 μm), 50°C (5 min) to 260°C at 10°C/min.

3. Results and Discussion

3.1. Identification and Synthesis. The total ion chromatogram (Figure 1) revealed several compounds, among which were the previously identified (*E*,*E*)-α-farnesene (**2**) and (*E*)-nerolidol (**3**) [6], plus compound **4**, the mass spectrum of which matched that of the unknown male-specific compound showing a molecular weight 220, previously found by Aldrich et al. [6]. In addition to these compounds, in the aeration sample from males we found a relatively large amount of hexyl hexanoate (**1**), a trace of which was

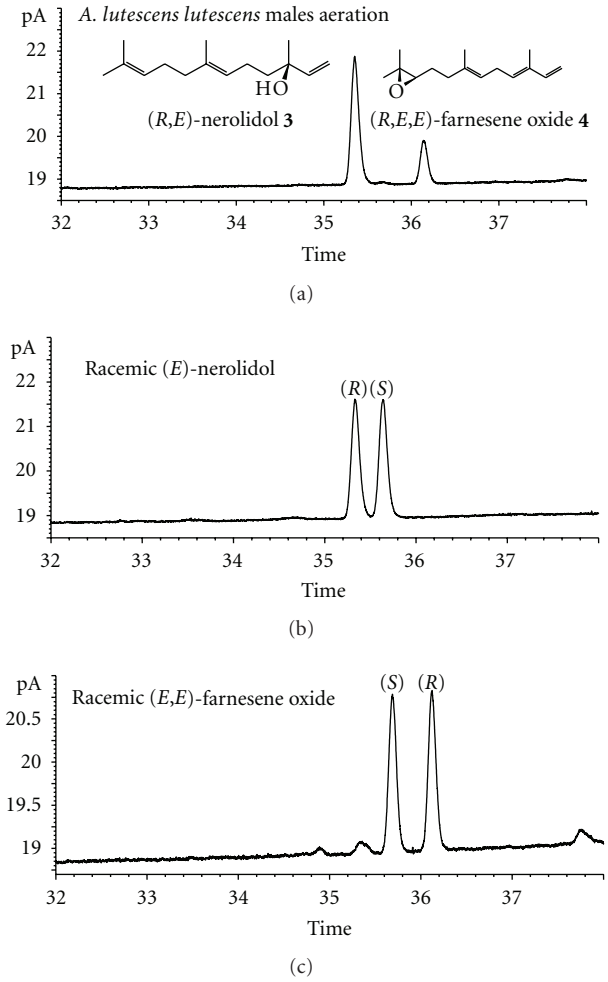
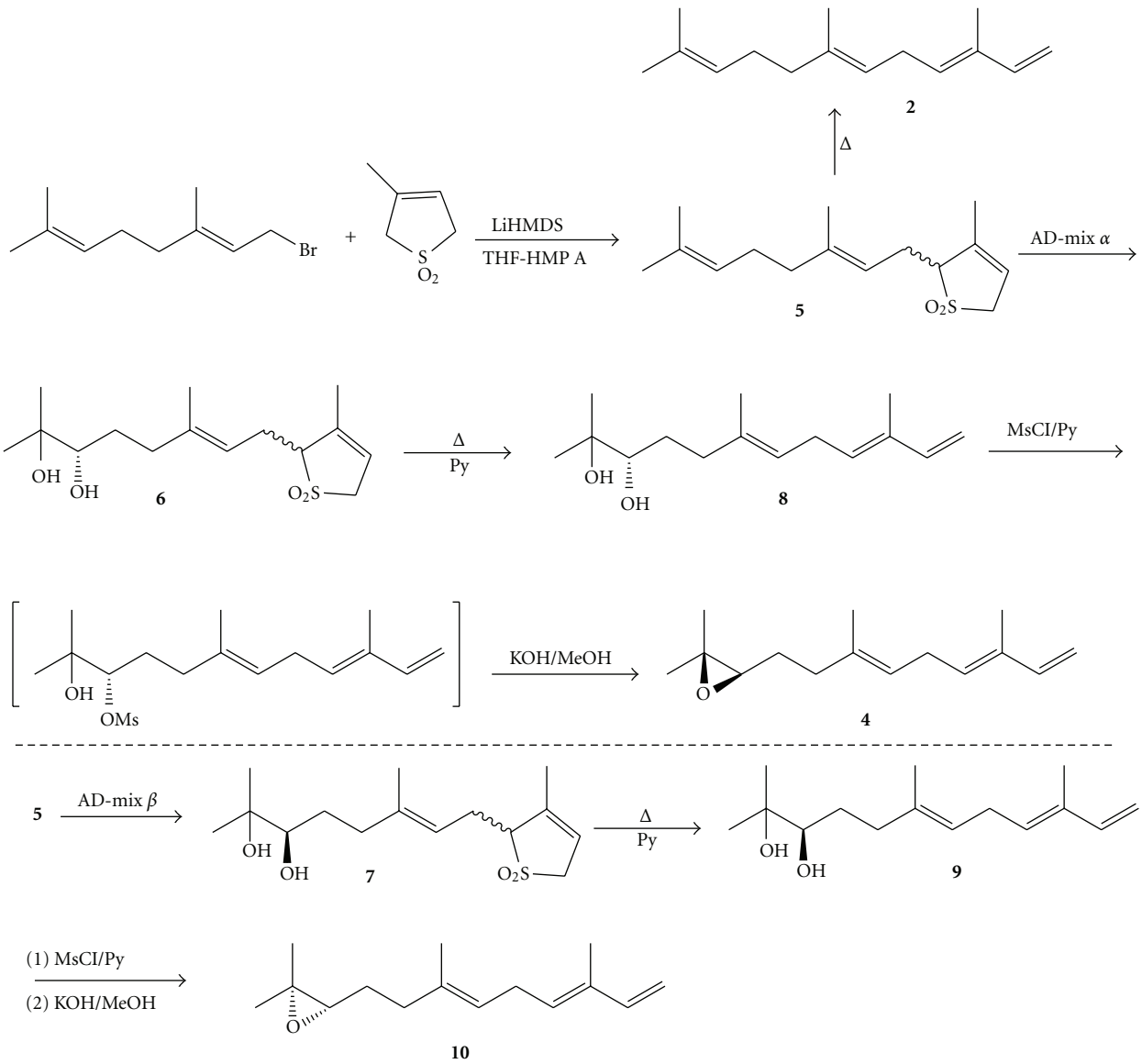


Figure 2: Sections of GC-FID traces of an aeration extract of male *Amblypelta lutescens lutescens* (a), racemic (*E*)-nerolidol (b), and racemic (*E*,*E*)-farnesene oxide (c). Column: Chiraldex B-DM (30 m × 0.25 mm i.d. × 0.12 μm), 100°C (2 min) to 180°C at 2°C/min.

previously found in female but not male *A. lutescens lutescens* [6]. Because the unidentified compound **4** conceivably could have been an epoxide of (*E*,*E*)-α-farnesene, we decided to start with a random epoxidation of (*E*,*E*)-α-farnesene that had already been reported [8, 15]. We repeated the *m*-chloroperbenzoic acid epoxidation of (*E*,*E*)-α-farnesene in methylene chloride and found that the monoepoxidation product at the 10,11 double bond matched compound **4** by GC retention time and mass spectrum. The Chiraldex B-DM GC column resolved (Figure 2) synthetic racemic farnesene oxide **4** (lower chromatogram) and racemic (*E*)-nerolidol to baseline (middle chromatogram). Analysis of the *A. lutescens lutescens* aeration sample of males on the Chiraldex B-DM column (upper chromatogram) revealed that only one enantiomer of (*E*)-nerolidol and farnesene oxide were produced by the bug. Aldrich et al. [6] had already determined that (*E*)-nerolidol produced by *A. lutescens lutescens* had the (*R*)-configuration, and we confirmed this assignment by showing that synthetic (*R*,*E*)-nerolidol had the same GC retention time as the natural compound, or the first eluting enantiomer of racemic (*E*)-nerolidol.

Determination of the enantiomeric composition of (*E*,*E*)-α-farnesene-10,11-epoxide **4** produced by *A. lutescens lutescens* necessitated synthesis of both enantiomers. Thus, we considered a route developed by Moore et al. [18] for synthesis of ocimene epoxides, which was based on a Sharpless asymmetric dihydroxylation and further stereoselective cyclization of intermediate chiral diols to epoxides through secondary mesylates. In fact, asymmetric dihydroxylation of (*E*,*E*)-α-farnesene **2** had been reported in the literature [14] but only in very low yields (11 and 15% for (−) and (+) diols, resp.) for a 10,11-dihydroxylation product. The low yield of the desired diol was a reflection of the multiple sites of unsaturation present in (*E*,*E*)-α-farnesene that were susceptible to asymmetric dihydroxylation. To overcome this shortcoming, we developed a new route presented in Scheme 1, which was essentially an enantioselective version of the synthesis of racemic (*E*,*E*)-α-farnesene-10,11-epoxide developed by Fielder and Rowan [15]. The synthesis started with alkylation of 3-methyl-3-sulfolene with geranyl bromide, as described in several reports (see Section 2). The key step, asymmetric dihydroxylation of the intermediate **5** with AD-mix α, was highly regioselective (as was MCPBA epoxidation of **5** [15]) and offered sulfolenediol **6** in 88% yield. ^{1}H NMR spectrum of diol **6** recorded at 600 MHz clearly shows the presence of two diastereomers with separation of several signals, including $(CH_3)_2COH$, even though the two chiral centers are separated by five carbon atoms (see Section 2). The observed regioselectivity of asymmetric dihydroxylation of **5** was in stark contrast with direct asymmetric dihydroxylation of (*E*,*E*)-α-farnesene [14] and was similar to the analogous dihydroxylation of ocimene [18] and squalene [19], whereby the reactions occurred primarily at the terminal trisubstituted double bonds.

Thermal elimination of sulfur dioxide in **6** was accomplished in pyridine [10] to minimize side reactions and polymerization due to the acidity of SO_2. For the same reason, mesylation of diol **8** was also conducted in pyridine, and in the last step, the crude mesylate was cyclized to epoxide **4** with potassium hydroxide in methanol [20]. Analogously, sulfolene **5** was dihydroxylated with AD-mix β to provide diol **7**, which was then thermally transformed to diol **9**. Cyclization of the latter as described above furnished enantiomeric farnesene epoxide **10**. Both **4** and **10** were prepared in ≥94% ee as judged by GC analysis on the Chiraldex B-DM column, which indicated that asymmetric dihydroxylation proceeded with high enantioselectivity, as anticipated from the original report by Sharpless et al. [13] and the subsequent results of Moore et al. [18]. High optical purities of epoxides **4** and **10** were also consistent with previous reports [18, 20] and indicative of highly stereoselective, base-catalyzed S_Ni substitution in the cyclization step. Overall, the route capitalizes on the much higher regioselectivity of dihydroxylation of sulfolene **5** compared to direct dihydroxylation of (*E*,*E*)-α-farnesene and, in fact, does not add additional steps to the synthesis of diols **8** and **9** (and epoxides **4** and **10**) because one of the best known synthetic routes to (*E*,*E*)-α-farnesene includes intermediate **5** [11].



Scheme 1: Syntheses of (R,E,E)- and (S,E,E)-α-farnesene-10,11-oxides **4** and **10**.

With both enantiomers of farnesene epoxide **4** and **10** available, we identified the later eluting peak in the gas chromatogram of the racemic epoxide on Chiraldex B-DM (Figure 2, lower chromatogram) as the (*R*)-enantiomer and, hence, determined that the natural farnesene epoxide (Figure 2, upper chromatogram) collected from *A. lutescens lutescens* had the (*R*)-configuration.

3.2. Field Bioassay. In preliminary trapping experiments pheromone lures were deployed on painted plywood panels covered with Tangle-Trap insect barrier gel (The Tanglefoot Co., Grand Rapids, MI, USA). When deployed in avocado and mango orchards, these traps baited with either the 3- or 4-component pheromone lures (see Section 2) only caught 0.01-0.02 *A. lutescens lutescens*/trap/day. It was observed that many bugs attracted to the pheromone lures were able to escape from the traps unless caught on their backs or sides.

The Corflute panels covered by adhesive tape were much more effective at trapping *A. lutescens lutescens* and, when deployed with the 3- and 4-component pheromone lures in mangoes, caught up to 0.90 ± 0.23 fruit-spotting bugs/trap/day (mean ± SE) (Table 1). When run in the same trial, the plywood panels with a tangle-trap gel caught a maximum of 0.10 ± 0.06 fruit-spotting bugs/trap/day.

There was no statistical difference in the total numbers of *A. lutescens lutescens* attracted to either the 3- or 4-component lures, and traps baited with both caught significantly more bugs than the untreated control traps (Table 1). Thus, hexyl hexanoate does not seem to be a component of the attractant pheromone for *A. lutescens lutescens*. Both pheromone treatments attracted adult males, adult females, and nymphs, without a clear dominance of one sex or stage over another. Hence, our results indicate that the blend of male-specific compounds **2**–**4** found in the male *A. lutescens*

Table 1: Mean numbers of *A mblypelta lutescens lutescens* trapped in a mango crop in north Queensland by sticky panels containing 3- and 4-component pheromone lures over a 4-day period in October 2011.

Panel traps	Mean no. of *A. lutescens lutescens*/trap/day (±SE)				Total bugs caught
	Adults		Nymphs	Total	
	♂♂	♀♀			
3-component lure	0.45 (±0.16) a	0.25 (±0.08) a	0.20 (±0.09) a	0.90 (±0.23) a	18
4-component lure	0.15 (±0.10) ab	0.20 (±0.05) a	0.10 (±0.06) ab	0.45 (±0.12) a	9
Untreated control	0 b	0 b	0 b	0 b	0

Means within columns followed by the same letter are not significantly different at $P = 0.05$.

lutescens aerations contained the aggregation pheromone for this species. Whereas (*R*,*E*)-nerolidol had been identified previously from at least two bug species [6] and (*E*,*E*)-*α*-farnesene was recently identified as a termite alarm pheromone [21], to our knowledge, (*R*,*E*,*E*)-*α*-farnesene-10,11-oxide **4** has not been reported as a pheromone component for any other insect species. More research is needed to determine whether the actual pheromone consists of one, two, or all three of these compounds, and for practical purposes, the optimal dose(s) and importance of chirality of the farnesene oxide must be determined. Preliminary experiments have indicated that doubling or tripling the emission rate of the 3-component lure as used above did not increase bug catches. The recent deregistration of endosulfan in Australia has significantly reduced the insecticidal options for the control of *A. lutescens lutescens*, and an effective monitoring tool based on a pheromone lure may become an essential tool in the management of the banana-spotting bug.

Acknowledgments

Geoff Waite, now retired from DEEDI, collaborated in much of the earlier work on *Amblypelta* pheromones and should be acknowledged for his contributions in this area. Robert Bauer (DEEDI) assisted with the field trials of the pheromones in north Queensland and the Australian Centre for International Agricultural Research provided funds in support of this work.

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Review Article

Host-Symbiont Interactions for Potentially Managing Heteropteran Pests

Simone Souza Prado[1] and Tiago Domingues Zucchi[2]

[1] *Laboratório de Quarentena "Costa Lima", Embrapa Meio Ambiente, Rodovia SP 340, Km 127,5, Caixa Postal 69, 13820-000 Jaguariúna, SP, Brazil*
[2] *Laboratório de Microbiologia Ambiental, Embrapa Meio Ambiente, Rodovia SP 340, Km 127,5, Caixa Postal 69, 13820-000 Jaguariúna, SP, Brazil*

Correspondence should be addressed to Simone Souza Prado, sprado@cnpma.embrapa.br

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Insects in the suborder Heteroptera, the so-called true bugs, include over 40,000 species worldwide. This insect group includes many important agricultural pests and disease vectors, which often have bacterial symbionts associated with them. Some symbionts have coevolved with their hosts to the extent that host fitness is compromised with the removal or alteration of their symbiont. The first bug/microbial interactions were discovered over 50 years ago. Only recently, mainly due to advances in molecular techniques, has the nature of these associations become clearer. Some researchers have pursued the genetic modification (paratransgenesis) of symbionts for disease control or pest management. With the increasing interest and understanding of the bug/symbiont associations and their ecological and physiological features, it will only be a matter of time before pest/vector control programs utilize this information and technique. This paper will focus on recent discoveries of the major symbiotic systems in Heteroptera, highlighting how the understanding of the evolutionary and biological aspects of these relationships may lead to the development of alternative techniques for efficient heteropteran pest control and suppression of diseases vectored by Heteroptera.

1. Introduction

Insects are the most cosmopolitan, polyphagous, and varied living organisms on Earth, and many species are involved in some kind of symbiotic association with microorganisms, mainly bacteria [1, 2]. Some insects associated with bacteria are also vectors of disease or are important crop pests which increase the relevance of these symbiotic interactions. The widespread distribution of insects may, in fact, be related to bacterial associations that allow host insects to exploit different nutritional sources, such as lignocellulose by termites, or to obtain essential nutrients from their symbionts, as in *Buchnera aphidicola*-aphid symbiosis [3, 4].

According to Moran [2], symbiosis is a "close relationship between two or more individuals." In insects, there are two major categories of symbiotic associations: obligatory and facultative [5]. Obligatory symbionts (also called primary symbionts) are nutritionally required for the survival of their insect hosts and usually inhabit specialized host cells. On the other hand, secondary (also known as facultative) symbionts can be beneficial or cause incidental or deleterious infections [1]. The obligatory symbiont, *B. aphidicola*, for example, has never been cultured outside its host and is present intracellularly within specialized cells termed bacteriocytes [1, 5, 6]. Transovarial transmission is often the mode of symbiont transfer from one generation to another, which is a bottleneck that shapes the genome characteristics of the symbiont [7]. Phylogenetic analyses for certain insect families have shown that insects and primary endosymbionts have coevolved for millions of years after a single initial infection [5]. In contrast, secondary symbionts are nonessential to their hosts, may be free living, may not have specialized tissue localization, and occur extracellularly [8]. These secondary symbionts may provide benefits to their hosts such as tolerance to heat stress, compensation for loss of primary symbionts, and resistance to parasites and pathogens [9–13]. Conversely, facultative symbionts can negatively impact the growth, reproduction, and longevity

of their hosts [14]. The evolutionary history of secondary endosymbionts often shows no coevolution with their hosts, suggesting multiple infections and/or horizontal transmission [5, 15]. For example, the secondary endosymbiont of tsetse flies, *Sodalis glossinidius*, can be cultured *in vitro* and apparently has not coevolved with the insect hosts [16]. *Sodalis glossinidius* is closely related to bacterial pathogens of insects suggesting, in this case, that the symbiont evolved from an insect pathogen [16].

In Heteroptera, many species adversely affect humans and their environment by causing direct damage to the crops, or acting as vectors of disease to crops, domestic animals, and humans [17] (however, there are also many agriculturally beneficial predatory heteropterans). For over 50 years, it has been known that insects of the suborder Heteroptera (order Hemiptera) harbor symbiotic microorganisms; however, the significance of the relationship and their role in the host's ecology and evolution are only now being unraveled [1, 9]. Symbiotic bacterial associations occur in all three hemipteran suborders: Sternorrhyncha (e.g., aphids, mealybugs, whiteflies, psyllids, etc.), Auchenorrhyncha (e.g., spittlebugs, planthoppers, leafhoppers, treehoppers, etc.), and Heteroptera (true bugs; Figure 1) [5, 18–21]. Experimental procedures for the phylogenetic tree analysis of Figure 1, the almost complete 16S rRNA gene sequences (1,300 nucleotides) were obtained from GenBank database and were aligned manually using MEGA version 5 software [22]. Phylogenetic trees were inferred by using the maximum-likelihood [23], maximum-parsimony [24], and neighbor-joining [25] tree-making algorithms drawn from the MEGA 5 and PHYML packages [22, 26]; an evolutionary distance matrix for the neighbor-joining algorithm was generated using the Jukes and Cantor [27] model. The topologies of the evolutionary trees were evaluated by a bootstrap analysis [28] of the neighbor-joining method based upon a 1,000 replicates using the MEGA 5 software.

Heteropteran symbionts are found in the gut lumen, as in Reduviidae, or in gastric caecae as in Acanthosomatidae, Alydidae, Coreidae, Parastrachiidae, Pentatomidae, Pyrrochoridae, Plataspidae, and Scutelleridae. As opposed to symbionts of Sternorrhyncha and Auchenorrhyncha, the symbionts of Heteroptera are not passed to the next generation from mother to offspring in a transovarial manner. The posthatch transmission mechanisms may involve egg surface contamination (= smearing on egg surface), coprophagy (= proctophagy: feeding on excrement), capsule transmission, or acquisition from the environment "*de novo*" every generation after the nymphs hatch [1, 18, 19, 21, 29–39]. This curious mode of transmission is challenging to both the host and symbiont. In particular for the symbiont, external transmission may require high genome stability to prevent the loss of genes required for living outside the host in a variable environment, and the challenge for the host bugs is to reinoculate themselves each generation. These challenges may be responsible for the multiple acquisition of the symbiont or low cospeciation observed in Heteroptera [21, 29, 40].

Not all heteropteran microbial associations are beneficial to the host; at times the bacteria may be pathogenic and reduce fitness (i.e., reproduction, mortality, and longevity) [41]. The beneficial and pathogenic aspects of symbiosis have been studied in the past, but now novel molecular approaches are being applied to these systems. With the advent of molecular approaches, it is increasingly clear that manipulation of symbiotic interactions can contribute to the development of new strategies for pest control, including the use of modified symbionts to control insects (paratrangenesis) [30, 42], replacement of native symbionts with genetically modified symbionts via genetic drive [43, 44], and a technique called "incompatible insect" [45]. Additionally, Broderick et al. [46] showed that *Bacillus thuringiensis*, widely applied in biocontrol projects, only kills the lepidopteran larvae if the insects harbor a gut-associated microbial community, highlighting the importance of gut-associated bacteria to pest control.

2. Reduviidae

The vectors of the Chagas disease pathogen are bloodsucking Reduviidae in the subfamily Triatominae. For example, *Rhodnius prolixus* (Stal) is a blood-sucking triatomine that is a common vector of Chagas disease, the incurable illness damaging the heart and nervous system that afflicts millions of people in Central and South America [42]. This insect acquires its bacterial symbiont, *Rhodococcus rhodnii,* soon after the first instar bug hatches. In triatomines, generally, the aposymbiotic (without symbiont) first instar nymphs hatch and probe for their bacterial symbionts that are acquired orally through "contamination" by feces on or nearby egg masses [1, 30, 47]. This insect-symbiont association has been exploited by paratrangenesis, in which the symbiont has been genetically transformed to negatively interfere with the survivorship of the Chagas disease agent, *Trypanosoma cruzi* [48]. Genetically modified *R. rhodnii* symbionts expressing a selectable gene product were stably maintained in *R. prolixus* without deleterious effects on host survival and fitness, thereby substantiating the paratransgenic approach.

According to Hurwitz et al. [49] the paratransgenic strategy has been used with other vector-borne disease systems such as sandfly-mediated leishmaniasis and sharpshooter-mediated Pierce's disease [50–52]. Additionally, the authors highlight the main strategies for the success of the paratrangenesis strategy.

(1) In order to perform genetic manipulation, it is necessary that the symbiont can be cultured.

(2) The symbiont must be identified within a certain disease-transmitting vector.

(3) After the genetic manipulation, insect and symbiont fitness should not be negatively impacted.

(4) The transgene product expressed must interfere with pathogen development in the vector, but should not affect the fitness of the vector.

(5) The technique used to spread the genetically modified symbiont/commensal to naturally occurring vector populations should minimize the spread of

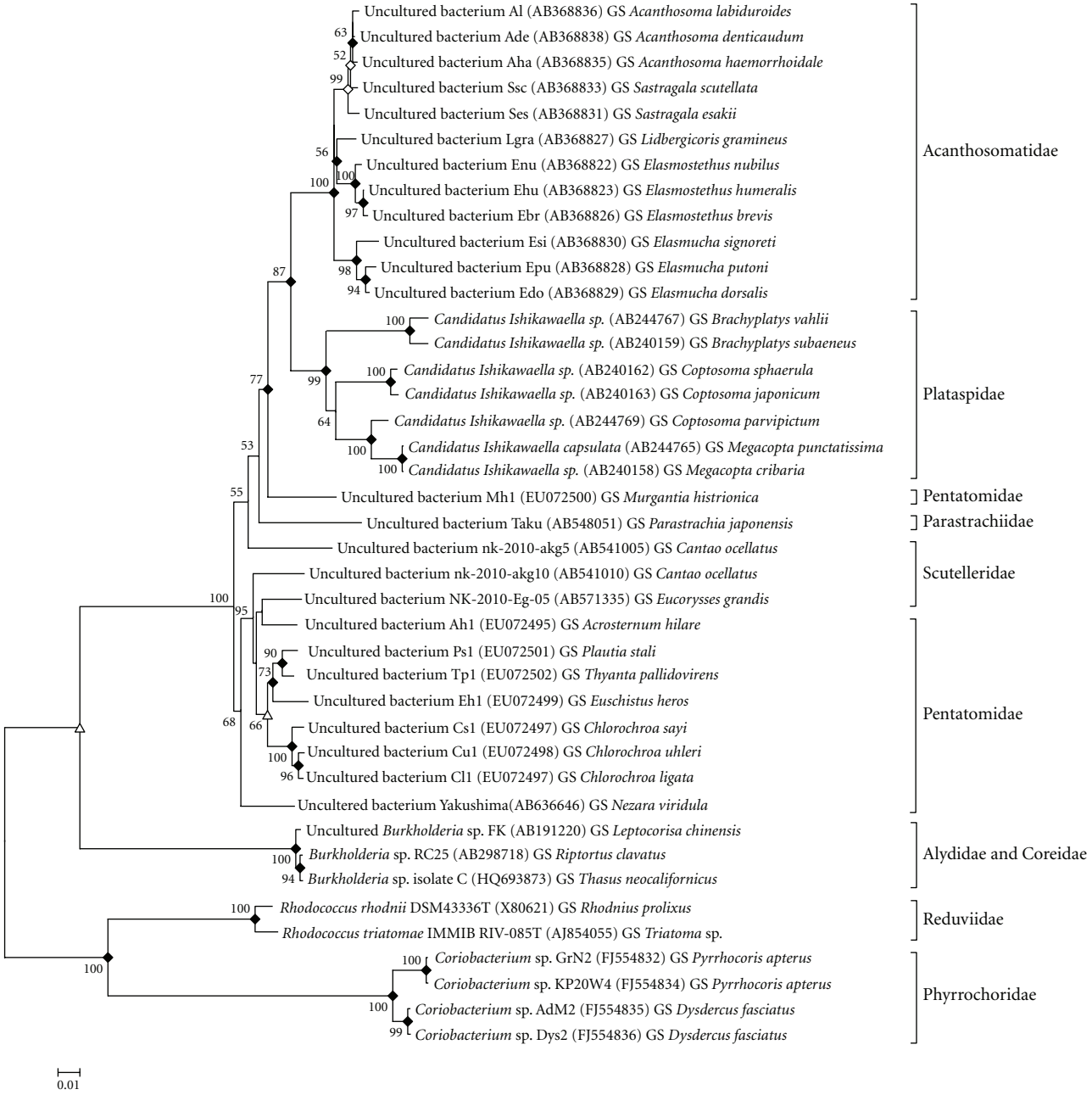

Figure 1: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences (1,300 bp) showing phylogenetic relationships between gut symbionts (GS) of heteropteran species. Insect families are indicated after brackets. Black diamonds indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; white triangle and diamonds stand for branches that were recovered by the maximum-likelihood or by the maximum-parsimony tree-making algorithms, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. Bar 0.01 substitutions per nucleotide position.

the transgene to other organisms in the vector's environment, which include both the nontarget microbes inside the host/vector and other organisms that live in the same ecological niche.

3. Acanthosomatidae

This family, a member of the stink bug or shield bug Pentatomoidea superfamily, is characterized by social behavior, which features the maternal instinct to guard eggs and nymphs against possible predators [53]. The genetic and evolutionary characterization of the caeca-associated symbionts in 14 different species of Acanthosomatidae, representing a total of five genera (*Elasmostethus*, *Lindbergicoris*, *Elasmucha*, *Sastragala*, and *Acanthosoma*), has been elucidated [33]. Acanthosomatid bugs harbor extracellular symbionts of a specific clade of Gammaproteobacteria in midgut crypts.

In Acanthosomatidae, both host and symbiont have co-speciated together, and the vertical symbiont transmission is via egg surface contamination [33].

4. Alydidae and Coreidae

Alydidae (called broad-headed bugs) and Coreidae (leaf-footed bugs) are relatively small, principally herbivorous families. Alydid and coreid bugs feed mostly on seeds, less often on the phloem, of various plants; alydids also occasionally exhibit coprophagy and carrion feeding [17]. The broad-headed bugs, *Riptortus clavatus* (Thunberg), *Leptocorisa chinensis* (Dallas), and the giant mesquite bug *Thasus neocalifornicus* Brailovsky and Barrera, harbor symbionts of the *Betaproteobacteria* type in the genus *Burkholderia*. These insects acquire symbionts from the soil (i.e., horizontally) in each generation and harbor the bacteria in the lumen of crypts situated along the midgut [15, 54, 55]. Inoculation of aposymbiotic nymphs with cultured symbiotic microorganisms and comparison with aposymbiotic adults reared under sterile conditions suggest that the absence of the symbiont decreases host fitness [15, 54]. Moreover, phylogenetic analysis shows that the *Burkholderia*-like sequences from the digestive tract of *T. neocalifornicus* are closely related to those found in *L. chinensis* and *R. clavatus*, data acquired by amplifying the 1.5-kb segment of the eubacterial 16S rRNA gene [15].

5. Parastrachiidae

In Parastrachiidae (another pentatomoid family), *Parastrachia japonensis* Scott is monophagous, feeding of drupes of the deciduous tree *Shoepfia jasminodora* (Santalales: Olacaceae) [56]. The parastrachiid mother provides food for her nymphs, and the ensuing adults enter into diapause for 9 months, surviving only on water. Molecular phylogenetic analyses of *P. japonensis* symbionts revealed that they constitute a distinct phyletic line in the Gammaproteobacteria 16S rRNA gene subclade. This parastrachiid symbiont has no close relatives, but is allied with gut symbionts of acanthosomatid and plataspid bugs, as well as with endocellular symbionts of sharpshooters, tsetse flies, and aphids [36]. According to Kashima et al. [57], this symbiont might be involved in the uric acid recycling system due to the increased mortality of the adults when they were treated with antibiotic during the nonfeeding period.

6. Pentatomidae

Within Heteroptera, the Pentatomidae (the true "stink bugs") is one of the largest families with over 4000 species [17]. Many pentatomid insects are polyphagous, feeding on a diverse range of plants. Stink bugs are economically important pests throughout the world on a multitude of crops, including soybeans, rice, pecan, cocoa, and macadamia nuts to name a few [17, 58]. They can cause direct and indirect damage, as can other heteropteran, by feeding on plant tissue with needle-like stylets, injecting digestive enzymes into plant tissue, or providing free access

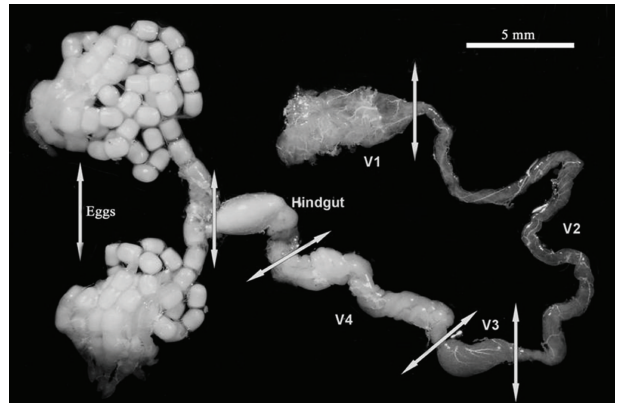

Figure 2: Detailed figure of the midgut of *Nezara viridula* divided into four ventricles. V4 is the gastric caeca, where the symbionts are located [21].

to microbial infection [17, 59]. The economic importance of stink bugs is magnified as they are usually difficult to control [60]. Recently, it was shown that the stink bugs, *Acrosternum hilare* (Say), *Chlorochroa ligata* (Say), *Chlorochroa sayi* (Stal), *Chlorochroa uhleri* (Stal), *Dichelops melacanthus* (Dallas), *Edessa meditabunda* (F.), *Euschistus heros* (Fabricius), *Loxa deducta* Walker, *Murgantia histrionica* (Hahn), *Nezara viridula* (L.), *Pellaea stictica* (Dallas, 1851), *Piezodorus guildinii* (Westwood), *Plautia stali* Scott, *Thyanta pallidovirens* (Stal), and, *Thyanta perditor* (F.) are associated with plant pathogens (*Pantoea* spp.) contained in the gastric caecal region (ventricula 4; Figures 2 and 3) of their midguts [21, 34, 35, 40, 61, Prado, S.S. unpublished data]. In general, stink bug symbionts are polyphyletic, although some degree of monophyly has also been observed suggesting that the symbionts were probably acquired and occasionally replaced by other bacteria over evolutionary time [34, 35]. Smearing of symbionts on the egg surface by ovipositing females and subsequent acquisition of the symbiont by aposymbiotic first instar nymphs appears to be the mechanism of vertical transmission.

The cosmopolitan pentatomid, *Nezara viridula*, is both generally and obligatorily associated with a gut symbiont; however, it seems that the type and duration of the association is somewhat different between populations based on the geographical region where the insect is found [20, 21, 39]. At 30°C, *N. viridula*'s symbiont maintenance is affected and insect development is accelerated [40]. Insects free of the symbionts reared at 20°C had longer mean nymphal developmental time, and females never laid eggs [21, 40].

In addition, *P. stali*, when deprived of its gut-associated symbiont, has a slower developmental time than individuals with the symbiont [29]. For *A. hilare*, the elimination of the symbiont by surface sterilization of egg masses negatively impacted development and reproduction [35]. Conversely, the absence of *M. histrionica*'s gut symbiont seems to have no effect on the development of the insect host; however, when both species (*A. hilare* and *M. histrionica*) were reared at 30°C, each lost their respective symbiont [61].

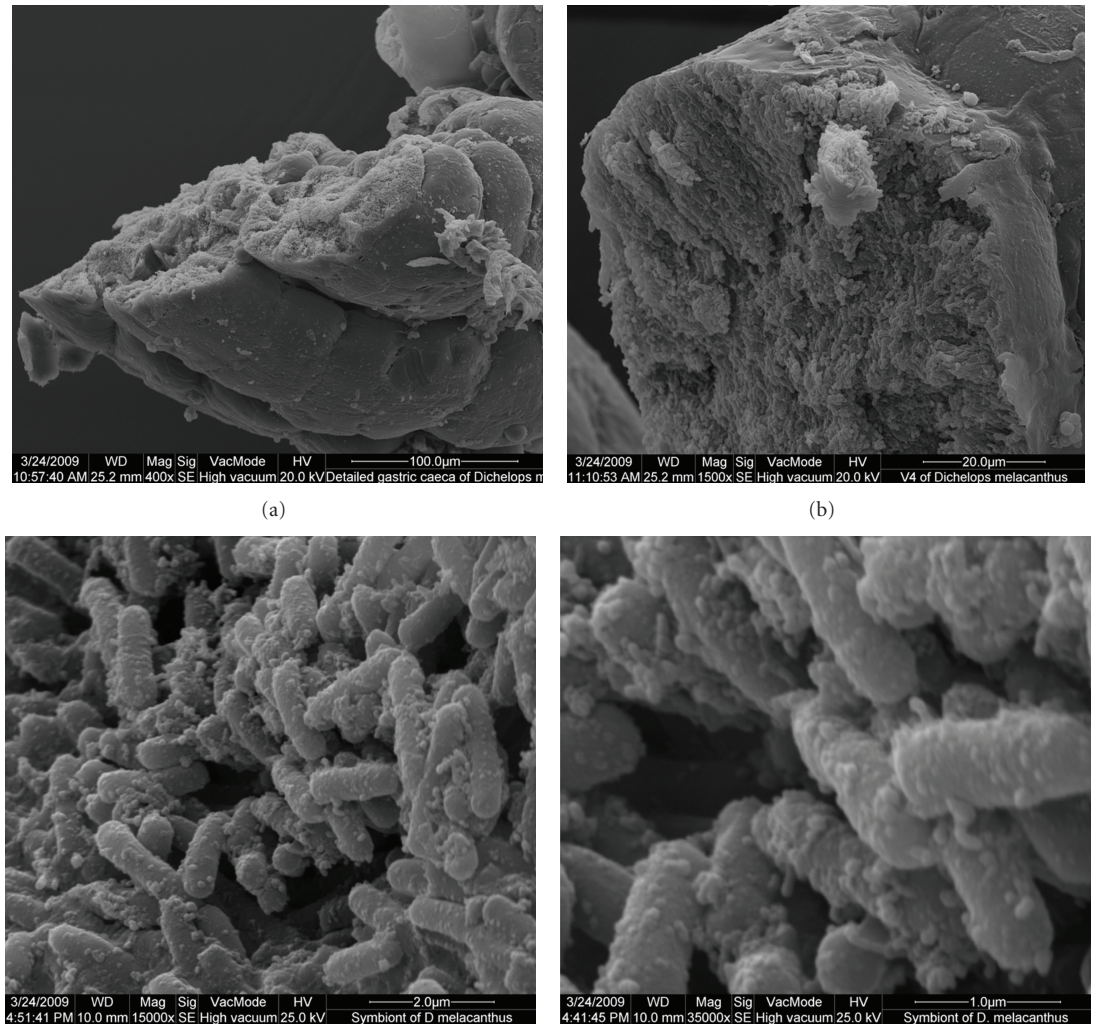

Figure 3: Detailed figure of scan electron microscopy (SEM) of the gastric caeca of *Dichelops melacanthus* in (a), detail of the amount of bacteria inside the gastric caeca in (b), and bacteria detail in (c) and (d).

The impact of surface sterilization on the maintenance of the symbionts and on the development of *E. heros*, *D. melacanthus,* and *P. stictica* is being evaluated by Prado et al. (unpublished). Data thus far has shown that a decrease in host fitness was associated with, and probably mediated by, symbiont loss at 30°C. This suggests that, not only egg mass sterilization, but also higher temperature may affect population performance of the insects directly or indirectly through mediated effects on their mutualists [35, 61]. The role of these vertically transmitted pentatomid gut symbionts, therefore, appears to vary for different bug host species. For instance, the cabbage stink bugs, *Eurydema rugosa* Motschulsky and *Eurydema dominulus* (Scopoli), also have symbionts associated with their gastric caecae; absence of the caeca-associated symbiont due to surface sterilization of egg masses caused retarded growth, reduced body weight, and abnormal body color [62].

Recently, using genus-specific primers and appropriate PCR conditions, Zucchi et al. [63] characterized an earlier unnoticed community of actinobacteria inhabiting the gastric caeca of several pentatomid species (Table 1). Although only a few insects species have been reported associated with *Actinobacteria* [64], the best-known case involving nutrient provision (see Section 2), studies on pentatomids have pointed to an alternative beneficial association in which the actinobacteria produce an antibiotic barrier against pathogens [65, 66]. The role of these actinobacteria in the midgut of the stink bug is still unknown, but Zucchi et al. [63] speculate that the actinobacteria byproducts may regulate the gastric caecal bacterial community.

7. Phyrrochoridae

There is at least 262 pyrrochorid species distributed in 29 genera, with *Dysdercus* being the most important and largest genus [17]. These bugs are called cotton stainers because their excreta, plus the disease organisms they admit, stain cotton fiber. An actinobacterium, *Coriobacterium glomerans,* has been described as the extracellular gut-associated

Table 1: Actinobacteria diversity inhabiting the midgut of Pentatomidae.

Family	Genus	Host
Actinomycetaceae	*Actinomyces* spp.	*Thyanta perditor*
Brevibacteriaceae	*Brevibacterium* spp.	*Dichelops melacanthus*
Corynebacteriaceae	*Corynebacterium* spp.	*Dichelops melacanthus*
		Edessa meditabunda
		Thyanta perditor
Dietziaceae	*Dietizia* spp.	*Dichelops melacanthus*
		Loxa deducta
		Pellea stictica
Intransporangiaceae	*Ornithinimicrobium* spp.	*Loxa deducta*
		Pellea stictica
Kineosporiaceae	*Kineococcus* spp.	*Dichelops melacanthus*
Microbacteriaceae	*Microbacterium* spp.	*Edessa meditabunda*
Micrococcaceae	*Arthrobacter* spp.	*Edessa meditabunda*
	Citrococcus spp.	*Dichelops melacanthus*
		Edessa meditabunda
Mycobacteriaceae	*Mycobacterium* spp.	*Loxa deducta*
		Pellea stictica
		Piezodorus guildinii
		Thyanta perditor
Propionibacteriaceae	*Propionibacterium* spp.	*Dichelops melacanthus*
		Piezodorus guildinii
Streptomycetaceae	*Streptomyces* spp.	*Nezara viridula*

Source: modified from Zucchi et al. [63].

symbiont in *Pyrrhocoris apterus* (L.) and *Dysdercus fasciatus* Sign. [32, 67, 68]. Recently, Kaltenpoth et al. [32] showed that the bacterial symbionts are located mainly in the third part of the midgut (V 3), with cells found connected to the epithelium and swimming freely in the gut. The symbionts are primarily transmitted vertically by egg smearing, but horizontal transmission also occurs [32]. The bacterial cells can form long chains in the gut of the insects, where they are assumed to aid in digestion [67].

8. Plataspidae

Insects of this family are almost entirely from the tropical old world most species are Oriental and; this is one of a few groups that feed most of the time on legumes [17]. The Japanese common plataspid, *Megacopta punctatissima* (Montandon), harbors the bacterial symbiont *Candidatus* Ishikawaella capsulata in its gastric caeca [19]. Fukatsu and Hosokawa [18] showed that after hatching, the aposymbiotic first instar nymphs immediately probe small brownish capsules attached to the eggs masses laid by the females in order to acquire their symbiont [18, 19, 41]. In addition, Hosokawa et al. [19] used phylogenetic reconstruction to show that both insect and symbiont have undergone cospeciation and when deprived of its symbiont, *M. punctatissima*'s growth and survival are negatively influenced [18, 19, 41]. The plataspid bug, *Megacopta cribraria* (Fabricius), also has an obligatory relationship with its primary endosymbiont, which is similar to that first described for *M. punctatissima* [69]. Recently, Hosokawa et al. [41] showed by experimentally exchanging the obligatory gut-associated symbiont between *M. punctatissima* and *M. cribraria*, that the success of the important pest species on legumes (*M. punctatissima*) was negatively impacted due to high nymphal mortality before or upon hatching. Conversely, *M. cribraria*, which is considered a nonpest species of the legume crop, when carrying *M. punctatissima*'s obligatory symbiont, exhibited the attributes of the naturally pestiferous species. These exciting findings raise new hypotheses on the evolutionary origin of an insect pest, which may lead to the development of alternative methods to control and manage species considered pests [41].

9. Scutelleridae

Within the Pentatomoidea, this family (commonly called shield bugs) is most closely related to Pentatomidae. All scutellerids are phytophagous, but only a few have been reported as pests [17]. The giant jewel shield bugs, *Cantao ocellatus* (Thunberg) and *Eucorysses grandis* (Thunberg), possess a gammaproteobacterial primary gut symbiont and a *Sodalis*-allied secondary symbiont [37, 38]. The specific

bacterium from *E. grandis* was consistently identified in insects from five different geographic regions and was detected in 100% of the insects surveyed from three host populations. Molecular phylogenetic analysis clearly showed that the primary gut-associated symbiont of *E. grandis* constitutes a distinct lineage in the *Gammaproteobacteria*, and is closely related neither to the gut symbiont associated with *C. ocellatus*, nor to gut symbionts of other stink bugs, suggesting that scutellerid symbionts have multiple evolutionary origins [38].

10. Concluding Remarks

In this paper, we reviewed major trends in symbiotic association for diverse members of the Heteroptera. These interactions have been primarily studied for their ecological interest regarding insect development. In the past decade, it has become increasingly clear that exploiting these relationships may be a fruitful alternative type of biological control; paratrangenesis [42] and specific elimination of the essential bacteria of the pest or vector insect [70] demonstrated promising results.

Endosymbiotic bacteria of insects have received considerable attention in the past few decades. Many studies focused on the intimate associations of intracellular symbionts, their hosts and the degree of mutual interdependence of these symbioses. Most of these studies focused on obligatory symbionts that are difficult or so far impossible to cultivate. Successful attempts at pest control or disease management, such as those demonstrated by the paratrangenesis of the endosymbiont actinobacteria from triatomine species, should become more common once efforts to identify other bacterial symbionts for other heteropteran hosts are more successful, particularly with secondary symbionts. Furthermore, recent studies have shown that extracellular gut symbionts of insects can engage in symbiotic interactions of similar intimacy and specificity with their hosts, and may exhibit similar evolutionary and genomic consequences of the symbiotic lifestyle [19].

There may also be negative impacts on the symbiont-host relationship caused by global warming changes and, consequently, interference in insect survivorship and ecology from elevated global temperatures, encouraging more research on these associations [9, 12, 14, 15, 71].

At the time of writing, only a few examples of insect-microorganism associations are effectively being explored for control of pests or human diseases. This is still an open area of research with great potential for control of insect pests and vectors of disease, as the cases mentioned earlier using the paratransgenic strategy in the systems of sandfly/leishmaniasis and sharpshooter/Pierce's disease represent [50–52]. In fact, it is only recently that considerable information has been gathered to permit the design of alternative methods of control. Studies on different bacterial groups, such as actinobacteria, reminds us how intricate and complex the associations between stink bugs and microorganisms are. Further comprehension of their biological, physiological, and ecological features is necessary to have a better picture of the evolution of these interactions and to devise a more effective pest and disease control programs.

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Research Article

A Male Aggregation Pheromone in the Bronze Bug, *Thaumastocoris peregrinus* (Thaumastocoridae)

Andrés González,[1] María Victoria Calvo,[1] Valeria Cal,[1] Verónica Hernández,[1] Florencia Doño,[1] Leticia Alves,[2] Daniela Gamenara,[2] Carmen Rossini,[1] and Gonzalo Martínez[3]

[1] *Laboratorio de Ecología Química, Facultad de Química, Universidad de la República, Avenida General Flores 2124, 11800 Montevideo, Uruguay*
[2] *Laboratorio de Síntesis Orgánica, Facultad de Química, Universidad de la República, Avenida General Flores 2124, 11800 Montevideo, Uruguay*
[3] *Estación Experimental INIA Tacuarembó, Instituto Nacional de Investigación Agropecuaria, Ruta 5 Km. 386, 45000 Tacuarembó, Uruguay*

Correspondence should be addressed to Andrés González, agonzal@fq.edu.uy

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Forest plantations in Uruguay have doubled in the past decade, with *Eucalyptus* spp. leading this growth. The bronze bug, *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae), originally restricted to Australia, is an important emerging pest of *Eucalyptus* plantations in the Southern hemisphere. *T. peregrinus* feeds on mature *Eucalyptus* leaves, causing them to turn brown and often fall from the tree. Although population dynamics and behavioural patterns are not clearly understood, circumstantial observations suggest that males and nymphs aggregate. We used gas chromatography coupled to mass spectrometry to analyze volatile organic compounds emitted by virgin males and females, and characterized a male-specific compound, 3-methylbut-2-enyl butanoate, based on mass spectral data and chromatographic comparison with a synthetic standard. We also performed Y-olfactometer bioassays to test the attraction of virgin males and females toward live virgin males, male volatile extracts, and synthetic 3-methylbut-2-enyl butanoate. Males were attracted toward conspecific males, while virgin females showed no preference, suggesting that male volatiles are not involved in sexual communication. Further olfactometer tests showed that males were attracted to male volatile extracts and to synthetic 3-methylbut-2-enyl butanoate. The ecological significance of this compound and its potential use for the management of *T. peregrinus* in *Eucalyptus* forests will be further investigated.

1. Introduction

The area of commercial forests in Uruguay reaches about 1 million hectares, of which more than 70% are covered by *Eucalyptus* plantations. This area has nearly doubled in the past decade, as part of broader trend in South America. In Uruguay, *E. globulus* and *E. grandis* are the most important species, representing 54% and 32% of the *Eucalyptus* planted area, respectively, while red gum trees such as *E. tereticornis* occupy about 7% [1]. Depending on whether the plantations are intended for pulp or timber production, the growth cycles take between 8 to 12 years, being, therefore, a clear example of monocultural practice of an exotic crop, which should favor the spread of alien invasive pests and diseases, such as the bronze bug, *Thaumastocoris pereginus* (Hemiptera : Thaumastocoridae).

T. peregrinus is a major emerging pest of eucalypt production in the Southern hemisphere. It is a small flattened bug (1–3 mm long) that feeds on *Eucalyptus* and some *Corymbia* species [2, 3]. It employs a lacerate-and-flush feeding strategy [4], causing the loss of photosynthetic surface area, defoliation, and even tree death [2]. This insect is native from Australia, and little research had been done on it until it became a pest of planted *Eucalyptus* trees in Sidney,

in 2002 [5, 6]. It was first recorded outside its natural range in South Africa in 2003, although it was misidentified as *T. australicus* [2]. Originating seemingly from independent introductions from Australia [7], it was first recorded in Argentina in 2005 [6], and it was recognized as the new species *T. peregrinus* [8]. It is now well established in Argentina, Brazil, Uruguay, Chile, and Paraguay [3, 9], and it is foreseen that it will have an important impact for *Eucalyptus* plantations in the region.

Information on the behavior and natural history of *T. peregrinus* is scarce. Our own observations of mating in captivity suggest short precopulatory times after adult emergence (G. Martínez, unpublished), and preoviposition times ranging from 7 to 10 days were recorded in Australia at 20°C [10].

Circumstantial observations suggest that males and nymphs tend to aggregate, possibly by means of semiochemicals. To begin unveiling the possibility of chemical communication in the bronze bug, we conducted a study that comprises twice a week five-instar nymphs emitted from virgin males and females, and Y-tube olfactometer bioassays to test for volatile-based intraspecific attraction. Specifically, we show that males produce a specific volatile compound which we characterized and synthesized, and that this compound acts as a male aggregation pheromone, attracting conspecific males.

2. Materials and Methods

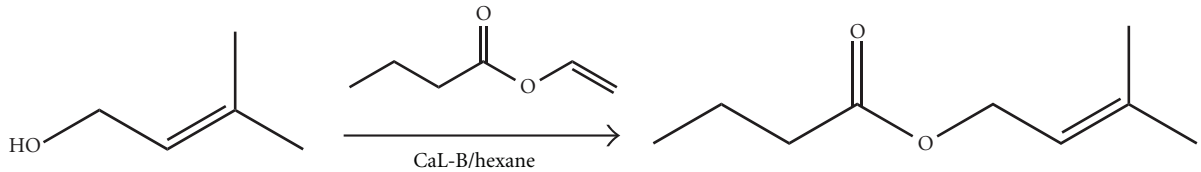
2.1. Insects. Virgin adult bugs were obtained from a laboratory colony reared on *E. tereticornis* (adapted from [10]). Males, females, and nymphs were kept together in mesh-covered cages (35 × 50 × 70 cm) in a greenhouse, on *E. tereticornis* potted plants, and with periodical introduction of field insects. From this stock colony, adults were periodically transferred to *E. tereticornis* branches in Erlenmeyer flasks with distilled water and kept in the laboratory under controlled conditions (20 ± 5°C, 55% RH). Egg clusters harvested from these adult cages were incubated in Petri dishes in a rearing chamber (25°C, 55% RH, 12 : 12 L : D) on leaf discs floating on distilled water. Hatching nymphs were transferred to maturation cages equal to those for adults, and twice a week-five instar nymphs were separated and checked once a day for adult emergence. Just-moulted adults were recognized by the unsclerotized cuticle and were immediately sexed and kept separate for the chemical and behavioral studies. They were kept with *ad libitum* access to *E. camaldulensis* leaves and water.

2.2. Volatile Collection and Analysis. Volatile organic compounds were obtained from virgin males and females enclosed in glass chambers (24 cm length, 4.6 cm diam.) with four *E. tereticornis* leaves. Volatiles from 20 to 40 males and females were adsorbed on 50 mg of Haysep-Q 80/100 mesh, with a current of charcoal-filtered humidified air (300 mL/min) during 72 h (24°C, 14 : 10 D : L photoperiod). The volatiles were eluted with 1 mL distilled hexane and concentrated to 100 μL for GC-MS analysis under a stream of Nitrogen. Volatiles from four *E. tereticornis* leaves were collected in the same fashion as a control. Male volatile extracts for bioassays were obtained similarly (from 29 males), except that only a portion of this extract (200 μL) was concentrated for GC-MS analysis, while most of it was used for bioassays without concentration.

GC-MS analyses were done using a QP-2010 Shimadzu GC-MS, equipped either with a polar (AT-WAX MS) or an apolar (AT-5 MS) column (Alltech) (30 m × 0.25 mm, 0.25 μm), and operated with a constant carrier flow of 1 mL/min (He). The temperature of the GC oven was programmed as follows: for the polar column, the initial temperature was 40°C (1 min), then raised to 250°C at 7°C/min, and held for 1 min at 250°C. For the apolar column, the initial temperature was 40°C (1 min), then raised to 300°C at 10°C/min, and held for 3 min. The injector temperature was 220°C and the interphase temperature 250°C. Injection (1 μL) was in the splitless mode, and mass spectra were acquired from *m/z* 30 to 350 (70 eV, scan mode). For retention index calculations, a mixture of n-alkanes (100 ppm each, in hexane) was injected in the splitless mode immediately after the samples.

2.3. Behavioral Bioassays. All experiments were performed during the day, using a glass Y-tube olfactometer (each arm 20 cm length, 4 cm diam.) with the stimuli placed in separate glass tubes (10 cm length, 4 cm diam.) and connected to the olfactometer by teflon tubing. The relative position of the tested stimulus and its corresponding control were alternated between replicates to prevent any positional bias in the behavior of the insects. Charcoal-filtered humidified air was pushed and pulled through the olfactometer at a total flow of 1200 mL/min. Tested insects were individually placed at the entrance of the central tube, and their behavior was observed for 10 min. First arm choice and time of residence in each arm were recorded, and the results were analyzed using the Chi-square and Wilcoxon tests, respectively. All tested insects were virgin adults and were used only once, and those that did not reach any of the olfactometer arms were not considered in the analysis. Tested stimuli were the following: (a) 10 live males (virgin, with two *E. tereticornis* leaves) versus two *E. tereticornis* leaves; (b) male volatile extracts versus hexane (5 μL, on filter paper, with two *E. tereticornis* leaves); (c) 3-methylbut-2-enyl butanoate versus hexane (1 μg in 5 μL, on filter paper, with two *E. tereticornis* leaves).

2.4. Synthesis. 3-Methylbut-2-enyl butanoate was synthesized from 3-methyl-2-buten-1-ol and vinyl butyrate (Scheme 1), using a biocatalyzed transesterification [11]. Lipase B from *Candida antarctica* (CaL B, 30 mg, Novozym 435) was added to a mixture of vinyl butyrate (0.15 g, 1.3 mmol) and 3-methyl-2-buten-1-ol (0.10 g, 1.2 mmol) in 2 mL of hexane. The mixture was stirred 2 h in an orbital shaker at 30°C. The enzyme was filtered, the solvent was distilled under reduced pressure, and the crude was purified by column chromatography (Hex:AcOEt 8 : 2). 3-Methylbut-2-enyl butanoate was obtained in 98% yield, and its was structure confirmed by NMR and mass spectrometry: ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 0.94 (t, J = 7.4 Hz, 3H); 1.65 (sext, J = 7.4 Hz, 2H); 1.71 (s, 3H); 1.76 (s, 3H); 2.28 (t, 3H, J = 7.4 Hz); 4.57



3-methyl-2-butenol

3-methyl-2-butenyl butyrate

Scheme 1: Biocatalytic synthesis of 3-methylbut-2-enyl butanoate from 3-methyl-2-buten-1-ol and vinyl butyrate.

(d, $J = 7.2$ Hz); 5.33 (tsept, $J_1 = 7.2$ Hz, $J_2 = 1.3$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 13.6; 17.9; 18.5; 25.7; 36.2; 61.1; 118.7; 138,9; 173.7. MS (IE; 70 eV): m/z = 157 (0.2%), 156 (2.1%), 128 (0.4%), 114 (1.9%), 96 (0.2%), 89 (2.7%), 86 (1.6%), 85 (7.4%), 83 (1.1%), 72 (4.6%), 71 (100%), 70 (4.7%), 69 (62.0%), 68 (89.3%), 67 (56.6%), 66 (1.3%), 65 (1.2%), 57 (2.6%), 56 (1.1%), 55 (3.6%), 54 (1.6%), 53 (14.3%), 51 (1.2%), 44 (2.4%), 43 (63%), 42 (6.5%), 41 (49.8%), 40 (5.1%), 39 (11.4%), 38 (0.6%), 31 (0.4%).

Commercial reactants were purchased from Sigma-Aldrich Inc., and Lipase B from *C. antarctica* (CaL B, Novozym 435) was obtained from Novozymes. Column chromatography was performed using silica gel flash (Kieselgel 60, EM reagent, 230–240 mesh) from Macherey-Nagel. NMR spectra (^{1}H and ^{13}C) were carried out in a Bruker Avance DPX 400 MHz equipment. All experiments were taken at 30°C; $CDCl_3$ was used as solvent and TMS as internal standard [abbreviations: sept(septet); sext (sextet); t(triplet); d(doblet); s(singlet)]. GC-MS analysis was performed as previously described for the volatile extracts.

3. Results

3.1. Volatile Analysis. Several volatile extracts were obtained from equal numbers of males and females, and their GC-MS comparative analyses consistently showed a male-specific compound (Figure 1). This compound eluted with a retention time of 10.1 min in the polar column (RI = 1379) and 7.4 min in the apolar column (RI = 1101). A coeluting compound in the polar column was present in the female volatile extracts and was clearly different from the male compound (Figure 1(b)). This compound was identified as nonanal from its mass spectrum and by comparison with a standard, and it was also found in the leaf volatile extracts (data not shown).

The mass spectrum of the male-specific compound (Figure 1(a)) showed a small molecular ion (2%) at m/z 156 and an M+1 ion of about 10%, suggesting 9 carbon atoms and a possible molecular formula of $C_9H_{16}O_2$. The base peak of m/z 71 suggested a fragment ion with a formula C_4H_7O, as expected from the α-cleavage of a butyric acid ester. In addition, the ion cluster at m/z 67, 68, and 69 is commonly found with varying relative intensities in prenyl esters. In accordance with this, the database search (NIST 08) for this mass spectrum suggested the ester 3-methylbut-2-enyl butanoate (similarity index 96%), which was, therefore, synthesized and compared by GC-MS with the male volatile extracts, resulting in identical retention times and mass spectra between the synthetic and natural compounds (Figure 2).

3.2. Behavioral Bioassays. When live *T. peregrinus* males were used as stimuli in the Y-tube olfactometer, males showed a significant preference toward conspecific males. The olfactometer arm bearing male volatiles was chosen more often (first arm choice: $P = 0.004$, χ^2 test, $N = 35$), and the males spent more time in this arm than in the control arm with *E. tereticornis* leaves ($P = 0.002$, Wilcoxon test, $N = 35$). The females showed no preference for either olfactometer arm, both regarding first arm choice and residence time in each arm ($P = 0.22$, χ^2 test, $N = 42$; $P = 0.69$, Wilcoxon test, $N = 42$, resp.) (Figure 3(a)).

The remaining behavioral experiments focused only in the response of males and in evaluating the chemical nature of the male attraction toward conspecific males. It is worth to note, nonetheless, that males did not show any preference in the Y-tube olfactometer in the presence of female volatile extracts as stimulus (first arm choice; stimulus = 27 males; control = 27 males). On the contrary, when male volatile extracts were used as stimulus, the males showed a clear attraction to male volatiles ($P = 0.005$, χ^2 test, $N = 43$; $P = 0.02$, Wilcoxon test, $N = 43$) (Figure 3(b)). When the results were separated according to male age, the results suggested that the attraction was more important for older males (two-week-old) than for those tested within one week after emergence ($P = 0.02$, χ^2 test, $N = 21$; $P = 0.09$, Wilcoxon test, $N = 21$; one-week-old males: $P = 0.66$, χ^2 test, $N = 22$; $P = 0.24$, Wilcoxon test, $N = 22$) (Figure 3(b)). The later, however, were significantly attracted to the synthetic male-specific compound (see below), indicating that they can respond to male odors as well as older males.

Synthetic 3-methylbut-2-enyl butanoate was also attractive to *T. peregrinus* males in Y-tube olfactometer tests. Tested in combination with *E. tereticornis* leaves, 1 μg of the male-specific compound absorbed in filter paper resulted in more males choosing the arm with the stimulus than the control arm ($P < 0.0001$, χ^2 test, $N = 88$; $P < 0.001$ Wilcoxon test, $N = 88$) (Figure 3(c)). Differently from the experiments with male volatile extracts, both older and just-emerged males showed a significant attraction toward the synthetic compound (two-week-old males: $P < 0.0001$, χ^2 test, $N = 42$; $P < 0.02$, Wilcoxon test, $N = 42$; one-week-old males: $P < 0.001$, χ^2 test, $N = 46$; $P = 0.002$, Wilcoxon test, $N = 46$) (Figure 3(c)).

Considering the number of males used for the male

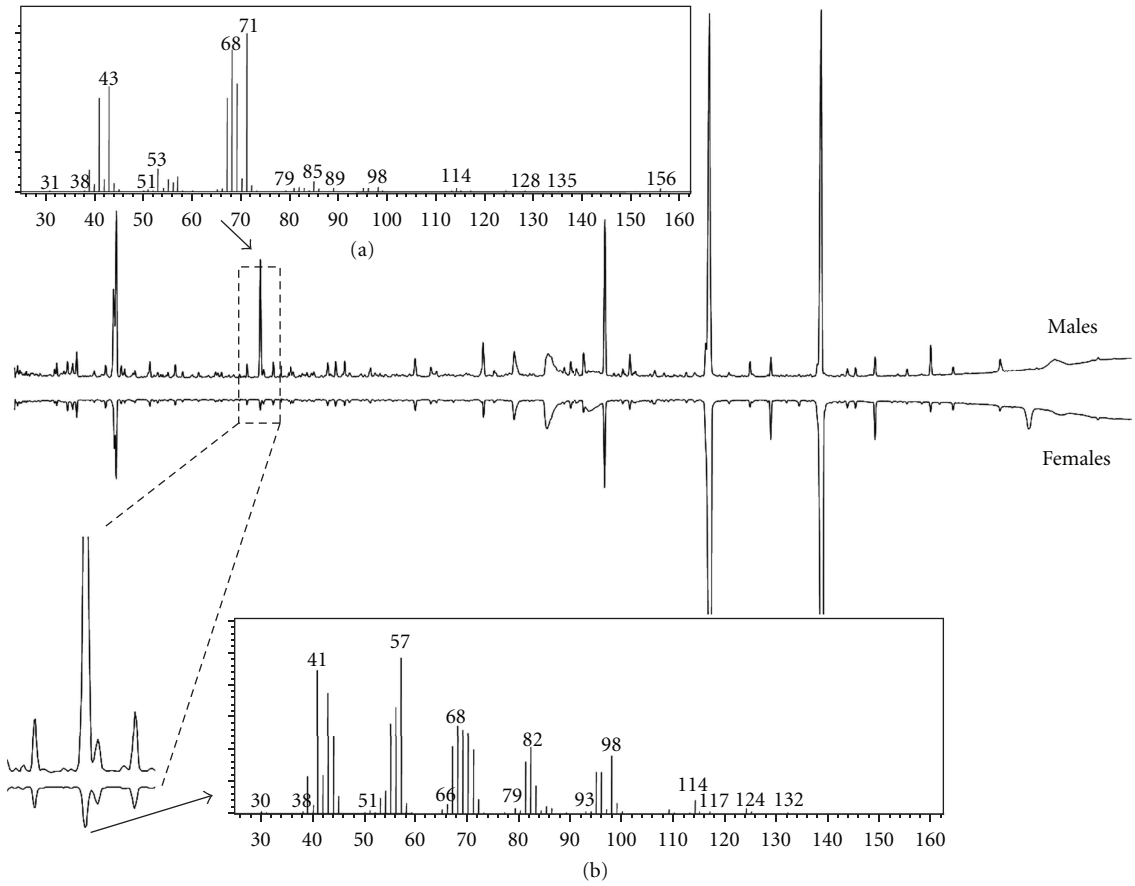


Figure 1: GC-MS traces (TIC) of *T. peregrinus* male (top) and female (bottom) volatile extracts analyzed in a polar column. A male-specific compound was present in male volatile extracts, with a retention time of 10.1 min (mass spectrum shown in insert (a)). A coeluting compound in female volatile extracts was identified as nonanal based on its mass spectrum (insert (b)) and comparison with a synthetic standard. This compound was also present in *E. tereticornis* leaf volatiles.

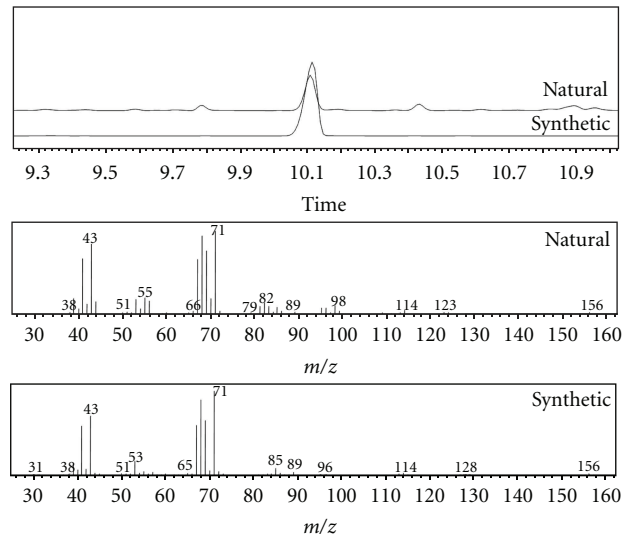


Figure 2: GC-MS traces (TIC) of *T. peregrinus* male volatile extracts (upper trace) and synthetic 3-methylbut-2-enyl butanoate (lower trace). The mass spectra of the natural and synthetic compounds match closely, with small ions in the natural sample corresponding to nonanal, a coeluting compound from *E. tereticornis* leaf volatiles.

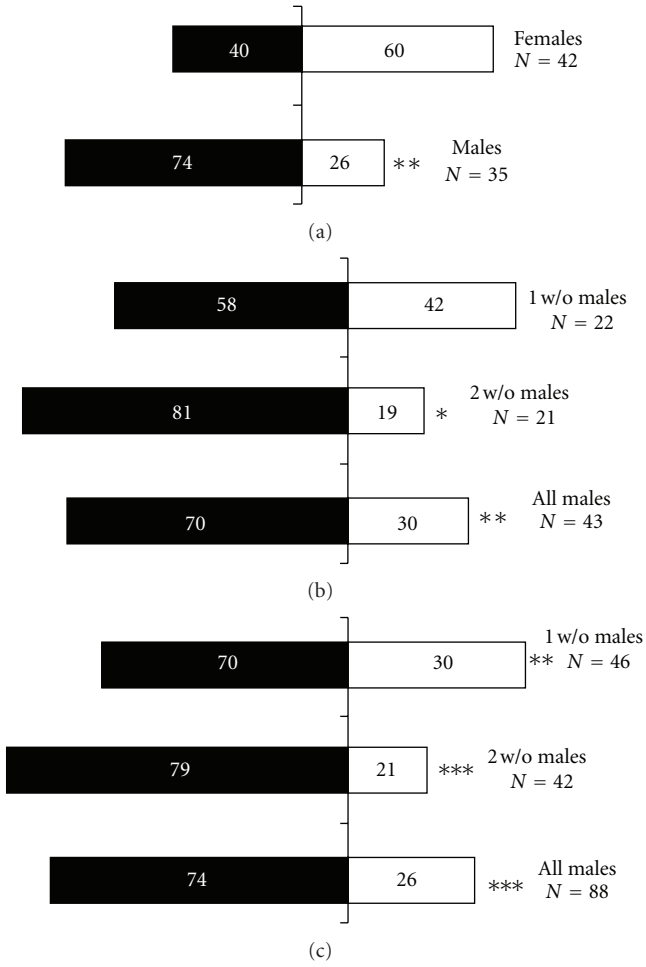


Figure 3: First arm choice of *T. peregrinus* adults in Y-tube olfactometer tests. Bars show the percent (numbers within bars) of insects choosing the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live males versus control. (b) Response of one- and two-week-old (w/o) males to male volatile extracts versus hexane. (c) Response of one- and two-week-old males to synthetic 3-methylbut-2-enyl butanoate versus hexane. All treatments and controls included 2 leaves of *E. tereticornis*. Asterisks indicate significance levels in Chi-square tests ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

volatile collections, and the time of collection, bioassays of the male volatile extracts were performed with 0.002 $\text{insect}_{\text{eq}} \cdot \text{h}^{-1}$. In addition, by comparing the peak areas of 3-methylbut-2-enyl butanoate in the tested extracts and in a 100 ppm solution of the synthetic compound, it can be estimated that the amount of 3-methylbut-2-enyl butanoate in 5 μL of the tested extract was 0.25 μg. The amount of synthetic material used in the bioassays was, therefore, larger, but in the same order of the estimated amount in the bioassays with the extracts. Moreover, in a parallel study, Martins et al. (2012, this issue) report that whole body extracts of *T. peregrinus* males contain up to 1 μg/insect of 3-methylbut-2-enyl butanoate, indicating that the amounts used in our bioassays are biologically relevant.

4. Discussion

To our knowledge, this is the first report of 3-methylbut-2-enyl butanoate as an insect semiochemical, and the first pheromone described in the small Thaumastocoridae family. The same compound is being reported in a simultaneous and independent study by Martins et al. (2012, this issue), confirming its occurrence in different populations of *T. peregrinus* in South America. These authors also found a small amount of the compound in female extracts, and another minor male-specific compound in male extracts, both of which we did not find probably due to differences in the sampling procedure (whole body extracts versus volatile extracts).

Short-chain aliphatic esters are common pheromone components in true bugs [12]. A positional isomer of 3-methylbut-2-enyl butanoate, (*E*)-2-methylbut-2-enyl butanoate, has been reported as a female-specific, male-attractant pheromone component in the broad-headed bug *Alydus eurinus* (Alydidae). Males, and to a lesser extent females and nymphs, were attracted to blends containing this and other butyrate esters produced in the metathoracic glands [13]. Aliphatic butyrate and hexanoate esters are also common pheromones in the Miridae family [12], which shares the superfamily Miroidea with the Thaumastocoridae [14]. The alcohol moieties of these esters are, however, clearly not of terpenic origin, which is most likely the case with the 3-methylbut-2-enyl portion of the male compound in *T. peregrinus*.

Different from the pentatomids, in which most sex or aggregation pheromones are emitted by the males, the few species for which pheromones have been identified in the closely related Miridae use sex pheromones produced by the females, or compounds emitted by both sexes but to which only males are attracted [12, 15–17]. Other than sex pheromones, a male-produced anti-sex pheromone (or male repellant), which has remarkably the same chemical *motiv* and anatomical origin of female phermones, has been reported in two mirid species, suggesting a mate-guarding strategy in these and possibly other species in the family [18, 19]. Our behavioral studies with *T. peregrinus* did not show any cross-gender attraction mediated by sex pheromones, but rather a volatile-mediated male attraction toward conspecific males. Although such male-male chemical interaction does not strictly fit the commonly used definition of an aggregation pheromone (both sexes attracted), we consider that the male-specific compound herein reported, 3-methylbut-2-enyl butanoate, can be regarded as a male aggregation pheromone, or pheromone component. Indeed, our results show that males were attracted to (a) odors from live males in Y-olfactometer bioassays (Figures 3(a) and 4(a)), (b) male volatile extracts (Figures 3(b) and 4(b)), and (c) synthetic 3-methylbut-2-enyl butanoate (Figures 3(c) and 4(c)). Of note is that all our behavioral experiments included *E. tereticornis* leaves in both arms of the olfactometer. We tested the attraction toward live insects with leaves to prevent the insects from desiccation; therefore, tests with volatile extracts and synthetic 3-methylbut-2-enyl butanoate were conducted similarly, in order to compare the results from the different

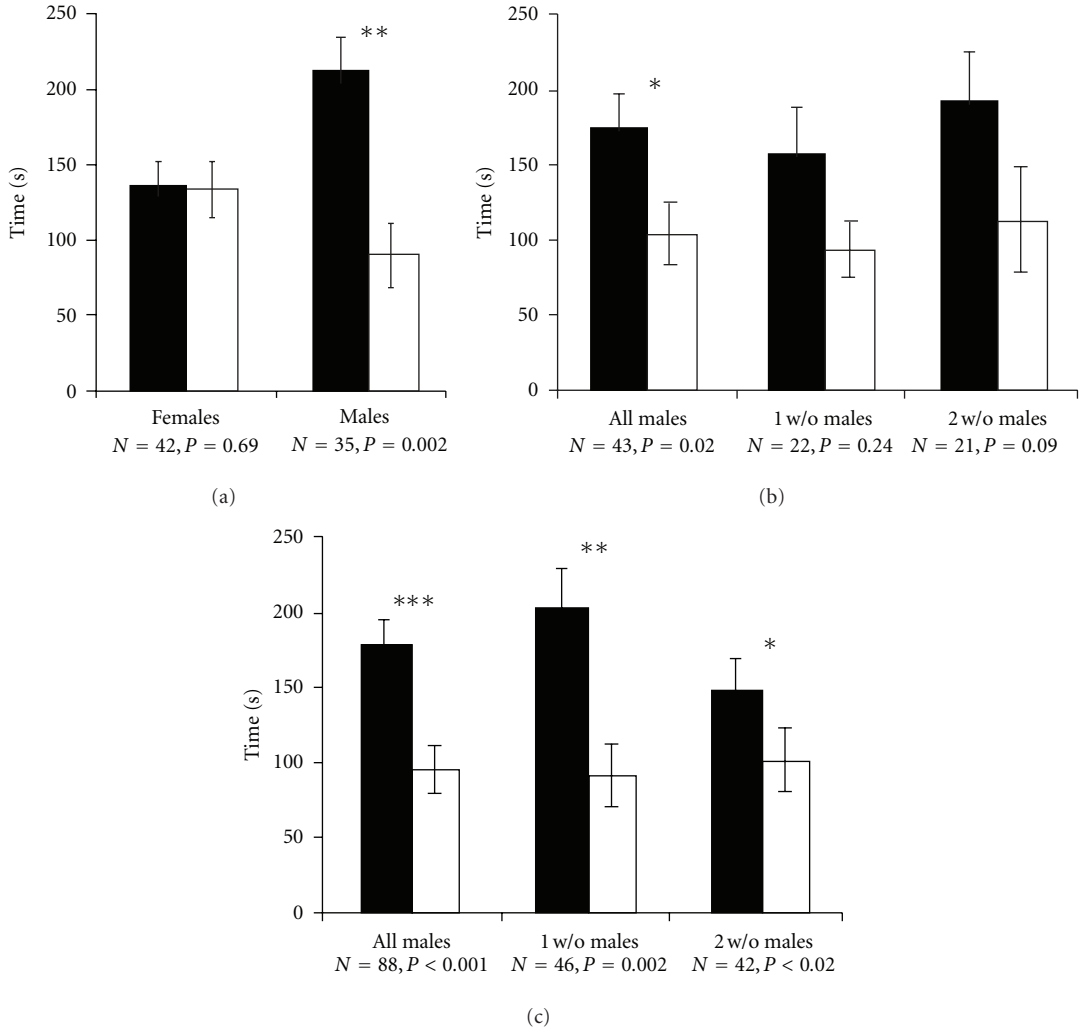


Figure 4: Time of permanence of *T. peregrinus* adults in the stimulus and control arms of the Y-tube olfactometer. Bars show the cumulative time (in 10 min.) that the insects spent in the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live males versus control. (b) Response of one- and two-week-old (w/o) males to male volatile extracts versus hexane. (c) Response of one- and two-week-old males to synthetic 3-methylbut-2-enyl butanoate versus hexane. All treatments and controls included 2 leaves of *E. tereticornis*. Asterisks indicate significance level in Chi-square tests ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

experiments. Although attraction to host plant odors cannot be ruled out from our results, the use of leaves in both olfactometer arms throughout our study allowed for an independent test of the added stimuli. It remains to be studied, nonetheless, if host plant volatiles play an additional, possibly synergic role in the attraction of males.

Finally, our olfactometer tests with male volatile extracts suggest an age-dependant difference in male attraction, since one-week-old males did not show a significant preference (Figure 3(b)). However, the trend in younger males was similar to that of older males, and one-week-old males clearly responded to synthetic 3-methylbut-2-enyl butanoate (Figure 3(c)). Therefore, a possible effect of age in the response of males needs further investigation. Interestingly though, chemical data published simultaneously to our study show that older males produce more 3-methylbut-2-enyl butanoate than younger ones (Martins et al., 2012, this issue), and one can speculate that a correlation between production of and response to this male aggregation pheromone may occur. The ecological significance of this aggregation pheromone, and its possible application for the management of *T. peregrinus* in *Eucalyptus* commercial plantations, will be further investigated.

Acknowledgments

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Research Article

Volatile Chemicals of Adults and Nymphs of the *Eucalyptus* Pest, *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae)

Camila B. C. Martins,[1] Rafael A. Soldi,[1] Leonardo R. Barbosa,[2] Jeffrey R. Aldrich,[3] and Paulo H. G. Zarbin[1]

[1] *Laboratório de Semioquímicos, Departamento de Química, Universidade Federal do Paraná (UFPR), Centro Politécnico, 81531-990, Curitiba, PR, Brazil*
[2] *Laboratório de Entomologia Florestal, Embrapa Florestas, Estrada da Ribeira, Guaraituba, 83411 000 Colombo, PR, Brazil*
[3] *Invasive Insect and Behavior Laboratory, ARS Biocontrol, USDA, Agricultural Research Center-West, B-007, Room 313, Beltsville, MD 20705, USA*

Correspondence should be addressed to Paulo H. G. Zarbin, pzarbin@gmail.com

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Thaumastocoris peregrinus is an introduced "true bug" that is now a severe pest in *Eucalyptus* plantations of various Southern Hemisphere countries. The semiochemicals of thaumastocorids are completely unknown. Therefore, volatile chemicals from *T. peregrinus* nymphs and adults were identified as possible leads for pheromones potentially useful for control. The contents of nymphal exocrine glands, which are shed at molting, were identified from extracts of exuviae. Adults lack functional metathoracic scent glands that are characteristic of most heteropterans; however, both males and females possess a glandular-appearing hold-fast organ that they quickly extrude posteriorly when disturbed. Whole body hexane extracts from males and females were prepared by freezing the insects in a flask so that they extruded the hold-fast organ, and then they were extracted with hexane. Volatiles from nymphal exuviae included benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid. Adult volatiles included 3-methylbut-2-en-1-yl butyrate and 3-methylbut-3-en-1-yl butyrate.

1. Introduction

Thaumastocoris peregrinus Carpintero and Dellapé (Heteroptera: Thaumastocoridae) is an introduced pest of nonnative *Eucalyptus* plantations in various countries in Southern Hemisphere (e.g., South Africa, Argentina, Uruguay, and Brazil) [1–3]. In 2005, it was first found in Buenos Aires, Argentina, on *Eucalyptus viminalis*, *E. tereticornis*, and *E. camaldulensis* [1]. In Brazil, *T. peregrinus* was first found in 2008, on a hybrid clone of *E. grandis x E. urophylla* in São Francisco de Assis, Rio Grande do Sul, and on *E. camaldulensis* trees in Jaguariaúna, São Paulo [4]. Initial studies on life history of *T. peregrinus* were done in Australia [5]; however, no investigation was performed on semiochemicals from these insects.

Heteropteran nymphs and adults characteristically produce allomones for defense; typically, the defensive secretions of nymphs are produced in dorsal abdominal glands (DAGs) [6]. The contents of DAGs are shed along with the exuviae each time the nymph molts, and extraction of exuviae is a convenient method to obtain the DAG secretion [7]. Adult heteropterans characteristically possess metathoracic scent glands from which they release irritating secretions [6]. However, examination of *T. peregrinus* adults by one of us (JRA) revealed that the metathoracic glands are vestigial (unpublished data). On the other hand, adults and nymphs of these unusual bugs possess a rectal organ, similar to that described for plant bugs (Miridae) [8] that is everted when the insects are disturbed. The *Thaumastocoris* rectal organ has a glandular appearance and instantly sticks the insects to the substrate when the insects are disturbed and can as quickly be released (JRA, personal observation) (Figure 1). Pheromones are known for members of several heteropteran families [9], but the semiochemicals of *T. peregrinus* and

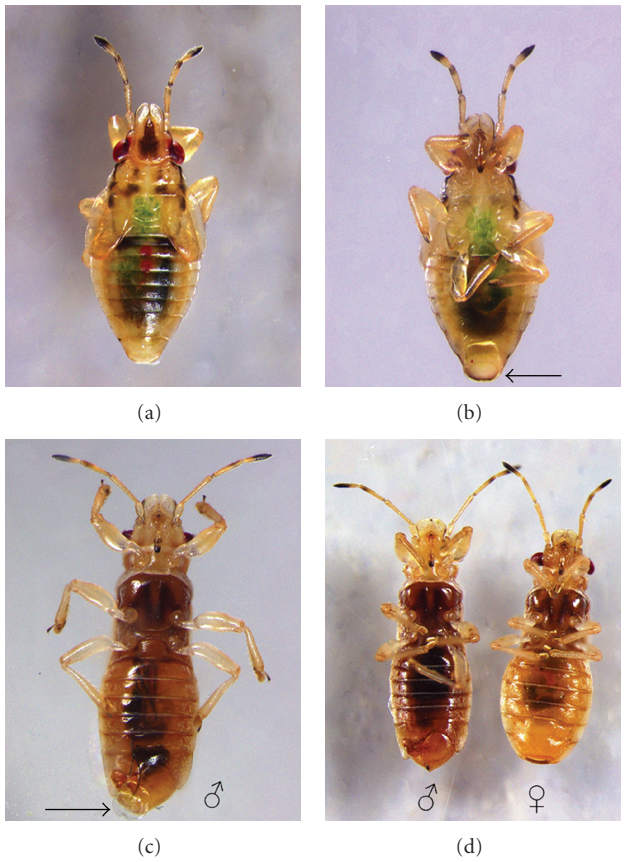

Figure 1: *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) male, female, and nymph. (a) Dorsal view of a 5th-instar nymph; (b) nymphal ventral view showing the rectal organ (arrow); (c) ventral view of the male showing the everted rectal organ (arrow); (d) male and female ventral view with the rectal organ not exposed.

other thaumastocorids are completely unknown. Therefore, the volatile chemical compounds present in the exuviae for the five nymphal instars, and both adult sexes were identified and quantified.

2. Materials and Methods

2.1. Insect Rearing and Extractions. Insects were obtained from a colony at EMBRAPA Florestas, Colombo, Paraná, maintained in the Laboratório de Semioquímicos, Departamento de Química of the Universidade Federal do Paraná (UFPR) under controlled laboratory conditions of 25°C ± 2 and 12 L: 12 D. Adults and nymphs were reared on branches of *Eucalyptus benthamii* in acrylic boxes (30 cm × 30 cm × 30 cm) until their use. To obtain exuviae and virgin adults for the extractions, nymphs of each instar were held individually in small round plastic containers (2.5 cm of diameter) with a gel (Hydroplan—EB/HyC, SNF S.A. Floger) in the bottom of the container for moisture and a leaf disc of *E. benthamii* on the gel. Leaf discs were changed every other day. After nymphs molted, exuviae were collected for extraction, and recently emerged males and females were isolated. Males or females of the same emergence date were grouped in Petri dishes (5 cm of diameter) containing gel and a leaf disc until the extraction. Fifth instar nymphs were grouped in cages provisioned as above to obtain mated males and females for extraction. Couples were formed within 2 days of emergence, and extractions of adults were performed only after eggs were present, which confirmed the mated status of adults.

2.2. Extraction of T. peregrinus Exuviae (1st–5th Instar). Exuviae were extracted with 180 μL of hexane for 24 hours. Each extraction was made with the exuviae available in that day, with a minimum of 12 and maximum of 24 exuviae. At least three repetitions were made for each instar, consisting of at least 45 exuviae in total. After extraction, tridecane (ca. 10 ppm) was added to each sample as an internal standard (IS); the final concentration of the IS was calculated for each extract. Extracts were concentrated and analyzed using a gas chromatograph (GC-2010—Shimadzu) and a gas chromatograph coupled with a mass spectrometer (GC-MS-QP 2010 Plus—Shimadzu). The detected compounds were quantified based on the area of the IS. The GC was equipped with a RTX-5 column (30 m × 0.25 mm i.d. and 0.25 mm film thickness; Restek, Bellefonte, PA, USA). One μL of extract was injected into the GC using the splitless mode with injector temperature at 250°C. The column oven temperature was maintained at 50°C for 1 min, then raised to 250°C at a rate of 7°C/min, and maintained in 250°C for 10 min. Helium was used as carrier gas at a column head pressure of 170 kPa. The same parameters were used for all analyses.

2.3. Extraction of T. peregrinus Adults. Extractions were made with mated and virgin males and females of different ages (3–9, 10–21, 22–34 days old), according to availability of insects. Quantified extracts were compared for virgin males and females (3–9 days), virgin and mated males (10–21 and 21–34 days old), and mated males and females (10–20 and 21–34 days old). There were at least two repetitions per treatment, with a minimum of 15 insects extracted in total. In both experiments, insects were separated by sex in glass Erlenmeyer flasks. The flasks with insects were put in a freezer for one hour so that they died with the rectal organ exposed while "glued" to the glass. Thus, the adults were extracted as complete adults with their rectal organ exposed. The extraction was made between 11:00 AM and 16:00 PM using 150 μL of double distilled HPLC-grade hexane for 10 minutes, then 150 μL of a tridecane solution was added as an IS. The samples were concentrated before injection into a GC-2010, a GC-MS-QP 2010 Plus, and a GC-Fourier transform infrared spectroscopy (GC-FTIR) (GC-2010 coupled to a DiscovIR-GC—Shimadzu). In the infrared analysis, the GC was operated in the splitless mode, and equipped with a DB-5 (0.25 μm, 0.25 m × 30 m) (J&W Scientific, Folsom, CA, EUA) capillary column with helium carrier gas. The column oven was maintained at 50°C for 1 min and then increased to 250°C at 7°C/min to 250°C. A liquid-nitrogen-cooled photoconductive mercury-cadmium-telluride (MCT) detector was used with FT-IR resolution of 8 cm^{-1}. As for nymphal extracts, the final concentration of the IS was calculated

for each extract, and extracted compounds were quantified based on the area of the IS.

2.4. Identification of Chemical Compounds and Synthesis of Esters. Compound identifications were based on coinjections with synthetic standards, Kovats indices (KI), mass spectra (MS), and GC-FTIR analysis. Benzaldehyde, octanol, octanoic acid, decanal, hexanoic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). (*E*)-2-octenol was purchased from Acros Organics (Geel, Turnhout, Belgium).

Twenty-one esters were synthesized by esterification of propionic acid, isobutyric acid, and butyric acid with the following alcohols: pentanol, 3-methylbutan-1-ol, 3-methylbut-2-en-1-ol, 3-methylbut-3-en-1-ol, (*Z*)-pent-2-en-1-ol, (*E*)-pent-2-en-1-ol, pent-4-en-1-ol (all from Aldrich Chemical Company, Milwaukee, WI, USA). 3-methyl-2-buten-1-ol (34.03 mmol, 3 g) and butyric acid (68.06 mmol, 6 g) were refluxed in a round-bottom flask with p-toluenesulfonic acid (TsOH) (1 mol%, 0.35 mmol, 0.06 g) and hydroquinone (5% (w/w) in relation to alcohol, 1.75 mmol, 0.191 g). The reaction medium was heated to 60°C with magnetic stirring for 3 h under argon. The product was purified by addition of aqueous solution NaOH 10% (m/V) until the pH was neutral. Afterwards, the product was extracted with ethyl ether; the combined organic solutions were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The crude product was purified by distillation at reduced pressure and collected from 150–160°C. The same experimental conditions were used in the syntheses of the other saturated esters; however, hydroquinone was not employed with saturated alcohols.

The following esters were coinjected with the natural extracts on three different GC columns (DB-5, DB-Wax and HP-1) for identifications: pentyl propionate (yielding 94%), 3-methylbutyl propionate (yielding 74%), pent-4-en-1-yl propionate (yielding 91%), (*Z*)-pent-2-en-1-yl propionate (yielding 83%), (*E*)-pent-2-en-1-yl propionate (yielding 83%), 3-methylbut-2-en-1-yl propionate (yielding 92%), 3-methylbut-3-en-1-yl propionate (yielding 90%), pentyl 2-methylpropanoate (yielding 90%), 3-methylbutyl 2-methylpropanoate (yielding 87%), pent-4-en-1-yl 2-methylpropanoate (yielding 92%), (2Z)-pent-2-en-1-yl 2-methylpropanoate (yielding 61%), (2E)-pent-2-en-1-yl 2-methylpropanoate (yielding 71%), 3-methylbut-2-en-1-yl 2-methylpropanoate (yielding 92%), 3-methylbut-3-en-1-yl 2-methylpropanoate (yielding 75%), pentyl butyrate (yielding 90%), 3-methylbutyl butyrate (yielding 92%), pent-4-en-1-yl butyrate (yielding 92%), (*Z*)-pent-2-en-1-yl butyrate (yielding 68%), (*E*)-pent-2-en-1-yl butyrate (yielding 74%), 3-methylbut-2-en-1-yl butyrate (yielding 92%), and 3-methylbut-3-en-1-yl butyrate (yielding 80%).

2.5. Statistical Analysis. Statistical analyses were performed using R version 2.13 [10]. To analyze the six main compounds found in the exuviae, the Kruskal-Wallis rank sum test was used followed by a nonparametric multiple comparisons test using the package "pgirmess" in case of significance. Data for the comparison of extracts of virgin and mated adults were tested for normality by the Liliefors and Shapiro-Wilk test. After the normality of the data was confirmed ($P > 0.05$), we performed a GLM (generalized linear model) procedure following Gaussian distribution, considering that mating status was an independent variable. For all analyses, P values >0.05 were considered not significant.

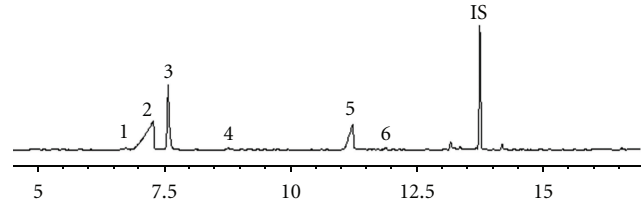

Figure 2: Typical gas chromatogram of a *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) exuvial extract. Numbers (1–6) correspond, respectively, to benzaldehyde, hexanoic acid, octanal, (*E*)-2-octenal, octanoic acid, and decanal. Extracts were analyzed on a Shimadzu GC MS-QP 2010; the internal standard (IS) was tridecane.

3. Results and Discussion

3.1. T. peregrinus Exuvial Extraction. Six compounds were present in the exuviae of *T. peregrinus* nymphs, including benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid (Table 1) (Figure 2). Fourth and fifth instars produced more hexanoic ($H_4 = 15.9$, P value =0.003) and octanoic acids ($H_4 = 15.9$, P value =0.003) than did first instars. All other compounds did not differ significantly by instar; benzaldehyde ($H_4 = 7.9$, P value =0.09), octanol ($H_4 = 6.1$, P value =0.19), (*E*)-2-octenol ($H_4 = 3.2$, P value =0.52), and decanal ($H_4 = 3.9$, P value =0.41) (Table 1).

Some of the compounds present in the exuviae of *T. peregrinus* have been found in other heteropteran species, either as repellents or attractants. For example, benzaldehyde from copulating pairs of *Triatoma infestans* (Klug, 1834) (Reduviidae) was highly attractive to conspecific females at low doses (0.05–0.1 μg) [11]. In the bed bug, *Cimex lectularius* (Linnaeus, 1758) (Cimicidae), decanal, (*E*)-2-octenal, and benzaldehyde are reportedly essential components of the airborne aggregation pheromone [12]. The hexanoic acid is produced in metathoracic scent gland secretions of many bugs (e.g., Scutelleridae: *Eurygaster maura* (Linnaeus, 1758)), along with (*E*)-2-hexanal, (*E*)-2-hexenyl acetate, *n*-tridecane, octadecanoic acid, and *n*-dodecane [13]. The alarm pheromone of *Leptoglossus zonatus* (Dallas, 1852) (Coreidae) adults includes hexyl acetate, hexanol, hexanal, and hexanoic acid [14]. Also, in Japan, a mixture of (*E*)-2-octenyl acetate and 1-octanol attracted the rice bug, *Leptocorisa chinensis* Dallas, 1852 (Alydidae) [15]. While the compounds identified here for *T. peregrinus* nymphs are commonly known exocrine compounds of Heteroptera, the combination of these compounds in these thaumastocorid nymphs is unique compared to the secretions of other heteropteran nymphs [6]. Other heteropterans produce some of these compounds (e.g., *Cimex lectularius*) but not

Table 1: Identification and quantification (ng) of compounds present in *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) exuviae. Different letters for each compound indicate significant differences between instars (KI: Kovats Index; SE: standard error). Statistical comparisons: Kruskal-Wallis rank sum test followed by a nonparametric multiple comparisons test ($P > 0.05$).

Chemical compounds	DB-5 column	1st instar	2nd instar	3rd instar	4th instar	5th instar
	KI	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE
(1) Benzaldehyde	962	0.1 ± 0.0^a	0.1 ± 0.0^a	0.2 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a
(2) Hexanoic acid	988	0.4 ± 0.0^a	1.7 ± 0.5^a	8.5 ± 3.9^a	21.2 ± 7.8^b	25.9 ± 6.7^b
(3) Octanal	996	2.1 ± 0.6^a	5.9 ± 1.2^a	5.7 ± 1.7^a	6.6 ± 2.6^a	3.6 ± 1.1^a
(4) (*E*)-2-octenal	1062	0.3 ± 0.1^a	0.4 ± 0.1^a	0.3 ± 0.0^a	0.3 ± 0.0^a	0.2 ± 0.1^a
(5) Octanoic acid	1209	0.2 ± 0.0^a	0.9 ± 0.3^a	2.6 ± 1.1^a	7.4 ± 2.9^b	7.3 ± 0.8^b
(6) Decanal	1239	0.3 ± 0.1^a	0.4 ± 0.1^a	0.3 ± 0.2^a	0.2 ± 0.1^a	0.1 ± 0.0^a
Total (ng)		3.4	9.5	17.7	35.7	37.2

all of them combined. The significance of this uniqueness is unknown.

3.2. Extracts of T. peregrinus Adults. The chromatographic profiles of extracts from *T. peregrinus* males and females revealed the presence of two esters, one minor (A) and one major (B). Their retention times (Rts) and Kovat's Indices (KIs) on the RTX-5 column were as follows: **A**: Rt = 8.867 min., KI = 1068 and **B**: Rt = 9.558 min, KI = 1103. The MS of **B** showed a base peak at *m*/*z* 71, fragments at *m*/*z* 68, *m*/*z* 85, *m*/*z* 128, and a molecular ion of 156 Da (Figure 3). When this spectrum was compared to the NIST library, it was evident that **B** might be a propionic, isobutyric, or butyric ester, with a molecular formula of $C_9H_{16}O_2$. The most important signals in the GC-FTIR spectrum of **B** (Figure 3) were a C-H vibration band (2962; 2935; 2871 cm^{-1}), an ester carbonyl band (1730 cm^{-1}), and multiple bands of C(CO)O characteristic of esters (1445, 1381, and 1189 cm^{-1}). These bands associated with a band of C–H stretching vibration of substituted double bond in 3023 cm^{-1}, and the presence of a band in 1674 cm^{-1}, characteristic of trialkyl-substituted alkenes, showed that compound **B** was an unsaturated ester with an internal double bond. In contrast, the molecular ion in MS of compound **A** was not obvious; however, the base peak at *m*/*z* 68 and a fragment at *m*/*z* 71 suggested **A** was an ester similar to **B**. Although the GC-FTIR spectra of **A** showed the same characteristic bands for esters that were detected for **B**, the presence of a band at 3080 cm^{-1} due to asymmetric stretch of a terminal double bond, demonstrating that **A** was an unsaturated ester with a terminal double bond. To positively identify the natural products **A** and **B**, the twenty-one above-mentioned esters were synthesized. Thus, the major compound **B** was identified as 3-methylbut-2-en-1-yl butyrate by coinjection of this standard with the natural extract on the three GC columns (RTX-5, RTX-WAX, and HP-1). Identification was based on coelution and MS. Additionally, the minor compound **A** was identified as 3-methylbut-3-en-1-yl butyrate by coinjection of this standard with the natural extract on the different GC columns.

Females and males produced the same esters, but their quantities varied by sex and age, particularly for the major compound, 3-methylbut-2-en-1-yl butyrate (Figure 4). Although the concentration of the esters in males increased with age (Table 2), reaching a maximum of approximately 1 μg per insect in 22-day-old mated males, this age difference could not be detected statistically. Only the amount of the major compound (**B**) of mated males was statistically different from that for mated females ($F_{1,3} = 10.3$, P value =0.048) (GLM). Ester concentrations of virgin males and females were not statistically different (GLM) for either the minor (**A**) ($F_{1,4} = 0.6$, P value =0.47) or major (**B**) ($F_{1,4} = 3.2$, P value =0.14) compounds. Likewise, ester concentrations of mated and virgin males (**A:** $F_{1,3} = 2.4$, P value =0.21; **B:** $F_{1,3} = 5.7$, P value =0.09), and of mated males and females (minor $F_{1,3} = 4.5$, P value =0.12) were not statistically different (GLM) (Table 2). The adults of 10–21 days old did not have enough repetitions to be compared. Thus, they were not considered for the concentration analysis.

Butyrates and isobutyrates are pheromone components for other Heteroptera, such as broad-headed bugs (Alydidae) [16], plant bugs (Miridae) [17, 18], and predacious stink bugs (Pentatomidae: Asopinae) [19]. Mirid bugs, particularly species of the genus *Phytocoris*, produce unsaturated butyrate and acetate semiochemicals. *Phytocoris* females attract males with sex pheromones based on butyrate and acetate blends [20–22], while males apparently release high concentrations of certain butyrates as antisex pheromones [17]. In *Alydus eurinus* (Say) (Alydidae), the sex pheromone of females is a blend of 2-methylbutyl butyrate and (*E*)-2-methyl-2-butenyl butyrate [16].

The biological function(s) of 3-methylbut-2-en-1-yl and 3-methylbut-3-en-1-yl butyrates in *T. peregrinus* remain to be elucidated. An aggregation function was attributed to the major compound through olfactometer experiments, in which males attracted only males (Gonzalez et al. 2012 this issue); however, we did field tests using delta traps with different concentrations of the major compound, and they all failed to attract insects in the field and in a greenhouse with a *T. peregrinus* population. Allomones and pheromones known for other heteropterans, such as those described above, undoubtedly originate from the dorsal abdominal glands of nymphs or the metathoracic scent glands that are characteristic of most true bug adults. In *T. peregrinus*, however, the metathoracic scent glands are vestigial. The butyrates from *T. peregrinus* appear to be associated with extrusion of the rectal organ (Figure 1) that has

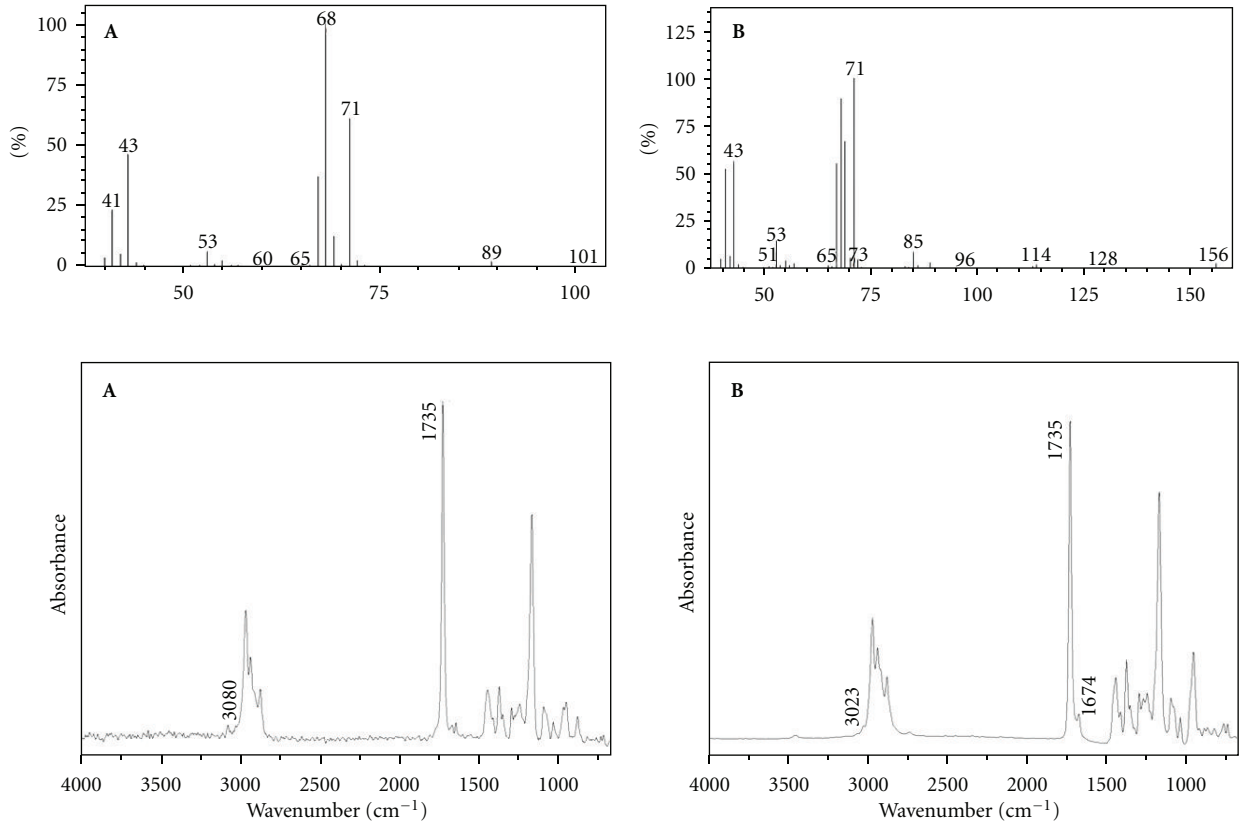

Figure 3: Mass and infrared spectra of the minor compound **A** and major compound **B** found in adults of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae). Extracts were analyzed on a Shimadzu GC MS-QP 2010 and GC-Fourier transform infrared spectroscopy (GC-FTIR) GC-2010 coupled to a DiscovIR-GC—Shimadzu.

Table 2: Quantification (μg) of esters identified in whole body extracts of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) virgin and mated males and females of different ages; 3–9 days (1) and 22–33 days (3). For each ester identified, ns: not statistically different, and $*$: statistically different; SE: standard error. Statistical comparisons: GLM (generalized linear model) with Gaussian distribution ($P > 0.05$).

		3-Methylbut-3-en-1-yl butyrate (A)		3-Methylbut-2-en-1-yl butyrate (B)
		Mean SE		Mean SE
Virgin males (1)	ns	0.1 ± 0.0	$*$	11.2 ± 3.8
Virgin females (1)		0.2 ± 0.1		0.5 ± 0.0
Virgin males (3)	ns	8.3 ± 0.2	$*$	191.8 ± 19.1
Mated males (3)		20.7 ± 5.4		743.5 ± 61.7
Mated males (3)	$*$	20.7 ± 5.4	$*$	743.5 ± 61.7
Mated females (3)		0.1 ± 0.1		0.5 ± 0.3

heretofore only been described within the Heteroptera for plant bugs (Miridae) [8]. Unequivocal verification that the rectal organ tissue is the source of these esters awaits further experimentation. Mated *T. peregrinus* males produce greater quantities of both esters, especially ester **B**, compared with virgin males and younger mated males. Moreover, these esters are produced by females, suggesting that these compounds are not involved in aggregating the sexes for mating. Speculating the differences of concentration, these esters could be indicators of sex and age recognition by conspecifics.

4. Conclusion

Benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid were present in the exuviae of *T. peregrinus* nymphs. Volatiles from adult males and females included 3-methylbut-3-en-1-yl butyrate and 3-methylbut-2-en-1-yl butyrate. Compounds identical or similar to those found in *T. peregrinus* exuviae and esters identified in the adults were found in other heteropterans with various functions. The possible pheromonal roles of these volatile blends are being studied.

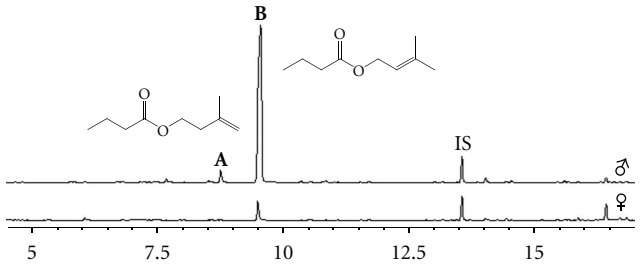

Figure 4: Representative gas chromatograms of body extracts of 21-day-old *Thaumastocoris peregrinus* (Heteroptera, Thaumastocoridae) males and females. Minor and major compounds, 3-methylbut-3-en-1-yl butyrate (**A**) and 3-methylbut-2-en-1-yl butyrate (**B**), respectively. The internal standard (IS) was tridecane; extracts shown here were analyzed on GCMS-QP 2010 Plus.

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Review Article

Chemical Ecology of Egg Parasitoids Associated with True Bugs

Eric Conti[1] and Stefano Colazza[2]

[1] *Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Perugia, Borgo XX Giugno, 06121 Perugia, Italy*
[2] *Dipartimento DEMETRA, Università degli Studi di Palermo, Viale delle Scienze, Building 5, 90128 Palermo, Italy*

Correspondence should be addressed to Eric Conti, econti@unipg.it

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Parasitoids representing some 15 families of Hymenoptera develop in insect eggs; three of these families, Platygastridae (= Scelionidae), Mymaridae, and Encyrtidae, are associated with Heteroptera. Several species of heteropteran egg parasitoids are or may be important for biological pest control. Successful parasitism of insect herbivores by insect parasitoids arises through several phases of host searching, which lead female wasps to the vicinity of, or in contact with, their hosts. During the host location process, females encounter and explore a variety of stimuli, among which chemical cues (i.e., semiochemicals or infochemicals) play a pivotal role. Female parasitoids are under selection pressure to efficiently invest their limited time on the location and exploitation of host-derived stimuli. In general, the levels of reliability and detectability of a particular stimulus are inversely correlated. Female parasitic wasps adopt differing strategies to solve this dilemma. In this paper we focus on the various host selection strategies employed by heteropteran egg parasitoids and possible means whereby the chemically mediated behavior of these wasps may be exploited to enhance biological pest control.

1. Introduction

Egg parasitoids are the largest group of entomophagous insects associated with Heteroptera. Thus, considering that they attack the host before it develops and inflict feeding damage, egg parasitoids show remarkable potential as biological control agents of Heteroptera, as well as other pests [1]. These parasitoids, however, also attack predaceous bugs which, of course, is counterproductive for the efficacy of these important natural enemies [2, 3].

Of the approximately 15 hymenopteran families that include egg parasitoids, those most commonly associated with Heteroptera are Platygastridae, Mymaridae, and Encyrtidae [1]. Most studies of their chemical ecology concern species associated with herbivorous bugs and, to a much lesser extent, predacious Heteroptera. The majority of semiochemical research on egg parasitoids associated with true bugs has been limited to species belonging to the genera attacking economically important pentatomid, scutellerid, mirid, alydid, and coreid plant pests (Table 1): *Trissolcus*, *Telenomus*, *Gryon* (Platygastridae = Scelionidae; see Sharkey [4] and Murphy et al., 2007 [5]), *Anaphes* (Mymaridae), and *Ooencyrtus* (Encyrtidae).

Generally, in order to reproduce, a female parasitoid must find its host at a stage suitable for parasitization. The host selection process involves a sequence of phases mediated by physical and chemical stimuli from the host, the substrate, and/or associated organisms, eventually leading to successful parasitism [6–9]. Because parasitoid foraging time is limited and the potential cues available are numerous, the parasitoid faces the need to optimize exploitation of available cues and discriminate those most reliable in indicating the presence of a suitable host [8, 10]. However, the location and recognition of a suitable host is a complex process, especially for egg parasitoids, because of major constraints due to the small sizes of both the host and the parasitoid itself. Eggs are usually unapparent, especially when they are small, dispersed in the habitat, and concealed in plant tissue. As such, cues that are directly related to the presence of eggs may have low detectability, but high reliability [8, 11, 12]. Additionally, suitable host eggs are generally available for only a short time due to their rapid development [7]. Therefore, egg parasitoids have developed specialized strategies to overcome the reliability-detectability dilemma in order to efficiently parasitize host eggs. Successful parasitism is accomplished

Table 1: Host or host-associated semiochemicals exploited by egg parasitoids of Heteroptera for host location or recognition (modified and updated from Colazza et al. [14]).

Parasitoid	Host	Plant	Function	Origin	Chemistry	Parasitoid response	References
Mymaridae							
Anaphes iole	*Lygus hesperus*	*Gossypium hirsutum* + other	Volatile induced synomone	Plants with feeding damage	Several HIPV, mostly α-farnesene, (Z)-3-hexenyl acetate, methyl salicylate	Attraction in olfactometer (effect of experience); response to EAG	[43, 44, 67]
			Short range synomones	Wounds in different substrates		Ovipositor probing	[52, 53]
			Short range kairomones	Host egg and adult		Ovipositor probing	[52–54]
Encyrtidae							
Ooencyrtus nezarae	*Riptortus clavatus*		Volatile kairomone	Host male attractant pheromone	(*E*)-2-Hexenyl (*Z*)-3-Hexenoate	Attraction in field, increased parasitism	[34–36]
Ooencyrtus telenomicida	*Nezara viridula*		Volatile kairomone	Host male pheromone + ovipositing female		Attraction in Y-tube olfactometer	[38]
Platygastridae							
Gryon boselli	*Gonocerus acuteangulatus*		Contact kairomone	Host footprints		Arrestment and increased searching in open arena	Colazza et al., unpubl.
Gryon pennsylvanicum	*Leptoglossus australis*		Volatile kairomone	Host pheromone		Attraction in field	[56]
Telenomus podisi	*Euschistus heros*	*Glycine max, Cajanus cajan*	Volatile induced synomone	Plants with feeding damage; *cis*-jasmone treatments on resistant plants	Quantitative differences especially (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3- hexenyl acetate, and (*E*)-2-octen-1-ol	Attraction in Y-tube olfactometer	[41, 42, 66]
			Volatile kairomone	Compounds from host defensive secretion	(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal	Attraction and increased searching	[33]
			Kairomone	Host male sex pheromone	Methyl 2,6,10-trimethyltridecanoate	Choice in closed arena	[85]
			Contact kairomone	Host footprints		Arrestment and increased searching in open arena	[46]
			Contact kairomone	Acetone extract of host eggs	2,6,10-Trimethyltridecanoate		[51]
	Euschistus conspersus		Volatile kairomone	Host male attractant pheromone	Methyl(E,Z)-2,4-decadienoate	No parasitism increase in field	[86]
Telenomus calvus	*Podisus neglectus*		Volatile kairomone	Host pheromone			[39]
	Podisus maculiventris		Volatile kairomones	Host male attractant pheromone; host female		Attraction in field allowing egg parasitism; phoresy	[2, 3, 87]
Trissolcus basalis	*Nezara viridula*	*Vicia faba, Phaseolus vulgaris*	Volatile induced synomone	Plants with oviposition + feeding	Increase of (*E*)-β-caryophyllene	Attraction in Y-tube olfactometer	[20, 21]

Table 1: Continued.

Parasitoid	Host	Plant	Function	Origin	Chemistry	Parasitoid response	References
			Volatile kairomone	Host defensive allomone	(*E*)-2-Decenal, 4-oxo-(*E*)-2-hexenal	Attraction in Y-tube olfactometer, increased searching	[30, 33]
			Volatile kairomone	Host attractant pheromone		Attraction in Y-tube olfactometer	[31]
			Contact kairomone (highly specific)	Host footprints (especially females)	Cuticular hydrocarbons	Arrestment and increased searching in open arena	[31, 71, 72]
			Contact kairomone	Follicular secretion and egg extracts	Mucopolysaccharide-protein complex	Host recognition	[50, 88, 89]
Trissolcus brochymenae	*Murgantia histrionica*	*Brassica oleracea*	Short-range induced synomone	Plants with feeding damage		Increased searching behavior	[22, 62, 63],
			Short-range induced synomone	Plants with feeding + oviposition (systemic induction)		Increased searching behavior	[22, 62, 63],
			Volatile kairomone	Pheromone from different host instars		Attraction in Y-tube olfactometer	[28]
			Contact kairomone (highly specific)	Host footprints (especially mated females)	Cuticular hydrocarbons	Arrestment and increased searching in open arena	[28, 32, 47]
			Volatile kairomone	Host egg masses		Attraction in Y-tube olfactometer	[28]
			Contact kairomone	Follicular secretion and egg extracts		Host recognition	[28]: Conti et al., unpubl.
Trissolcus euschisti	*Euschistus conspersus*		Volatile kairomone	Male attractant pheromone	Methyl(*E,Z*)-2,4-decadienoate	No parasitism increase in field	[86]
Trissolcus grandis	*Eurygaster integriceps*		Volatile kairomones	Host male attractant and female sex pheromones		Attraction in the field	[90]
	Eurygaster spp.		Contact kairomone	Host egg			[91]
Trissolcus simoni	*Eurydema ventrale*		Volatile kairomone	Host attractant pheromone		Attraction in Y-tube olfactometer	[92]
			Contact kairomone	Host footprints		Arrestment and increased searching in open arena	[32]
Trissolcus utahensis	*Euschistus conspersus*		Volatile kairomone	Host sex pheromone	Methyl(*E,Z*)-2,4-decadienoate	No parasitism increase in field	[86]

through the combined exploitation of cues that are directly and indirectly related to host eggs [7, 8, 13, 14]. First, parasitoids may detect volatiles from nontarget instars of the host, that is, adults or juveniles, to reach the vicinity of the host eggs (infochemical detour *sensu* Vet & Dicke [8]), eventually enabling them to pin-point eggs using additional long- and/or short-range cues. A particular and interesting example of such detour behavior of egg parasitoids is phoresy on adult host females; via this strategy, not only are relevant cues more detectable, but the adult itself is also exploited by the parasitoid as a vehicle to arrive at host eggs [15–17]. Second, parasitoids may exploit plant volatiles induced as a consequence of herbivory, which are emitted in large quantities and are, therefore, easily detectable by foraging parasitoids but not necessarily highly reliable [13]. For example, recent investigations have shown that some egg parasitoids are capable of exploiting plant chemicals emitted as a result of egg deposition, thus rendering such highly detectable cues also highly reliable [18–24]. Third, egg parasitoids have been observed to associate, through learning, highly detectable but less reliable cues with the presence of suitable hosts, thus increasing reliability of such cues in experienced wasp females [25, 26].

The complex of stimuli that are used by parasitoids for host seeking and acceptance, originating from the host, the associated substrate and/or organisms, and their possible interactions, has been called the "host-egg unit" [27–29]. The host-egg unit is often quite complex and is related to the parasitoid strategies described above. Thus, the same host might represent different host units for different parasitoids, depending on whether they are specialist versus generalist parasitoids, or whether they have evolved capabilities to exploit adult kairomones or induced plant synomones, or whether they have developed phoretic strategies. In this paper we will focus on the behavioral steps of host selection strategies and the chemical cues exploited by egg parasitoids of emerging pest species of Heteroptera.

2. Exploitation of Indirect versus Direct Host-Related Chemical Cues

The most common indirect host-finding tactic known thus far for egg parasitoids is the infochemical detour strategy based on the parasitoid ability to detect chemical cues associated with stages other than the egg [7, 8]. Exploitation of pheromones and/or allomones from host adults has been demonstrated for both Platygastridae [28, 30–33] and Encyrtidae [34–38] (Table 1). These stimuli provide indirect information on the presence of the host community, leading the wasp female to the vicinity of host eggs. In spite of their low reliability, pheromones and allomones are produced in large amounts, and, therefore, these cues are relatively easy to detect by the female wasps from long to medium distances [39].

Phoresy is a different, but highly specialized bridge-in-time (and bridge-in-space) strategy exploited by egg parasitoids to reduce the spatial and temporal discontinuity between where host adults mate and where host females oviposit [7, 40]. One well-documented case of phoresy by a heteropteran egg parasitoid is that of *Telenomus calvus* Johnson (Platygastridae) females that parasitize eggs of the predacious spined soldier bug, *Podisus maculiventris* (Say) (Pentatomidae). Female *T. calvus* wasps go to the male-produced *P. maculiventris* attractant pheromone, wait nearby for a conspecific soldier bug female to arrive and mate, and then become phoretic on the mated host female until she eventually oviposits [2, 3].

Another indirect means to locate host eggs is exhibited by *Trissolcus basalis* (Woll.) and *Telenomus podisi* Ash. (Platygastridae), which exploit plant synomones induced by oviposition and/or feeding of their hosts, *Nezara viridula* L. [20] and *Euschistus heros* (Fabr.) [41, 42] (Pentatomidae) (Table 1). In a hierarchical context, whether volatiles from host adults or from the host plants of host adults are exploited from the furthest distance has yet to be elucidated. In the case of *Tr. basalis*, because host oviposition is necessary for volatile induction in bean plants, such synomones appear more reliable compared to kairomones from nontarget instars or to feeding-induced synomones [20, 21]. Therefore, it can be hypothesized that kairomones from adults and feeding-induced plant synomones act as a long-distance, indirect cue used to localize the host community (or host habitat), whereas oviposition-induced plant synomones are shorter-range cues used to find plants that actually have host eggs.

Induced plant volatiles are also exploited by mymarids [43, 44] (Table 1). Feeding on cotton, and several other plants, by either sex of *Lygus hesperus* Knight (Miridae) results in the induction of volatiles that are behaviorally and physiologically active towards *Anaphes iole* Girault (Mymaridae). Oviposition appears unnecessary to induce active volatiles [44]; therefore, these synomones should also be considered as indirect cues, exploited to localize the host community.

Once close to a potential host, female parasitic wasps in flight preferably should alight on a plant that probably has host eggs and then commence searching on the substrate for suitable eggs. Different strategies, and cues, are used during this phase (Table 1). For example, *Trissolcus brochymenae* (Ashm.) exploits short-range chemicals that are induced in cabbage plants by *Murgantia histrionica* (Hahn) (Pentatomidae) during oviposition [22]. Such a directly host-related synomone that is systemically emitted by the plant provides reliable information on the presence of suitable host eggs, but not necessarily precise information on where the eggs have been laid [22].

While searching for the host eggs on the plant, different Platygastridae were shown to use chemical traces left behind from the adults and/or juveniles (Table 1). The typical response to such cues, which are perceived through gustatory sensilla [45], is arrestment behavior followed by increased searching intensity [28, 31, 32, 46]. These cues are not directly related to the host eggs, but because of finely tuned adaptations of the parasitoid, these cues may become quite reliable. In fact, although responding to the "footprints" of both males and females of the host species, *Tr. basalis* and *Tr. brochymenae* are able to discriminate host adult sex and

the physiological conditions of host adults (e.g., virgin versus mated females) [28, 31, 47, 48]. Interestingly, in addition to chemical footprints, *Te. podisi* also uses vibratory signals mediating sexual behavior of the host species, *E. heros* [49].

Short-range host location is the next and final step, and female wasps may use visual cues at this point or short-distance kairomones directly related to the host to finally reach the target egg. The presence of volatiles from *M. histrionica* eggs is detectable by *Tr. brochymenae* whereas, in this species, visual cues do not appear to play an important role [28] (Table 1). When a host egg mass is encountered, host recognition by *Trissolcus* species is elicited by contact kairomones present on the egg surface, although physical factors such as shape and size may also affect wasp behavior [28, 50, 51] (Table 1). Short-range physical and chemical stimuli from the plant and host egg are also exploited by *A. iole* to locate and recognize *L. hesperus* eggs embedded in plant tissue [52–54].

3. Long-Range Kairomones from Nontarget Instars of the Host

As mentioned above, a well-known solution for the egg parasitoids to overcome the low detectability of host egg cues is to eavesdrop on the pheromonal communication (sex and other attractant pheromones), or the allomonal defenses, of nontarget stages of their hosts. This strategy was initially studied for egg parasitoids of Lepidoptera (reviewed by Fatouros et al. [13]; Colazza et al. [14]), but several cases are also known under laboratory and field conditions for species associated with Heteroptera (Table 1).

In early laboratory experiments using Y-tube olfactometers, *Tr. basalis* was found to be attracted by volatiles from adults of *N. viridula* [55]. A subsequent study revealed that (*E*)-2-decenal, a component of the defensive secretion from the metathoracic scent gland of *N. viridula*, is responsible for this dose-dependent attraction [30]. More recently, both *Tr. basalis* and *Te. podisi* were found to be attracted to and increase their searching behavior in the presence of defensive compounds from metathoracic scent gland secretions of their hosts, *N. viridula* and *E. heros* [33]. *Trissolcus basalis* showed a significant preference for (*E*)-2-decenal and 4-oxo-(*E*)-2-hexenal, while *Te. podisi* responded positively to (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal [33]. In addition to volatiles from metathoracic glands, those from the dorsal abdominal glands of nymphs also appear to be exploited as kairomones by *Tr. basalis*, but nymphal secretions may be attractive at intermediate range rather than long range [39].

Detailed investigations of *Tr. basalis* responses to volatiles from both sexes of *N. viridula* showed that the female egg parasitoid is attracted to the males and to preovipositional females, whereas it is not attracted to virgin females [31]. When males and preovipositional females were assayed in a two-choice test, the parasitoid preferred females [31]. Similar results were obtained in a Y-tube olfactometer with *Tr. brochymenae*, as this parasitoid was attracted by cues from differing stages (eggs, nymphs, and adults) and sexes but showed significant preference for gravid females when compared to males [28]. Therefore, volatile allomones and sex pheromones from host males can direct female wasps toward host aggregates, whereas volatiles from gravid females act hierarchically on a subsequent step, representing a more reliable indicator of the potential presence of host eggs [28, 31].

The generalist egg parasitoid, *Ooencyrtus telenomicida* (Vassiliev) (Encyrtidae), is attracted in Y-tube olfactometer to odors of virgin male and, less intensely, of mated *N. viridula* females in preovipositional state, suggesting that the parasitoid exploits the host male-produced attractant pheromone [38]. When exposed to tomato plants treated with *N. viridula* or untreated control plants, *O. telenomicida* females did not respond to healthy or damaged plants, but only to plants with adult bugs, indicating that active volatiles originate from the host rather than the plant [38]. Field experiments confirm parasitoid attraction towards host adults. Traps baited with the synthetic attractant pheromone of male *Riptortus clavatus* (Thunberg) (Alydidae) captured females of the encyrtid egg parasitoid *Ooencyrtus nezarae* Ishii [34]. Males of *R. clavatus* emit an aggregation pheromone, composed of a blend of three compounds that attract adults of both sexes and nymphs. One compound, (*E*)-2-hexenyl (*Z*)-3-hexenoate, attracts females of *O. nezarae* and resulted in higher parasitism in treated fields compared with untreated fields [35, 36]. This tiny parasitoid has the remarkable ability to fly just above the plant canopy in nonhost habitat, while exploiting the above cues to reach the host habitat [37], although the exact flight mechanisms are unknown. In a different system, the use of traps baited with live adult *Leptoglossus australis* F. (Coreidae) resulted in increased parasitism efficacy by *Gryon pennsylvanicum* (Ashmead) (Platygastridae) [56].

Field confirmation of egg parasitoid response to the attractant pheromone of *P. maculiventris* [2] was also achieved [3]. The phoretic females of *Te. calvus* Johnson parasitized significantly more host eggs in pheromone-baited versus nonbaited traps, whereas the generalist *Te. podisi* did not show any significant differences. As described earlier, *Te. calvus* females exploit the male attractant pheromone of *P. maculiventris* to locate females during mating and then become phoretic on mated female bugs [2, 3].

4. Plant Synomones Induced by Feeding or Oviposition

Host-induced plant synomones are reliable and readily available cues for foraging parasitoids that attack feeding stages of hosts [8, 57, 58]. Egg parasitoids may respond to plant synomones induced by feeding [22, 41, 43]. However, not only feeding but also oviposition by herbivores induces emission of plant compounds acting as synomones between the primary and tertiary trophic levels towards their respective egg parasitoids [18–22, 59–65] (Table 1).

Each of the above systems has unique characteristics *vis-à-vis* induced plant defenses. Important differences are the cause of induction (i.e., oviposition, feeding, or a combination of the two, i.e., direct versus indirect cues),

the type of oviposition (i.e., exposed or embedded), and the relationship between egg and plant (i.e., magnitude of synomone emission, local or systemic emission, and activity range of synomone). Other important differences involve timing of synomone release (often reliably related to host suitability), the elicitor source, and, if known, the chemistry of the induced synomone.

4.1. Egg Parasitoid Exploitation of Feeding-Induced Synomones. Feeding likely induces higher synomone levels than does egg deposition. Regardless, exploitation of such indirect host-related cues seems to have evolved because it allows parasitoids to minimize the searching area, thus maximizing efficiency.

Tritrophic plant/bug/egg parasitoid systems involving Heteroptera have been described for *Glycine max* and *Cajanus cajan* (Leguminosae)/*E. heros*/*Te. Podisi*/[41, 42, 66]; *Brassica oleracea* (Cruciferae)/*M. histrionica*/*Tr. brochymenae* (including oviposition-induced synomones) [22, 62, 63]; *Gossypium hirsutum* (Malvaceae) and other plants/*L. hesperus*/*A. iole* [43].

In olfactometer tests, *Te. podisi* responded to volatiles from soybean and pigeon pea fed upon by adults and nymphs of *E. heros* [41]. Application of *cis*-jasmone elicited a similar volatile profile from soybean plants after 96 hours with quantitative, rather than qualitative, chemical differences and resulted in the concomitant attraction of egg parasitoids [66]. Remarkably, when resistant versus susceptible soybean cultivars were compared, parasitoids were only attracted to resistant cultivars; volatile profiles in damaged plants differed between cultivars. In addition, volatiles from oviposition-damaged plants did not attract *Te. podisi* females [42].

Trissolcus brochymenae females' response to plant volatiles induced by host feeding is different than that of *Te. podisi* females. What is known for the former parasitoid indicates that females mainly exploit plant compounds systemically induced by a combination of oviposition, feeding punctures, and footprints of its host, *M. histrionica*, to elicit host searching. Nevertheless, failure to actually oviposit by the host still induces emission of leaf-surface volatiles but, in such cases, parasitoid response was observed only on the damaged leaves [22]. These types of cues appear less reliable compared with oviposition-induced cues, and would act hierarchically at a lower level than other semiochemical cues.

Olfactometer research with *Anaphes iole* Girault (Mymaridae) demonstrated that this species of egg parasitoid also employs volatiles from cotton and other herbaceous plants infested by adults of its host, *L. hesperus* [43]. Eggs of *Lygus* spp. are embedded through an incision made with the ovipositor in plant tissue, with only the operculum exposed, and compounds from these wounds elicit parasitoid behavioral responses even if no eggs have been laid [52, 53]. Both oviposition and feeding damage cause volatiles to be emitted from cotton plants although oviposition appears to induce release of constitutive terpenes from specific glands in cotton leaves adjacent to the oviposition incision, whereas feeding resulted in systemic induction of different volatiles [67]. The volatile blend induced by *L. hesperus* salivary gland extracts is similar to that induced by volicitin, an elicitor isolated from the regurgitant of moth larvae [68], although chemical analyses of the salivary glands from *Lygus* spp. have shown no evidence of a volicitin-type of fatty acid-amino acid conjugate [67]. In electroantennogram (EAG) assays, *A. iole* responded to the majority of herbivore-induced plant volatiles tested, but most intensely to (*Z*)-3-hexenyl acetate, and methyl salicylate; females responded more than males [44]. Olfactometer and wind tunnel bioassays showed that the female wasps were positively stimulated by (*Z*)-3-hexenyl acetate, methyl salicylate and α-farnesene, although response to (*Z*)-3-hexenyl acetate was exhibited only after preconditioning females to blends of host-plant odors. In field trials, host eggs baited with α-farnesene and (*Z*)-3-hexenyl acetate were more heavily parasitized than were untreated eggs [44].

4.2. Egg Parasitoid Exploitation of Oviposition-Induced Synomones. Oviposition-induced synomones, which are directly related to the target stage, are highly reliable and detectable for egg parasitoids. Herbivore oviposition induces emission of plant compounds that act as synomones towards a variety of egg parasitoids [18–22, 59–65]. This "early herbivore alert" [69] by the plant denotes a particular type of indirect induced defense, which, among parasitoids of Heteroptera, has been observed in two tritrophic systems: *Vicia faba* and *Phaseolus vulgaris* (Leguminosae)/*N. viridula*/*Tr. basalis* [20, 21] and, *Brassica oleracea* (Cruciferae)/*M. histrionica*/*Tr. brochymenae* [22, 62, 63] (Table 1).

In the Heteroptera, the first case of plant volatile induction by a bug gluing eggs on leaves without causing mechanical damage is that of *Nezara viridula* ovipositing on legumes [20]. *Trissolcus basalis* was attracted by oviposition-induced volatiles in an olfactometer, and the volatile emission was systemic (i.e., from damaged and adjacent undamaged leaves) [20]. By maximizing the release surface, the plant may have evolved to increase synomone volatilization, thereby increasing herbivores apparency to egg parasitoids. Over time, synomone activity seems to be finely tuned to parasitoid behavior and biology since the attraction fades when host eggs are near to eclosion. Oviposition by *N. viridula* females on *Vicia faba* L. and *Phaseolus vulgaris* L., combined with feeding punctures, induced release of (*E*)-β-caryophyllene, as well as two other sesquiterpenes. Only the fraction containing (*E*)-β-caryophyllene attracted *Tr. basalis* [21]. Whether the elicitor originates from the eggs, follicular tissue, or elsewhere is unknown; however, the combined presence of feeding punctures is necessary for synomone induction [21].

The other known case of an oviposition-induced synomone for an egg parasitoid of true bugs is quite different from the one just described because the induced compounds act at a very short distance. *Tr. brochymenae* perceives the induced synomone only when it alights on a damaged plant [22, 62]. Compared to healthy plants, females of this parasitoid intensely antennated and searched on leaves having a host egg mass, plus nearby feeding punctures and

chemical footprints (treated surface). Parasitoid response was tested on the upper (adaxial) leaf surface, opposite to the treated (abaxial) surface. Female wasps also responded in a static olfactometer at near contact range to volatiles perceived through olfaction, but host-damaged plants in a Y-tube olfactometer were not attractive to wasps. As with *N. viridula* on leguminous plants, the induction is both local and systemic, but the origin of the elicitor and the mechanisms involved remain unknown. However, in the case of *M. histrionica* on cabbage, egg mass deposition is sufficient, as are feeding punctures, to elicit parasitoid response, although the combination of oviposition, feeding, and footprints increases parasitoid response [22]. Parasitoid reaction to compounds emitted as a consequence of host feeding appears to be a response to damaged host plants [22]; leaves with feeding punctures exhibit alteration of tissues and photosynthesis [70].

5. Short-Range Kairomones from Nontarget Host Stages

Indirect host-related cues originate from adults or juveniles of the host and, in general, elicit arrestment and searching behavior in the parasitoid. Trichogrammatidae and Platygastridae species responses to lepidopteran moth scales were studied earlier (reviewed by Colazza et al. [14]). However, a comparable strategy was also discovered for the heteropteran egg parasitoids, *Trissolcus* spp. [28, 31, 32, 71] *Te. podisi* [46], and *Gryon boselli* Mineo & Szabo (Colazza, Lo Bue, and Cusimano, personal observation), which respond to chemical footprints of pentatomid bugs (Table 1). Both *Tr. basalis* and *Tr. brochymenae* females are able to discriminate chemical footprints left by host females, to which they respond more strongly than to chemical traces left by walking males or nymphs [28, 31, 47, 72]. In addition, *Tr. brochymenae* is able to detect cues from mated females in the preovipositional state, which are preferred to virgin females and parous females (i.e., those that have already produced offspring), thus finely tuning their searching to the host stage most likely to lead to host eggs [47]. This preference was strictly related to the transfer of sperm and associated substances from the conspecific male bug to the female during copulation. The compounds mediating arrestment of *Tr. brochymenae* females are from host cuticle, and those that play a role as gender-specific cues are most abundant on the legs of the host adult [47].

Associative learning plays an important role in host footprint recognition behavior. Oviposition experience increased the arrestment response of *Tr. basalis* females to footprints of *N. viridula* females, whereas prior experience not followed by oviposition led to the gradual fading of the learned behavior [25]. In contrast, previous experience with the footprints of host males did not result in a change of parasitoid response, indicating that residues from males only provide general information for the parasitoid, which is not directly associated with host eggs [25]. There is significant variation in the learning ability of *Tr. basalis* females as a function of environment and spatial distribution, but learning always helps make foraging more successful [26].

Footprint chemistry was investigated in the *Tr. Basalis-N. viridula* relationship. Analysis of extracts of cuticular lipids from *N. viridula* revealed the presence of normal alkanes, with quantitative and qualitative differences between the sexes. One compound, *n*-nonadecane, was recovered only from the cuticle and footprints of males. When added to cuticular extracts of *N. viridula* females, *n*-nonadecane caused *Tr. basalis* females to significantly reduce their residence time in the arena, similar to the behavior of female wasps in the presence of hexane extracts of male hosts [72]. Parasitoid response to host footprints is mediated by adsorption of the contact kairomone in the epicuticular wax layer of plants walked upon by host bugs [48, 73].

6. Short-Range and Contact Kairomones from Host Eggs

Semiochemicals from or on host eggs are likely present in faint amounts and, thus, are probably exploited by egg parasitoids only at close range or upon contact. Short-range and contact kairomones are best known for egg parasitoids of Lepidoptera [14]. Among the Heteroptera, this semiochemical level has been investigated for the egg parasitoid of the harlequin bug (*M. histrionica*), *T. brochymenae*, in both a Y-tube olfactometer and an open arena. In the olfactometer the *T. brochymenae* females were attracted to volatiles from host egg masses, whereas, in the open arena, the female parasitoids oriented towards egg clusters or dummies treated with chemical extract of host eggs. When the egg extract was applied without dummies, it elicited the same response, whereas dummies without extract did not influence parasitoid behavior, indicating that visual factors are unnecessary for this last step in host location [28] (Table 1).

Parasitoid host recognition to egg contact kairomones is much more obvious than are responses to egg volatiles (Table 1). When in contact with the heteropteran host egg mass, *Trissolcus* spp. use both physical and chemical cues; egg size and the shape are important cues, but chemicals on the egg surface are fundamental for host acceptance [50]. The recognition kairomones are contained in the adhesive secretion from the follicular cells of heteropteran hosts [28, 50, 51], composed of mucopolysaccharide-protein conjugants [50], but their chemical nature has not been defined yet. Surprisingly, (*E*)-2-decenal, a component of the defensive secretion from the metathoracic scent gland of *N. viridula* that was attractive to *Tr. basalis* in an olfactometer, also elicited parasitoid antennation and ovipositor probing of egg-sized glass beads [30].

When host eggs are embedded in plant tissue, semiochemicals may originate from the egg, the damaged plant, or from their interaction. Females of the mymarid, *Anaphes iole*, respond to *Lygus* eggs inserted into plant tissue with arrestment, increased antennation, and ovipositor probing even if oviposition wounds do not contain eggs, although probing is much more intense if the incision contains a host

egg. Artificial wounds are also probed by the parasitoid, and so are eggs removed from the substrate and placed on a surface [52, 53]. Although chemical cues appear to play a major role, physical cues are also important in this phase; *A. iole* females adopt a probing posture when chemicals from host or plants are combined with appropriate shapes [52–54]. Electroantennogram assays showed that the parasitoid antennae sense several plant volatiles, including green leaf volatiles, confirming the importance of plant chemicals during host searching in this species [44]. Ovipositor probing behavior is the final step of host searching by *A. iole*, rather than merely host recognition; perhaps this explains why these mymarid females insert their ovipositor, although less frequently, even in artificial wounds made in a parafilm substrate containing neither host eggs nor contaminated by host material [52]. Moreover, learning host and plant cues increases *A. iole* oviposition efficiency [53]; indeed, a preference for (*Z*)-3-hexenyl acetate occurs only after preconditioning with host-induced plant volatiles [44].

7. Conclusions

The importance of egg parasitoids as biological control agents for herbivorous insect pests is widely recognized. Nevertheless, their success in classical biological control programs is slightly lower than that for other kinds of introduced parasitoid species [74]. Several Mymaridae and Platygastridae species have been used for augmentative biological control of Heteroptera [74], the most successful of which involved releases of *Tr. basalis* against *N. viridula* on soybean in Brazil [75, 76].

While several egg parasitoids of Heteroptera are potentially effective biological control agents, there are still constraints preventing the realization of this goal. Incomplete behavioral and ecological knowledge for most parasitoid species remains problematic and, perhaps most importantly, mass production of egg parasitoids remains inefficient [77–79]. Understanding the host selection strategies of egg parasitoids and the chemical stimuli involved could lead to improved biological control efficacy through behavioral manipulation of egg parasitoids in the field and to development of better *in vivo* and *in vitro* rearing methods. An estimated 16 million ha of cropland worldwide currently receives inundative releases of egg parasitoids, primarily involving species in the genus *Trichogramma* [77]. Efficient mass rearing of *Trichogramma* spp., as well as intensive, focused research preceded this achievement, including elucidation of the chemicals necessary for acceptance of artificial host media and the parasitoid development [79]. The success of *Trichogramma* biocontrol programs is a model of what may be achieved with other parasitoids given the required research investment.

Successful implementation of egg parasitoids against true bugs will depend on judicious applications of synthetic semiochemicals, particularly synomones and kairomones, and appropriate strategies to overcome existing constraints, including treatments facilitating parasitoid rearing, conditioning, and manipulation [61, 74, 80, 81]. Other interesting possibilities include the development of plants with elevated expression of indirect induced resistance factors (i.e., the induction of plant synomones exploited by the herbivores' parasitoids) or spraying fields with resistance elicitors [61]. Accurate knowledge of parasitoid chemical ecology will be important in all phases of biocontrol and integrated pest management [82]; important elements toward implementation include procedures for selection of egg parasitoids, evaluation of their specificity [27, 32, 71], risk assessment of new introductions [83], release methods [80], and quality control [80, 84]. Implementation of knowledge gleaned from laboratory studies must finally be scaled up and transferred to growers.

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Research Article

Attractant Pheromone of the Neotropical Species *Neomegalotomus parvus* (Westwood) (Heteroptera: Alydidae)

Raul Alberto Laumann,[1] Miguel Borges,[1] Jeffrey R. Aldrich,[2] Ashot Khrimian,[2] and Maria Carolina Blassioli-Moraes[1]

[1] *Embrapa Recursos Genéticos e Biotecnologia, Avenida W5 Norte (Final), CEP 70770-900 Brasília, DF, Brazil*
[2] *USDA-ARS and Invasive Insect Biocontrol and Behavior Laboratory, Agricultural Research Center-West, Building 007, Room 313, Beltsville, MD 20705, USA*

Correspondence should be addressed to Maria Carolina Blassioli-Moraes, mcbmorae@cenargen.embrapa.br

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The Neotropical broad-headed bug, *Neomegalotomus parvus* (Westwood), is adapted to various leguminous crops and is considered a pest in common bean and soybean. The chemical communication of this species was studied in order to identify an attractant pheromone. Males and females of *N. parvus* produce several short-chain esters and acids, and their antennae showed electrophysiological responses to five of these compounds, three common to both sexes (hexyl butanoate, 4-methylhexyl butanoate, and hexyl hexanoate), and two female-specific compounds (4-methylhexyl pentanoate and hexyl pentanoate). Both aeration extracts of females and a solution containing five synthetic compounds mimicking the natural blend were attractive to males and females *N. parvus* in a laboratory bioassay. Aspects of the chemical ecology of the broad-headed bugs and the possibility to use pheromone-baited traps in the field for monitoring are discussed.

1. Introduction

Neomegalotomus parvus, or broad-headed bug (Heteroptera: Alydidae), subfamily Alydinae, is native to South America. As other alydines, *N. parvus* is an oligophagous bug that feeds on immature seeds of legumes [1, 2]. The taxonomic status of Neotropical alydine bugs was reviewed by Schaefer and Panizzi [3], Schaffner and Schaefer [4], Schaefer [5], and Schaefer and Ahmad [6]. From these works, Neotropical species formerly classified in the genus *Megalotomus* are now grouped in the genus *Neomegalotomus*, and South American species of *Neomegalotomus* were synonymized as *N. parvus*.

N. parvus has been adapted to various leguminous crops such as lablab beans, *Dolichus lablab* L. [7], pigeon pea, *Cajanus cajan* (L.) Mill., pig bean, *Canavalia ensiformis* (L.) DC., and indigo, *Sesamum indicum* L. [8, 9]. However, it is the common bean, *Phaseolus vulgaris* L. [10, 11] and soybean, *Glycine max* (L.) Merrill [12], that this bug is an economically important pest.

Insect feeding causes direct damage to crops and, in beans, is responsible for reduction of seed mass and high seedling mortality [9, 11]. Santos and Panizzi [12] showed that artificial infestation of soybean plants during the pod-filing stage causes a reduction in seed vigor and viability and has a negative effect on seed quality when infestation is in advanced stage. However, asynchrony between vulnerable stages of soybean seed development and *N. parvus* populations allows soybean crops to usually escape severe injury from this insect in the field [9].

Currently, control of *N. parvus* is exclusively insecticidal, and application timing is not based on the accurate population monitoring. These insects are easily disturbed and highly mobile, so the sampling cloth technique normally used to survey heteropteran populations in the field [13] is not a reliable. In Brazil, sweep-netting is the recommended monitoring method but it is laborious and time consuming; therefore, most growers prefer to use calendar-based application of insecticides. Pheromone-baited traps would be

an alternative, more precise sampling technique to help minimize pesticide applications and increase efficacy.

Semiochemicals of Alydidae have been described for *Alydus* [14, 15], *Megalotomus* [14], *Riptortus* [16–18], and *Leptocorisa* [19] species, and for some of these bugs both adults and nymphs are reportedly attracted. For example, field experiments showed that *Riptortus clavatus* (Thunberg) could be efficiently captured in traps baited with their aggregation pheromone [20–22].

For *N. parvus*, traps baited with live males captured significantly more males that unbaited traps [23]. In addition, it is known from field observations that some bugs are attracted to cow urine [24], and traps baited with cow urine or NH_4OH solutions captured *N. parvus* in the field [25]. Thus, traps are potentially useful for population monitoring of *N. parvus*, but the efficiency of trap-based monitoring could be greatly improved if more specific and powerful attractants were available. The objective of this work was to determine if *N. parvus* males and/or females produce specific compounds that could be used as pheromone.

2. Materials and Methods

2.1. Insects Rearing. A laboratory colony of *N. parvus* was established from adults and nymphs field-collected from 2009 to 2011 from beans fields near Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil (15° 47′ S and 47° 55′ W). Bugs were reared in 8 L plastic containers, on a diet of green beans pods (*Phaseolus vulgaris* (L.)), branches with flowers, and pods of pigeon pea (*Cajanus cajan* (L.)) Millsp, dried pigeon pea seed, and water at 26° ± 1°C and 65% r.h. a 14 light: 10 dark photoperiod (light 06:00–20:00 h). The food supply was renewed twice a week. Males and females were grouped for mating, with pieces of cotton placed in containers for oviposition. Eggs were conditioned in plastic containers and, after emergence, nymphs were maintained similarly to adults. Males and females used in the experiments were separated after the imaginal molt and cuticular hardening to prevent mating. Sexually mature 8–15-day-old adults were used for all experimental bioassays and volatile collections since at this age insects started to mate.

2.2. Collection of Volatiles. Volatiles were collected ($N = 6$ extracts) from groups of 20–30 males or females *N. parvus*. To minimize emission of defensive compounds [26] the insects were carefully introduced into 1 liter glass containers shortly after the end of scotophase when they were quiescent. Air was drawn into the container through a bed of 4–12 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out of the container through two traps (15 cm × 1.5 cm OD) containing Super Q (100 mg each; Alltech Associates, Inc., Deerfield, IL, USA) by a suction pump (~1 L/min). Insects were fed fresh green beans daily, and aerated continuously for 7–10 d, and a sample taken every 24 h. The Adsorbent traps were eluted with 0.5 mL hexane, and the eluates were stored at −20°C until needed for chemical analysis or behavioral bioassays. Extracts were concentrated under a gentle stream of N_2 to yield a solution of approximately 0.1 bug-equivalent/μL/24 hours of solution (~500 μL) to be tested.

2.3. Analysis and Derivatization of Extracts. For quantitative analysis, 1 μL crude extracts and fractions thereof were analyzed by gas chromatography flame ionization detector ((GC-FID), Shimadzu 17A GC) (Kyoto-Japan) equipped with DB5 column (30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA) on a temperature ramp of 50°C/2 min, then 8°C/min to 250°C/10 min. Injections were made in splitless mode. To quantify the pheromone released per insect, 5 aeration samples of females were selected, and 1 μL of isobutyl acetate (1 mg/mL hexane solution) was added as internal standard (IS) at a final concentration of 0.02 μg/mL. One microliter of each sample was injected into the GC in the splitless mode, with helium as carrier gas. Amounts of volatiles released by the insects per day were calculated in relation to the area of the internal standard. Data were collected with Class GC software (Class CG-10 Version 2.01, Shimadzu (Kyoto, Japan)) and were handled using Excel (Microsoft Corporation 2003).

For qualitative analysis, selected extracts were analyzed using an Agilent MSD 5975 instrument equipped with a quadrupole analyzer coupled to a GC 7890, a splitless injector, and helium as the carrier gas. Ionization was by electron impact (70-eV, source temperature 200°C) using the same column and conditions described above for GC-FID analysis. Chemical ionization (CI) MS spectra were obtained using the same GC-MS equipment using methane (CH_4) as the reagent gas with the same column and conditions described above for GC-FID analysis.

Compounds were identified comparing their mass spectra with database spectra (NIST 2008 library), retention indices, and coinjection with authentic standards.

Five aeration samples of females were combined, and concentrated to dryness under gentle N_2 flow, and submitted to alkaline hydrolysis by adding 50 μL of methanol and 50 μL of 2 M NaOH in a 1.5 mL glass conical vial. The sample was kept at room temperature for 2 hours. After, water (100 μL) was added and organic phase was extract with hexane (200 μL tree times). The combined organic phases were concentrated under nitrogen flow to ~50 μL, and the extract was analyzed by GC-MS by electron impact as described above.

2.4. Coupled Gas Chromatography-Electrophysiology. GC-electroantennography (EAG) was used to pinpoint compounds within mixtures that were detected by the antennae of males and females.

A GC Perkin Elmer Autosystem XL (NY, USA) was coupled to an EAG detector (Syntech, Inc., Hilversum, The Netherlands). The GC was equipped with a nonpolar DB-5 column (30 m × 0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA), and a splitless injector with helium as the carrier gas (1 mL/min). The column temperature was programmed to 80°C (2 min), then heated to 250°C at 8°C/min, and held for 10 min. The effluent temperature to EAG system was kept at 195°C. The antenna of one male or one female were cut and immediately placed in stainless steel

electrodes, the base of the antenna was placed in the reference electrode and the distal ends of the antenna was placed in the recording electrode, the electric connection was achieved using conductive gel. The electrodes were connected to an Autospike interface box and an AC/DC amplifier IDAC-2 (Syntech, Inc.). Preparations were held in a continuous humidified air flow (1 L/min) with a Stimulus Controller CS-55 (Syntech, Inc.). The females and males antennae of *N. parvus* were tested using a female extracts of *N. parvus* ($N = 5$) containing all compounds identified in the volatiles collections and for a solution containing the synthetic compounds, that showed electrophysiology response from the antenna in crude extract, hexyl butanoate (0.05 mg/mL), (*S*)-4-methylhexyl butanoate (0.005 mg/mL), hexyl pentanoate (0.0075 mg/mL), (*S*)-4-methylhexyl pentanoate (0.002 mg/mL), and hexyl hexanoate (0.0075 mg/mL) ($N = 3$). Only peaks that showed the polarization and depolarization of the antenna were considered as EAG responses, and only compounds that elicited response in all antennas tested ($N = 5$) were considered electrophysiologically active.

2.5. Synthesis of 4-(S)-Methylhexyl Pentanoate and 4-(S)-Methylhexyl Butanoate. (*S*)-4-methyl-1-hexanol (TCI America, Boston, MA, USA) 11.6 mg (0.1 mmol) was treated with butyryl chloride (10.4 μL, 0.1 mmol) in the presence of pyridine (8 μL,/500 μL methylene chloride). The mixture was poured into ice-water, extracted with methylene chloride ($3 \times 100\,\mu$L), the organic extract was washed with 0.1 M HCl, water, and dried with Na_2SO_4. A similar procedure was conducted using valeryl chloride to prepare 4-(*S*)-methylhexyl pentanoate. The structures of synthesized compounds were confirmed by mass spectrometry analysis.

4-(S)-Methylhexyl Butanoate MS. m/z = 129 (12), 115(4), 98 (28), 89 (82), 83(8), 71 (82), 70 (100), 69(49), 57 (58), 56 (36), 55(31), 43(55), 42(15), 41(49).

4-(S)-Methylhexyl Pentanoate MS. m/z = 143(10), 115(5), 103(79), 98(35), 97(6), 85(58), 83(9), 70(100), 69(44), 57(95), 56(34), 55(31), 43(16), 42(13), 41(43).

Source of Compounds. Super *Q* (80/100 mesh) was purchased from Alltech (PA, USA). The sources of chemical as follows: camphene, 6-methyl-5-hepten-2-one, hexanoic acid limonene, undecane, nonanal, dodecane, decanal, tridecane (Sigma Aldrich, Steinheim, Germany), hexyl acetate (TCI-America, portland, USA). Butyl butanoate, pentyl butanoate, hexyl butanoate, hexyl pentanoate, and hexyl hexanoate were provided by Jeffrey Aldrich (USDA-ARS, Invasive Insect and Behavior Laboratory, MD, USA).

2.6. Olfactometer Bioassays. A two-choice olfactometer modified from Borges and Aldrich ("W-olfactometer"; [27]) was used to test the biological activity of *N. parvus* aeration extracts and synthetic compounds. The olfactometer release chamber was a 500-mL three-neck, round-bottom flask (all 24/40 joints, Kontes, Vineland, New Jersey). Two 250 mL rotary evaporator trap adapters (24/40 joints) were attached to each side arm of the release flask (the treatment and control arms). A charcoal (20/40 mesh) filter column (130 mm $\times$ 10 mm ID) was attached to the side arms using two 40 cm long pieces of a silicone tubing (3/16 I.D. $\times$ 5/15 E.D. VWR Scientific Corporation, Darmstadt, Germany), inserted in a "Y" connector of the same diameter and connected to adapters (24/40 joint) on each side arm of the olfactometer. The air was humidified by passage through a container of distilled water between the charcoal filter and the arms of the olfactometer. The middle neck of the flask was connected to the vacuum pump with an adapter, and the air flow was adjusted with a "Clear Flow Rotameter" (Accura Flow Products, Warminster, Pennsylvania 18974-0100) to a flow of 0.8 L/min. The apparatus was positioned horizontally on a countertop in a room with bright fluorescent lights (2×36 W, daylight (6500 K) lamps Sylvania Activa 172, Sylvania, Danvers, MA, USA) during photophase. The temperature in the bioassay room was maintained at $26.0 \pm 1.0^\circ$C. The positions of the olfactometer arms were inverted between control and treatments after each three repetitions to avoid any positional bias. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried at 120°C after every five replicates. The insects were placed in the round-bottom flask (release chamber), and the treatments were placed at the end of the reducing adapter chamber (treatment arms).

A single *N. parvus* adult (male or female) was gently introduced into the release chamber of the Y-tube olfactometer with the aid of an artist's paint brush (Camel Hair, number 1), and its pattern of behavior (response) was recorded for 10 min/replicate. The duration of each bioassay replicate was monitored using a stopwatch. Prior to testing, the insects were allowed to acclimate for a short period (ca. 3 min) in the release chamber while assembling the treatment chambers. The first choice of the insect was recorded, that is, the first arm of olfactometer that the insect chose, entered and remained in for at least 100 sec. The test insects were used only once during the bioassays.

The bioassay procedures described above were used to compare the biological activity of aeration extracts of females, and synthetic standards prepared in proportions matching that produced by *N. parvus* females. For aeration extracts, the solution of test stimulus was 1 individual equivalent/24 hours (IE) spotted on a strip of filter paper (1.5 cm long and 0.5 cm wide); controls consisted of filter papers treated with hexane. Forty-five bioassays were performed for each sex (males and females). Bioassays were conducted using a 5 μL of a synthetic solution containing the five synthetic compounds that showed EAG responses ($N = 35$ for females and $N = 40$ form males) (hexyl butanoate (0.005 mg/mL), (*S*)-4-methylhexyl butanoate (0.0005 mg/mL), hexyl pentanoate (0.00075 mg/mL), (*S*)-4-methylhexyl pentanoate (0.0002 mg/mL), and hexyl hexanoate (0.00075 mg/mL)).

2.7. Statistical Analysis. Choices made by the insects in the bioassays were analysed using logistic regression. The fitted model contained a factor for the side (left or right) on which the stimuli were presented to control for this variability. We tested the hypothesis of no preference (50% first choice to

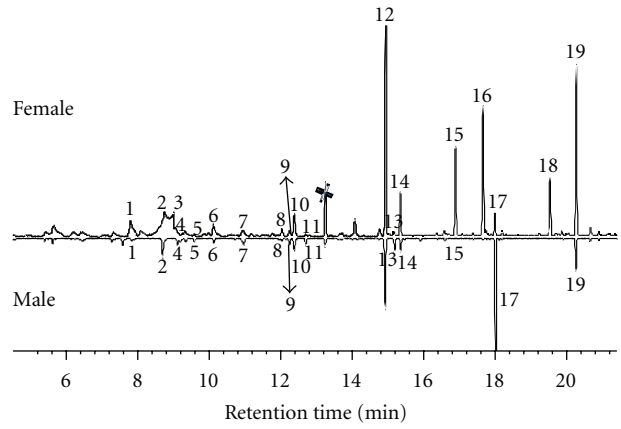

Figure 1: Gas chromatogram profile of an air-entrainment extract of males and females of *Neomegalotomus parvus*. (1) Camphene, (2) 6-methy-5-hepten-2-one, (3) hexanoic acid, (4) butyl butanoate, (5) hexyl acetate, (6) limonene, (7) (*E*)-2-octen-1-ol, (8) pentyl butanoate, (9) undecane, (10) nonanal, (11) unknown compound, (12) hexyl butanoate, (13) dodecane, (14) decanal, (15) 4-methylhexyl-butanoate, (16) hexyl pentanoate, (17) tridecane, (18) 4-methylhexyl-pentanoate, and (19) hexyl hexanoate.

each vibratory signal) using a chi-square Wald test. All tests were conducted using the *R* programming language [28].

3. Results

3.1. Chemical Analysis. The chemical analysis of males and females of *N. parvus* extracts obtained from volatile collection showed quantitative and qualitative differences between the extracts. Quantification of extracts showed that females produce higher amounts of several compounds (Table 1, Figure 1) compared to males and females also release some specific compounds that were not found in the extracts of males, such as hexyl pentanoate and 4-methylhexyl pentanoate.

The mass spectra and the retention times of compounds **15** and **18** (Figure 1) did not match those of any compound from the database and/or the literature. The mass spectra of **15** (*m/z*, relative abundance): 129(11), 115(4), 98(28), 89(78), 83(9), 71(76), 70(100), 69(47), 57(53), 56(34), 43(49), 41(42), and compound **18**: 143(9), 115(5), 103 (76), 98(35), 97(6), 85(57), 83(7), 70(100), 69(30), 57(89), 56(32), 55(28), 43(15), 42(12), 41(40) suggested ester homologues. CI-MS analysis of the female crude extract showed that compounds **15** and **18** had a molecular adduct ions ($[M+H]^+$) at 187 and 201, thus providing additional evidence that these two compounds could have a similar chemical structure differing by one methyl group. In order to obtain more information about the chemical structure of these two esters, a pooled female extract was submitted to alkaline hydrolysis. The GC-MS analysis of the hydrolyzed crude extract showed that the peaks corresponding to the esters disappeared and new peaks were generated, one of which matched the synthetic standard of 4-methyl-1-hexanol. These results, combined with retention index data (Table 1), suggested that compounds 15 and 18 could be 4-methylhexyl butanoate and 4-methylhexyl pentanoate, respectively. Indeed, the mass spectra and retention indices of synthetic (*S*)-4-methylhexyl butanoate and (*S*)-4-methylhexyl pentanoate matched those of esters found in female crude extract.

The other volatile compounds identified from both males and females are common to Alydidae; mainly short-chain esters and acids, and one alcohol [(*E*)-2-octen-1-ol] that is a common defensive compound in several Pentatomidae (Table 1).

3.2. Coupled Gas Chromatography-Electrophysiology. In GC-EAG experiments the antennae of *N. parvus* males and females responded to only five components present in the extract of females (Figure 2) that were identified as hexyl butanoate, 4-methylhexyl butanoate, hexyl pentanoate, 4-methylhexyl pentanoate, and hexyl hexanoate. When a blend containing these five synthetic compounds (hexyl butanoate, (*S*)-4-methylhexyl butanoate, hexyl pentanoate, (*S*)-4-methylhexyl pentanoate and hexyl hexanoate) was tested, the antennae of males and females responded in a similar way as to aeration extracts of females.

3.3. Bioassays. In W-olfactometer bioassays with aeration extract of females, both *N. parvus* males and females were significantly attracted to the extract treatment arm of the olfactometer (Figure 3). Similarly, males and females were significantly attracted to the treatment arm containing the five-component synthetic blend active in EAG experiments (Figure 4).

4. Discussion

Males and females of *N. parvus* produce several short chain esters and acids, most of which were previously reported for others species of Alydidae from the metathoracic scent glands [14, 15, 17, 18, 22] (Table 2); however, this is the first report of pentanoates from Alydidae. The antennae of *N. parvus* showed electrophysiological responses to five of these esters, three common to both adult sexes (hexyl butanoate, 4-methylhexyl butanoate and hexyl hexanoate), and two female-specific compounds (4-methylhexyl pentanoate and hexyl pentanoate). Both males and females were attracted to aeration extracts of females, and to the synthetic blend of the five EAD-active compounds in proportions mimicking those of the compounds produced by females.

Interestingly, in the Alydidae either the male [17, 18] or female [15–19] can emit the attractant pheromone, depending on the genus. In the rice alydid bug, *Leptocorisa chinensis* (Dallas), although there were no detectable qualitative differences in aeration extracts of males versus females, only males were attracted to a 5 : 1 blend of *(E)*-2-octenyl acetate and octanol [19]. In the alydid *R. clavatus*, the attractant pheromone is produced by males and attracts females, males, and nymphs, plus an egg parasitoid [17]. Furthermore, it has been established for *R. clavatus* that two of the three essential pheromone components ((*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate) are produced in the enlarged lateral accessory glands of males that are attached to the metathoracic scent gland reservoir. However, the third

Table 1: Amounts of the compounds identified in extracts obtained from air-entrainment of males and females of *N. parvus* ($N = 5$).

Compounds	Retention index (DB-5)*	(ng/24 hours/insect)	
		Males	Females
(1) Camphene	954	0.02 ± 0.01	1.7 ± 0.02
(2) 6-Methyl-5-hepten-2-one	980	1.12 ± 0.85	1.05 ± 0.78
(3) Hexanoic acid	981	0.59 ± 0.37	1.19 ± 0.74
(4) Butyl butanoate	995	1.11 ± 0.73	0.01 ± 0.01
(5) Hexyl acetate	1008	1.26 ± 0.91	1.37 ± 0.79
(6) Limonene	1035	0.06 ± 0.01	1.2 ± 0.52
(7) (*E*)-2-Octen-1-ol	1059	0.06 ± 0.02	0.01 ± 0.01
(8) Pentyl butanoate	1093	1.23 ± 1.17	0.66 ± 0.16
(9) Undecane	1100	2.14 ± 0.98	1.67 ± 0.92
(10) Nonanal	1104	1.28 ± 0.51	1.48 ± 0.45
(11) Unknown compound	1165	0.14 ± 0.13	0.33 ± 0.12
(12) Hexyl butanoate	1193	2.15 ± 1.92	43.58 ± 10.12
(13) Dodecane	1200	2.21 ± 1.49	2.89 ± 1.48
(14) Decanal	1207	1.68 ± 0.49	2.08 ± 0.64
(15) 4-methyl hexyl-butanoate	1262	0.11 ± 0.07	4.49 ± 1.64
(16) Hexyl pentanoate	1288	—	6.84 ± 2.11
(17) Tridecane	1300	15.06 ± 14.05	5.11 ± 2.21
(18) 4-methylhexyl-pentanoate	1364	—	0.49 ± 0.15
(19) Hexyl hexanoate	1386	1.20 ± 0.84	1.50 ± 0.52

* Retention index was calculated using the retention time obtained in GC-FID analysis using a DB-5 column with a temperature program of 50°C/2 min, then 8°C/min to 250°C/10 min.

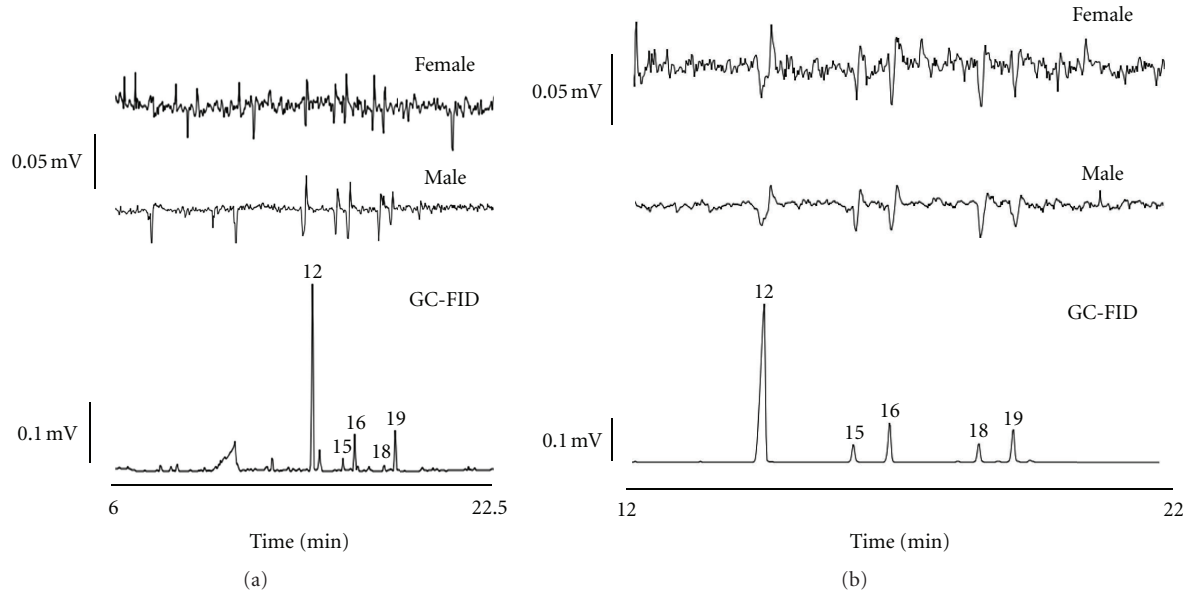

Figure 2: (a) A typical response to crude extracts of female aerations of antenna of *Neomegalotomus parvus* female and male in GC-EAG analyses. (b) A typical response to a synthetic mixture of compounds found in *N. parvus* aerations and scent glands of antenna of *N. parvus* female and male in GC-EAG analyses. (12) hexyl butanoate, (15) 4-methylhexyl-butanoate, (16) hexyl pentanoate, (18) 4-methylhexyl-pentanoate, and (19) hexyl hexanoate. In (b) the synthetic mixture tested was (12) hexyl butanoate (0.05 mg/mL), (15) 4-*S*-methylhexyl-butanoate (0.005 mg/mL), (16) hexyl pentanoate (0.0075 mg/mL), (18) 4-*S*-methylhexyl-pentanoate (0.002 mg/mL), and (19) hexyl hexanoate (0.0075 mg/mL).

Table 2: Principal compounds found in metathoracic scent gland and volatile collection of Alydidae species.

Compounds	*M. scutellaris* [18]	*R. serripes* [17]	*R. clavatus* [21, 22]	*M. quinque-spinosus* [14]	*A. eurinus* [14, 15]	*A. pilosulus* [14]	*L. chinensis* [19]	*N. parvus* (this work)
Butanal					MF	MF		
Nonanal							MF	
2-Methyl-butanal				MF				
Methyl propanal				MF				
(*E*)-2-Hexenal	MF	MF		MF	MF	MF		
(*E*)-2-Octenal	F						MF	MF
Octanol							MF*	
(*E*)-2-Hexen-1-ol	MF				MF	MF		
(*E*)-2-Octen-1-ol	MF						MF	MF
2-Methyl-butanoic acid				MF				
Butanoic acid	M			MF				
Hexanoic acid	M	M			MF	MF		MF
(*E*)-2-Hexanoic acid		M						
(*Z*)-3-Hexanoic acid		M						
(*E*)-2-Octanoic acid	M							
Hexyl-acetate								MF
Octyl acetate							MF	
(*E*)-2-Octenyl acetate							MF*	
(*Z*)-3-Octenyl acetate							MF	
Isobutyl 2-methyl-propanoate				MF				
2-Methyl-butyl 2-methyl-propanoate				MF				
2-Methyl-butyl butanoate				MF	MF			
(*E*)-2-hexenyl butanoate	M	M						
Butyl butanoate				MF	MF	MF		
(*E*)-2-Methyl-butenyl-butanoate					F*			
2-Methyl-butyl-butanoate					F*			
Butyl-butanoate								MF
Pentyl-butanoate								MF
Hexyl-butanoate					MF	MF		MF*
4-Methyl-hexyl-butanoate								MF*
(*E*)-2-Hexenyl-butanoate	F	F						
Hexyl-pentanoate								F*
4-Methyl-hexyl-pentanoate								F*
Butyl-hexanoate				MF				
2-Methyl-butyl-hexanoate				MF				
(*E*)-2-Hexenyl-(*Z*)-3-hexenoate	M	M	M*					
(*E*)-2-Hexenyl-(*E*)-2-octenoate		M						
(*E*)-2-Hexenyl-(*E*)-2 hexenoate		M	M*			MF		
(*E*)-2-Hexenyl-(*Z*)-2-hexenoate		M	M*					
Hexyl hexanoate								MF*
Tetradecyl-2-methyl-propanoate			M*					
Octadecyl-2-methyl-propanoate			MF					

Note: compounds labeled with F are female specific, labeled with M are male specific, and labeled with MF were identified in both gender. Compounds labeled with asterisk are those for which attraction function were confirmed in laboratory or field assays. Aldrich et al. [18], Leal et al. [17], Endo et al. [21], Yasuda et al. [22], Aldrich and Yonke [14], Aldrich et al. [15].

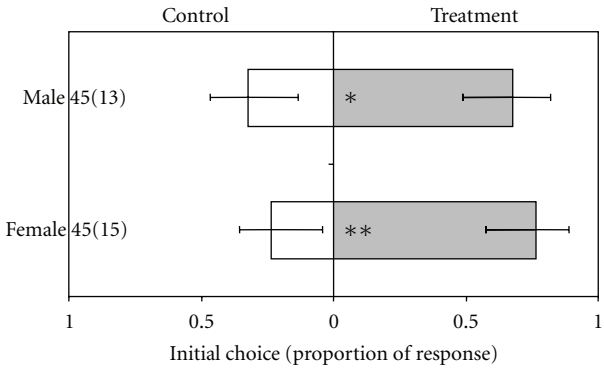

Figure 3: Initial choice (mean ± confidence interval (CI) 95%) of *Neomegalotomus parvus* males and females to crude extract of female aeration. Analyses of initial choices were carried out by logistic regression and χ^2 Wald test. *indicates $P < 0.05$ and **indicates $P < 0.01$. Numbers at left indicate total number of insects tested and numbers in brackets indicate the number of insects that did not make a choice after 5 minutes.

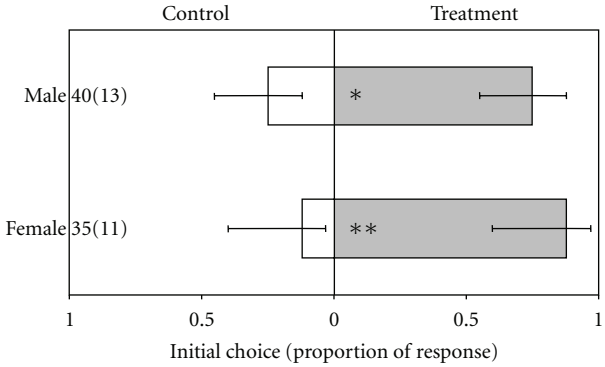

Figure 4: Initial choice (mean ± confidence interval (CI) 95%) of *Neomegalotomus parvus* males and females to 5 uL of a solution containing the synthetic authentic standards found in females extracts (hexyl butanoate (0.005 mg/mL), 4-(*S*)-methylhexyl-butanoate (0.0005 mg/mL), hexyl pentanoate (0.00075 mg/mL), 4-(*S*)-methylhexyl-pentanoate (0.0002 mg/mL), hexyl hexanoate (0.00075 mg/mL). Analyses of initial choices were carried out by logistic regression and χ^2 Wald test. * indicates $P < 0.05$ and ** indicates $P < 0.01$. Numbers at left indicate total number of insects tested and numbers in brackets indicate the number of insects that did not make a choice after 5 minutes.

essential component (myristyl isobutyrate) is produced from cells in the abdominal sternum of males [15].

Of the known alydid pheromone systems, *N. parvus* attractant pheromone appears most similar to that of *Alydus eurinus* (Say), where females produce a pheromone from the nonsexually dimorphic lateral accessory glands of the metathoracic scent gland complex with essential components being (*S*)-(-)-2-methylbutyl butanoate and *(E)*-2-methyl-2-butenyl butanoate. Although the chirality of 4-methylhexyl hexanoate and 4-methylhexyl pentanoate in *N. parvus* has not been unequivocally established ((*R*)-4-methyl-1-hexanol was not available), the biological activity of a blend containing synthetic esters with *S* absolute configuration combined with the fact that only (*S*)-(-)-2-methylbutyl butanoate was active for *A. eurinus* is suggestive that the 4-methylhexyl esters in *N. parvus* may also have *S* configuration. Dissections of adult *N. parvus* by one of us (J. Aldrich) indicated that the lateral accessory glands are enlarged in females and contain the key pheromone components (unpublished data). In *A. eurinus*, the female-produced pheromone attracts males, and to lesser extents females and nymphs; further tests are needed to establish whether the *N. parvus* pheromone will exhibit a similar pattern in the field.

Interestingly, a communication system similar to that of alydids occurs in plant bugs (Miridae) where sex pheromones consisting of aliphatic esters are produced in metathoracic scent glands of females [29–31], and in certain seed bugs (Lygaeidae) where males produce chemically similar aggregation pheromones from sexually dimorphic metathoracic scent glands [32].

In summary, *N. parvus* females produce two specific compounds that were not identified in male extracts and these compounds are pentanoates, the first time observed in Alydidae species, the other esters identified, hexanoates, and butanoates, were previously reported for others species of Alydidae. Five of these esters, hexyl butanoate, (*S*)-4-methylhexyl butanoate, hexyl pentanoate, (*S*)-4-methylhexyl pentanoate, and hexyl hexanoate stimulate an electrophysiological and behavioral response from males and females, indicating a possible function as aggregation pheromone. The identification of an attractant pheromone for *N. parvus* may lead to a more effective monitoring system for this pest, to more accurately guide insecticidal programs against this pest. Further field testing is necessary to make this potential application a reality.

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Review Article

The Sexual Behaviour of Chagas' Disease Vectors: Chemical Signals Mediating Communication between Male and Female Triatomine Bugs

Gabriel Manrique[1] and Marcelo Lorenzo[2]

[1] *Laboratorio de Fisiología de Insectos, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428EHA Ciudad Autónoma de Buenos Aires, Argentina*
[2] *Laboratory of Triatomines and Chagas' Disease Epidemiology, René Rachou Institute, FIOCRUZ, 30190-002 Belo Horizonte, MG, Brazil*

Correspondence should be addressed to Gabriel Manrique, gabo@bg.fcen.uba.ar

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Chemical communication mechanisms that mediate sexual behaviour in triatomine bugs are reviewed with regard to source, identity, and function of sex pheromones. Males attempt to copulate but may be rejected, depending on female age and nutritional status. Triatomine males locate partners through sex pheromones emitted by the metasternal glands (MGs) of females. These activate males, inducing them to leave their refuges and initiate flight. Wandering males display anemotactic orientation modulated by chemical signals emitted from female MGs. Analyses of the MG secretions of several species resulted in the identification of numerous ketones, acetals, and alcohols. Occlusion experiments showed that Brindley's gland products were not required for mating. Metasternal gland volatiles are emitted by virgin male and female bugs, with detection over females occurring more consistently, especially during the early scotophase, suggesting female calling behaviour. Mating triatomine females have been reported to attract males that tend to copulate successively with them. Mating males prolong mating and postcopulatory mate guarding in the presence of other males. This is indicative of a polyandrous mating system in several triatomine species. Its potential advantages remain unknown, and comparative studies are required to increase our understanding of triatomine reproductive strategies.

1. Introduction

Triatomine bugs have great epidemiological relevance because they transmit *Trypanosoma cruzi* Chagas 1909, the etiological agent of Chagas' disease [1–3]. This disease afflicts ~15 million people, with a further 75–90 million at risk from potential transmission in Latin America [4]. The vectorial capacity of these insects relies largely on their ability to invade habitations and develop large colonies, coupled with their being efficient intermediate hosts of trypanosomes. The risk of transmission is greatly enhanced in the case of poorly maintained thatch and mud houses where cracks provide abundant shelter in close proximity to humans and their domestic animals. Vector control is currently the only feasible means of reducing Chagas' disease transmission because of the lack of vaccines and effective therapeutic drugs [4].

Triatomine bugs have been shown to use a diverse range of chemical cues and signals to detect their hosts and communicate with conspecifics, respectively [5]. Host-related cues include stimuli such as body heat, CO_2, and volatile compounds from the skin and breath [6]. Insects in this group also utilize pheromones for marking refuges, locating mates, and for alerting conspecifics to danger [5]. The following sections review our current knowledge of triatomine chemical communication, particularly with regard to reproductive behaviours.

2. Triatomine Mating Behaviour

The mating behaviour of several triatomine species has been described in detail [7–12]. The general mating pattern is similar across species and consists of several behavioural steps performed by males. Initially, a male approaches and either jumps onto or slowly mounts a female. The male then places itself in a dorsolateral position in relation to the female. Once in this position, the male grasps the female, both dorsally and ventrally using all three pairs of legs, releases its paramers, and immobilizes the genitalia of the female to allow the introduction of the aedeagus. The duration of copulation varies between species, ranging from *ca.* 6 min for *Triatoma infestans* Klug, 1834, to *ca.* 50 min for *Rhodnius prolixus* Stål, 1859 [12]. Even after the genitalia of both insects are separated, the males occasionally remain on top of females for several minutes.

Males may perform several copulatory attempts with the same female in order to engage in mating. These copulatory attempts are not always successful because female receptivity depends on age and nutritional status, among other factors [8–10]. Females can display different rejection behaviours in response to copulation attempts [8–10]. These include flattening (the female lowers her body against the substrate to prevent a male from moving into the copulatory position), abdominal movements (females shake their bodies up and down), evasion (females walk away), and stridulation (females rub the tip of their rostrum against the prosternal groove) [12]. The term male-deterring stridulation has been used to describe this behaviour by nonreceptive females. This behaviour has been reported as source of a substrate-borne signal which plays a role in intraspecific communication in *T. infestans* and *R. prolixus* [13, 14].

It has been demonstrated that odours from the metasternal glands promote mating in *T. infestans* and *R. prolixus* [15, 16]. In addition to the volatile compounds secreted by the metasternal glands, it has been suggested that epicuticular lipids may play a role as female recognition signals (i.e., contact pheromones) in *T. Infestans*, but this needs to be confirmed [17].

3. Signals Mediating Copulation Behaviours

Following moulting to the adult stage, triatomine bugs search for host cues to obtain blood meals for nutrition and egg development by females, but they also need to find mates as they become sexually mature. The various species in this subfamily inhabit diverse sylvatic habitats where they associate with vertebrates. Both immatures and adults usually feed at night on a wide array of warm- and cold-blooded animals such as birds, mammals, reptiles, and amphibians. Generally, ecotopes such as birds' nests offer limited and unpredictable access to blood, for example, due to seasonal chick rearing. In addition, coinhabiting the den of a mammal or a bird's nest with a host represents an increased predation risk. Such circumstances might have induced the evolution of the strong tendency of triatomines to hide in cracks and crevices, at the cost of simultaneously affecting the chances of finding mates.

The location, frequency, and timing of mating in triatomines remain unknown, but it is likely that mating occurs around dusk when general locomotion patterns are enhanced [18]. Similarly, bugs probably leave their refuges to search for mates around this time, and it is likely that chemical signals could mediate mate location (see the following). In fact, without long distance signals to mediate encounters between males and females, reproduction would be limited to small sylvatic colonies where adult pairs coexist.

Recently it has been shown that males of two triatomine species are stimulated to leave their shelters by female-produced volatile signals [19, 20]. Interestingly, males may attempt copulation with other males, and according to these authors, this tendency is greatly increased in the presence of chemical signals emitted by the female. These results indicate that odours from the female act as activators, attractants, and aphrodisiacs.

Many authors have attempted to determine whether triatomine bugs use chemical signals to communicate between sexes, often with contradictory results which in many cases are difficult to interpret or place in context due to methodological flaws. For example, Antich [21] reported the attraction of adult *R. prolixus* to volatile pheromones. Later, Neves and Paulini [22] presented similar results describing sexual attraction mediated by chemical signals in adult *T. infestans* and *Panstrongylus megistus* Burmeister, 1835. Additionally, Ondarza and collaborators [23, 24] presented results indicating that a volatile signal mediated the sexual behaviour of *T. mazzotti* Usinger, 1941, but conflicting results were subsequently presented by Rojas and collaborators [25]. Closer inspection of some of these pioneering studies suggests that the experimental designs might not have been sufficient to be able to conclude that chemical signals were mediating encounters between males and females.

Many years later, Brindley's glands became the target of several studies aimed at elucidating communication between adult triatomine bugs. Fontan and collaborators [26], as well as Guerenstein and Guerin [27], presented results showing that a subset of the compounds in Brindley's glands could be recovered from the headspace over groups of copulating *T. infestans* and *R. prolixus*, respectively. The first report provided evidence of behavioural or electrophysiological effects of single components from the secretion of Brindley's glands on adult triatomines. However, it remains unclear whether these effects are related to reproductive behaviours, given that the elicitation of an electrophysiological response or a change in the level of activity does not necessarily indicate a sexual role for the compounds tested. Even attraction of adults to single compounds is not a certain demonstration of a sexual role, unless only one sex is attracted and immatures do not display similar behavioural responses. Two additional points weaken the argument that Brindley's gland products are related to sexual behaviour. First, Brindley's glands are the proposed source of alarm pheromones in all species in the subfamily [28–30], including *R. prolixus*, *T. infestans*, *T. phyllosoma*, *P. megistus*, *T. maculata*, *T. brasiliensis,* and *T. vitticeps*. Therefore, the inadvertent release of Brindley's gland compounds by handling bugs before or during bioassays may have confounded the results of those

assays, and in fact, some of the studies suggesting a sexual role for Brindley's glands secretions did not report how they avoided the emission of the alarm pheromone while handling bugs. Second, reports proposing such a role for Brindley's gland odours have used groups of adults for odour collection; however it is uncertain whether similar results can be obtained when working with pairs of insects, the basic unit of reproductive behaviour. Addressing these questions may help to clarify unresolved issues in the study of triatomine sexual communication.

4. Chemical Signals Mediating Possible Attraction between Adult Triatomine Bugs

It had been suggested that males of several triatomine species might be captured more frequently in light traps than females [31], and this proved to be the case with light trapping of *T. infestans* in Northern Argentina [32]. A recent report also suggested a link between male flight initiation and sexual communication for *R. prolixus* [33], indicating that males may undergo dispersal flights as a mechanism for locating females via volatile chemical signals. Zacharias and collaborators [33] have shown that *R. prolixus* females emit volatile compounds that induce males to initiate flight, and that these compounds are likely associated with the metasternal glands. In addition, these authors showed that excised metasternal glands elicited similar responses from males, confirming that these glands are the source of a volatile signal from the female. A similar increase in take-off frequency was also observed for male *T. brasiliensis* Neiva, 1911, when presented airstreams containing female odour [19]. Females of both species showed no response to male odours. Preliminary results with *T. vitticeps* Stål, 1859, have shown that males of this species only engage in flight if volatile signals emitted by females are present (H.H.R. Pires, *personal communication*). It is also worth noting that flight initiation by males has a clear directional component, with males typically flying upwind [33]. The introduction of female metasternal gland odours into an airstream only induces an increase in male take-off frequency. However, the female-produced chemicals that cause this increase remain unknown.

5. Attraction between Walking Adult Triatomine Bugs

In addition to flying, triatomine adults may detect and walk towards sexual partners from a distance. Whereas the reaction of males to the presence of odours from females has already been described, two recent publications investigated the anemotactic responses of wandering males. Vitta and collaborators [34] used an olfactometer to show that male *T. brasiliensis* move upwind in airstreams laden with the odour of females, exhibiting odour-modulated anemotaxis in response to a sexual pheromone. In addition, they reported that the bioactive compounds were associated with metasternal gland products from females. Interestingly, males also exhibited similar responses in the presence of airstreams containing odours from males. However, blocking the metasternal gland orifices of the males used as the stimulus source did not impair the response of tested males, suggesting that the test males were responding to odours from a different source, and possibly being used in a different context. In contrast, *T. brasiliensis* females showed no response to odours from either sex.

A different experimental approach allowed Pontes [20] to show that *R. prolixus* males are attracted and orient towards airstreams laden with the odour of females. This study used a locomotion compensator [35] to evaluate the responses of males and females presented with odours from adult bugs of either sex. The study showed that *R. prolixus* males were attracted by volatile compounds from the metasternal glands of females.

6. The Role of Triatomine Exocrine Gland Secretions

Most Reduviidae have several exocrine glands in the thorax and abdomen, such as the metasternal glands, Brindley's glands, dermal glands, ventral glands, and abdominal glands [38, 39]. Ventral and abdominal glands are apparently absent in the subfamily Triatominae, and only adult insects possess both metasternal and Brindley's glands [40]. The paired metasternal glands are widespread among the Heteroptera. They are ventrally located at the anterior margin of each metacoxal cavity [41]. Each gland consists of an unbranched secretory tubule and a small pear-shaped reservoir opening laterally to the sternal apophyseal pit [42, 43]. The sac-like paired Brindley's glands are dorsally located, extending into the lateral portion of the second abdominal segment and opening onto the metathoracic epimeron [39, 41, 44, 45]. These glands, which secrete isobutyric acid as their most abundant product, are likely to be associated with alarm and defense functions [29, 30, 45, 46]. As stated previously, it has also been suggested that compounds produced by Brindley's glands are involved in sexual signalling in triatomines [26, 27, 45]. However, no behavioural evidence has been reported that associates compounds from Brindley's glands with behavioural responses specifically related to sex. Further investigation is required to clarify the role of Brindley's glands secretions in the reproductive behaviour of triatomines.

The secretion from the metasternal glands contains several compounds, some of which are highly volatile aliphatic ketones and alcohols (Table 1). The first compound identified from these glands, 3-methyl-2-hexanone, from *Dipetalogaster maxima* (Uhler, 1894), may function as an alarm pheromone or defensive secretion [36] and can be detected when bugs of this species are disturbed [27]. Volatile components emitted from these glands have now been identified in other triatomine species such as *T. infestans, R. prolixus*, and *T. brasiliensis*. Table 2 summarizes metasternal gland compounds detected in the headspace odours of triatomine bugs in different behavioural contexts. As mentioned, it has been proposed that metasternal gland components may mediate sexual communication between

Table 1: Compounds identified from metasternal glands of different triatomine species (references cited in brackets).

	D. maxima [36]	*T. infestans* [30]	*R. prolixus* [16]	*T. brasiliensis* [34]	*T. brasiliensis* *T. infestans* [37]
2-butanone		+	+	+	
2-pentanone			+		
(*S*)-2-butanol			+	+	
(*R*)-2-pentanol				+	
2-methyl-3-buten-2-ol			+		
3-methyl-2-butanol			+		
3-pentanone		+		+	
3-methyl-2-hexanone	+				
3-pentanol		+	+	+	
(*S*)-2-pentanol			+		
(*E*)-2-methyl-3-penten-2-ol			+		
(*S*)-4-methyl-2-pentanol			+		
(*S*)-3-hexanol			+	+	
(*R*)-3-hexanol				+	
3-methyl-2-hexanol				+	
(*R*)-4-methyl-1-hexanol				+	
3-hexanol		+			
2-methyl-1-propanol				+	
2-methyl-1-butanol		+	+		
(*S*)-2-methyl-1-butanol				+	
4-methyl-3-penten-2-ol			+		
1-heptanol				+	
6-methyl-1-heptanol				+	
(*R*)-4-methyl-1-heptanol				+	
(*R*)-1-phenylethanol				+	
(4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane					+
(4*S*,5*S*)-2,4-diethyl-2,5-dimethyl-1,3-dioxolane					+trace
(2*R*/*S*,4*S*,5*S*)- and (2*R*/*S*,4*R*,5*R*)-4-ethyl-5-methyl-2-(1-methylethyl)-1,3-dioxolane					+
(2*R*/*S*,4*S*, 5*S*)-4-ethyl-5-methyl-2-(1-methylpropyl)-1,3-dioxolane					+trace
(2*R*/*S*,4*S*, 5*S*)-4-ethyl-5-methyl-2-(2-methylpropyl)-1,3-dioxolane					+trace

Table 2: Compounds associated with metasternal gland secretions of different triatomine species emitted in different behavioural contexts (references cited in brackets).

	D. maxima (disturbed adults: [27])	*T. infestans* (disturbed adults: [30])	*T. infestans* (mating: [30])	*R. prolixus* (female calling: [16])	*R. prolixus* (mating: [16])
2-butanone		+		+	
2-pentanone				+	+
3-pentanone		+	+		
3-methyl-2-hexanone	+				
(*S*)-2-butanol				+	
2-methyl-3-buten-2-ol					+
3-methyl-2-butanol				+	
(S)-2-pentanol				+	
2-methyl -1-butanol		+		+	+

adults of these species [30, 34, 47]. In *T. infestans,* the metasternal glands produce a number of volatiles, including the main component 3-pentanone, which was detected in the odours from copulating pairs [30]. This suggests that *T. infestans* may use this and possibly other compounds for signalling during mating. The quantities of 3-pentanone in the metasternal glands of *T. infestans* varied between 10 and 100 μg per adult [30], whereas other compounds of the secretion such as 2-methyl-1-butanol and 3-pentanol were present at 1 and 10 μg per insect, and 3-hexanol between 0.1 and 1 μg per insect. Surprisingly, no apparent differences were found in the composition of the scent produced by these glands between males and females [30]. More recently, further compounds have been identified from two triatomine species, including the acetal (4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane and related compounds [37, 48]. Occlusion of female metasternal gland orifices resulted in a significant decrease in copulation frequency and prevented the male aggregation behaviour described for this species, suggesting that metasternal gland odours mediate the sexual behaviour of *T. infestans* [15]. Because the attractiveness of mating pairs decreased after occluding female metasternal glands, it was suggested that females emit volatile compounds that promote both copulation and male aggregation behaviour. Similar results were obtained with *R. prolixus*, in which occlusion of male or female metasternal gland orifices induced a decrease in copulation success [16]. Recently, Pontes and Lorenzo [47] have shown that the occlusion of *R. prolixus* female metasternal glands also prevented male aggregation around mating pairs, whereas the occlusion of male metasternal glands did not affect male aggregation. In addition, occlusion experiments also showed that Brindley's gland products are not required for normal mating and male aggregation [15]. In sum, these experiments suggest that volatile compounds from metasternal glands may be among the key signals used in mating interactions.

The metasternal glands of *R. prolixus* produce a variety of volatile compounds, including at least 12 ketones and alcohols. Of these, the most abundant are 2-methyl-3-buten-2-ol, 2-pentanol, (*E*)-2-methyl-3-penten-2-ol, 4-methyl-3-penten-2-ol, and the enantiomers of 4-methyl-3-penten-2-ol [16]. Further analysis suggested that the minor compounds 2-butanol, 2-pentanol, 4-methyl-2-pentanol, and 3-hexanol were produced as the (*S*)-enantiomers, whereas 4-methyl-3-penten-2-ol (mesityl alcohol) was a mixture of the (*R*)- and (*S*)-enantiomers. As with *T. infestans*, the metasternal gland components of male and female *R. prolixus* appeared to be the same. For *R. prolixus,* these volatile compounds were also detected in the odours of virgin male and female bugs [16], with detection being more frequent from headspace odours from females. Furthermore, females released these substances more frequently during the early hours of the scotophase. In addition, their detection, albeit in limited quantities (e.g., 10–100 pg for 2-pentanol), over copulating pairs of *R. prolixus* suggests that these compounds may be involved in sexual communication [16]. Interestingly, 2-methyl-1-butanol is the most consistently detected compound found over mating pairs, even though it is not the most abundant product of metasternal gland secretions.

The metasternal gland secretions of *T. brasiliensis* were found to contain at least 16 ketones, acetals, and alcohols [34, 37, 48], with 3-pentanone being one of the most abundant compounds, as seen for *T. infestans* [30]. In addition to 3-pentanone, other abundant components included 3-pentanol and (*R*)-4-methyl-1-heptanol. The configurations of the chiral compounds varied; 2-methyl-1-butanol was present as the (*S*)-enantiomer, whereas 4-methyl-1-hexanol, 4-methyl-1-heptanol, and 1-phenylethanol were present as (*R*)-enantiomers, and 3-hexanol and 3-methyl-2-hexanol were present in all isomeric forms [34]. GC-EAD recordings performed with *T. brasiliensis* showed that male antennae responded to a number of volatile compounds in the metasternal glands of females, including 3-pentanone, (*R*)-4-methyl-1-heptanol, (4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane, (*S*)-2-methyl-1-butanol, and (*R*)-1-phenylethanol [34]. Electrophysiological experiments still need to be carried out with other important vector species such as *T. infestans, T. dimidiata*, and *R. prolixus* in order to determine which of the components of the secretions have associated receptors on the bugs' antennae.

Paired glandular areas have been described on the intersegmental membrane between abdominal segments 8 and 9 of male *T. infestans* and *Triatoma rubrofasciata* [49, 50], along with three areas of glandular structures associated with the basal articulatory apparatus in *T. rubrofasciata* [50]. The identities and roles of the products of these glands have not been determined.

7. Male Aggregation Signals and Their Emission during Copulation

The existence of a chemical signal that is released during mating and promotes the aggregation of males around mating pairs has been consistently reported for *R. prolixus* and *T. infestans* [47, 51–53]. In these species, females were not observed to aggregate around a mating pair, suggesting that female behaviour is not influenced by the presence of copulating pairs [47, 53]. The mating and aggregation behaviour of males is not consistent across all triatomines. *Panstrongylus megistus* females are receptive to copulation only once during their imaginal life, perhaps because the genitalia of males and females remain coupled for an extended period, thus preventing mating with other males [54]. In addition, the apparent absence of signals promoting the aggregation of other males around a mating pair contrasts with the behaviour of *T. infestans* and *R. prolixus* [54]. For the latter two species, pairs remain coupled for shorter intervals, allowing subsequent copulations. For example, aggregated males copulate successively with the same female for *T. infestans* [53], *T. brasiliensis* [55], and *R. prolixus* [47]. Thus, the aggregation of males could be related to a polyandrous mating system [56].

Copulating *T. brasiliensis* males are known to modify their behaviour in response to the presence of other males [55], with the duration of mating being prolonged in pairs copulating in the presence of other males. In addition, long postcopulatory associations were observed with *T. brasiliensis*

and *R. prolixus* males mating in similar circumstances [47, 55]. These postcopulatory associations are likely a form of mate guarding geared towards impeding subsequent copulation by other males [57]. The observation of multiple mating in several triatomine species suggests the likelihood of sperm competition mechanisms, and the possible costs and benefits of multiple mating in triatomines deserve further analysis.

8. Perspectives

Recent advances in identifying and determining the roles of chemical signals in triatomine bugs will aid the development of semiochemical tools for controlling these important disease vectors, but much further work needs to be done to determine which specific compounds mediate sexual behaviours, and their precise roles. If synthetic chemicals are shown to attract males, they could find immediate use as baits for traps to monitor and control populations, thereby limiting the transmission of Chagas' disease. Further detailed analyses of gland contents, including the complete identification of minor components, are essential. The compounds identified will then need to be methodically tested in both laboratory and field bioassays to elucidate their roles as mediators of behaviour. These experiments will need to be followed up with practical field bioassays to determine whether these compounds are sufficiently biologically active that they can be used to trap triatomine bugs effectively. The problem is compounded by the fact that these compounds only affect the behaviours of adult bugs and, in many cases, may only affect one sex. Thus, it remains to be seen whether mass trapping males only, or possible pheromone-based mating disruption, could result in effective control of these bugs. Other aspects, such as cost-effectiveness, can only be evaluated once the key compounds are known. However, because control of triatomine bugs hinges on sensitive detection of low-density populations and foci of vectors, even effective methods of monitoring triatomine bugs would represent a major advance in minimizing disease transmission.

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Research Article

Temporal Dynamics and Electronic Nose Detection of Stink Bug-Induced Volatile Emissions from Cotton Bolls

David C. Degenhardt, Jeremy K. Greene, and Ahmad Khalilian

School of Agricultural, Forest, and Environmental Sciences, Edisto Research and Education Center, Clemson University, 64 Research Road, Blackville, SC 29817, USA

Correspondence should be addressed to David C. Degenhardt, ddegenh@clemson.edu

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Management decisions for stink bugs (Pentatomidae) in *Bt* cotton are complicated by time-consuming sampling methods, and there is a need for more efficient detection tools. Volatile compounds are released from cotton bolls in response to feeding by stink bugs, and electronic nose (E-nose) technology may be useful for detecting boll damage. In this study, we investigated the temporal dynamics of volatile emissions in response to feeding by stink bugs and tested the ability of E-nose to discriminate between odors from healthy and injured bolls. Feeding by stink bugs led to an approximate 2.4-fold increase in volatile organic compound (VOC) emissions. Principal components analysis of E-nose sensor data showed distinct (100%) separation between stink bug-injured and healthy bolls after two days of feeding. However, when E-nose was used to randomly identify samples, results were less accurate (80–90%). These results suggest that E-nose is a promising technology for rapid detection of stink bug injury to cotton.

1. Introduction

Phytophagous stink bugs (Heteroptera : Pentatomidae) are major pests of food and fiber crops worldwide [1]. In the USA, stink bugs are increasingly destructive in cotton, *Gossypium hirsutum* (L.). Prior to the introduction of transgenic cotton varieties expressing insecticidal proteins from *Bacillus thuringiensis* (*Bt*), growers were afforded coincidental control of stink bugs through the use of broad-spectrum insecticides for major pests such as bollworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.). With the widespread adoption of *Bt* varieties, eradication of the boll weevil, *Anthonomus grandis grandis* (L.), and the subsequent reduction in the use of broadspectrum insecticides, stink bugs have emerged as important pests threatening cotton production, especially in the southeastern USA [2]. Since 1995, insecticide use targeting these pests has risen from zero to millions of applications, and crop losses recently exceeded 50 million dollars [3]. Stink bugs feed directly on cotton bolls resulting in boll abscission and lint staining and reduced fiber quality and yield [2, 4–6]. Scouting techniques for stink bugs require destructive sampling of cotton bolls with visual inspection for internal lint staining and callus warts on internal carpal walls [7]. Due to difficulties in assessment and time-consuming scouting practices, there is a need for a more reliable, rapid, and nondestructive method for determining boll injury from these pests.

It is well known that herbivory results in the induced synthesis of volatile organic compounds (VOCs) from plant tissues including a variety of terpenoids, phenylpropanoids, and fatty-acid derivatives [8, 9]. In cotton, terpenoids are stored in lysigenous glands, and herbivore feeding causes the release of stored terpenes, as well as the induction of novel compounds through a combination of physical damage and elicitors from salivary components of the attacking herbivore [10–13]. Stink bug feeding has been shown to induce VOC emissions from cotton [14], corn [15], and soybean [16]. Recently, it was shown that stink bug feeding on cotton bolls led to a 2-3-fold increase in VOC emissions compared with healthy bolls and that induced emissions were similar in response to feeding by different hemipteran species, including southern green stink bug, *Nezara viridula* (L.), and brown stink bug, *Euschistus servus* (Say) [17].

In the last decade, electronic nose (E-nose) technology has been developed to detect VOC emissions at the level of parts-per-million to parts-per-billion [18]. These devices characterize the overall profile of an odor as a digital "smellprint" generated by the change in resistance of several nonspecific gas sensors when exposed to components of the VOC mixture [18]. E-noses have been used for a range of applications, including detection of diseases [19], microbes [20], hazardous chemicals [21], and changes in food quality [22]. E-nose technology has also been successfully applied to monitoring pest damage in several systems. Based on brief (7 to 12 sec) samples from the headspace of tomato plants, E-nose was capable of discriminating pathogen infection, herbivore damage, and mechanical damage based on changes in VOC emissions among treatments [23]. In a laboratory study, it was demonstrated that an E-nose was 90% accurate at differentiating excised healthy boll from those injured by stink bugs [24].

Because stink bugs are known to induce VOC emissions from cotton bolls [17], E-nose technology could potentially serve as a rapid, nondestructive tool for monitoring stink bug injury to cotton bolls; however, this technology has yet to be tested for this purpose under field conditions. The overall goal of this study was to determine the feasibility of using an E-nose to detect and differentiate between VOCs from healthy (undamaged) bolls and those exposed to stink bug feeding (damaged) under field conditions. The specific objectives were to (1) determine the temporal dynamics in VOC emissions from cotton bolls in response to feeding by stink bugs, (2) determine the ability of an E-nose to differentiate between undamaged bolls and those damaged by stink bugs, and (3) determine if an E-nose can discriminate between VOCs induced in response to feeding by *E. servus* and *N. viridula*.

2. Materials and Methods

2.1. Plants and Insects. Experiments were conducted during August 2010 at the Edisto Research and Education Center (EREC) in Blackville, SC, USA. Field trials were carried out in a 1.5-hectare field planted with *G. hirsutum* var. Delta and Pine Land 161 B2RF.

Adults and nymphs of *E. servus* and *N. viridula* were initially collected from field populations in soybean and maintained separately in an insect rearing chamber at EREC. Insects were fed on a source of fresh green beans and provided with water on moistened cotton pads until initiation of experiments. Male and female adult stink bugs (5–7 d in stage) were used in all experiments.

2.2. E-Nose Instrumentation. The E-nose used in all experiments was the Cyranose 320 (Smiths Detection, Inc., Pasadena, CA, USA). The Cyranose 320 is a conductimetric, chemiresistive E-nose containing 32 sensors constructed of an aluminum substrate coated with a thin film of variable carbon composite polymers. Preliminary tests indicated that four sensors were sensitive to water vapor, and these sensors were deactivated for all experiments to improve discrimination capability. A typical sample cycle for the E-nose consisted of a sensor baseline purge, sample collection, and a sample purge. In this study, a high pump speed ($180\,\text{mL}\cdot\text{min}^{-1}$) was used for baseline and sample purges, and a medium pump speed ($120\,\text{mL}\cdot\text{min}^{-1}$) was used for all sample collections. When exposed to chemical vapors during sample collection, the film on each sensor swells causing an increase in resistance. The change in resistance for each sensor ($\Delta R/R$) is represented as the difference between electrical resistances (ΔR) recorded during sample collection and baseline purge during the sample cycle and the resistance (R) recorded at the end of the baseline purge. The resistance from all 32 sensors is measured, and the overall composition of individual sensor responses represents a "smellprint" for an odor. The resistance data from all sensors is analyzed by on-board pattern recognition algorithms including K-nearest neighbor, K-means clustering, and canonical discriminant analysis. The canonical algorithm was used for all analyses in this study.

2.3. VOC Collection and Analysis. A randomized complete block design was used to evaluate the temporal dynamics in VOC emissions and E-nose detection (described below) of undamaged and damaged bolls. In each block, three white blooms at the same node on individual plants were enclosed in cages to protect developing bolls from insect damage prior to experiments. Cages were constructed from polystyrene foam cups with the base of the cup removed and nylon stocking stretched over the cup. Cages were placed over blooms, and the nylon stocking was secured around the peduncle using a 24-gauge steel wire. The nylon stocking was also secured around the top of each cage using another piece of 24-gauge steel wire. For analysis of stink bug-induced VOC emissions, a single adult *N. viridula* or *E. servus* was placed in cages on bolls 10–12 d postanthesis and allowed to feed *ad libitum*. Stink bugs were removed in 24 hr intervals to collect VOC emissions from damaged and undamaged bolls. Volatiles were sampled using a dynamic headspace sampling method. A polyacetate oven bag (Reynolds, Inc., Richmond, VA) modified to a volume of 300 mL was placed over a boll and loosely fastened with a small cable tie at the base of the boll to permit airflow through the bag. A volatile trap was connected to the top corner of each bag using a small cable tie. Volatile collection traps were constructed from glass Pasteur pipettes (10 cm long, 0.5 cm [o.d.]) and contained 35 mg of Porapak Q adsorbent polymer (Alltech Assoc., Deerfield, IL, USA) held in place by two small plugs of glass wool. A battery-operated air-sampling pump (SKC, Inc. Eighty Four, PA, Model 224-44XR) fitted with an independently controlled, adjustable, 4-way splitter (SKC, Inc. Model 224-26-04) was used to draw ambient air through the bag across the boll and directly onto the trap at a rate of $300\,\text{mL}\cdot\text{min}^{-1}$. Volatiles were collected for 3 hr during each sampling interval. Ambient air blanks were collected simultaneously with boll volatile collections to correct for any volatile contaminants contained in the air pulled through the collection bag. Emissions from damaged and undamaged bolls were collected in 24 hr intervals over a 4 d period.

Volatiles were extracted from adsorbent traps by washing with 150 μL of analytical grade hexane and collecting the extract directly into a 2 mL autosampler vial containing a 150 μL insert. Two μL of each sample were analyzed by gas chromatography (GC) on a Hewlett-Packard 6890 gas chromatograph equipped with a RTX-5 column (30 m $\times$ 0.25 mm [i.d.], 0.25 μm film thickness) (Restek, Bellefonte, PA). Injections were made in the splitless mode for 0.5 min with an inlet temperature of 250°C. The oven was held at 50°C for 10 minutes and then increased to 150°C at 5°C·min^{-1}, followed by an increase to 250°C at a rate of 15°C·min^{-1}, and a final increase to 300°C at a rate of 10°C·min^{-1}. Helium was used as a carrier gas at a flow rate of 1 mL·min^{-1}. Samples were subsequently analyzed by mass spectrometry (MS) using a Varian VG-70S (Waters Corp., Milford, MA) operated in electron impact mode. Compound identities were confirmed by comparison with mass spectra and retention indices of the library of essential oil components identified by GCMS [25], with spectra obtained from known standards (Sigma-Aldrich, Inc., Milwaukee, WI), and from high-resolution mass spectra from solvent extracts of boll material. Quantification of compounds was based on external standard curves of α-pinene for monoterpenes and β-caryophyllene for sesquiterpenes. The C11 homoterpene, DMNT, and the C16 homoterpene, TMTT, were quantified based on the standard curve of α-pinene and β-caryophyllene, respectively.

2.4. E-Nose Training on Damaged and Undamaged Bolls. Before an E-nose can be used for discrimination between treatments, it must be trained to recognize the odor profile among replicates of treatment groups. The temporal dynamics in E-nose detection of stink bug feeding damage were examined by training the E-nose on VOC emissions from bolls damaged by *N. viridula*, as well as undamaged bolls, in 24 hr intervals over a 4 d feeding period. During each 24 hr training period, cages and stink bugs were removed, and bags were placed over bolls (described previously) prior to E-nose sampling. Headspace VOCs were allowed to accumulate inside the bag for 30 minutes prior to E-nose training. To train the E-nose, the snout of the E-nose was inserted into the top corner of a bag, and VOCs were sampled using a 10 sec baseline purge, a 15 sec sample collection, and a 30 sec postsample purge. VOCs were sampled from 8 damaged and 8 undamaged bolls during each training period, and the same bolls were sampled on consecutive training periods. Following E-nose training, bugs and cages were placed back over bolls. This process was repeated for each 24 hr training set. Smellprint data from each temporal training set were analyzed separately by canonical discriminant analysis followed by cross-validation to assess the accuracy of each training set.

2.5. Identification Accuracy of E-Nose Temporal Training Sets. To evaluate the accuracy of E-nose training data, each temporal training set was used to randomly identify a separate set of 10 bolls damaged by *N. viridula*, and 10 undamaged bolls immediately following collection of training data. Bolls used for E-nose identification were exposed to stink bugs for an equivalent amount of time as those used for training. The same bolls were used for consecutive training set evaluations. The identification quality of the E-nose was set to "highest," which included a range of potential responses including correct identifications (undamaged or damaged), as well as incorrect identifications (incorrect treatment, confused, or unknown). The number of correct and incorrect identifications was recorded after each sample exposure, and the identification accuracy was determined based on the percentage of correct identifications.

2.6. E-Nose Discrimination of Species-Specific Feeding Damage. To examine the ability of E-nose to discriminate between feeding damage by different species of stink bugs, a single adult of *E. servus* or *N. viridula* (5–7 d in stage) was placed on individual 10–12 d old bolls inside cages (described previously). Stink bugs were allowed to feed *ad libitum* for 3 d, after which cages and stink bugs were removed and a polyacetate oven bag was placed over the bolls. E-nose was used to randomly sample VOCs from undamaged bolls and those damaged by *E. servus* and *N. viridula* using the sampling protocol described previously.

2.7. Statistical Analysis. A mixed model, repeated measures analysis of variance (PROC MIXED) [26] was used to test for differences in total and individual volatile emissions using plant treatment (control, *N. viridula*-damaged, and *E. servus*-damaged) and time (0, 24, 48, 72, and 96 hr) as main effects in the model statement, with plant assigned as the repeated subject. Volatile emissions data were $\log(x + 1)$ transformed prior to analysis to satisfy assumptions of normality. Differences in the emissions of individual compounds among treatments were analyzed by Tukey post hoc comparisons. On-board data analysis software was used for canonical discriminant analysis and cross-validation analysis of E-nose training sets. The proportions of correct identifications of damaged and undamaged bolls following each temporal training session were statistically analyzed using *chi*-square analysis (PROC FREQ) [26].

3. Results

3.1. Volatile Emissions from Cotton Bolls in Response to Stink Bug Feeding. Herbivory by *N. viridula* and *E. servus* had a significant effect on the emission of VOCs over time (Table 1; Figure 1). A significant interaction between treatment and time (sampling period) indicated that the effect of stink bug feeding on VOC emissions varied significantly among sampling periods (Table 1). Prior to placement of stink bugs in cages (time 0), total VOC emissions were not significantly different among treatment (Figure 1). Significantly greater emissions were detected from bolls exposed to *N. viridula* and *E. servus* compared with controls 48 hr after feeding (Figure 1). Total emissions showed the strongest increase between the 48 and 72 hr sampling period, and emissions remained significantly elevated 96 hr after initial exposure (Figure 1). Feeding by stink bugs resulted in approximately a 1.3-, 1.9-, 2.2-, and 2.4-fold increase in total VOC emissions

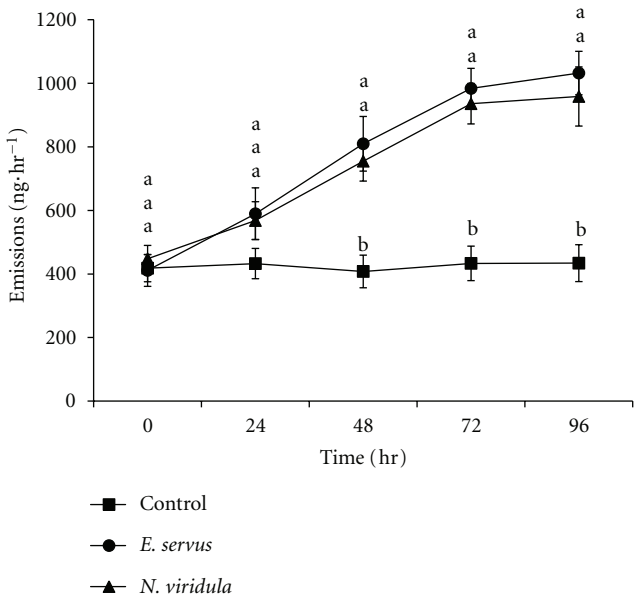

Figure 1: Temporal dynamics of total VOC emissions from cotton bolls exposed to *Euschistus servus and Nezara viridula* and unexposed (control) bolls under field conditions. Data points represent mean VOC emissions (±1 SE; $n = 5$). Different letters indicate a significant difference in total VOC emissions between treatments at each sampling interval (repeated measures ANOVA $P < 0.05$).

Table 1: Repeated measures analysis of variance on total volatile emissions released from cotton bolls exposed to *Euschistus servus* and *Nezara viridula* and unexposed bolls collected over a 4 d feeding period.

Effect	Num DF	Den DF	*F* value	*P* value
Treatment	2	12	41.54	<0.0001
Time	4	48	16.1	<0.0001
Treatment ∗ Time	8	48	3.98	0.0011

24, 48, 72, and 96 hr, respectively, from initial exposure to bolls (Figure 1). No significant difference was detected in total VOC emissions between *E. servus*- and *N. viridula*-damaged bolls at any sampling period (Figure 1).

Herbivory by *E. servus* and *N. viridula* resulted in a strong increase in the emission of several acyclic terpenes compared with controls, including the C10 monoterpene β-ocimene, the C15 sesquiterpene β-farnesene, and the C11 and C16 homoterpenes 4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), respectively (Figure 2). Emissions of β-ocimene, β-farnesene, and DMNT increased significantly in bolls after 48 hr of exposure to stink bugs and remained elevated 72 and 96 hr after introduction of bugs (Figure 2). A significant increase in emissions of TMTT was not detected until 72 hr after exposure to stink bugs (Figure 2). Furthermore, while small quantities of β-ocimene, β-farnesene, and DMNT were detected in controls, TMTT was not detected in the headspace emissions of controls during any sampling interval (Figure 2).

3.2. E-Nose Training on Damaged and Undamaged Bolls. According to principal components analysis and cross-validation of training data, smellprints of damaged bolls were separated from smellprints of undamaged bolls with 81.25% accuracy after 24 hr of exposure under field conditions (Figure 3(a); Table 2). Smellprints of bolls exposed to *N. viridula* for 48, 72, and 96 hr were separated with 100% accuracy from smellprints of undamaged bolls (Figures 3(b), 3(c), and 3(d); Table 2). Interclass Mahalanobis distances (*M*-distance) provided an indication of the degree of separation between classes following each training session (Table 2). Longer durations of exposure led to greater separation of smellprints from damaged and undamaged bolls according to the interclass *M*-distance values generated from the cross-validation analysis (Table 2).

3.3. Identification Accuracy of E-Nose Temporal Training Sets. Regardless of the length of feeding exposure, identification accuracy was always lower compared with training set accuracy (Tables 2 and 3). When using the smellprints from training sets collected after 24 and 48 hr of feeding, the E-nose correctly identified bolls (damaged or undamaged) 60 and 65% of the time, respectively (24 hr: *chi*-square $P = 0.37$; 48 hr: *chi*-square $P = 0.18$; Table 3). Identification accuracy was markedly improved when using the smellprints from training sets collected after 72 and 96 hr of exposure to stink bugs. The E-nose correctly identified bolls (damaged or undamaged) with 95% accuracy after 72 hr of feeding exposure (*chi*-square $P < 0.0001$; Table 3) and 90% accuracy after 96 hr of exposure (*chi*-square $P = 0.0003$; Table 3).

3.4. E-Nose Detection of Stink Bug Species-Specific Feeding Injury. Overall, nine of the 28 active sensors showed a strong change in resistance in response to headspace emissions from cotton bolls (Figure 4). Smellprints collected from the headspace of unexposed bolls (Figure 4(a)) were distinct compared with smellprints from bolls exposed to *E. servus* and *N. viridula* (Figures 4(b) and 4(c)). Differences among smellprints were analyzed by canonical discriminant analysis followed by cross-validation (Figure 5; Table 4). Treatment groups were separated mainly along the first canonical component, with smellprints from unexposed bolls located along positive values of the first component and exposed bolls along negative values (Figure 5). A greater separation was observed between control and exposed bolls than between bolls exposed to *E. servus* or *N. viridula* (Figure 5). Cross-validation of the dataset indicated that all treatment groups were separated with 70% accuracy, but smellprints from bolls exposed to either stink bug species were separated with much less accuracy (65%) than control and damaged bolls (87.5%) (Table 4).

4. Discussion

In this study, it was demonstrated that E-nose technology can readily detect and distinguish VOCs emitted from cotton bolls damaged by stink bugs under field conditions. Herbivory by *N. viridula* and *E. servus* resulted in a significant

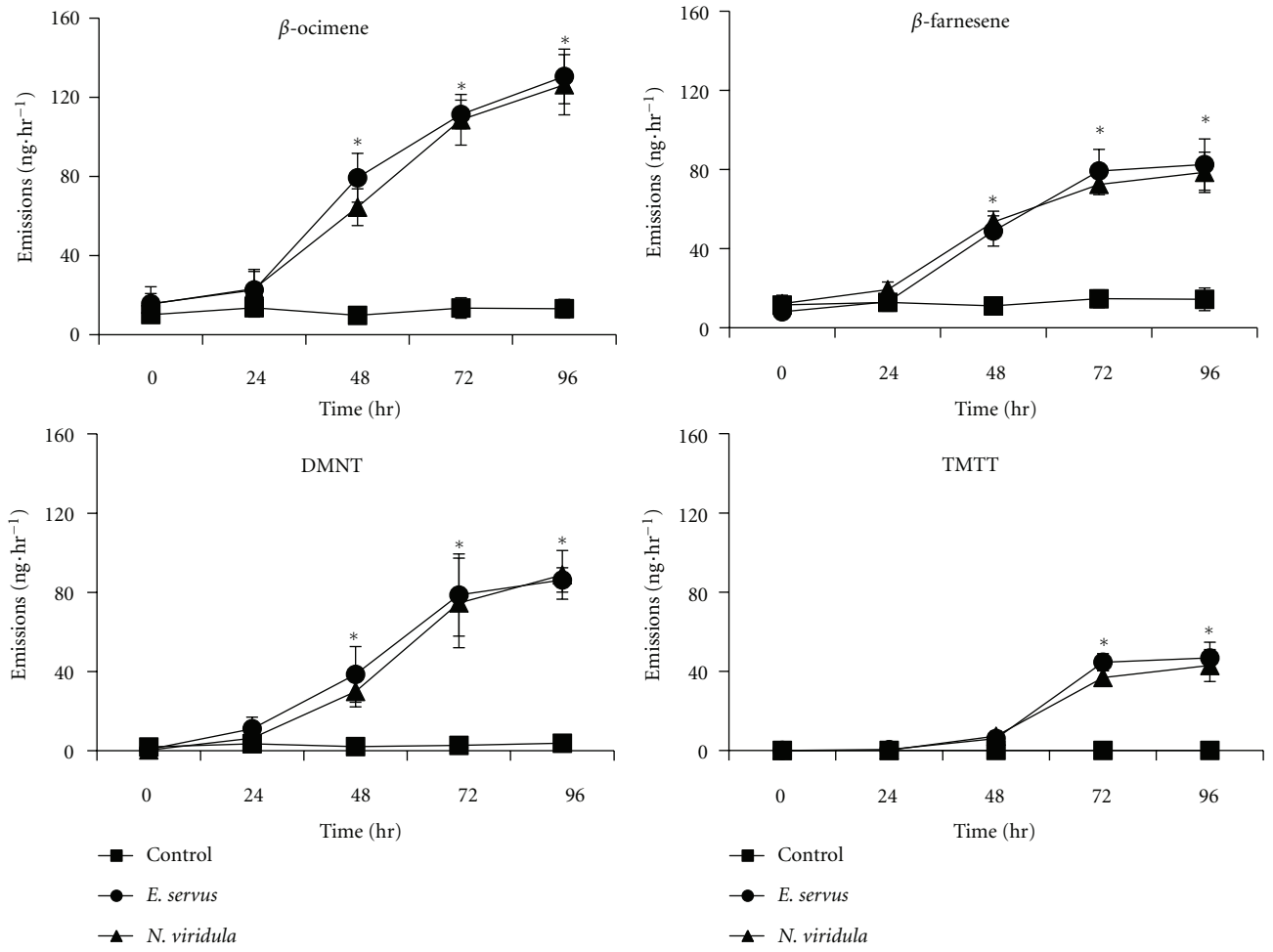

Figure 2: Temporal dynamics of induced VOC emissions from cotton bolls exposed to *Euschistus servus* and *Nezara viridula* and unexposed (control) bolls under field conditions. Data points represent mean VOC emissions (±1 SE; $n = 5$). Asterisks indicate significant difference ($P < 0.05$) between control and infested bolls.

Table 2: Cross-validation, training accuracy, and interclass Mahalanobis distance of training sets based on E-nose sensor response to headspace VOC emissions from 8 unexposed (control) bolls and 8 bolls exposed to *Nezara viridula*.

	Time		Identified as		Training accuracy	Interclass M-distance
			N. viridula	Control		
Trained as	24 hours	*N. viridula*	7	1	81.25%	2.339
		Control	2	6		
			N. viridula	Control		
	48 hours	*N. viridula*	8	0	100%	5.117
		Control	0	8		
			N. viridula	Control		
	72 hours	*N. viridula*	8	0	100%	5.125
		Control	0	8		
			N. viridula	Control		
	96 hours	*N. viridula*	8	0	100%	5.837
		Control	0	8		

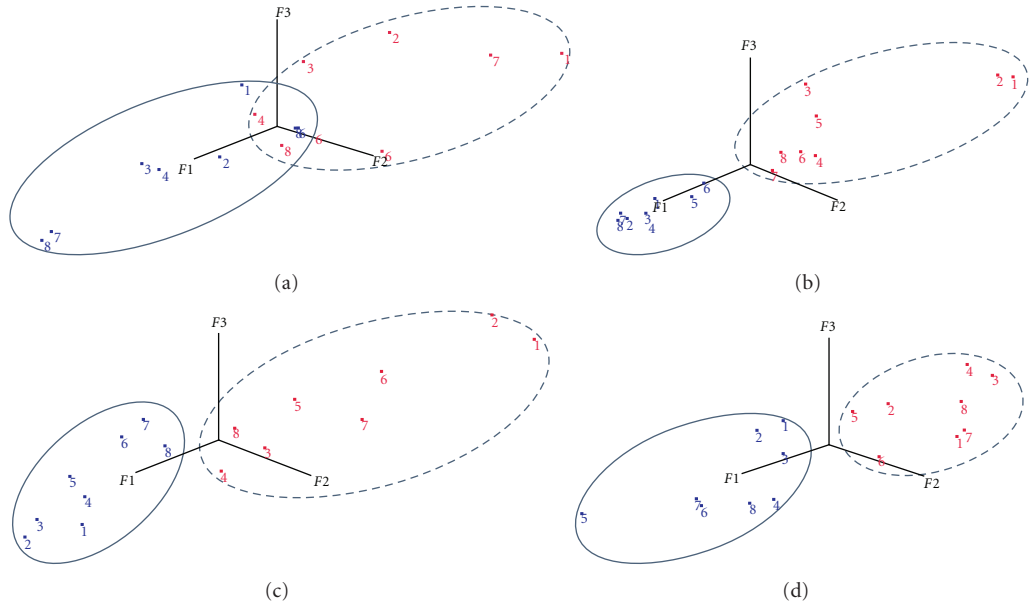

Figure 3: Principal components plot of E-nose sensor responses to headspace VOC emissions from unexposed cotton bolls (solid ellipsoids) and bolls exposed to *Nezara viridula* (dashed ellipsoids) after 24 (a), 48 (b), 72 (c), and 96 (d) hr of feeding.

Table 3: Accuracy of E-nose training sets used to identify 10 unexposed bolls (control) and 10 bolls exposed to *Nezara viridula* collected during a 96 hr feeding trial.

Time	Treatment	Correct[a]	Incorrect[b]	Identification accuracy
24 hr	*N. viridula*	5	5	50%
	Control	7	3	70%
48 hr	*N. viridula*	4	6	40%
	Control	9	1	90%
72 hr	*N. viridula*	9	1	90%
	Control	10	0	100%
96 hr	*N. viridula*	8	2	80%
	Control	10	0	100%

[a]Classification includes total number of bolls correctly identified by E-nose (damaged or control).
[b]Classification includes total number of bolls incorrectly identified by E-nose (false, confused, unknown).

Table 4: Cross-validation of the training set used to train the E-nose to recognize headspace VOC emissions from undamaged (control) bolls and bolls damaged by *Euschistus servus* and *Nezara viridula* following 3 days of feeding damage.

		Identified as		
		Control	*E. servus*	*N. viridula*
Trained as	Control	8	2	0
	E. servus	0	8	2
	N. viridula	1	4	5

increase in VOC emissions 24–48 hr after initial exposure, and temporal dynamics in VOC emissions were similar between the two species. Furthermore, E-nose was capable of accurately (90% success) identifying bolls damaged by stink bugs from undamaged bolls, and the degree of separation between treatments increased with increasing exposure of stink bugs. Finally, E-nose was much less accurate (65% success) at differentiating VOC emissions induced by different species of stink bugs.

In response to stink bug feeding injury, cotton bolls released VOC emissions in significantly greater quantities compared with controls between 24–48 hr of exposure. Herbivory by stink bugs has been shown to induce VOC emission from leaves of several plant species [14–16], as well as from cotton bolls [17]. This study provides a more detailed analysis of the timing of induced VOC emissions in response to stink bug feeding damage. Similar quantitative changes were observed in overall emissions as well as the emissions of specific volatiles in response to feeding by both *N. viridula* and *E. servus*, suggesting that induced VOCs were not species-specific for stink bugs, at least for the level of sensitivity in this experiment. The specificity of induced VOC emissions is determined in part by the feeding mode of herbivores [27, 28] but also by the presence of elicitors in the oral secretion of insects [29]. Oral secretions from stink bugs applied to leaves in the absence of physical injury caused a 2-fold increase in sesquiterpene emissions in corn seedlings [3], indicating that stink bugs contain bioactive compounds in their regurgitant that induces VOCs. The similarities of induced VOC emissions between bolls damaged by *N. viridula* and *E. servus* suggest that, in addition to similar types of physical damage from piercing-sucking mouthparts, these species may also contain bioactively similar elicitors.

E-nose was not capable of accurately discriminating between VOC profiles induced in response to feeding damage from closely related stink bug species. In a previous study, it was shown that E-nose could differentiate between the

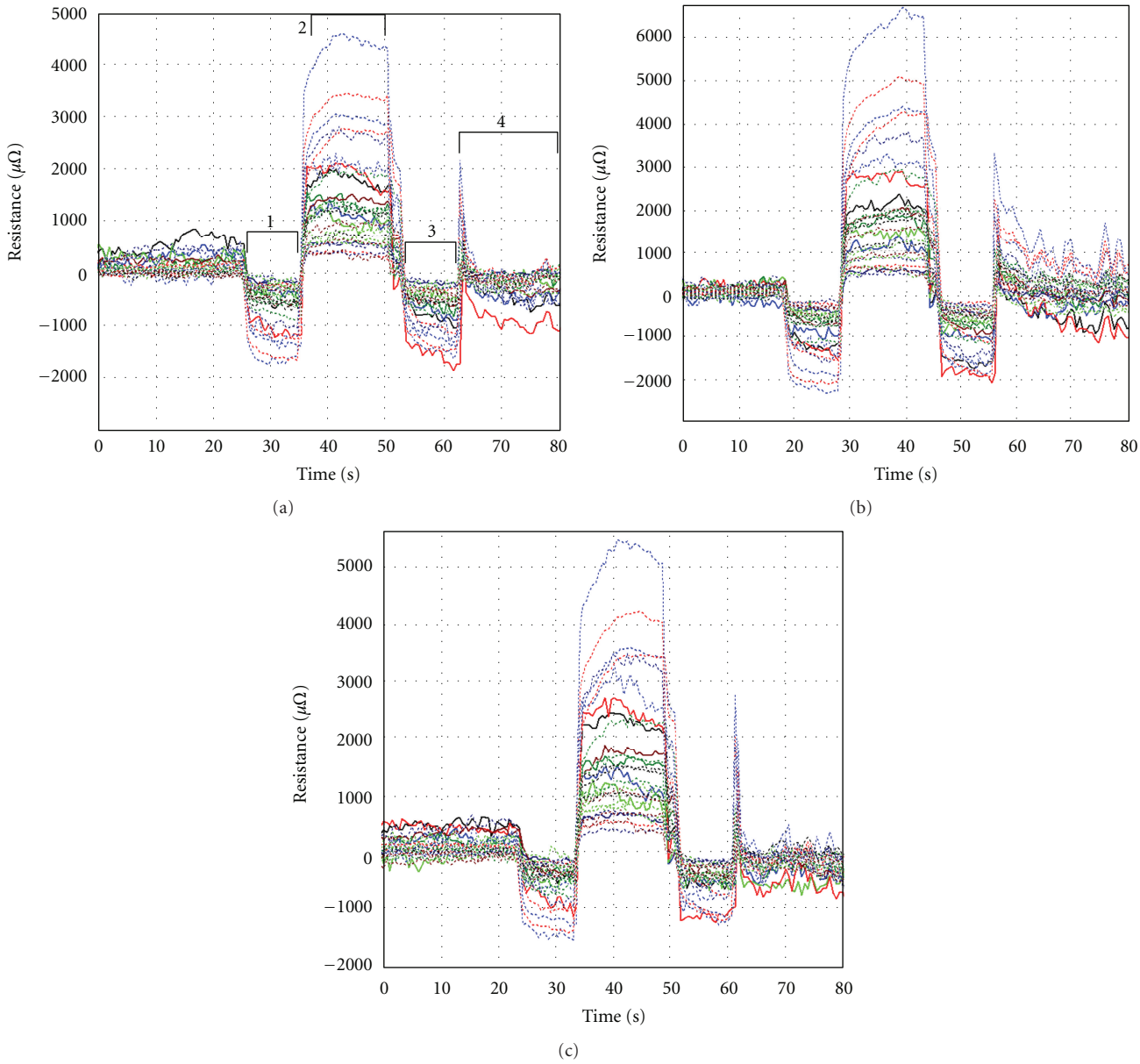

FIGURE 4: Representative E-nose sensor response patterns (smellprints) based on a 15 sec sample of headspace VOC emissions from unexposed (control) cotton bolls (a), or bolls exposed to *Euschistus servus* (b), and *Nezara viridula* (c) following 3 d of feeding damage. 1: baseline purge; 2: sample draw; 3: air purge; 4: sample purge.

defensive secretions released by *N. viridula* and green stink bug, *Acrosternum hilare* (Say) [24]. While E-nose technology was sufficiently sensitive to discriminate species-specific stink bug odors, the results presented here suggest that E-nose is not capable of accurately differentiating sources of damage based on plant VOCs induced by different stink bug species. This is most likely due to the lack of differences in VOC emissions from bolls damaged by *N. viridula* and *E. servus* detected in this study. These results are similar to those reported in a previous study, which indicated that VOC emissions were similar in response to feeding by three hemipteran species [17]. It has been suggested that sufficient specificity in VOC emissions may enable E-nose to detect particular host plant-pest interactions [23]. In a study investigating different types of damage to rice plants, it was demonstrated that E-nose could discriminate between damage by striped stem borer, *Chilo suppressalis* (Walker), and the rice brown plant hopper, *Nilaparvata lugens* (Stäl) [30]; however, these herbivores are from different feeding guilds (leaf chewer versus piercing sucking) and likely cause significant differences in VOC emissions due to differences in elicitors and physical damage inflicted during feeding [28, 31]. The results presented here indicate that feeding by different species of stink bugs does not result in sufficient

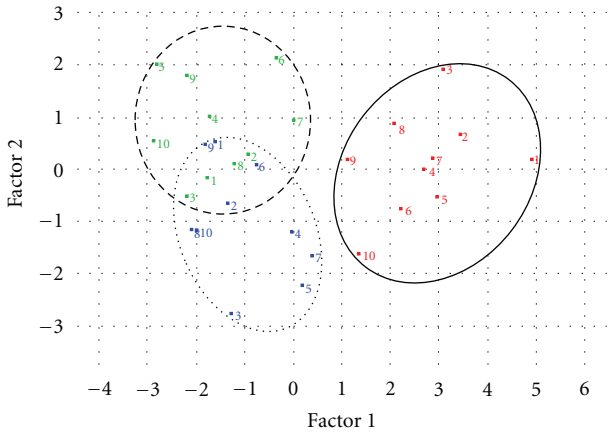

Figure 5: Canonical projection plot of E-nose sensor responses after training the E-nose to recognize VOC emissions from 10 unexposed (control) bolls (solid ellipsoid) and 10 bolls exposed to *Euschistus servus* (fine-dashed ellipsoid) and *Nezara viridula* (broad-dashed ellipsoid) following a 3 d exposure.

specificity in VOC emissions to allow E-nose to discriminate between species-specific sources of damage when the attacking herbivores have similar feeding modes.

While E-nose could not accurately differentiate between plant VOCs released in response to feeding by different species of stink bug, it was highly accurate when discriminating between damaged and undamaged bolls. Prolonged feeding exposure led to greater separation between treatments based on E-nose training sets. Training set data indicated that E-nose was capable of separating damaged and undamaged bolls with 100% accuracy. Identification of known (damaged or undamaged) samples based on E-nose training sets revealed that identification accuracy was consistently 10–15% less than training set accuracy over the course of the experiment. This is likely due to the modest separation of treatment groups as indicated by the low interclass M-distances. In previous research, it was demonstrated that E-nose was capable of identifying damaged bolls with 90% accuracy using bolls excised from plants and measured under laboratory conditions [24]. In this study, all tests were performed on intact plants under field conditions to more accurately demonstrate the potential of this technology as a nondestructive, in-field assessment tool for stink bug damage. In several cases, E-nose technology has been successfully applied to discriminate between healthy and pest-damaged plants [23, 24, 30], and, to our knowledge, this is the first study to demonstrate that E-nose technology is capable of accurately distinguishing between stink bug-damaged and undamaged bolls under field conditions based on a rapid sample of headspace VOC emissions.

While the results of this study are promising, it remains to be determined how the temporal dynamics in VOC emissions (and subsequently E-nose detection) observed from these feeding assays relate to natural variation in stink bug feeding dynamics. Under field conditions, cotton bolls are likely not injured by a single individual over 96 hr, but, rather, visited by one or multiple foraging stink bugs over time. As a result, VOC emissions under broader spatiotemporal variation in feeding dynamics may inhibit the ability to accurately distinguish damaged and healthy bolls using a predetermined training set. Whether induced VOC emissions reach significantly different levels from bolls exposed to much broader (and variable) spatiotemporal feeding dynamics from stink bugs and, subsequently, whether E-nose training sets are capable of differentiating those emissions remain to be determined. Preliminary data suggest that this may not be a major complication, as training sets based on 96 hr of feeding damage were successful in identifying stink bug damage in naturally infested fields (unpublished data); however, this is still under investigation.

The ability to differentiate pest injury based on brief samples of VOC emissions makes E-nose an attractive technology for monitoring stink bug feeding damage in cotton because it could potentially serve as a non-destructive monitoring tool, increase the accuracy of monitoring, and reduce the time and effort associated with current techniques. Further separation of treatment groups by E-nose could be achieved by optimizing sensor technology for the detection of stink bug induced VOCs. For example, sensor chemistries have been designed to respond specifically to VOCs induced by bark beetle attack [32]. In addition to specific sensor chemistries, research also suggests that longer durations of absorption and desorption cycles from E-nose may increase the ability to differentiate among treatments [23]. In this study, E-nose discrimination accuracy was achieved using standard sampling protocols that were not optimized specifically for detecting stink bug damage. Further sampling modifications and/or incorporation of VOC-specific sensor technology would likely improve the discrimination accuracy of E-nose. Nevertheless, the results of this study support the conclusion that E-nose is a promising technology for development of a rapid, nondestructive monitoring tool for stink bug feeding damage in cotton.

Acknowledgments

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Research Article

Reproductive Biology, Mating Behavior, and Vibratory Communication of the Brown-Winged Stink Bug, *Edessa meditabunda* (Fabr.) (Heteroptera: Pentatomidae)

Cleonor Cavalcante A. Silva,[1] Raul Alberto Laumann,[1] Jonatas Barbosa Cavalcante Ferreira,[1] Maria Carolina Blassioli Moraes,[1] Miguel Borges,[1] and Andrej Čokl[2]

[1] *Laboratorio de Semioquimicos, EMBRAPA Recursos Genéticos e Biotecnologia, Avenida W5 Norte (Final), 70770-900 Brasília, DF, Brazil*
[2] *Departament of Entomology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*

Correspondence should be addressed to Andrej Čokl, andrej.cokl@nib.si

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We describe different aspects of the reproductive biology, mating behavior, and vibratory communication of the pentatomid *Edessa meditabunda* (Fabr.). This species shows lower copulation frequency and reproductive potential with longer sexual maturation period compared to other species of pentatomids. Females with multiple mating show increased fecundity when compared with single-mated females and both increased fecundity and reduced longevity when compared with virgin females. Courtship and mating behavior and vibratory signals are typical and similar to what was observed in other species of pentatomids, except that males started the courtship. These results constitute the first paper on biology, behavior, and vibratory communication among species of the subfamily Edessinae.

1. Introduction

The brown-winged stink bug, *Edessa meditabunda* (Fabr.) (Heteroptera: Pentatomidae), has been reported as a minor component of the stink bug species complex that is economically important pest in many crops mainly of families Solanaceae and Leguminosae [1]. In Brazil, *E. meditabunda* is present in the central-west and southern states [2–4]. Although *E. meditabunda* has been reported on a large number of plant families [5–7], soybean plants (*Glycine max* L.) seem to be a preferred host for adult feeding and reproduction [2].

The life cycle of *E. meditabunda* has been reported by several authors [8–11], however, with no detailed information about the reproductive and sexual behavior of the species.

Reproductive behavior of pentatomids is mediated by communication signals of different modalities among which sex pheromones are involved in female-male encounters, courtship, and matting [12–16]. Sex pheromones of stink bugs show high variability in the chemical structure, but in some cases, as in *Nezara viridula* (L.) and *Chinavia* spp. the pheromones consist of isomers of the same compound (trans and cis (*Z*)-bisabolene epoxide) in different ratios [16]. Communication with vibratory signals transmitted through the plant is used among others for mate location and recognition [17–20]. Communication with species-specific substrate-borne vibratory signals as the key element of mating behavior has been demonstrated in different species of Heteroptera, for example, in *Nezara viridula* [21], *Acrosternum hilare* (Say) [22], *Lygocoris pabulinus* (L.) [23], *Chinavia impiciticornis* (Stål) (= *Acrosternum impicticorne*), *Euschistus heros* (Fabr.), *Piezodorus guildinii* (Westwood), *Thyanta perditor* (Fabr.) (Heteroptera: Pentatomidae) [17], and many other species [19].

No data on sex pheromones or communication signals of any other modality and general mating behavior among species of Edessinae have been described. Therefore, we investigated different aspects of *E. meditabunda* reproductive biology, mating behavior, and communication signals.

2. Material and Methods

2.1. Insect Rearing. Adults of *E. meditabunda* were collected from soybean fields in Brasilia, DF, Brazil, and maintained in a colony in the Semiochemicals Laboratory at Embrapa Genetic Resources and Biotechnology (Brasilia, DF). The insects were reared in plastic cages (8 L) and fed on bean pods (*Phaseolus vulgaris*), soybean pods, and stalks of "boldo-brasileiro," *Plectranthus barbatus* (Lamiaceae). Stalks of *boldo* were placed in 7.5 cm high × 7.5 cm diameter plastic containers, half-filled with vermiculite (sterilized at 120°C for one hour), and sprayed with tap water to simulate a wet soil conditions to the plants and provide humidity.

The containers were placed inside the rearing cages (one vial/cage), which were kept in a climatic room at 26 ± 1°C, 60 ± 10% RH, and photophase of 14 L : 10 D. Twice a week the containers were sprayed with tap water to keep the vermiculite moist and the plants replaced every week. Most of the eggs were oviposited on leaves of *boldo*, and, after oviposition the eggs mass attached to the leaves were transferred to new rearing cages. Egg masses were examined daily for hatching and molting, and nymphs were fed in the same way as adults. After completing the immature development (<24 h after the final molt), adults were collected and managed as described above for the experiments and for colony maintenance.

2.2. Sexual Maturity and Longevity of Males and Females. To determine the sexual maturation age and longevity for each sex separately, one virgin male (1 to 2 days after the final molt) was placed together with three 15-day-old females in plastic cup (500 mL) and fed as previously described. Subsequently, one virgin female (1 to 2 days after the final molt) was placed together with three 15-day-old males. The groups ($N = 10$ for each adult combination) were observed daily every 30 min between 13:00 and 18:30 h that was the time interval when major frequency of copulation was previously observed. When a female or a male of each group (3 females or 3 males) dies, it was replaced by a new insect at the same age.

2.3. Mating Frequency Experiments

(1) Multiple-Mated Females. One virgin female and one virgin male (<24 h after final moult) were placed into plastic pots of 500 mL ($N = 20$) to record the mating sequence. The insects were observed daily every 30 min during the period of higher frequency of copula (determined in previous observations) during the photophase (from 8:30 to 18:30 hours). The couples were allowed to mate throughout their lifetime. For this group of females, only those observed in copula at least two times ($N = 18$) during their life span were considered for data analysis.

(2) Single-Mated Females. One virgin female and one virgin male (<24 h after final moult) ($N = 20$) were placed into plastic pots of 500 mL ($N = 20$) and observed every 30 min for the first mating (from 8:30 to 18:30 hours). Once mating had finished males were removed and females were observed daily until they died. Only females observed in copula ($N = 15$) were included in data analyses.

(3) Virgin Females. Single virgin females (<24 h after final moult) were isolated in plastic pots of 500 mL ($N = 20$) and observed for egg production and longevity.

Insects of the different treatments were fed as described above. Reproductive parameters (number of egg mass/female, number of eggs/mass, number of eggs/female, and number of nymphs/number of eggs (fertility)) were recorded for each tested female. The duration of copula was estimated from couples that start and finish the mating during the observation period (from 8:30 to 18:30 hours). We measured the time to start oviposition (i.e., the preoviposition period), the intervals between the first mating and first oviposition, the time between consecutive ovipositions, the total reproductive period (time between first and last oviposition), and the females longevity.

2.4. Courtship Behavior and Vibratory Communication. The behavioral sequences of courtship and mating in *E. meditabunda* were observed simultaneously with the records of male and female vibratory signals (Figure 3). Insects were separated by sex in the first 24 hours after the final moult and maintained in different rooms until they reach the sexual maturation. A pair of virgins and sexual mature male and female (15–20 days old) was placed on the membrane of a 10 cm diameter low-midrange loudspeaker (40–6000 Hz frequency response, 8′Ω impedance; RadioShack, Taiwan). An acrylic box (9 cm diameter × 4 cm high) was placed over the speaker without contacting the loudspeaker membrane to prevent the insects moving away from the membrane surface. The loudspeaker was placed into a sound-insulated room to decrease environmental noise. All observations were conducted between 13:00 and 18:30 when most of the mating activities have been previously observed. If the insects did not display any courtship behavior within 20 minutes of the observations, they were classified as failed courtship. In this experiment, 62 couples were observed. To describe the courtship and mating behavior, the previously determined behavior categories in pentatomids were used [24, 25] (Table 1). The sequence of behavioral categories was registered in each observed couple.

The vibratory signals captured by the loudspeaker were amplified by a home-made operational amplifier TL081CN, digitized (Aardvark-Direct Pro 24/96 (Aardvark Computer Systems, Washington, USA), and stored on a computer using Cool Edit Pro software (Syntrillium Software 2001—Fort Wayne, Indiana, USA). Signals were followed in real time with headphones and recorded until the insects stopped singing.

Vibratory signals were analyzed by the Sound Forge 4.5 software (Sonic Foundry http://www.sonicfoundry.com/). A pulse was defined as a unitary homogeneous parcel of vibration of finite duration [26]; pulse trains as repeatable and temporally distinct groups of pulses and a song as a sequence of pulses and/or pulse trains with distinct beginning and end.

Table 1: Courtship sequence performed by *Edessa medidabunda* described in the ethogram.

Code of the behavior	Description of the behaviors
MFRG	Males and females at rest and grooming
MS1	Males spontaneously emit song type 1
MS2	Males spontaneously emit song type 2
MstS1	Males interrupt the emission of MS1
Mw	Males walk around the arena
FS1	Females emit song 1 in response to MS2
D	MS2/FS1 duet
MApFf	Male approaches the female from the front
MApFb	Male approaches the female from the back
MAnFAn	Male antennates female's antennae
MAnFt + p	Male antennates female's thorax and moves to the posterior side of her abdomen
Mb180° + RT	Male puts the head behind the female abdominal tip and butt her abdomen until she adopts the copulatory position At the time turning 180° from the female posterior, the insects are oriented end to end in copulatory position
Pr + 180°	Male rotates its pygophore 180° so that it is inverted
PM	Pair mate
MRF	Male rejects female and walks away from her
FRM	Female rejects male and runs away from male
NM	Pairs not mate

Spectra were described by the dominant, first harmonic, and other subdominant frequency peaks, by the spectral width 20 dB below the amplitude of dominant frequency value and by frequency modulation described as downward or upward-orientated frequency sweeps quantified by the frequency difference per signal duration (Hz/s). Songs were classified, according to their order of appearance in a duetting couple [22].

2.5. Statistical Analyses. Reproductive parameters were analyzed by generalized linear models (GLM) and deviance analyses (ANODEV). The models have a factor for treatments and Gaussian distribution of errors for time variables, Poisson distribution of errors for fecundity parameters (number of egg masses/female number of eggs/mass and number of eggs/female), and binomial distribution of errors for fertility (number of nymphs/number of eggs). Contrast analyses were used to multiple comparisons of means. To test the relation between successive mating and cumulate fecundity, a linear model was used, with the cumulate fecundity (cumulate numbers of eggs/female) as dependent variable.

Data from observations of all courtship sequences were used to create a first-order Markovian behavioral transition matrix of total frequency of transitions (i.e., moving from one behavioral step to the next). The repetition of a single behavior (self-transition) was not included in the records to avoid the possible influence in the relative weight of transitions between behaviors. Transition probabilities were calculated from the observed frequency of a transition between two events divided by the total number of occurrences of the first event [27]. The expected values of the matrix cells were found using the iterative proportional fitting method of [28], and the statistical significance of the individual transitions were evaluated using a log-likelihood ratio test (G test) and the results presented graphically in the ethogram. Data are shown as means ± SD, together with the number of signals analyzed (N) and the numbers of individuals (n).

3. Results

The age at which females reached the sexual maturity (18.08 ± 1.26 days) was estimated from the first copulation of virgin females maintained with old males. It was significantly different from the age at which males reached the sexual maturity (15.92 ± 0.86) (estimated from first copulation of virgin males maintained with old females) (ANODEV χ^2_1 = 30.15, $P < 0.001$).

Multiple mating with the same male increased female's fecundity of *E. meditabunda* (ANODEV χ^2_2 = 51.95 $P <$ 0.001) but not its longevity (Table 2). The reproductive period also increased ($\chi^2_2 = 651.1$ $P < 0.001$) with the increase of the number of copulations (Table 2). For multiple-mated females, we observed 3.92 ± 0.79 copula (N = 20) with duration of 222.55 ± 60.02 min (N = 11) and intervals between copulations of 6.35 ± 3.8 days. The pre-oviposition period of virgin- and single-mated females was significantly longer when compared with multiple-mated females (ANODEV $\chi^2_2 = 81.54$ $P < 0.001$) (Table 2). However, the interval between the first mating and first oviposition was similar for both groups of mated females and females with multiple mating showed shorter intervals between consecutive ovipositions (Table 2). Females with multiple mating laid a higher number of egg mass than did virgin and once-mated females (ANODEV χ^2_2 = 86.51 $P <$ 0.001)

Table 2: Effect of mating frequency on the reproductive biology and longevity of *Edessa meditabunda* females in the laboratory.

	Preoviposition period (days)	Days between 1° mating and 1° oviposition	Days between consecutive oviposition	Number of egg mass/female	Number of eggs/mass	Total eggs/female	Reproductive period (days)	Fertility[b] (%)	Female longevity (days)
Multiple-mated females	18.50 ± 2.06^{a} ($N = 20$)	1.35 ± 0.48^{a} ($N = 20$)	6.26 ± 3.02^{a} ($N = 95$)	5.70 ± 1.17^{a} ($N = 20$)	13.50 ± 0.79^{a} ($N = 115$)	77.65 ± 16.11^{a} ($N = 20$)	30.45 ± 10.22^{a} ($N = 20$)	96.11 ± 9.70^{a} ($N = 115$)	56.65 ± 7.51^{a} ($N = 20$)
Single-mated females	21.27 ± 1.53^{a} ($N = 15$)	1.53 ± 0.74^{a} ($N = 15$)	9.21 ± 2.67^{b} ($N = 14$)	2.07 ± 0.70^{b} ($N = 15$)	$13.\ 32 \pm 1.88^{a}$ ($N = 31$)	26.87 ± 9.30^{b} ($N = 15$)	9.14 ± 5.17^{b} ($N = 15$)	95.16 ± 13.47^{a} ($N = 31$)	57.87 ± 6.32^{a} ($N = 15$)
Virgin females	21.25 ± 2.76^{a} ($N = 9$)			0.70 ± 0.77^{c} ($N = 17$)	9.45 ± 2.58^{b} ($N = 11$)	5.78 ± 6.44^{c} ($N = 18$)	1.22 ± 0.44^{c} ($N = 19$)		64.50 ± 9.53^{b} ($N = 18$)

Letters in each column followed by the same letter are not significantly different (ANODEV and contrast analyses $P > 0.05$).

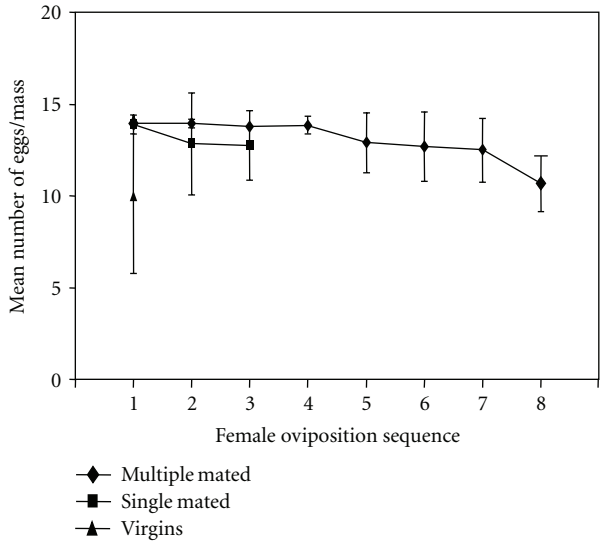

Figure 1: Mean number of eggs oviposited by *Edessa meditabunda* females in successive ovipositions. Multiple-mated females: females maintained with males during all their reproductive life. Single-mated females: females maintained with males until complete one mating. Virgins: females maintained isolated of males during all their reproductive life. Vertical lines indicate the standard deviation of the means.

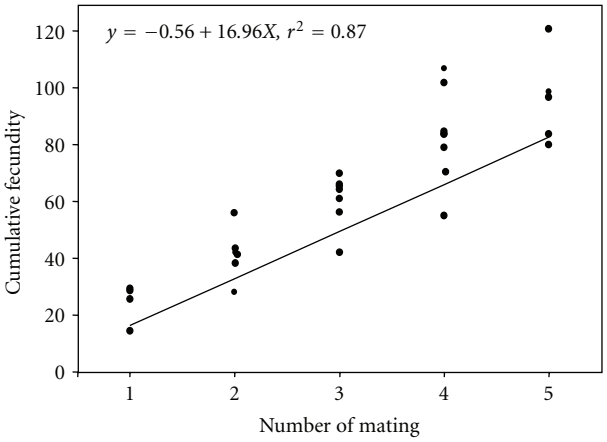

Figure 2: Relation between number of mating and cumulative fecundity (cumulated number of eggs/female after successive mating). A linear model was adjusted to the variables. Points indicate the cumulative number of eggs of each individual observed and line the values estimated from the adjusted model.

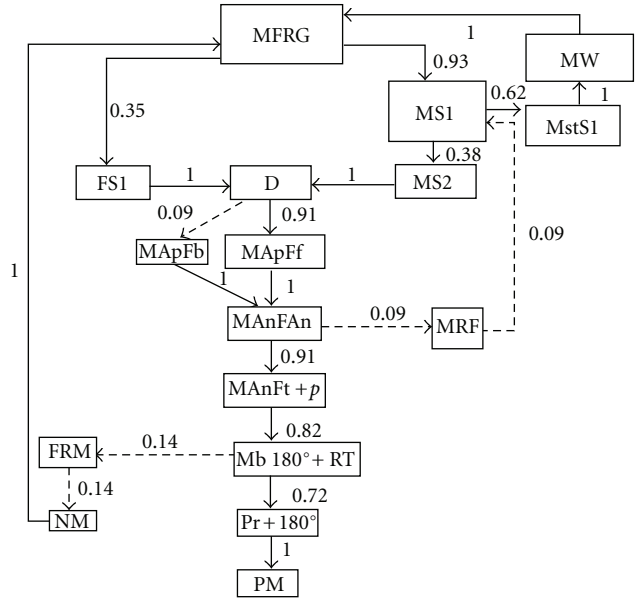

Figure 3: Sequence of courtship and mating behavior of *Edessa meditabunda*. Values represent the probability of transitions between behaviors. Solid-line arrows indicate the significant transitions ($P < 0.05$) and dashed-line arrows not significant transitions ($P > 0.05$). Boxes representing behavioral categories are in relative size to the frequency-observed behaviors from 62 pairs (male and female) observed. Codes for behavioral categories are listed in Table 1.

(Table 2). However, there was no significant difference in the number of eggs per mass in multiple- or single-mated females (Table 2) but a significant difference in the number of eggs/mass from these groups of females in comparison with the lower number determined in virgin females (Table 2).

Most eggs were laid at night in two rows of approximately seven eggs per row, generally, under the surface of *bold* leaves and never on the smooth surface of the cages. Despite the increased fecundity of multiple-mated females there was no significant difference in the fertility (number nymphs/number of eggs) of the single- or multiple-mated females (Table 2). Thus, multiple mating was required for fecundity but not for egg viability.

Despite of higher fecundity, the number of egg/mass was constant and no effect of female age was observed for multiple- or single-mated females; the number of eggs/mass did not show significant differences in consecutive ovipositions in these groups of females (ANODEV $\chi^2_7 = 4.08$ $P = 0.77$ for multiple-mated females and $\chi^2_2 = 0.64$ $P = 0.72$ for single-mated females) (Figure 1). In the same way and for multiple-mated females, the cumulative fecundity (mean number of cumulative eggs oviposited by females) after one to five mating shows a significant linear increase ($F_{1-67} = 443.6$ $P < 0.001$ $r^2 = 0.87$) (Figure 2).

At close range (i.e., below 10 cm between mates), successful copulation followed the usual behavioral steps described until now in most stink bugs as resting, grooming (i.e., rubbing the antennae, thorax, or abdomen with a par of leg), approaching, antennation during male-female interaction, abdominal vibration, genitalia contact, and copulation [15]. Courtship was initiated by male approaching the female by emission of vibrational signals before any physical contact, indicating that at close range vibratory signals are involved in the first encounter. The courtship steps are characterized and coded in the Table 2, and their transitional probabilities are shown in the ethogram (Figure 4).

Vibratory communication started with the emission of the first male song (MS1) (Figure 4(a)). This song was produced when a male was alone in the arena, in the presence of a

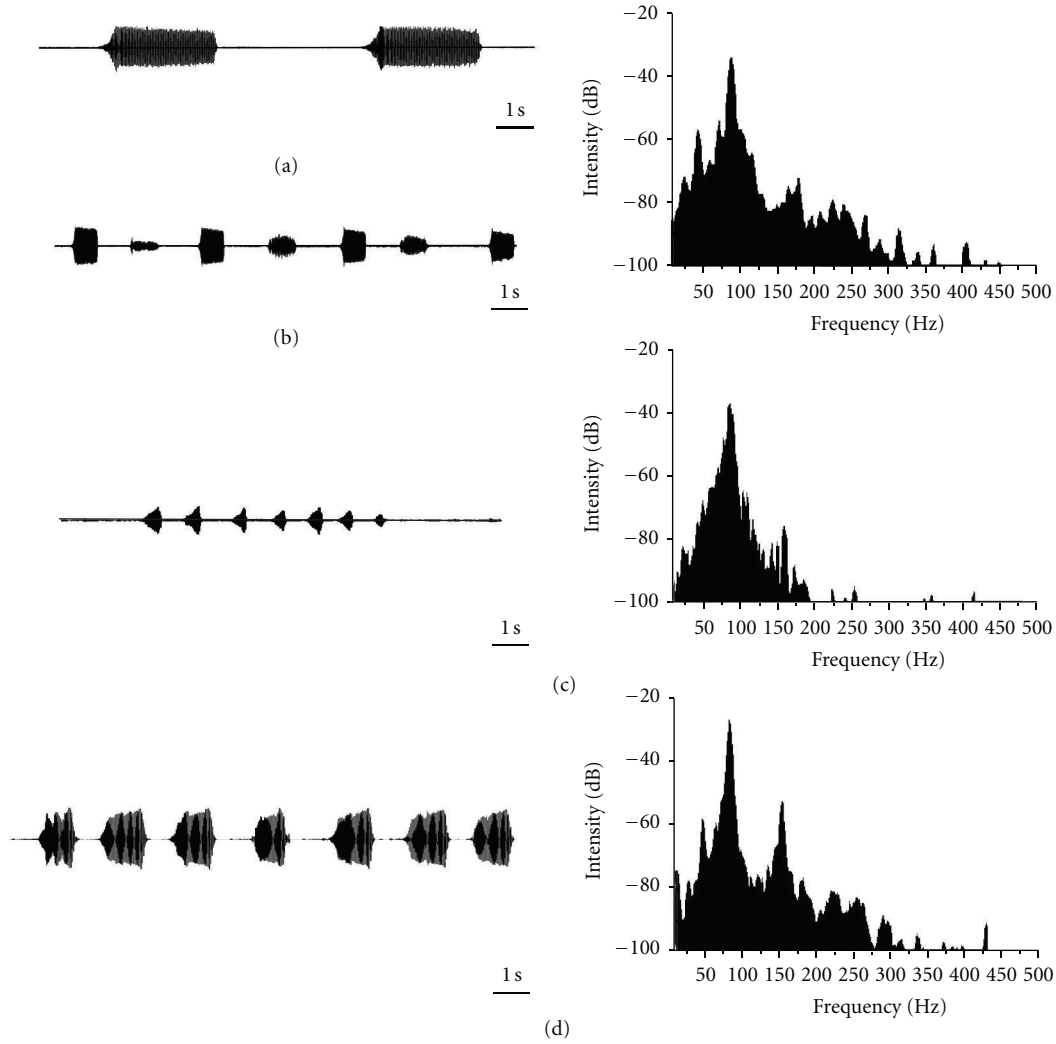

Figure 4: Oscillogram and frequency spectrum of one pulse of *Edessa meditabunda* vibratory songs: (a) male song 1 (MS1); (b) male rivalry song (MR); (c) male song 2 (MS2); (d) female song 1 (FS1).

female or as response to a female song. During courtship, the MS1 may be changed to MS2 or interrupt it for some minutes (Figure 4(c)). In response to MS2 females emitted their first song (FS1), and, after the emission of some pulses, the couple started to exchange FS1 and MS2 first as intercalated and later with superimposed pulses (Figure 4). After some seconds of duet song emission, the male approached the female and started to antennate her body and antennae. The female responded with a slow antennation of male's head and antennae. Subsequently, the male antennated the posterior end of female's abdomen. During this mutual stimulatory antennation phase, both proceeded with the FS1 and MS2 song emission. In the final courtship step, both mates stopped singing, and the male lifted the female with the head to get the copula position, then turned its body 180° to female's back and its phygophore, so that male and female in copula faced each other in opposite direction, and finally the copulation occurred (Figure 4). We recorded no vibratory emissions of copulated male and female. The male rival song (MR) was emitted when two males were placed with a female in the arena, these males emitted pulses similar (same temporal and spectral parameters) to MS1 pulses in an alternated a-b-a-b-a-b fashion. The pulses of one of the males were emitted with higher amplitude, until the other was silenced (Figure 4(a)).

Vibratory signals showed typical temporal and spectral characteristics described until now for most Pentatominae species (Figure 4, Table 3). The male song MS1 was a typical calling song, with relatively long pulses (~1 sec in duration) that may be repeated for several minutes. The dominant frequency and band width were also in the typical range of pentatomid vibratory signals (Table 3). The second song, MS2, was composed by a pulse trains of 5 to 11 short pulses with similar frequency characteristics as those of the MS1 (Table 3). Females emitted only one song type (Figure 4(d)) with specific temporal parameters and similar dominant frequency and band width as males' songs (Table 3). FS1 showed a clear frequency modulation with the dominant

Table 3: Temporal and spectral characteristics of male and female songs of *Edessa meditabunda*. Mean ± SD are show with the number of signals test (N) and the number of insect tested (n).

Song	Duration (ms)	Repetition time (ms)	Dominant frequency (Hz)	Bandwidth −20 dB (Hz)
MS1	1666,74 ± 147,38 (76/11)	4590,09 ± 1834,26 (65/11)	73,75 ± 2,60 (76/11)	20,08 ± 4,77 (76/11)
MS2	175,71 ± 83,91 (75/12)	305,97 ± 210,92 (64/12)	80,27 ± 5,68 (75/12)	57,87 ± 19,39 (75/12)
FS1	433,04 ± 78,81 (80/11)	677,19 ± 104,48 (80/11)	76,93 ± 5,82 (80/11)	17,73 ± 7,29 (80/11)

MS1: male song 1, MS2: male song 2, FS1: females song 1.

frequency decreasing throughout the duration of each pulse (Table 3). The duet emission of MS2 and FS1 shows a clear courtship function initiating all the sequential behaviors that lead to copula.

4. Discussion

Results presented here are the first report on the *E. meditabunda* reproductive biology, behavior, and vibratory communication. Adults of *E. meditabunda* showed lower copulation frequencies, reproductive potential, and longer sexual maturation period if compared with other pentatomid species reared under similar [29, 30] or different [25, 31, 32] laboratory conditions. The preoviposition period (time between emergence and first egg mass) for females with one or multiple matting was also longer when compared with other pentatomids [29, 32].

Repeated mating increased female fecundity in *E. meditabunda*, but it was costly in terms of reduced longevity compared with virgin females. Studies with some pentatomids reported similar correlation as a result of mating frequency [29, 32–34]. Decreased longevity and increased reproduction associated with multiple mating have been also reported for other insect species, like *Coccinella septempunctata* (L.) and *Propylea dissecta* (Mulsant) (Coleoptera: Coccinellidae) [35]. This seems to be the general negative cost of multiple mating [36].

Arnqvist and Nilsson [36] suggested that, in many species, especially among insects, exists an optimal remating rate for females so that one or a few matings are necessary to increase the offspring production. In such a way, additional matings are not necessary. Results of works with Heteroptera showed that in some species exists an optimal number of mating [31, 32, 37, 38] but in others not [39, 40]. In *E. meditabunda*, multiple-mated females have increased fecundity but did not show any fecundity peak along successive mating.

The fecundity of multiple-mated *E. meditabunda* could be considered low when compared with previous studies [11] or with data on other stink bug species [30, 31]. The low fecundity could be a characteristic of the central Brazil populations and may be related to the reproductive biology of the insect, since both adults showed long sexual maturation period and females a short period of oviposition. This may be the one of the reasons for the low population densities of *E. meditabunda* observed in the field.

At close range, the main behavioral steps of courtship in *E. meditabunda* did not differ from those observed for other species of pentatomids [17, 23–25, 31], except that, in all couples that emitted vibratory signals, males started the courtship by approaching females, antennate them, and emit vibratory signals. The sequence of steps was highly stereotyped, suggesting that once a male starts the courtship, the subsequent steps will most likely follow. Copulation was successful when the female remained stationary after the first contact. High percentage of courted females (53.06%) refuses copulation and run away from males during the antennation phase of courtship behavior. A similar failed courtship behavior was observed in *Murgantia histrionica* [25]. This fact could be a characteristic of the male selection behavior of females during courtship in some species of stink bugs or an effect of the artificial arenas used in the experiments.

The temporal and spectral patterns of vibratory signals of *E. meditabunda* were similar to the characteristics described previously for species of Pentatominae. However, some differences were found in the emission of signals and in the songs repertoire. In most Pentatominae species, two or three different male and female songs have been described [17–19] with calling and courtship songs of different temporal and spectral characteristics. In *E. meditabunda*, the repertoire of signals appears to be less complex with just one female and two male songs. The MS-2 has been emitted in the calling and courtship phase of the reproductive behavior. In most until now investigated Pentatominae species vibratory communication starts by female songs that trigger males to sing and move towards her [17–19]. The absence of a female song initiating the vibratory communication in *E. meditabunda* could be a characteristic of vibratory communications in Edessinae or could be related to the chemical communication in this species.

As sex pheromones of *E. meditabunda* was not identified, vibratory communication could have a central role to the sexual behavior in this species and males could use the vibratory signals to attract females.

Further observations on a plant are needed to confirm behavioral data and the role of communication signals of other modality described in this study for couples mating on a loudspeaker membrane. A possible influence of the size of arena on the vibratory communication cannot be discarded. Because the communication on stink bugs normally start on plants at distances of several cm (sometimes reaching 1 m or more) [18, 19], the reduced dimensions of the arenas used in our experiments (9 cm) could influence or inhibit the emission of some signals. In addition, the male calling song seems to act also as a rivalry song when a second male is present as reported by Shestakov [41] for Asopinae bugs.

Results here presented describe the mating biology, behavior, and vibratory signals of *E. meditabunda*. Multiple

mating showed to be advantageous for *E. meditabunda* females. During courtship, *E. meditabunda* communicates with signals produced by abdominal vibration. Songs are similar to those of other stink bugs studied with the exception that the courtship is initiated by males rather than by females as reported for other stink bugs.

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Review Article

Sex Pheromones of *Stenotus rubrovittatus* and *Trigonotylus caelestialium*, Two Mirid Bugs Causing Pecky Rice, and Their Application to Insect Monitoring in Japan

Tetsuya Yasuda and Hiroya Higuchi

National Agriculture and Food Research Organization, Agricultural Research Center (NARC), Tsukuba, Ibaraki 305-8666, Japan

Correspondence should be addressed to Tetsuya Yasuda, tyasuda@affrc.go.jp

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Two mirid bugs, *Stenotus rubrovittatus* and *Trigonotylus caelestialium* (Heteroptera: Miridae), are important pests that infest rice crops in many regions of Japan. Males of each species were attracted to traps baited with conspecific, unmated females. Hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal were identified as possible female-produced sex pheromone components for *S. rubrovittatus*, whereas hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate were found to be sex pheromone components for *T. caelestialium*. Pheromone doses and ratios were optimized for attraction of males of each species. Sticky traps set up close to or below the top of the plant canopy were optimal for monitoring these species, and trap catches were almost constant when traps were placed 7 or more meters in from the edge of a paddy field. Mixed lures, in which the six compounds from both species were loaded onto a single septum, or separate lures for each species, deployed in a single trap, were equally effective for monitoring both species simultaneously.

1. Introduction

The sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (Figure 1(a)) and the rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae) (Figure 1(b)) are major pests of rice, *Oryza sativa* L., in Japan [1]. They reproduce on graminaceous plants and invade rice fields after rice plant heading. Damage from bug feeding causes stained grains or kernel spotting, known as pecky rice (Figure 2) [2, 3]. Pecky rice contamination in brown rice, even in very small amounts (more than one stained grain per 1,000 brown rice grains), reduces rice quality under the Japanese rice quality regulations. This reduction in rice quality resulted in a price reduction to farmers of 8–16% in 2010. Damage due to heteropteran bugs has occurred in 30% of rice cultivation areas in Japan since 1999, and the total area of rice fields requiring pecky rice control currently amounts to 1,700,000–1,900,000 ha [1]. The range of *S. rubrovittatus* in Japan has been spreading since the 1990s [4], and this bug is now distributed from the southern part of Hokkaido to Kyushu [1]. *Trigonotylus caelestialium* is found in most parts of eastern Japan, but its damage to rice occurs mainly in the northern part of Japan.

Sweeping of vegetation with an insect net is one of the conventional methods of surveying for insect pests in rice. However, this is a time- and labor-intensive method and requires some knowledge and experience to determine the types of insects captured. In contrast, pheromone-baited traps are easy to use and can provide similar data on seasonal population dynamics and densities of specific species. Species-specific pheromone traps also eliminate the need for specialized training to detect and identify the target insects.

Attractant pheromones have now been identified for three major true bug species that cause pecky rice in Japan: *Leptocorisa chinensis* Dallas (Heteroptera: Alydidae) [5] and the two mirid bugs which are the subject of this paper. Here, we summarize the identification of the sex pheromones for these two mirid species, and the testing of their pheromones for insect monitoring.

(a) Sorghum plant bug *Stenotus rubrovittatus* (b) Rice leaf bug *Trigonotylus caelestialium*

Figure 1: Sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (a) and rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) (b) (Heteroptera: Miridae).

2. Sex Pheromones of the Mirid Bugs, *Stenotus rubrovittatus*, and *Trigonotylus caelestialium*

2.1. Mate Attraction and Mating Behavior. In the true bug family Miridae, orientation of males to conspecific females has been observed in several species [6–12], including *T. caelestialium* [13] and *S. rubrovittatus* [14]. In *T. caelestialium* and *S. rubrovittatus*, males were attracted only to conspecific females, and females were not attracted by either sex [13, 14]. These results indicated that females of these species probably produced female-specific sex attractant pheromones.

In *S. rubrovittatus*, courtship behavior by males consists of four steps: approaching a female, antennation (touching with the antennae), grasping (holding with the antennae), and mounting [15]. Male *T. caelestialium* exhibits similar behavioral steps, except for antennation [13]. In both species, calling behavior in females, as observed in another mirid bug, *Campylomma verbasci* [8], was not observed [13, 15].

2.2. Identification of Sex Pheromone Components

2.2.1. Stenotus rubrovittatus. Whole-body extracts of *S. rubrovittatus* females were attractive to conspecific males [16], and 16 peaks were detected from hexane extracts of whole female bodies by coupled gas chromatography-mass spectrometry (GC-MS) analysis (Table 1) [16]. The three most abundant components elicited responses from antennae of male bugs in gas chromatography-electroantennographic detection (GC-EAD) analyses (Figure 3) [17]. These three compounds were identified as hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal (Figure 4(a)). When the attractiveness of a 100 : 40 : 200 (μg) combination of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal and subsets thereof were examined, a few males were attracted to the binary blend of hexyl butyrate and (*E*)-2-hexenyl butyrate, and no males were attracted to lures lacking either hexyl butyrate or (*E*)-2-hexenlyl butyrate [18]. Significantly more

Figure 2: Damaged (pecky rice; top row) and nondamaged (bottom row) brown rice grains.

S. rubrovittatus males were caught in traps baited with the three-component blend ($P < 0.05$) than in unbaited controls [18]. Extracts of female *S. rubrovittatus* contained at least 13 minor components, but lures impregnated with female extracts were no more attractive to males than the three-component blend [16], and none of the minor components enhanced the attractiveness of the lure when added individually to the three-component blend. These results suggest that the minor components, in the amounts found in the extracts of females, are not part of the sex attractant pheromone [16].

2.2.2. Trigonotylus caelestialium. Whole-body hexane extracts of *T. caelestialium* females were not attractive to conspecific males [19], even though live females had been shown to attract males. This suggested that attraction of males to extracts was being masked by other components of the extracts. Thus, extracts were fractionated by liquid chromatography on Florisil, successively eluting with hexane and 5%, 15%, 25%, and 50% ether in hexane. Ten components in the 5% ether in hexane fraction elicited responses from antennae of conspecific males in GC-EAD analyses [20].

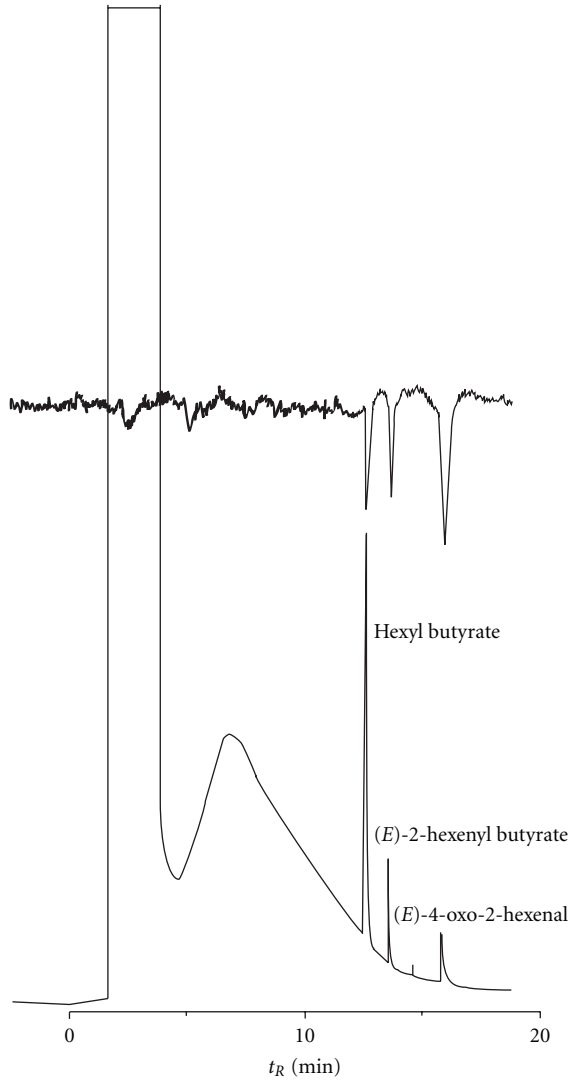


Figure 3: Coupled gas chromatography-electroantennographic detection (GC-EAD) chromatograms showing the responses from an antenna of a male *Stenotus rubrovittatus* (top trace) to a crude extract of unmated females.

Partial reconstruction of the mixture of EAD-active compounds determined that a six-component mixture of 5 μg hexyl hexanoate, 2.5 μg (*E*)-2-hexenyl hexanoate, 5 ng hexyl (*E*)-2-hexenoate, 150 ng octyl butyrate, 275 ng octyl hexanoate, and 275 ng (*E*)-2-octenyl hexanoate attracted males, whereas lures lacking either hexyl hexanoate or (*E*)-2-hexenyl hexanoate were not attractive [20]. Furthermore, a two-component blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (Figure 4(b)) was attractive to males, and adding octyl butyrate (Figure 4(b)) enhanced the attraction [20].

2.3. Chemicals. The components identified as possible sex attractant pheromones of *S. rubrovittatus* [18] and *T. caelestialium* [20] are all commercially available in high purity, with the exception of (*E*)-4-oxo-2-hexenal. (*E*)-4-Oxo-2-hexenal was obtained readily in one step from commercially available 2-ethylfuran [21], in high chemical purity (96.9% pure, as custom synthesized by Shin-Etsu Chemical Co., Ltd.). Although this compound is unstable in impure form, in our hands, pure (*E*)-4-oxo-2-hexenal was relatively stable in a freezer and could be used in a mixture of synthetic pheromones without further purification.

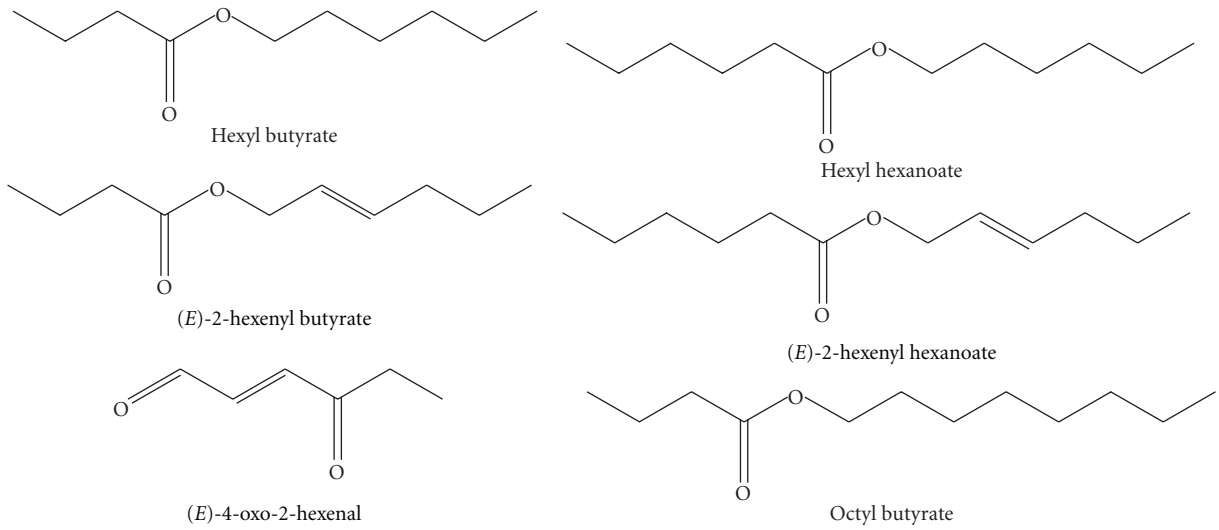
Table 1: Compounds identified in extracts of female *Stenotus rubrovittatus* [16].

Compounds	$KI^{a}_{\text{HPINNOWax}}$	KI^{a}_{HP1}	Relative amount (%)[b]
Hexyl acetate	1,276	996	0.8
Pentyl butyrate	1,320	1,076	0.5
(*E*)-2-Hexenyl acetate	1,337	995	0.2
Hexyl propionate	1,342	1,091	0.2
4-Methylpentyl butyrate	1,374	1,142	0.8
Hexyl butyrate	1,417	1,192	100
Hexyl isopentanoate	1,447	1,228	0.2
(*E*)-2-Hexenyl butyrate	1,475	1,195	46.2
(*Z*)-3-Hexenyl butyrate	1,466	1,146	0.5
Hexyl pentanoate	1,516	1,274	0.2
Heptyl butyrate	1,520	1,276	0.1
Hexyl (*E*)-2-butenoate	1,562	1,224	0.1
(*E*)-4-Oxo-2-hexenal	1,599	958	5.4
Hexyl hexanoate	1,613	1,370	0.1
Octyl butyrate	1,620	1,374	0.1
Methyl tetradecanoate	2,014	1,684	2.0

[a] Kováts retention index [22] using HP-INNOWax ($KI_{\text{HPINNOWax}}$) and HP-1 (KI_{HP1}) columns.
[b] Values are percentages relative to the amount of hexyl butyrate.

2.4. Lures. The rubber septa which are often used as pheromone dispensers for lepidopteran insects are not the most suitable pheromone dispensers for pheromones of some mirid bugs because most of the volatile pheromone components for mirid bugs are of relatively low molecular weight, and the components evaporate from septa too quickly. For example, for *Phytocoris relativus* and *Phytocoris californicus*, rubber septum lures that had been exposed in the field for 2 weeks were significantly less attractive than fresh lures [23, 24]. For *Lygus rugulipennis*, the effective lifetime of a rubber septum lure loaded with the same pheromone compounds as those of *S. rubrovittatus* was only a few hours, whereas polyethylene vials or sachets were found to give sustained release for at least 2 weeks [25]. Nevertheless, because rubber septa are easy to work with, they can be used as dispensers for short-term experiments testing variables such as pheromone blend ratio.

Experiments were carried out with both *S. rubrovittatus* [16, 18] and *T. caelestialium* [20, 26] to determine the optimal doses and ratios of pheromone compounds required for male attraction. For *S. rubrovittatus*, using a rubber septum pheromone dispenser, the release rate of (*E*)-4-oxo-2-hexenal from the lure was much faster than that of the butyrates [27]. Owing to the loss of significant quantities of



(a) Sorghum plant bug *Stenotus rubrovittatus* (b) Rice leaf bug *Trigonotylus caelestialium*

Figure 4: Structures of the pheromone components of *Stenotus rubrovittatus* (a) and *Trigonotylus caelestialium* (b).

(*E*)-4-oxo-2-hexenal during impregnation into the septum, the amount of (*E*)-4-oxo-2-hexenal released from the septum appeared to be substantially less than the amount that was loaded onto the septum. Thus, the ternary mixture formulated with the ratio found in the female extract (a 5 : 2 : 0.5 blend of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal) was not attractive in preliminary bioassays, but ratios containing higher proportions of (*E*)-4-oxo-2-hexenal than found in the female extracts were found to attract males in subsequent bioassays [16]. As a result, a 5 : 1 : 10 blend of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal at a total dose of 64 μg per septum was found to be most effective for attraction of males [16]. Analyses of the volatiles released from septa loaded with this blend showed that the ratio of compounds released from the lure was approximately 5 : 1 : 0.3, substantially different than the 5 : 1 : 10 loading rate.

Innocenzi et al. [27] observed that the release rate of (*E*)-4-oxo-2-hexenal drastically decreased when this compound was mixed with hexyl butyrate. They suggested that this phenomenon might be caused by chemical interaction between hexyl butyrate and (*E*)-4-oxo-2-hexenal, so they suggested that the butyrates and (*E*)-4-oxo-2-hexenal should be applied separately [25]. However, an experiment with *S. rubrovittatus* comparing catches in traps baited with lures containing the three-component blend versus catches in traps baited with a lure loaded with the two butyrates and a separate lure loaded with (*E*)-4-oxo-2-hexenal revealed that mixing the butyrates and (*E*)-4-oxo-2-hexenal made no difference [18]. Although the reason for this discrepancy between experiments is not clear, it may have been influenced by the purity of the (*E*)-4-oxo-2-hexenal used in the two experiments.

Extracts from female *T. caelestialium* were found to contain hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate in a ratio of 1000 : 414–491 : 5–11 [20]. Lures loaded with a 100 : 40 : 3 ratio of hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate at 4.29–14.3 μg per glass capillary tube (5 μL, 0.021 mm ID, 125 mm long) [20] or 10 μg per rubber septum (gray sleeve stopper, 8 mm outside diameter) [26] were most effective for attraction of male *T. caelestialium*.

The effective lifetime of rubber septum lures for these two species (*S. rubrovittatus*, 14 d [16]; *T. caelestialium* 30 d [26]) was generally shorter than those of the rubber septum lures used for many Lepidoptera. Experiments with alternate dispensers are in progress, with the aim of developing lures with longer effective field lifetimes.

3. Application of Synthetic Pheromone Lures for Monitoring Mirid Bug Populations

As a possible alternative to sweep-net sampling of vegetation in and around paddy fields, we have been investigating the potential for using pheromone-baited traps for monitoring *S. rubrovittatus* and *T. caelestialium*. As expected, we found that the pheromone traps attracted only conspecific males, and not females or nymphs, nor did they attract significant numbers of nontarget insects.

3.1. Trap Design. Two types of pheromone traps, a water-pan trap and a double-sided sticky trap (Figure 5), were tested for capturing both mirid species. Possible effects of trap color have not yet been examined. The water pan trap consisted of a plastic pan (~40 cm diam × 12 cm deep) filled with water, with a small amount of surfactant added to prevent trapped bugs from escaping. The lure was hung above the water on a wire frame. Double-sided sticky traps made up of two sticky boards (24 cm × 30 cm) were hung vertically, with the lure placed at the top [28].

(a) Water pan trap (b) Double-sided sticky trap

Figure 5: Typical water pan trap (a) and double-sided sticky trap (b) tested for catching mirid bugs.

For *S. rubrovittatus*, double-sided sticky traps were more effective in capturing males than water pan traps. Sticky traps caught an average of 2.1 ± 0.4 (±SE; $n = 12$) males per trap over 4 days, whereas significantly fewer males (0.8 ± 0.3, $n = 12$; t-test, $P < 0.01$) were captured in water-pan traps during the same time period.

For *T. caelestialium*, there was no significant difference in the effectiveness of the water pan or sticky traps [29, 30]. Water pan traps are cheaper than sticky board traps, but this cost saving is negated by the need to replenish the water frequently, especially in hot and/or dry areas. Thus, for practical use, sticky traps may be more suitable for monitoring both species than water pan traps. However, to be most effective, sticky board traps need to be replaced weekly. In field experiments with *S. rubrovittatus*, significantly more (2.4 ± 0.9; $n = 12$) males per trap were caught on new sticky traps than on sticky traps kept outdoors for 1 week before the experiments (0.3 ± 0.2 males; t-test, $P = 0.036$).

3.2. Optimizing Trap Location. Sticky traps placed below or near the top of the plant canopy were more effective than traps placed 30 cm above the canopy, for both *T. caelestialium* [28] and *S. rubrovittatus*. However, traps set below the canopy picked up a large amount of leaf litter and other detritus, rendering them less effective and more difficult to count. The effects of trap height have also been evaluated with the mullein bug *C. verbasci* in apple orchards [31], where it was found that more males were captured with traps higher (at 2.5-m) than lower (1.5-m) in the canopy.

The effect of trap position within a paddy field was tested by placing traps 0, 3, 7, 15, or 25 m in from the edge of the field (85 m long × 55 m wide). More males were captured in traps placed 3 m in from the edge of the field than in traps placed at the edge of the field. When the traps were set at or over 7 m in from the edge, the numbers of captured males were almost constant regardless of the distance from the edge [32]. Therefore, we recommend that monitoring traps be placed 7 or more meters in from the edge of a field.

3.3. Application for Monitoring the Seasonal Numbers of Bugs. We directly compared the effectiveness of pheromone trapping versus sweep-net sampling for monitoring mirid bug populations. Thus, insects were sampled with a 36-cm diameter sweep net, using 40 sweeps around a trap. For *T. caelestialium*, the number of adults captured by sweep-net sampling at 5-day intervals throughout the season increased from the middle of June to early or mid-July, then decreased, and increased again at the heading time of each rice variety. The seasonal trend of males caught in a trap set at the center of a paddy field (27 m long × 13 m wide) and the trend in the numbers of males captured by sweep-net sampling were similar (Figure 6) [28].

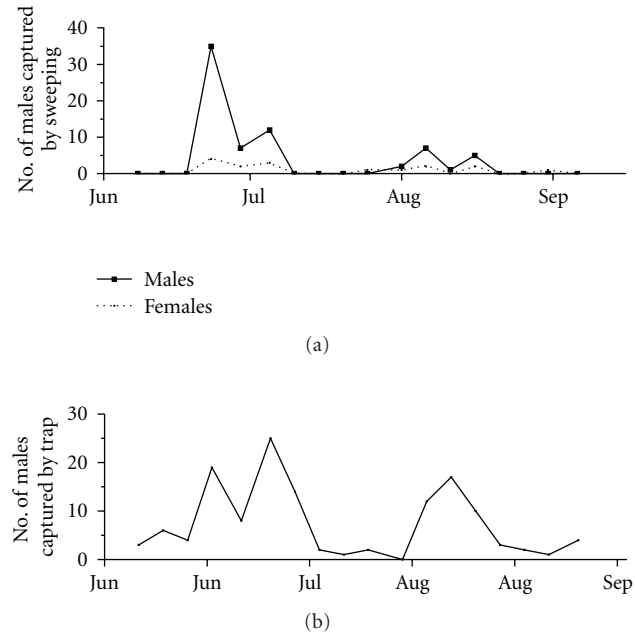


Figure 6: Comparison of the numbers of *Trigonotylus caelestialium* males caught by sweep-net sampling (a) or by a pheromone-baited sticky trap placed in the center of the paddy field (b). Sweep-net samples were taken 18 times at 5 d intervals. Data were modified from [28].

For *S. rubrovittatus*, catches in a trap set 10 m in from the edge of a paddy field (110 m long × 70 m wide) and

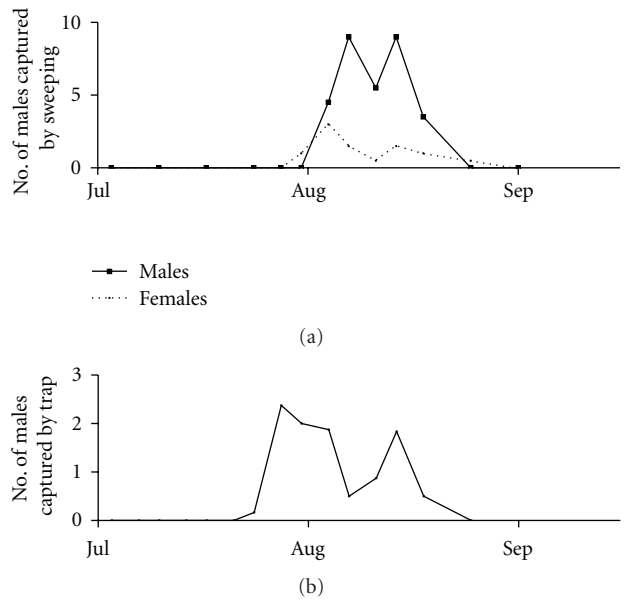


Figure 7: Comparison of the numbers of *Stenotus rubrovittatus* males caught by sweep-net sampling (a) or by a pheromone-baited sticky trap placed in the center of the paddy field (b). Sweep-net samples were taken 13 times at 7 d intervals. Data were modified from [32].

weekly sweep-net samples also showed similar seasonal patterns, with bugs being caught by both methods from about mid-July through late August (Figure 7) [32]. These results suggest that pheromone traps can be used as a tool for monitoring the seasonal population trends of these two mirid bugs in paddy fields.

Stenotus rubrovittatus and *T. caelestialium* are sympatric pests that infest rice crops in many regions of Japan. Synthetic pheromone lures for *S. rubrovittatus* do not attract *T. caelestialium* and vice versa. However, baiting traps with two separate lures (one for each species; combination lures) proved to be as effective as deploying separate traps for each species [33]. Even better, a mixed lure in which all six synthetic pheromone components (three from each species, see above) were loaded onto a single septum was as attractive to males of both species as the separate lures for each species, indicating no inhibition of attraction by any of the mixed pheromone components. These results suggest that mixed lures or combination lures can be used to monitor both species simultaneously, with a single trap. Furthermore, if necessary, *S. rubrovittatus* and *T. caelestialium* can be easily distinguished from each other by the color of the body and hind legs (Figure 1), even when stuck on sticky traps.

4. Chemical Ecology of Mirid Bugs

4.1. Acquisition of Pheromone Components. Crude pheromone extracts of mirid bugs can be prepared easily by brief soaking of individuals in a solvent such as hexane. However, the amounts recovered may be quite small, and, of course, the bugs are killed by the extraction process. Thus, as an alternative, a method for sampling pheromone components from living organisms may be more useful for qualitative and/or quantitative analyses of insect-produced semiochemicals. Adsorbents such as Porapak Q, Tenax TA, or activated charcoal have been widely used for collection of volatiles from living organisms. In a recent innovation to this general method, magnetic stir bars coated with polydimethylsiloxane (Twister, Gerstel, Mülheim an der Ruhr, Germany; 1 mm film thickness × 10 mm length) have been used to adsorb headspace odors released by a variety of organisms [34]. The Twister was originally designed for solventless sample collection followed by thermal desorption and online analysis by GC or GC-MS. However, analytes also can be recovered from the Twister by extraction with small volumes of organic solvents. For *S. rubrovittatus*, pheromone components were collected on the Twister for 1 d, followed by extraction in hexane (1 mL) [35]. Any desired number of replicate samples can be collected simultaneously by simply setting up the appropriate number of sampling chambers, each with its own Twister. Patterns of pheromone release from live individuals can be determined simply by changing the Twister at any desired time interval.

4.2. Effect of Age and Mating Status of Females on Their Attractiveness. Males of *S. rubrovittatus* were more frequently attracted to young virgin females than to old virgin females and were rarely attracted to mated females [14], probably due to differences in the release rates of pheromone between the different classes of females. That is, mated females released less pheromone (~0.67 μg in total) than unmated females (~1.54 μg), and young unmated females released more pheromone (~1.48 μg) than older unmated females (~0.79 μg) [35].

Interestingly, the amounts of volatile compounds released by females and the levels of compounds extracted from whole bodies with solvents did not appear to be correlated. The amounts of pheromone extracted from unmated females (3-d old) totaled about 5 μg and decreased with age to about 0.2 μg extracted from 18-d old females [35]. In contrast, the amount of pheromone extracted from mated females remained constant after mating until 18 d (total about 6–8 μg) [35].

For *T. caelestialium*, there was no evidence for daily periodicity in male attraction to females or mating [36], whereas male *S. rubrovittatus* were most attracted to females at night and in the morning [37]. Mating behavior of *S. rubrovittatus* was observed at any time of day, and males courted females regardless of the time of day [37]. However, female mating receptivity was higher in the morning than in the afternoon [38]. For *S. rubrovittatus*, mating behavior was sometimes initiated even when attraction of males to females was not observed, suggesting that, over shorter ranges, other signals may mediate the initiation of copulation.

4.3. Pheromone-Based Control. The efficacy of pheromone-based mating disruption has been investigated with *C. verbasci* in Canada [31] and *T. caelestialium* in Japan [39]. Captures of male *C. verbasci* in pheromone-baited traps were

greatly reduced when fields were treated with the complete, two-component sex pheromone blend, but reductions in trap captures were not observed when fields were treated with only one component of the pheromone [31]. In this experiment, decrease in trap captures was correlated with increased densities of pheromone dispensers.

In pheromone-based mating disruption experiments with the rice leaf bug *T. caelestialium*, treatment of grassy fields with pheromone-reduced captures of male *T. caelestialium* in traps baited with pheromone lures or with virgin females, and lowered population levels of *T. caelestialium* [39]. The total numbers of adults captured in the treated fields were 0–45% of those in the untreated fields, and the total numbers of nymphs sampled in the treated fields were 0–2.2% of those in the untreated fields [39].

However, *T. caelestialium* and *S. rubrovittatus* are polyphagous, and their host plant range includes a variety of graminaceous plants. Therefore, mated females of these species can invade treated areas from outside, even if mating behavior in treated areas such as paddy fields has been suppressed. Pecky rice damage results from mirid bugs invading paddy fields during the period when the rice ears are sprouting. The nymphs and adults present at the middle to end of the grain-filling period are the offspring of the adults that invaded early in the grain-filling period [39]. Decreasing the number of nymphs and adults during the grain-filling period should minimize pecky rice damage, and so pheromone-based control measures must remain effective for the duration of this period (~2 months). Furthermore, pheromone treatments are expensive (~9,300 yen/ha for the compounds alone, at a rate of 60 g/ha) and must be reapplied approximately monthly [39]. Thus, under the conditions used in the present study, mating disruption of *T. caelestialium* is not economically feasible for preventing pecky rice damage. It remains to be determined whether larger-scale application of mating disruption, and the resulting economies of scale, might make it possible to develop mating disruption of *T. caelestialium* and *S. rubrovittatus* as cost-effective management tools.

5. Conclusion

Our results suggest that pheromone-baited traps for two mirid bugs, *S. rubrovittatus* and *T. caelestialium*, may be able to replace sweep-net sampling with monitoring seasonal population dynamics of these two important pests of rice, particularly as traps baited with mixed or combination lures can be used to sample both species simultaneously. Sticky traps set up near the top of the plant canopy, and 7 or more meters in from the edge of the paddy field were optimal for monitoring. For *T. caelestialium*, mating disruption experiments showed that although the pheromones interfered with male attraction to lures and to females and appeared to suppress populations, the costs of treatment and the relatively small decrease in damage to the rice crop suggest that mating disruption of this bug may not be a practical technique for preventing pecky rice damage.

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Review Article

A Paratransgenic Strategy for the Control of Chagas Disease

Ivy Hurwitz,[1] Annabeth Fieck,[1] Nichole Klein,[1] Christo Jose,[1] Angray Kang,[2] and Ravi Durvasula[1]

[1] *Department of Internal Medicine, Center for Global Health, Health Science Center, University of New Mexico and New Mexico VA Health Care System, Albuquerque, NM 87131, USA*
[2] *Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London E1 4AT, UK*

Correspondence should be addressed to Ravi Durvasula, ravi.durvasula@va.gov

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Chagas disease results from infection with the parasite *Trypanosoma cruzi*. This disease remains a significant cause of morbidity and mortality in central and south America. Chagas disease now exists and is detected worldwide because of human migration. Control of Chagas disease has relied mainly on vector eradication however, the development of insect resistance to pesticides, coupled with cost and adverse health effects of insecticide treatments, has prompted our group to investigate novel methods of transmission control. Our laboratory has been instrumental in the development of the paratransgenic strategy to control vectorial transmission of *T. cruzi*. In this paper, we discuss various components of the paratransgenic approach. Specifically, we describe classes of molecules that can serve as effectors, including antimicrobial peptides, endoglucanases, and highly specific single chain antibodies that target surface glycoprotein tags on the surface of *T. cruzi*. Furthermore, we address evolving concepts related to field dispersal of engineered bacteria as part of the paratransgenic control strategy and attendant risk assessment evaluation.

1. Chagas Disease

American trypanosomiasis, or Chagas disease, is caused by the protozoan *Trypanosoma cruzi.* Between 8–11 million people worldwide are infected, and, of these, approximately 50,000 will die annually [1]. In 2000, the annual cost of morbidity and mortality attributed to Chagas disease in endemic countries was estimated to be close to US$8 billion [2]. Two years later, the WHO estimated the burden of Chagas disease to be as high as 2.7 times the combined burden of malaria, schistosomiasis, leishmaniasis, and leprosy [3]. Though traditionally a disease endemic to Mexico, central, and south America, human migration has resulted in reported cases of *T. cruzi* infection worldwide [4]. For example, cases of Chagas disease have been reported in Portugal [5], Spain [6, 7], France [8, 9], and Switzerland [10], countries that are favored for immigration from Latin America. Reports from Australia estimate *T. cruzi* infection in 16 of every 1,000 Latin American immigrants [1, 5], and Chagasic heart disease was reported in Brazilian immigrants of Japanese origin in Japan [6].

There have been numerous reported cases of Chagas disease resulting from unscreened blood transfusions and organ donation. Further, the parasite can be congenitally passed from mother to child. However, this disease is most often transmitted to humans by *T. cruzi*-infected blood-sucking triatomine bugs. These insects are members of the heteropteran family Reduviidae. The major vectors for Chagas disease in central and south America are *Rhodnius prolixus* (Figure 1(a)) and *Triatoma infestans* (Figure 1(c)), respectively. These bugs thrive in thatch and adobe of poorly constructed homes during the heat of the day, coming out in the cooler hours of the night to feed. Carbon dioxide emanating from the breath of the sleeping vertebrate victims as well as ammonia, short chain amines, and carboxylic acids from skin, hair, and exocrine glands are among the volatiles that attract triatomines. These insects are often dubbed "kissing bugs," from their common habit of biting the face, which is often exposed during sleep. The bite of triatomine bugs is painless, allowing the insect to feed without interruption. As the insect engorges, it defecates. If the insect is infected with *T. cruzi*, the parasite will be in the

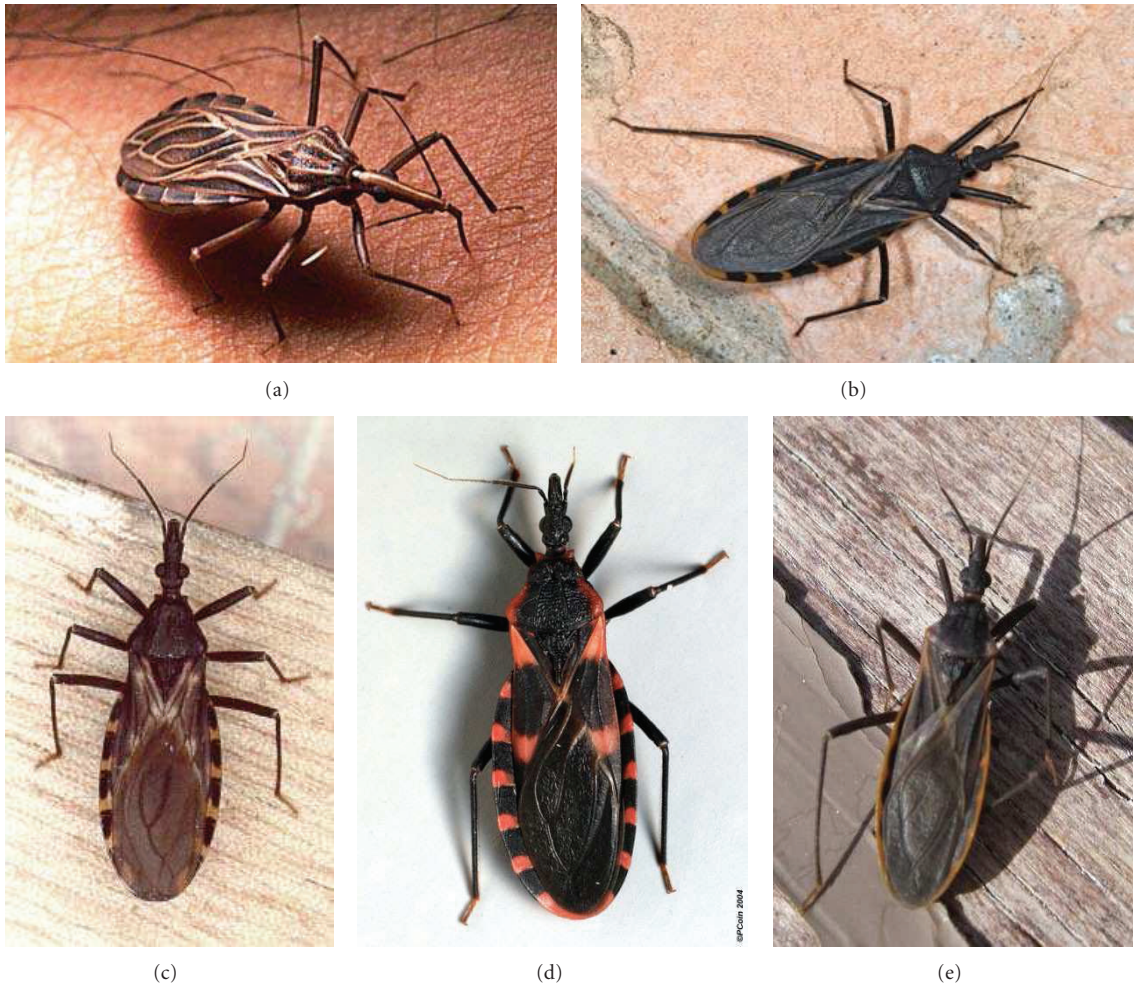

Figure 1: Triatomine bugs. (a) *R. prolixus*, picture adapted from http://www.jyi.org/articleimages/185/originals/img0.jpg; (b) *T. gerstaeckeri*, picture adapted from http://theearlybirder.com/insects/hemiptera/reduviidae/index.htm; (c) *T. infestans*, picture adapted from http://www.k-state.edu/parasitology/546tutorials/ARTHFIG01.JPG; (d) *T. sanguisuga*, picture adapted from http://bugguide.net/node/view/5164; (e) *T. rubida*, picture adapted from http://bugguide.net/node/view/185220.

fecal material and will then enter the bloodstream when the victim scratches the irritated bite wound.

There are currently more than 300,000 people infected with *T. cruzi* living in the United States. Most acquired the disease while residing in endemic areas [11]. Although *T. cruzi*-infected vectors and animals are found in many parts of this country [12], there have only been 5 documented cases of autochthonous (indigenous) transmission in the US [13]. Surveys of Reduviid bugs in the American Southwest have shown high rates of *T. cruzi* infection in *T. rubida* (Figure 1(e)), *T. protracta, T. sanguisuga* (Figure 1(d)), and *T. gerstaeckeri* (Figure 1(b)) [12, 14–16]. Species-specific behavior of the north America triatomines may explain why more autochthonous transmission is not observed. In a study by Klotz et al. [17], field caught *T. protracta* and *T. rubida* were allowed to feed on live immobilized white mice and the defecation pattern was observed for one hour. Of the 71 triatomines observed, only 30 (42 percent) produced a fecal droplet within one hour after feeding. Sixty-seven percent of these defecated 1.5–6 cm from the mouse, whereas 33 percent defecated 7–10 cm away from the mouse. None of the bugs defecated on the mouse.

The transmission cycle of *T. cruzi* is complex. Reduviid vectors become infected with *T. cruzi* when they feed on vertebrate blood containing bloodstream trypomastigotes (Figure 2). The trypomastigotes differentiate into epimastigotes in the midgut and then to infective metacyclic trypomastigotes as they move further into the hindgut of the insect. When the triatomine bug takes its next blood meal, the trypomastigotes are defecated onto the bite wound of its victim. Transmission of the parasite therefore occurs through contamination of the bite site. Upon entry into the blood stream of the vertebrate host, the trypomastigotes colonize muscle and neuronal tissue where they form intracellular amastigotes. These proliferating cells will form pseudocysts, which after several successive cell divisions, will asynchronously transform into trypomastigotes. The trypomastigotes then escape from the pseudocysts into the blood

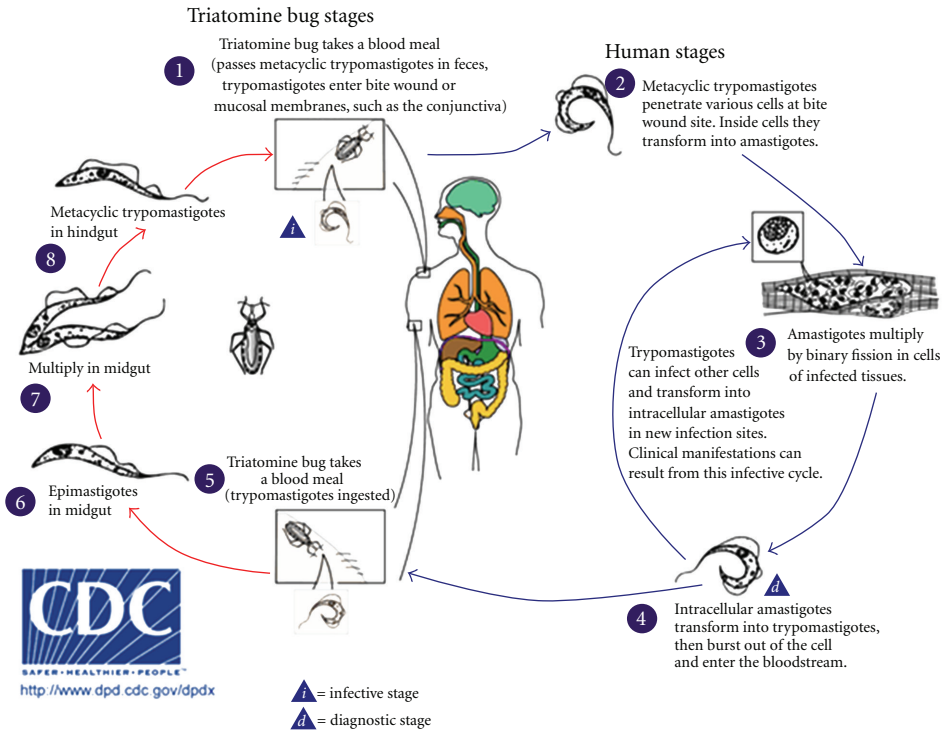

Figure 2: An infected triatomine bug takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the wound (1). Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes (2). The amastigotes multiply by binary fission and form pseudocysts (3). Following several cycles of division, these cells will asynchronously differentiate into trypomastigotes, which are then released into the circulation as bloodstream trypomastigotes where they can start infecting cells from other tissues (4). The cycle of infection continues as another triatomine bug becomes infected by feeding on human blood containing the circulating parasites (5). The ingested trypomastigotes transform into epimastigotes in the vector's midgut (6). The parasites multiply in the midgut (7) and differentiate into infective metacyclic trypomastigotes in the hindgut (8). This figure is adapted from http://www.dpd.cdc.gov/dpdx.

and lymph to invade new cells. The cycle of transmission continues when another triatomine bug takes a blood meal from the infected vertebrate.

Chagas disease is characterized by three successive stages. The acute stage, often characterized by generalized malaise, fever, swelling of the lymph nodes, and enlargement of the liver and spleen, is minimally symptomatic and lasts from 4 to 8 weeks. The hallmark of acute infection is the chagoma, an inflammatory skin lesion that develops at the site of a triatomine bug bite. The lesion is a result of lymphocytic infiltrate, intracellular edema, and adjacent reactive lymphadenopathy due to the intramuscular presence of *T. cruzi* at the site of inoculation. When the bite is on the face near the eye, the characteristic Romana's sign occurs (Figure 3). The acute phase is then followed by an indeterminate phase that can last between 10 to 20 years. During this period, there are few clinical manifestations but active replication of *T. cruzi* and periodic release of bloodstream forms of the parasite into the circulation occurs. Approximately, 30 percent of these patients progress to symptomatic chronic Chagas disease, which often manifests in the 4th or 5th decade of life [18]. Hallmarks of chronic infection include inflammation of the heart muscles, enlargement of the esophagus, and enlargement of the colon. For the most part,

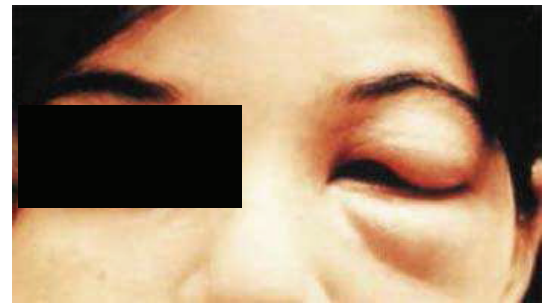

Figure 3: Romana's sign is characterized by unilateral palpebral edema, conjunctivitis, and lymphadenopathy. Photograph is adapted from WHO/TDR 2011.

the gastrointestinal manifestations of chronic Chagas disease are geographically restricted and play a lesser role in the overall disease burden of Chagas disease. However, progressive heart disease is a leading public health concern throughout much of central and south America. The chronic phase of Chagas disease is incurable and on average is associated with a ten-year shortening of life span.

Whereas acute Chagas disease is treatable with appropriate and timely antiparasitic medication, the chronic phase of

this debilitating disease often goes undiagnosed. Antiparasitic treatment of chronic disease is of questionable clinical benefit and is often limited by adverse drug reactions and side effects. In the absence of vaccines and effective drug therapies, control of Chagas disease had relied largely on measures aimed at vector eradication. To date, several large-scale insecticide-based efforts have been undertaken with considerable success. In 1991, an international coalition of governmental agencies from Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay, and Peru started The Southern Cone Initiative to minimize transmission of *T. cruzi* by *T. infestans*. This coalition developed educational programs aimed at reducing human contact with *T. infestans,* as well as blood bank screening programs to eliminate transmission of *T. cruzi*. Successes of this program include a Pan-American Health Organization awarded certificate for the disruption of Chagas disease transmission by *T. infestans* to Uruguay, Chile, and Brazil [19]. However, sustainability of this program has been called into question due to the need for continued application of pyrethroid insecticides and possibility of reinfestation of domestic structures [19]. The poor effects of pyrethroid insecticides are mainly caused by their short-lasting residual effects in outdoor sites exposed to sunlight, high temperatures, rain, and dust [20, 21]. The reduced effectiveness of pyrethroids was further compounded by the development of triatomine populations that were resistant to a variety of insecticides in Chagas disease endemic areas [22]. This, coupled with recent surveillance data indicating resurgence of human infections, particularly in the Argentinan Gran Chaco [23], would suggest that current insecticidal programs for control of vectorial transmission of Chagas disease are failing, and that novel, effective, and sustainable supplementary tactics are critically needed to maintain suppression of Chagas disease transmission.

2. The Paratransgenic Approach, an Alternative Strategy to Control Vectorial Transmission of *T. cruzi*

Triatomine bugs subsist on a blood-restricted diet. To supplement their basic nutritional and developmental needs, these insects have developed a symbiotic relationship with nocardioform actinomycetes [24]. These soil-associated bacteria are thought to aid in the processing of B complex vitamins and are essential to the survival of the bug. The symbiosis of these bacteria with several triatomine species and the amenability of these cells to genetic manipulation are the cornerstones of the paratransgenic strategies aimed at interrupting vectorial transmission of *T. cruzi*.

Rhodococcus rhodnii is a soil-associated nocardioform actinomycete. It also lives extracellularly in the gut lumen of *R. prolixus* in close proximity to *T. cruzi*. This microbe is transmitted from adult triatomine bugs to their progeny through coprophagy, the ingestion of fecal material from other bugs. *Rhodococcus rhodnii* is critical to the growth and development of *R. prolixus* [24]. *Rhodnius prolixus* nymphs that lack gut-associated symbionts (aposymbiotic) do not reach sexual maturity and most die after the second developmental molt. Introduction of the bacteria to the first or second instar nymphs permits normal growth and maturation. In 1992, we transformed *R. rhodnii* with pRr1.1, a shuttle plasmid containing a gene encoding resistance to the antibiotic thiostrepton, to support the hypothesis that a transgene-carrying symbiont could be introduced into *R. prolixus* [25]. These transformants were introduced into aposymbiotic first instar *R. prolixus* nymphs via artificial membrane feeding and reared in the presence and absence of thiostrepton. Examination of bacteria from the gut of insects carrying the transformed symbionts demonstrated that thiostrepton resistant colonies could be recovered from these vectors, regardless of antibiotic presence, for up to 6.5 months after infection. These studies demonstrated that genetically modified *R. rhodnii* symbionts expressing a selectable gene product could be stably maintained in *R. prolixus* without adverse effects on insect survival and fitness, thereby substantiating the paratransgenic approach.

For the paratransgenic strategy to work, it is imperative that a population of symbiotic bacteria, that is, amenable to culture and receptive to genetic manipulation, be identified within a given disease-transmitting vector. The fitness of these symbionts should not be compromised nor should their normal functions within the vector be affected following genetic manipulation. The transgene products, when expressed in proximity to the target pathogen, should interfere with pathogen development in the vector, and should not be detrimental in any way to the vector. Finally, the dispersal technique used to spread the genetically modified symbiont/commensal to naturally occurring vector populations should minimize the spread of the transgene to other organisms in the vector's environment, which include both the nontarget microbiota inside the vector and other organisms that live in the same ecological niche.

Since our initial experiments, we have adapted the paratransgenic strategy to numerous other vector-borne disease systems, including sand-fly-mediated leishmaniasis [26, 27] and sharpshooter-mediated Pierce's disease [28]. The strategy is also being developed and extrapolated into shrimp mariculture [29]. In this paper, we will focus on work relating to the paratransgenic control of Chagas disease with a number of effector molecules, specifically, antimicrobial peptides (AMPs), recombinant endoglucanases that disrupt the surface of the parasite, and functional transmission-blocking single-chain antibodies.

2.1. Antimicrobial Peptide Genes. Cecropin A is an AMP that was isolated from the giant silk-worm moth *Hyalophora cecropia* [30]. This AMP is 38 amino acids in length. Cecropin A lyses cells by binding to and covering the parasite membrane surface, effectively dissipating transmembrane electrochemical gradients [31]. We cloned the DNA sequence for cecropin A into the pRr1.1 shuttle vector to produce pRrThioCec. This plasmid was then used to transform *R. rhodnii* [32]. Paratransgenic *R. prolixus* were generated with the cecropin A-expressing symbionts and allowed to engorge on *T. cruzi*-laden human blood until they reached sexual maturity. Hindgut contents from paratransgenic insects carrying pRrThioCec-transformed *R. rhodnii* were

either devoid of *T. cruzi* trypomastigotes (65 percent) or maintained markedly reduced titers of the parasite (35 percent) [32]. In contrast, all control insects harboring untransformed *R. rhodnii* or *R. rhodnii* transformed with pRr1.1 (original shuttle vector without cecropin gene) that were infected with *T. cruzi* in the same manner carried mature trypomastigotes. This study provided proof of concept for the paratransgenic strategy, and suggested that other AMPs might be employed singly or in concert to elicit complete elimination of infective parasites in the hindgut of *T. cruzi* vectors.

A large number of AMPs have been and are being discovered that function in a variety of ways, including disruption of cell membranes similar to cecropin A, interference with host metabolism, and inactivation of cytoplasmic components [31]. Many AMPs are also capable of discriminating between host and invading organisms, thereby permitting the expression of recombinant AMP's from certain cell lines without deleterious effects to a host insect. *In vitro* studies were carried out with six AMPs selected from different insect sources to determine their differential toxicity profiles against host bacterial strains and *T. cruzi* parasites [33]. AMP's were identified that displayed high toxicity against *T. cruzi* (LC_{100} < 10 μM) compared to *R. rhodnii* (MBC > 100 μM) in single synthetic peptide treatment regimens. These peptides; apidaecin, cecropin A, magainin II, and melittin, were employed in pairwise treatment protocols against *T. cruzi.* Dual peptide treatments of *T. cruzi* showed synergistic or additive effects between different AMP's resulting in increased toxicity over any single AMP treatment. The best example for this was observed with apidaecin. When administered alone to *T. cruzi*, apidaecin killed the parasite at the 10 μM dose, but when used in combination with melittin, magainin II, or cecropin A, complete lethality to *T. cruzi* was seen at 1.0 μM—a tenfold decrease in the necessary lethal concentration. While all combinations exhibited additive activity compared to single AMP treatments, synergistic activity was observed when magainin II was applied in combination with apidaecin or melittin (Figure 4). It has been inferred from the pair-wise treatment data that the additive and synergistic effects observed could improve the 65 percent rate of *T. cruzi* elimination seen in the initial *in vivo* studies with cecropin A. Furthermore, the use of peptides in combination could reduce the development of peptide resistance in target *T. cruzi* populations.

Rhodococcus rhodnii has been transformed with expression plasmids for the four peptides (melittin, magainin II, apidaecin, and cecropin A) and expression of these molecules was confirmed by ELISA and western blot (Fieck et al., in prep.). The shuttle vector employed for these studies expressed AMP gene sequences from the *Mycobacterium kansasii-α* antigen promoter and export signal sequence. Selection of positive transformants was achieved for both the *E. coli* cloning host and *R. rhodnii* symbiont by growth on carbenicillin and confirmed by colony PCR. Cell lysates from AMP-transformed *R. rhodnii* have been shown to be toxic to *T. cruzi* in single- and pairwise *in vitro* toxicity assays. *In vivo* experiments are currently underway at the Centers for Disease Control (CDC) to test the toxicity of products from single and dual peptide-carrying symbionts to *T. cruzi* in aposymbiotic *R. prolixus* nymphs.

2.2. Single-Chain Antibodies. Antimicrobial peptides act as direct effectors in the paratransgenic model by physically damaging cell structure or metabolic function, resulting in parasite death. Single-chain antibodies (scFv) comprise a second class of effector molecules selected to negatively impact *T. cruzi* development and transmission by acting through an indirect mechanism. In this design, scFvs with binding specificity to *T. cruzi* surface proteins interfere with the physical contact between trypanosomes and the vector that is essential for parasite development. This interference model predicts that the activity of the effector scFv molecules would be specific to parasite development and elicit fewer negative effects on the vector or transformed symbiont. Parasite maturation, which involves metacyclogenesis of *T. cruzi* from noninfective epimastigotes to infective trypomastigotes, occurs in the gut of the triatomine bugs and is an important step in the transmission of Chagas disease [34]. This maturation process is dependent on interactions between the surface epitopes of *T. cruzi* and the gut lumen of the insect vector [35] and would be the target of scFv effector activity.

Single-chain antibodies usually consist of variable regions of heavy and light chains of immunoglobulins connected by a flexible linker, (Gly4Ser) n = 3–5, that permits the two protein domains to interact effectively with their corresponding antigen [36]. The fact that DNA sequences for scFvs can be cloned into expression plasmids and expressed from bacterial transformants renders these molecules uniquely suited to this system of insect paratransgenesis.

To test the ability of a scFv to be expressed and functional within the gut of the Reduviid vector, the pRrMDWK6 expression shuttle plasmid was constructed with a marker gene coding for a murine antiprogesterone antibody fragment, rDB3 [37]. Constitutive expression of rDB3 from pRrMDWK6-harboring symbionts was under control of the *M. kansasii-α* antigen promoter/signal sequence and could be quantified by ELISA for progesterone binding activity. Aposymbiotic *R. prolixus* nymphs were exposed to DB3-expressing *R. rhodnii* symbionts and allowed to develop on blood meals. Subsequent examination revealed that the rDB3 antibody fragment was synthesized by the transformed *R. rhodnii* and secreted into the gut lumen throughout the development of the nymphs to the adult stage (6 months). Protein extracts from the gut of paratransgenic *R. prolixus* bound progesterone suggesting that the presence and activity of scFvs could be maintained in the environment of the insect gut [37]. Further evidence for this was provided by similar experiments carried out with *T. infestans*, another major vector of Chagas disease. A *Corynebacterium* sp., a bacterium closely related to *R. rhodnii*, was identified as a symbiont in this vector. We successfully transformed this bacterium to express rDB3 from the pRrMDWK6 shuttle vector, and generated paratransgenic *T. infestans* lines [38]. ELISA analysis of gut extracts from these paratransgenic bugs

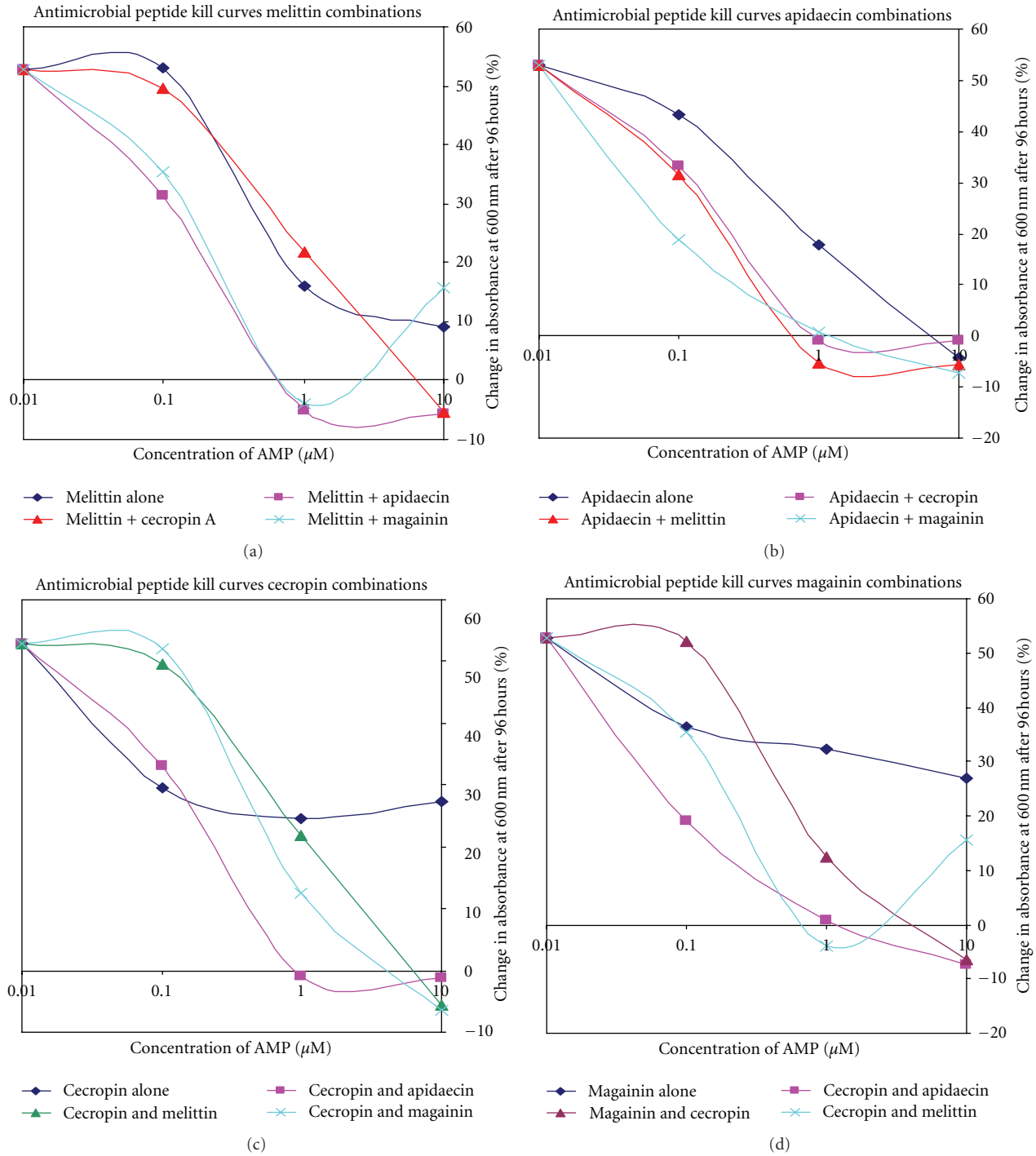

FIGURE 4: Antimicrobial peptide kill curves for dual-combination treatments of *T. cruzi* cultures. Results averaged from triplicate samples in three separate experiments and displayed as the percent change in absorbance at 600 nm compared to untreated controls. Figure is adapted from Fieck et al. *Trypanosoma cruzi*: synergistic cytotoxicity of multiple amphipathic antimicrobial peptides to *T. cruzi* and potential bacterial hosts [33].

showed the presence of rDB3 antibodies capable of binding to progesterone [38].

Progression from the paratransgenic system employing a marker scFv to one utilizing effector scFv's required the development of antibodies with strong binding affinities to parasite surface coat proteins. *Trypanosoma cruzi* does not synthesise or catabolise free sialic acid, but expresses a developmentally regulated sialidase which is used for surface sialylation by a trans-sialidase mechanism [39]. If the appropriate galactosyl acceptor is available the sialyltransferase activity of the *T. cruzi* sialidase is greater than its hydrolytic activity. This implies a mechanism that is capable of remodelling the *T. cruzi* glycan surface using host glycoconjugates as the sialyl donor. Such sialylation might

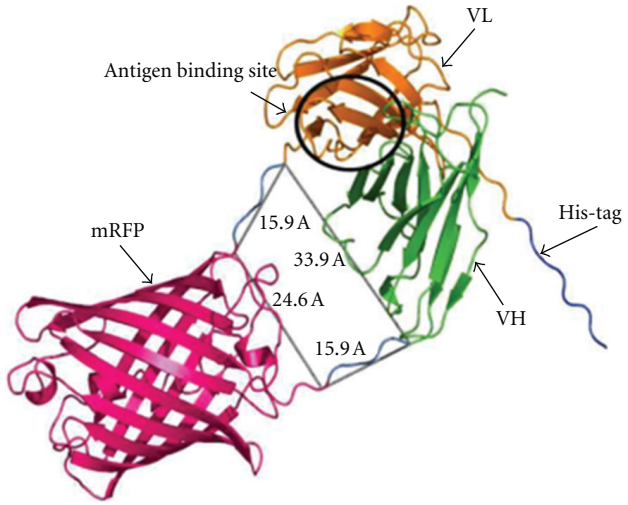

Figure 5: Molecular assembly of an affinity fluorescent protein. The REDantibody shown here has mRFP engineered between the VH and VL domains of the scFV, resulting in a highly stable red fluorescent targeted affinity probe. Figure adapted from Markiv et al. Module based antibody engineering: a novel synthetic REDantibody [36].

provide protection for *T. cruzi* from the innate immune responses. This large family of cell surface sialyated mucin-like glycoproteins plays an essential role in the parasite's life cycle [35], and, therefore is excellent targets for scFv binding. Two well characterised murine monoclonal antibodies, B72.3 [40] and CA19.9 [41], that bind sialyl-Tn and sialyl-(le)a surface glycans, respectively, were selected for application to the paratransgenic system incorporating scFv's for control of Chagas disease transmission [36]. Synthetic DNA sequences encoding the variable regions of the heavy and light chains of these monoclonal antibodies were used to assemble the complete coding sequences for the scFvs. In place of the standard 15 amino acid linker between the heavy and light chain fragments, a monomeric red fluorescent protein (mRFP) derived from the red fluorescent protein cloned from the *Discosoma* coral, DsRed [42], was inserted as a rigid linker that conferred extra stability and fluorescence to the scFvs (Figure 5). Binding to fixed *T. cruzi* epimastigotes and fluorescent optical properties of these scFvs were demonstrated using confocal microscopy (Figure 6). The gene sequences for these scFvs are currently being inserted into the *E. coli/R. rhodnii* shuttle vector for the generation of traceable scFv-expressing symbionts and subsequent generation of effector scFv containing paratransgenic *R. prolixus*.

2.3. β-1,3-Glucanase. Endoglucanases comprise a third class of trypanocidal molecules expected to function as effector molecules in the paratransgenic system for Chagas disease control. *β*-1,3-glucanase is part of an endoglucanase complex isolated from *Arthrobacter luteus* called lyticase. This molecule functions by breaking the 1–3 and 1–6 glycosidic linkages of surface glycoproteins [43]. The surface of *T. cruzi* is covered by a thick coat of glycoproteins proposed to play a role in the binding of the cell body and flagellum

Figure 6: Confocal image of anti-sialyl-Tn REDantibody targeting glycan structures on the surface of *T. cruzi* epimastigotes. Figure adapted from Markiv et al. Module based antibody engineering: a novel synthetic REDantibody [36].

to membranes in the vector gut [44]. This binding is necessary for *T. cruzi* to complete its development and, as a consequence, is essential for its transmission. In unpublished work, we showed that *A. luteus* lyticase was efficient in lysing *T. cruzi* while being nontoxic to *R. rhodnii* and *R. prolixus*. We inserted the cDNA for *β*-1,3-glucanase into our shuttle plasmid and isolated extracts from the transformed *R. rhodnii* for use in toxicity assays against *T. cruzi*. Although *β*-1,3-glucanase was originally described as being effective only as a part of the lyticase complex in the presence of a complementary alkaline protease [43], we have demonstrated that recombinant *β*-1,3-glucanase is biologically active and clears *T. cruzi* at low concentrations even in the absence of the protease (Jose et al., in prep.). Toxicity of the recombinant *β*-1,3-glucanase against *T. cruzi* is comparable to *A. luteus* lyticase complex. These results emphasize the potential for use of *β*-1,3-glucanase as another effector molecule in a paratransgenic strategy to control Chagas disease transmission. The *in vivo* toxic effects of recombinant *β*-1,3-glucanase expressed from symbiotic *R. rhodnii* transformants in *R. prolixus* will be determined using the previously described experimental approach.

The three classes of molecules described above target *T. cruzi* differentially. An effective paratransgenic strategy for field application would involve the delivery of these effector molecules in combination, for example: AMPs with scFvs or AMP's with endoglucanases. This strategy should reduce not only transmission of *T. cruzi*, but also the development of resistance resulting from prolonged treatment with a single effector.

3. Preparations for Field Trials

In anticipation of field trials, we have tested the efficacy of a simulated triatomine-fecal preparation, CRUZIGARD, consisting of an inert guar gum matrix dyed with India ink, as a method for delivery of engineered *R. rhodnii* to closed colonies of *R. prolixus* [45]. The CRUZIGARD preparation was mixed with 10^8 colony forming units (CFU)/mL of genetically modified *R. rhodnii* and used to impregnate cages constructed of thatch and adobe building materials

from Chagas-endemic regions of Guatemala (Olopa) [45]. In these experiments, field caught adult *R. prolixus* from the Olopa district were placed in the cages and removed after eggs were laid. Nymphs were allowed to mature in the CRUZIGARD-treated cages. Nine months later, genetically altered *R. rhodnii* were detected in approximately 50 percent of F_1 adults and comprised nearly 95 percent of total CFUs in these bugs, demonstrating that CRUZIGARD may be useful as a gene dispersal strategy even in environments where competing microbes are present. To increase the volume and duration of CRUZIGARD ingestion, and consequently increase rates of vector inoculation with transformed symbiont, on-going collaborations to develop triatomine attractants and semiochemicals to supplement the current CRUZIGARD formulation are underway.

We realize that deployment of genetically altered *R. rhodnii* into the field may have profound environmental consequences. In a recent publication, we evaluated the risks of horizontal gene transfer (HGT) between R. rhodnii and Gordona rubropertinctus, a closely related nontarget Gram-positive actinomycete [46]. We developed a model that treats HGT as a composite event whose probability is determined by the joint probability of three independent events: gene transfer through the modalities of transformation, transduction, and conjugation. Genes are represented in matrices, with Monte Carlo method and Markov chain analysis used to simulate and evaluate environmental conditions. The model is intended as a risk assessment instrument and predicts an HGT frequency of less than 1.14×10^{16} per 100,000 generations at the 99 percent certainty level [46]. This predicted transfer frequency is less than the estimated average mutation frequency in bacteria, 10^{-1} per gene per 1,000 generations. These predictions were further substantiated when laboratory studies that involved coincubation of R. rhodnii and G. rubropertinctus in conditions highly conducive to HGT resulted in no detectable HGT. These results would suggest that even if HGT were to occur between *R. rhodnii* and *G. rubropertinctus*, the transgene would likely not persist in the recipient organism, and that the likelihood of these unwanted events is vanishingly small [46].

To further minimize gene spread to nontarget arthropods, we are engaged in the development of a strategy to determine the minimum amount of transformed symbionts necessary to prevent *T. cruzi* transmission to humans, and an effective method of CRUZIGARD application suitable for at-risk domiciles in the endemic region. We are also engaged in the development of second-generation paratransgenic delivery systems that utilize microparticle encapsulated, genetically altered *R. rhodnii* for targeted release in the gut of the triatomine bug. Finally, we realize that the field release of engineered bacteria cannot occur until a risk assessment framework is in place. Because such information will not be readily available through field release trials, we are working with collaborators to develop a framework involving rigorous mathematical modeling and simulations. Outputs of these models will be integral to informing risk assessment and regulatory oversight of the paratransgenic program, and, ultimately, to permit field trials of the paratransgenic strategy.

4. Conclusion

Chagas disease affects the lives of millions of people worldwide and remains a major cause of mortality and morbidity, as well as economic loss [47]. Increased attention from the World Health Organization and interest from governments of endemic regions have yielded desirable results for control of Chagas disease transmission. However, success of disease control with large-scale insecticide-based approaches, as demonstrated through the Southern Cone [48], central American [49], Andean Pact [50], and Amazonian Initiatives [49], has been dimmed by the looming possibilities of environmental toxicity, human health impacts, cost of repeated applications, and development of vector resistance.

We describe a novel and potentially environmentally friendly method to control vectorial transmission of Chagas disease. This paratransgenic approach is based upon genetically manipulating symbionts of the triatomine vectors to express effector molecules that would kill or prevent the development of the parasite within the gut of the insect. We have demonstrated the feasibility of this approach in a number of laboratory-based experiments, using effector molecules such as AMPs, endoglucanases and highly specific scFvs. A number of collaborations are underway to evaluate environmental risk related to field release of the genetically modified symbionts. We believe that eventual field application of the paratransgenic approach could provide a more effective and feasible alternative to current strategies of Chagas disease control in endemic regions of the world.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgment

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Research Article

Interactions among Carbon Dioxide, Heat, and Chemical Lures in Attracting the Bed Bug, *Cimex lectularius* L. (Hemiptera: Cimicidae)

Narinderpal Singh,[1] Changlu Wang,[1] Richard Cooper,[1] and Chaofeng Liu[2]

[1] *Department of Entomology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901, USA*
[2] *Department of Statistics, Purdue University, West Lafayette, IN 47907, USA*

Correspondence should be addressed to Narinderpal Singh, nsingh@aesop.rutgers.edu
and Changlu Wang, cwang@aesop.rutgers.edu

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Commercial bed bug (*Cimex lectularius* L.) monitors incorporating carbon dioxide (CO_2), heat, and chemical lures are being used for detecting bed bugs; however, there are few reported studies on the effectiveness of chemical lures in bed bug monitors and the interactions among chemical lure, CO_2, and heat. We screened 12 chemicals for their attraction to bed bugs and evaluated interactions among chemical lures, CO_2, and heat. The chemical lure mixture consisting of nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil was found to be most attractive to bed bugs and significantly increased the trap catches in laboratory bioassays. Adding this chemical lure mixture when CO_2 was present increased the trap catches compared with traps baited with CO_2 alone, whereas adding heat did not significantly increase trap catches when CO_2 was present. Results suggest a combination of chemical lure and CO_2 is essential for designing effective bed bug monitors.

1. Introduction

Hematophagous arthropods use a variety of visual, mechanical, chemical, and thermal cues to detect vertebrate hosts [1]. Host searching behavior in unfed bont tick, *Amblyomma hebraeum* Koch [2, 3], and *Glossina* spp. (Diptera: Glossinidae) [4] is stimulated by carbon dioxide (CO_2) emitted by mammalian hosts. Odors from human skin [5], sweat, breath and body odors from cattle, birds, and mice [6], bird feathers or skin [7], and bird uropygial glands [8] play a major role in attracting different families of hematophagous mosquitoes. R-(−)-1-octen-3-ol, an enantiomer of 1-octen-3-ol, was found attractive to field populations of adult mosquitoes [9]. Geranyl acetone (E and Z enantiomers), a component of human sweat, elicited strong electroantennogram responses in female *Anopheles gambiae* Giles [10].

The resurgence of bed bugs (*Cimex lectularius* L.) in recent years stimulated research on bed bug behavior [11, 12] with the goal of developing effective bed bug monitoring tools. It is known that bed bugs use CO_2 [11–13], heat, and chemical odors to locate their hosts [11, 12, 14, 15]. Among the chemical lures, geranyl acetone, 1-octen-3-ol, and L-lactic acid have been reported to be attractive to bed bugs [16, 17]. Bed bug airborne aggregation pheromones including (E)-2-hexenal, (E)-2-octenal, (2E, 4E)-octadienal, benzaldehyde, nonanal, decanal, sulcatone, (+)-limonene, (−)-limonene, and benzyl alcohol were attractive to bed bug nymphs in olfactometer bioassays [18]. These chemicals could potentially be used for monitoring bed bugs; however their effectiveness has not been tested yet in arenas or under conditions that simulate field conditions.

Anderson et al. [11] demonstrated the effectiveness of a trap baited with CO_2 (50–400 mL/min), heat (37.2–42.2°C), and a chemical lure comprised of 33.0 μg propionic acid, 0.33 μg butyric acid, 0.33 μg valeric acid, 100 μg 1-octen-3-ol (octenol), and 100 μg L-lactic acid. In a separate study, Wang et al. [12] confirmed the effectiveness of CO_2 (169 mL/min) and heat (43.3–48.8°C) in their attraction to bed bugs. Until

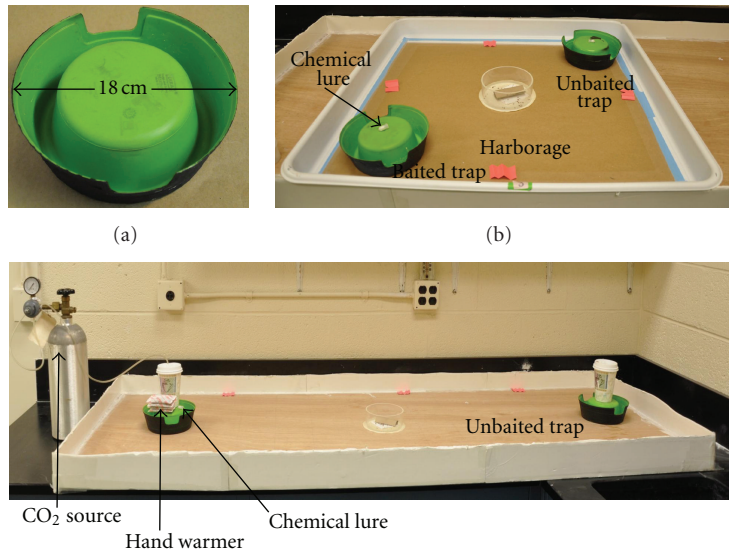

Figure 1: Experimental setup for determining bed bug attraction to nonchemical and chemical lures: (a) pitfall trap used in all bioassays; (b) a plastic tray arena with a baited and an unbaited trap; (c) a wooden door arena with a baited trap and an unbaited trap.

present, there are no studies investigating the interactions among chemical lures, heat, and CO_2.

Bed bugs hide during the day and are difficult to locate as they are small and elusive. Therefore, developing effective monitoring tools has been recognized as a critical component in the current campaign for fighting the bed bug resurgence [19]. Most of the available monitors incorporate one or several nonchemical and chemical lures to attract and capture hungry bed bugs foraging for blood meals. However, the data on the role of various lures in the effectiveness of monitors are very limited. Studying the interactions among nonchemical and chemical lures has immediate practical significance in designing more effective monitors which can be used to detect the presence of small numbers of bed bugs or as an alternative control method. The objectives of this study were (1) screening for chemical lures that are attractive to bed bugs, (2) testing the effect of CO_2 release rate and heat source on trap catches and (3) determining the interactions among chemical lures, CO_2, and heat in attracting bed bugs.

2. Material and Methods

2.1. Insects. Bed bugs were collected from an infested house in Lakewood, NJ. They were maintained in plastic containers (4.7 cm height and 5 cm diameter) with folded paper as harborages at 26°C ± 1°C, 40 ± 10% relative humidity, a 12 : 12-hour (L : D) photoperiod, and were deprived of food for the entire duration of the study. There was a great variation in their hunger levels ranging from very hungry to very well fed at the time of collection. We immediately started the experiments after collection using hungry bugs based on color of the insect abdomen. Only males and large bed bug nymphs were used in this study. Females were not tested to avoid mating and laying eggs in the arenas. All bioassays were conducted within 3 months after bed bugs were collected.

2.2. Pitfall Trap and Experimental Arenas. Pitfall traps were used to evaluate the attractiveness of various lures. The pitfall trap was an inverted plastic dog bowl (600 mL volume, 18 cm diameter, 6.4 cm depth, and 1 mm thickness) (IKEA, Baltimore, MD, USA) (Figure 1(a)). The outer wall of the trap was covered with a layer of paper surgical tape (Caring International, Mundelein, IL, USA), which was painted black with ColorPlace spray paint (WalMart Stores Inc., Bentonville, USA). Bed bugs preferred black color to white color in our preliminary bioassays.

Two types of experimental arenas were used: (a) wooden door arenas (200 by 76 cm by 6.4 cm) (length by width by height) with wooden floor and (b) plastic tray arenas (80 by 75 by 5 cm) (length by width by height) with bottom lined with brown paper (Figure 1(b)). The brown paper was never changed during the entire study. A layer of fluoropolymer resin (DuPont Polymers, Wilmington, DE, USA) was applied to inner walls of the experimental arenas to prevent the bugs from escaping. A layer of this resin was also applied to inner walls of the pitfall traps in a similar fashion to confine the bed bugs that fell into the traps. A filter paper (15 cm diameter) was placed on the floor in the center of each arena, and then a plastic ring (13.3 cm diameter and 6.4 cm height) was placed on the filter paper for confining the bed bugs. A piece of folded cardboard and folded fabric was placed on the filter paper inside the ring to provide harborages for bed bugs. Six and four additional paper harborages measuring 5.1 cm long and 3.3 cm wide were placed along the edges of the floor of each wooden and tray arena, respectively. Two wooden door arenas were located at least 6 m away from each other in a 15 m long and 9 m wide room at 23–25°C. Two additional wooden door arenas were located in two 4 m long and 2.3 m wide rooms at 24-25°C. These rooms had air current through vents on the ceilings or through the open door. In experiments using plastic tray arenas, four arenas

were placed simultaneously in a nonventilated, closed room measuring 4 m long and 2.3 m wide at 24-25°C. A 12 : 12-hour (L : D) cycle was maintained in all the rooms that were used for bioassays.

2.3. Effect of CO_2 Release Rate on Bed Bug Trap Efficacy. Four door arenas were used and each arena had an unbaited control pitfall trap and a pitfall trap baited with CO_2. The two traps were placed at opposite ends equidistant (85 cm) from the center. The experiment was tested over 4 consecutive days. On each day, a different CO_2 release rate was used in each arena following a Latin square design. The CO_2 source was 5 lb cylinders (Airgas East Inc., Piscataway, NJ, USA). The tested release rates were 200, 300, 400, and 500 mL/min. The rate was determined as mL of bubble fluid displaced by CO_2 per unit of time using a Bubble-O-Meter (Bubble-O-Meter, Dublin, Ohio, USA). The CO_2 was introduced into 240 mL plastic cups that were placed on the pitfall traps (Figure 1(c)). Two holes were made on the lid of each plastic cup for CO_2 to escape. Fifty bed bug nymphs and adult males were released into the center of each arena and confined with a plastic ring. The bugs were acclimated for approximately 15 hours prior to the start of the experiment. At 1 hour after dark cycle, CO_2 was released and the plastic ring confining the bugs was removed. The numbers of bed bugs trapped in the pitfall traps and those in the arenas were collected and counted only after 8 hours with the aid of a flashlight. An 8-hour period has been observed to be sufficient for observing the effect of lures on bed bug behavior in preliminary bioassays. After counting, dead and moribund bugs were replaced with healthy bugs in each arena. All bugs were placed back to the center of the arenas and confined with plastic rings for 15 hours before starting the next bioassay.

2.4. Effect of Heat on Bed Bug Trap Efficacy. This experiment was conducted in four plastic tray arenas. Mini hand warmers were used as the heat source (Grabber, Grand Rapids, MI, USA). Two pitfall traps were placed at opposite corners of each arena equidistant (25 cm) from the center. One trap received either two or four mini hand warmers, and the other trap was used as an unbaited control. The surface temperature of the hand warmer was 40–48°C during the first 6 hours. The air temperatures on the floor of arenas 1 cm away from the pitfall trap baited with 2 and 4 hand warmers were 0.2–0.3°C and 0.5–0.6°C, above the ambient temperature, respectively. The air temperatures at the lip of pitfall trap baited with 2 and 4 hand warmers were 0.8–0.9°C and 1.3–1.6°C, above the ambient temperature, respectively. These temperatures were based on hourly recordings of one monitor during the first 6 hours after trap placement using a thermocouple thermometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). The ambient temperature was recorded in the center of each arena equidistant (25 cm) from all traps and 3 cm above arena floor. Each treatment was replicated 6 times over 3 consecutive days. Fifty bed bugs were released into each arena and the testing procedure was the same as that in Section 2.3.

2.5. Effect of Heat on Bed Bug Trap Efficacy When CO_2 is Present. CO_2 at 200 mL/min was selected based on results from Section 2.3. This rate is similar to the respiration rate of an adult human at rest (250 mL/min) [20]. CO_2 alone or in combination with 2, 3, or 4 mini hand warmers was tested in four wooden door arenas on the same day under similar conditions to those in Section 2.3. Each treatment was assigned to a different arena, and the experiment was repeated four times over four consecutive days following a Latin square design. Each arena had an unbaited control trap and a baited pitfall trap placed on opposite ends of the test arena. Fifty bed bugs were released into each arena and the testing procedure was the same as that in Section 2.3.

2.6. Screening of Chemical Lures for Attraction to Bed Bugs in Four-Choice Bioassays. Twelve known or potential bed bug chemical lures (Table 1) were evaluated for their attractiveness to bed bugs in plastic tray arenas. Most of them were provided by Bedoukian Research Inc. (Danbury, CT, USA). Three chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). One chemical was purchased from New Directions Aromatic (Ontario, Canada). Among them, styralol, benzyl alcohol, 6-methyl-5-hepten-2-one, and Insect Biting Lure, were potentially attractive to bed bugs (Robert Bedoukian, personal communication). The chemicals were randomly divided into 4 groups. Each group was tested in the same arenas to evaluate the attractiveness of the chemicals. A 50 μL aliquot of each chemical was dispensed on cotton within a 0.7 mL microcentrifuge tube. The lid of each tube had a 2 mm diameter opening to allow for slow release of the chemical. Four pitfall traps were placed at four corners equidistant (25 cm) from the center. Three traps in each arena were baited with three different chemical lures belonging to the same group listed in Table 1 and the fourth trap was an unbaited control. Each group of chemical lures was tested 8 times over two consecutive days. Fifty bed bugs were released into each arena and the testing procedure was the same as that in Section 2.3.

2.7. Attractiveness of Chemical Lures to Bed Bugs in Two-Choice Bioassays. Nonanal, 1-octen-3-ol, spearmint oil, coriander Egyptian oil, L-lactic acid, and L-carvone exhibited significant attraction to bed bugs in Section 2.6. These chemicals were further evaluated to confirm their attractiveness to bed bugs using two-choice bioassays. The experimental setup and testing procedure were similar to Section 2.6. The difference was that only two traps were placed at opposite corners of each arena (Figure 1(b)). One trap was used as an unbaited control and the other trap received a chemical lure. Each chemical lure was evaluated 8 times over two consecutive days. The baited and unbaited trap positions in each arena were switched on the second day to eliminate any positional effect that could influence the trap catch.

2.8. Relative Attractiveness of Chemical Lures to Bed Bugs in Four-Choice Bioassays. The relative attractiveness of four most effective chemicals, nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil identified from Section 2.7,

Table 1: Percent bed bugs in pitfall traps baited with three chemical lures and an unbaited control in each arena.

Group	Chemical lure	N	Mean (%) ± SE	F	P value	Source of material
I	1-Octen-3-ol	8	28.3 ± 2.5*	8.60	0.0001	Bedoukian Research Inc.
	L-Lactic acid	8	25.7 ± 2.7*			Bedoukian Research Inc.
	Coriander Egyptian oil	8	24.2 ± 4.8*			New Directions Aromatic
	Control	8	12.0 ± 1.3			
	Arena	8	10.0 ± 3.9			
II	L-carvone	8	27.5 ± 3.5*	6.90	0.0001	Bedoukian Research Inc.
	Spearmint oil	8	25.0 ± 2.2*			Bedoukian Research Inc.
	Styralol	8	16.4 ± 2.4			Bedoukian Research Inc.
	Control	8	14.6 ± 1.1			
	Arena		16.4 ± 2.2			
III	Nonanal	8	27.7 ± 3.2*	4.84	0.002	Sigma-Aldrich Co.
	Benzyl alcohol	8	25.1 ± 3.4*			Sigma-Aldrich Co.
	6-Methyl-5-Hepten-2-one	8	20.9 ± 2.5			Sigma-Aldrich Co.
	Control	8	15.1 ± 1.8			
	Arena		11.2 ± 2.6			
IV	Insect Biting Lure	4	16.8 ± 1.6	0.57	0.63	Bedoukian Research Inc.
	R-Octenol + NH_3HCO_3	4	15.3 ± 3.3			Bedoukian Research Inc.
	Z-Geranyl Acetone	4	13.0 ± 2.4			Bedoukian Research Inc.
	Control	4	12.0 ± 3.7			
	Arena		42.7 ± 3.6			

* Indicates significantly different from the unbaited control within each group ($P < 0.05$).

was evaluated using the same method as that in Section 2.6. Each of the four traps in each arena was baited with one of these chemicals. Four arenas were used to obtain four replicates.

2.9. Attractiveness of a Chemical Lure Mixture to Bed Bugs. Nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil were confirmed with significant attraction to bed bugs from Section 2.7. We examined the attractiveness of a mixture of these four chemical lures. Ten microliter of each chemical was dispensed onto cotton within a 0.7 mL microcentrifuge tube. The experimental setup was similar to Section 2.7 (Figure 1(b)). Each plastic tray arena had two traps: one trap was used as an unbaited control and the other trap received the chemical lure mixture. Four tray arenas were used. The experiment was repeated the next day. The baited and unbaited trap positions in each arena were switched on the second day. Other procedures were the same as those in Section 2.3.

The attractiveness of the four-chemical lure mixture was also compared with each individual lure component. A 40 μL of individual chemical lure was dispensed on cotton within a 0.7 mL microcentrifuge tube. Two traps were placed at opposite corners. One trap received one of the four chemicals and the other trap received the four-chemical lure mixture. Four arenas were used. On each day, a different chemical was tested in each arena. The experiment was repeated four times over four consecutive days following a Latin square design. Other procedures were the same as those in Section 2.3.

2.10. Attractiveness of a Chemical Lure Mixture When CO_2 and Heat Are Present

2.10.1. Comparison between CO_2 Alone and CO_2 + Chemical Lure + Heat. Two door arenas were baited with CO_2 (200 mL/min) and two arenas were baited with a combination of CO_2 (200 mL/min), heat (4 mini hand warmers), and the chemical lure mixture as discussed in Section 2.9 (Figure 1(c)). The experiment was repeated four times over four consecutive days to obtain 8 replicates. The baited and unbaited trap positions in each arena were switched after two days.

2.10.2. Comparison between CO_2 Alone and CO_2 + Chemical Lure. Two door arenas were baited with CO_2 (200 mL/min) and two arenas were baited with a combination of CO_2 (200 mL/min) and the chemical lure mixture. The experiment was repeated three times over three consecutive days to obtain 6 replicates. The baited and unbaited trap positions in each arena were switched after the second day. The experimental procedures were the same as those in Section 2.3.

2.11. Statistical Analyses. Bed bug distribution among traps in each arena was summarized as percentage of bed bugs in traps and percentage of bugs that remained in the arena. Generalized mixed linear models (PROC GLIMMIX) were used to analyze the count data [21]. The model accommodates random effects (cohort), repeated measures, and overdispersion. In all experiments, only those bed bugs

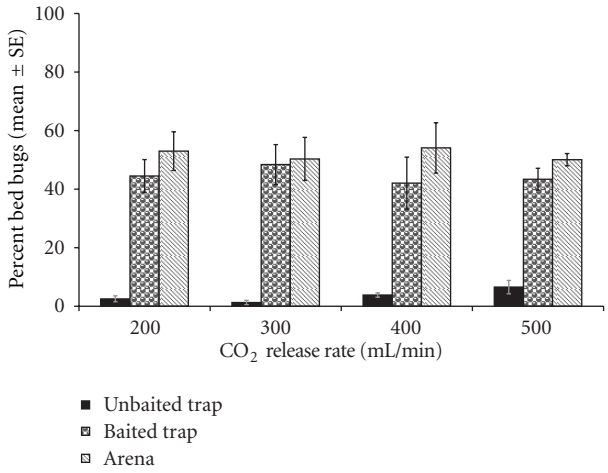

Figure 2: Effect of CO_2 release rate on bed bug trap efficacy.

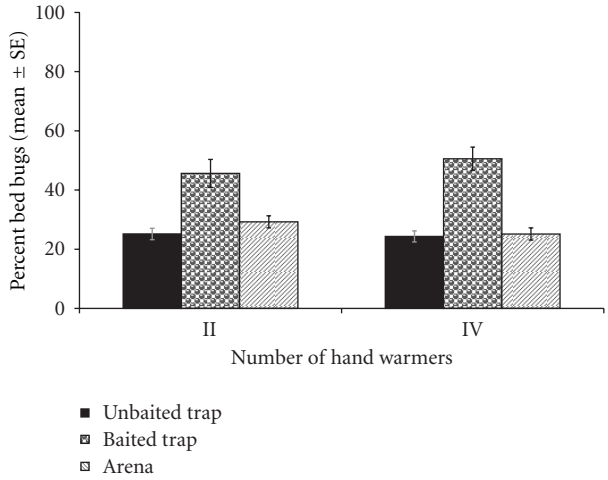

Figure 3: Effect of heat on bed bug trap efficacy.

that appeared in the traps were analyzed. Those bugs that remained in the arenas at the end of the experiments were weak, inactive, or behaviorally different from those actively seeking for a host. Previous observations indicate that the presence of bed bugs in a trap had no significant effect on the probability of trapping additional bed bugs. Therefore, the bed bugs in the traps were considered independent events and were not related to gregarious behavior. The data for Sections 2.10.1 and 2.10.2 were pooled for analyzing differences among treatments.

3. Results

The different CO_2 release rates had no significant effect on trap catches ($F = 2.23$, df = 3, $P = 0.08$) (Figure 2). In each test arena, the probability (mean ± 95% confidence interval) of bed bugs being caught in a trap baited with 200, 300, 400, and 500 mL/min CO_2 was 94.6 ± 2.6, 97.4 ± 1.8, 91.7 ± 3.2, and 85.9 ± 4.1%, respectively. Heat (two or four mini hand warmers) significantly increased trap catches ($P < 0.05$) although there were no significantly differences between the two heat sources ($F = 0.08$, df = 1, $P = 0.77$) (Figure 3). The probability of bed bugs being caught in traps baited with two and four hand warmers was 64.7 ± 4.3 and 66.4 ± 3.9%, respectively. There were no significant differences among pitfall traps baited with CO_2 alone or in combination with 2, 3, or 4 hand warmers in door arenas (Figure 4) ($F = 0.61$, df = 3, $P = 0.60$). The probability of bed bugs being caught in traps baited with 200 mL/min alone and in combination with 2, 3, and 4 hand warmers was 93.2 ± 2.6, 95.8 ± 2.0, 92.2 ± 2.8, and 91.0 ± 2.8%, respectively.

Out of the twelve bed bug attractants evaluated in four-choice bioassays, nonanal, 1-octen-3-ol, spearmint oil, coriander Egyptian oil, L-lactic acid, L-carvone, and benzyl alcohol baited traps caught a significantly higher number of bugs than their corresponding controls ($P < 0.05$) (Table 1). In two-choice bioassays, nonanal, spearmint oil, 1-octen-3-ol, and coriander Egyptian oil baited traps caught significantly more bugs than L-lactic acid and L-carvone baited

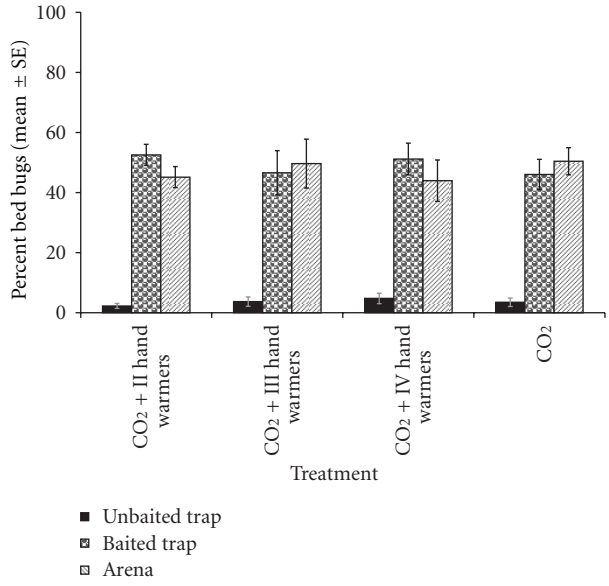

Figure 4: Effect of heat on bed bug trap efficacy when CO_2 is present.

traps ($F = 10.02$, df = 5, $P = 0.0001$) (Figure 5). Nonanal, spearmint oil, 1-octen-3-ol, and coriander Egyptian oil were not significantly different from each other ($P > 0.05$). The probability of bed bugs caught in traps baited with nonanal, spearmint oil, 1-octen-3-ol, coriander Egyptian oil, L-lactic acid, and L-carvone was 75.1 ± 3.3, 73.9 ± 3.0, 69.0 ± 3.7, 67.3 ± 4.0, 55.2 ± 3.8, and 51.9 ± 4.3%, respectively. Further analysis in four-choice experiments showed that pitfall traps baited with nonanal captured a significantly higher number of bed bugs than spearmint oil, 1-octen-3-ol, and coriander Egyptian oil ($F = 6.43$, df = 3, $P = 0.0002$). In each arena, the probability of bed bugs being trapped in nonanal, coriander Egyptian oil, 1-octen-3-ol, and spearmint oil baited traps was 41.5 ± 4.0, 19.6 ± 3.0, 18.3 ± 4.0, and 20.6 ± 4.0%, respectively.

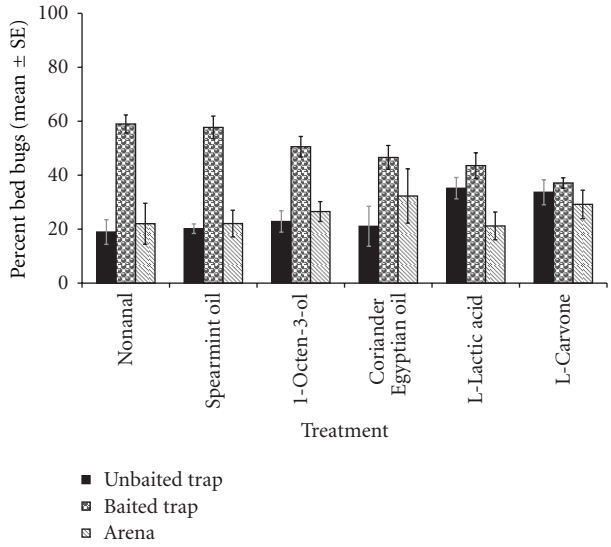

Figure 5: Attractiveness of chemical lures to bed bugs in two-choice bioassays.

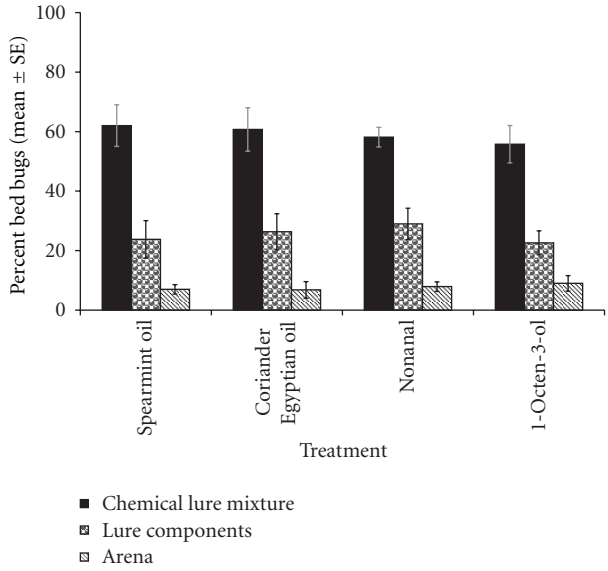

Figure 6: Attractiveness of a lure mixture compared with single lures.

The traps baited with a chemical lure mixture comprising nonanal, spearmint oil, 1-octen-3-ol, and coriander Egyptian oil captured significantly higher numbers of bed bugs than the unbaited control traps ($P < 0.05$). The probability of bed bugs trapped in chemical lure mixture baited traps was 71.0 ± 2.8%. These chemical lure mixture baited traps were significantly more attractive to bed bugs than any of the four individual lure components ($P < 0.05$) (Figure 6). The probability of bed bugs trapped in chemical lure mixture baited traps when compared with nonanal, coriander Egyptian oil, 1-octen-3-ol, or spearmint oil baited traps was 66.9 ± 3.6, 70.4 ± 3.5, 71.1 ± 3.6, and 72.6 ± 3.4%, respectively. Traps with a combination of either chemical lure mixture + CO_2, or chemical lure mixture + CO_2 + heat captured significantly more bed bugs when compared to the traps baited with CO_2 only ($F = 24.81$, df = 2, $P = 0.0001$). However, bed bug counts in traps baited with chemical lure mixture + CO_2 were not significantly different than those in traps baited with chemical lure mixture + CO_2 + heat ($P > 0.05$). The probability of bed bugs being caught in traps baited with CO_2, chemical lure mixture + CO_2, and chemical lure mixture + CO_2 + heat was 71.7 ± 1.9, 87.5 ± 2.0, and 88.8 ± 1.7%, respectively (Figure 7).

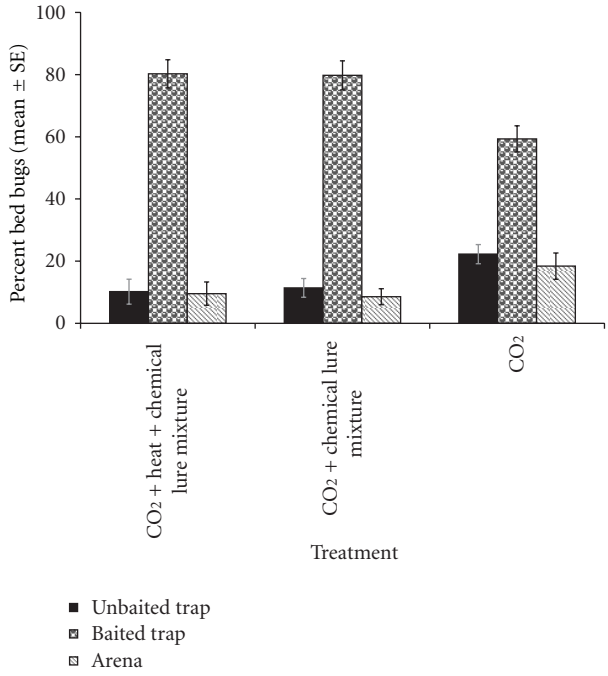

Figure 7: Effect of chemical lure mixture and heat + lure mixture when CO_2 is present.

4. Discussion

Our experiments demonstrated the attractiveness of four chemical lures to bed bugs: nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil. Among these, nonanal was the most attractive chemical lure. Nonanal has been reported to play a major role in the chemical ecology of triatomine bugs [22], *Aedes aegypti* L. [23], and *Anopheles gambiae* [24]. Nonanal was also the major compound found in odorant profiles of humans, chicken, and pigeon and elicited strong response in antenna of southern house mosquito, *Culex pipiens quinquefasciatus* Say [25]. Traps baited with nonanal and CO_2 caught higher number of southern house mosquitoes than traps baited with CO_2 alone [25]. 1-Octen-3-ol has been reported to attract different blood sucking insects including bed bugs [11, 12], *Triatoma infestans* Klug [26], *Glossina* spp. [27], and *Aedes* and *Culex* spp. mosquitoes [28, 29]. Spearmint oil and coriander Egyptian oil are plant derived. L-carvone is the major component (51%) present in spearmint oil [30]. However, L-carvone did not significantly increase trap catch in two-choice bioassays. Its enantiomer,

D-carvone, has been patented as an attractant for Culicidae mosquitoes [31]. Spearmint oil and carvone (L and D enantiomers) were found very attractive to both nymphs and adults of spot clothing wax cicada, *Lycorma delicatula* White [32]. Coriander Egyptian oil has the aroma similar to odors emitted by bed bugs [33].

CO_2 was very attractive to bed bugs regardless of the CO_2 release rates being used when tested in door arenas, indicating that 200 mL/min rate is sufficient for attracting bed bugs in a room that is 2 m in length. Marx [13] and Anderson et al. [11] reported that bed bugs can locate a host that is 150 cm and 86 cm away. The 200 mL/min rate seems to have exceeded the bed bug response threshold and any higher concentrations above that were not helpful in enhancing their responses in door arenas. Measuring the CO_2 gradient at various locations of the arenas might be helpful to establish the relationship between CO_2 release rate and bed bug responses. Under field conditions where a typical room is much larger, the minimum effective CO_2 release rate might be larger. Moreover, bed bug hunger levels, air current, and presence of a human host will affect the minimum effective CO_2 rate.

Adding a mixture of four attractants (nonanal, 1-octen-3-ol, spearmint oil, and coriander Egyptian oil) increased bed bug trap catches when CO_2 was present, indicating the additive effect of chemical lures and CO_2 on bed bug host searching behavior. Similarly, Allan et al. [7] found greater attraction in *Culex* spp. by the combined use of feathers and CO_2 than by using each component alone. Mixture of 1-octen-3-ol with CO_2 was reported to be more attractive than CO_2 alone in *Culex salinarius* [34, 35]. Tropical bont ticks, *Amblyomma variegatum* F., were found to be more attracted to pheromone + CO_2 than CO_2 alone [36]. Host seeking in *A. variegatum* involves activation and a nondirectional searching activity by CO_2 and a directional movement to pheromone and to other host emanating odors [36]. Hematophagous hemipteran, *Triatoma infestans* Klug, which is closely related to *C. lectularius*, also uses a combination of host cues to locate a host. CO_2 served as a long range cue in its nonoriented searching behavior and when a bug arrives in close proximity of its host, then radiant heat and chemical odors from the host oriented it to the exact host location [37]. It is possible that bed bugs host searching behavior follows a similar sequence to that of *T. infestans* or *A. variegatum*.

The presence of either two or four hand warmers (or a 0.8–1.6°C difference in temperature between the lip of the trap and the ambient air) attracted bed bugs from a distance of 25 cm. The role of heat became insignificant when used in combination with CO_2 in wooden door arenas, indicating adding heat when a gradient of CO_2 concentration was present in the environment was not helpful in increasing trap catches. In contrast, the role of chemical lure mixture was significant even when CO_2 was present.

The arena substrates were never cleaned or changed during the study period. They could retain natural attractant/chemical cues, which also persist in natural infestations. We wanted to mimic field conditions and determine if the traps can attract the bugs that were already acclimated to the arenas with feces and their associated pheromones present. Results from such experimental conditions would more likely correspond well to those obtained under field conditions.

Wang et al. [38] showed the effectiveness of pitfall traps baited with CO_2 alone for detecting very low level bed bug populations. But none of the bed bug monitors provide 100% assurance of the presence/absence of bed bugs in field environments. Results from this study suggest adding an inexpensive chemical lure to a trap may significantly improve the trap efficacy and provide more accurate monitoring of bed bug infestations. Wang et al. [38] suggested that an effective monitor can be used in unoccupied infested rooms to trap the hungry bed bugs and for reducing the probability of bed bugs dispersing into adjacent uninfested rooms. An effective monitoring/trapping system for bed bugs could also be combined with insecticides to kill bed bugs that are attracted to lures or baited traps.

It is noteworthy to mention that the bed bug strain, hunger level, arena size, and test room conditions had significant impacts on test results in our preliminary experiments. Even within a test arena, there could be a location effect. In a ventilated room, bed bugs that are downwind of the baited trap are more likely to be exposed to the plume of CO_2, chemical lure, or heat. When testing the effect of chemical lures or heat alone, we used small plastic arenas and a room with still air. Using a door-sized arena or in a ventilated room could not detect the attractiveness of chemical lures or heat. When testing CO_2 or combination of CO_2 and heat and/or chemical lure, we used door arenas in ventilated rooms, which mimic the field conditions. Field conditions are usually much more complex than laboratory environments. The presence of a human host, clutter, furniture, and various odors from food and household cleaning agents could significantly affect the performance of a bed bug monitor. Further research is needed to optimize the chemical lure release rate and CO_2 release rate and to evaluate the effectiveness of baited monitors under various field conditions.

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Research Article

Age-related and Individual Variation in Male *Piezodorus hybneri* (Heteroptera: Pentatomidae) Pheromones

Nobuyuki Endo,[1] Tetsuya Yasuda,[2] Takashi Wada,[1] Shin-etsu Muto,[3] and Rikiya Sasaki[3]

[1] *Agro-Environment Research Division, NARO Kyushu Okinawa Agricultural Research Center (KARC), 2421 Suya, Koshi, Kumamoto 861-1192, Japan*
[2] *Division of Plant Protection, NARO Agricultural Research Center (NARC), 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan*
[3] *Ecomone Division, Fuji Flavor Co., Ltd., 3-5-8 Midorigaoka, Hamura, Tokyo 205-8503, Japan*

Correspondence should be addressed to Nobuyuki Endo, enobu@affrc.go.jp

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Males of the *Piezodorus hybneri* stink bug produce a pheromone comprising β-sesquiphellandrene (Sesq), (*R*)-15-hexadecanolide (R15), and methyl (*Z*)-8-hexadecenoate (Z8). We collected airborne volatiles from individual *P. hybneri* males and analyzed them by GC-MS. Daily analysis from 1 to 16 days after adult emergence showed that pheromone emission started around 3 to 6 days after adult emergence and peaked ($\sim$1 μg/male/day) on day 11. The proportion of Sesq tended to increase with age to about 80% on days 12 to 16. On the other hand, the proportion of R15 tended to decrease with age. The proportion of Z8 reached a maximum of about 34% on day 9 but otherwise remained below 20%. The total amount of pheromone emitted by individual males varied considerably: three males emitted more than 10 μg, whereas another three males emitted little or no pheromone and failed to survive by the end of the experiment. These results suggest that the amount of *P. hybneri* pheromone and its blend ratio could be affected by the male's physical conditions, such as vitality and age.

1. Introduction

The stink bug *Piezodorus hybneri* (Heteroptera: Pentatomidae) is an important soybean pest in southern Japan [1, 2]. Male adults of *P. hybneri* attract conspecific adults of both sexes [3] via a pheromone comprising β-sesquiphellandrene (Sesq), (*R*)-15-hexadecanolide (R15), and methyl (*Z*)-8-hexadecenoate (Z8) [4]. These synthetic chemical mixtures attract conspecific adults, especially females in fields [5]. Our previous study [6] revealed that *P. hybneri* males produce the pheromone simultaneously with their development to sexual maturity, and that diapausing males produce no pheromone components; thus, the pheromone is likely to play a role in sexual communication. The average amount of Sesq in whole-body extracts increased steadily until day 30 after adult emergence, whereas the other two components peaked at day 10 and then decreased somewhat [6]. Consequently, the proportions of the pheromone components, especially Sesq, changed with age. These findings suggest that the proportions of components in emissions also change with age. However, pheromone production might not coincide with emission. In addition, marked variation in the pheromone component ratio among individuals of the same population was found the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae) [7]. Therefore, it is necessary to monitor the pheromone emission of *P. hybneri* over time from the same males to examine variation in the pheromone blend.

Mating behavior of *P. hybneri* males began on day 4 after adult emergence and showed high mating activity between days 5 and 15 [6]. Development of the ectodermal accessory gland, which is involved in reproduction and an indicator of male sexual maturity, showed that males fully matured by day 10 [6]. Thus, in this study, we collected and analyzed the volatiles from individual *P. hybneri* males from 1 to 16 days after adult emergence.

Table 1: Individual variation of pheromone titer and extraction from *Piezodorus hybneri* males.

Male	Days of first detection	Pheromone emission (μg)			Amount extracted at 16 d (μg)
		Daily maximum	15-16 d	Sum (1–16 d)	
1	5-6	0.91	0.09	2.91	12.07
2	4-5	3.20	0.58	14.54	27.49
3	4-5	0.75	0.26	4.22	10.51
4	5-6	1.81	1.51	12.41	48.81
5	5-6	0.31	0.00	0.62	0.00
6	—	0.00	—	0.00	—
7	5-6	0.09	—	0.09	—
8	4-5	0.32	—	1.03	—
9	3-4	1.03	0.29	5.68	5.18
10	3-4	3.47	0.50	18.62	27.02
Average		1.32	0.46	6.01	18.73

2. Materials and Methods

2.1. Insects. Adults of *P. hybneri* were caught in soybean fields of the NARO Kyushu Okinawa Agricultural Research Center (32°52′5″ N, 130°44′2″ E), Kumamoto, Japan, in 2005. Their progeny were kept in the laboratory (24 ± 1°C, 16L–8D photoregime) and used for experiments in March 2006. The bugs were reared on a diet of soybean (*Glycine max*) seeds, red clover (*Trifolium pratense*) seeds, and water.

2.2. Collection of Airborne Volatiles with Glass Beakers. Following the method of Yasuda et al. [8] with some modifications, we collected and analyzed the volatiles from individual *P. hybneri* males ($n = 10$) in glass beakers. Collection started 1 day after adult emergence. A single adult male was confined in a 50 mL glass beaker with a few soybean seeds and moist cotton. The beaker was placed upside down and sealed with aluminum foil. The male was kept in the beaker for 24 h under laboratory conditions. After 24 h, the male was removed and the beaker surface was rinsed with 3 mL hexane containing 2 μg octadecane as an internal standard. After this treatment, any pheromone component as well as internal standard was not detected from the hexane rinsing the beaker. The hexane was collected for analysis of volatiles. The male was placed in a new beaker with food and the process was repeated until day 16. After the final collection, the males that survived were extracted with 2 mL hexane containing 2 μg octadecane as an internal standard and then rinsed once with 1 mL hexane. All extracts were stored in glass vials with Teflon-lined screw caps at −20°C until analysis. Extracts were concentrated to ca. 100 μL in an evaporator just before gas chromatography—mass spectrometry (GC-MS) analysis.

2.3. GC-MS Analysis. Quantitative GC-MS analysis was done on an Agilent 6890 N GC with an HP-5 ms column (30 m × 0.25 mm ID × 0.25 μm film thickness; Agilent Technologies) and an Agilent 5975i Network Mass Selective Detector using an internal-standard method. Mass spectrometry data by selected ion monitoring (SIM) and full scan (range: 35–350 *m/z*) were acquired synchronously. Quantitative (selected) and reference ions for SIM were *m/z* 254 and 57, respectively, for octadecane, *m/z* 204 and 69 for (±)-β-sesquiphellandrene, *m/z* 210 and 55 for (±)-15-hexadecanolide, and *m/z* 268 and 55 for methyl (*Z*)-8-hexadecenoate. Injection was performed in splitless mode with a split/splitless injector using an Agilent 7683 series automatic liquid sampler at 250°C. Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The GC oven temperature was an initial 50°C (2-min hold), increased to 240°C at 15°C/min, and then held for 5 min. To determine the quantity of each component, standard curves obtained using known amounts of authentic chemicals with the internal standard (octadecane) were used.

2.4. Chemicals. (±)-β-Sesquiphellandrene, (±)-15-hexadecanolide, and methyl (*Z*)-8-hexadecenoate were synthesized according to a previous report [4].

3. Results

The temporal patterns and the amounts of pheromone emission by males varied greatly among individuals (Figure 1). Nine out of the 10 males emitted pheromone (Table 1). Emission started 3 to 6 days after adult emergence, peaked (∼1 μg/male/day) on day 10 or 11, and remained high until day 16. The maximum average emission was 1.32 μg/day, and two bugs exceeded 3 μg/day. The total amount of pheromone varied more than 30-fold among surviving individuals (0.62–18.62 μg). Male No. 6 did not emit any pheromone and died on day 11. Males No. 7 and 8 emitted little pheromone and died on days 7 and 15, respectively. There was a strong correlation between the amount of pheromone collected on day 16 and the amount extracted on day 16 ($r^2 = 0.885$, $P = 0.0016$).

The proportions of the three pheromone components, especially Sesq, showed great variability (Figures 1 and 2). The proportion of Sesq tended to increase with age to about 80% on days 12 to 16. On the other hand, the proportion of R15 tended to decrease with age. The proportion of Z8 reached a maximum of about 34% on day 9, but otherwise remained below 20%.

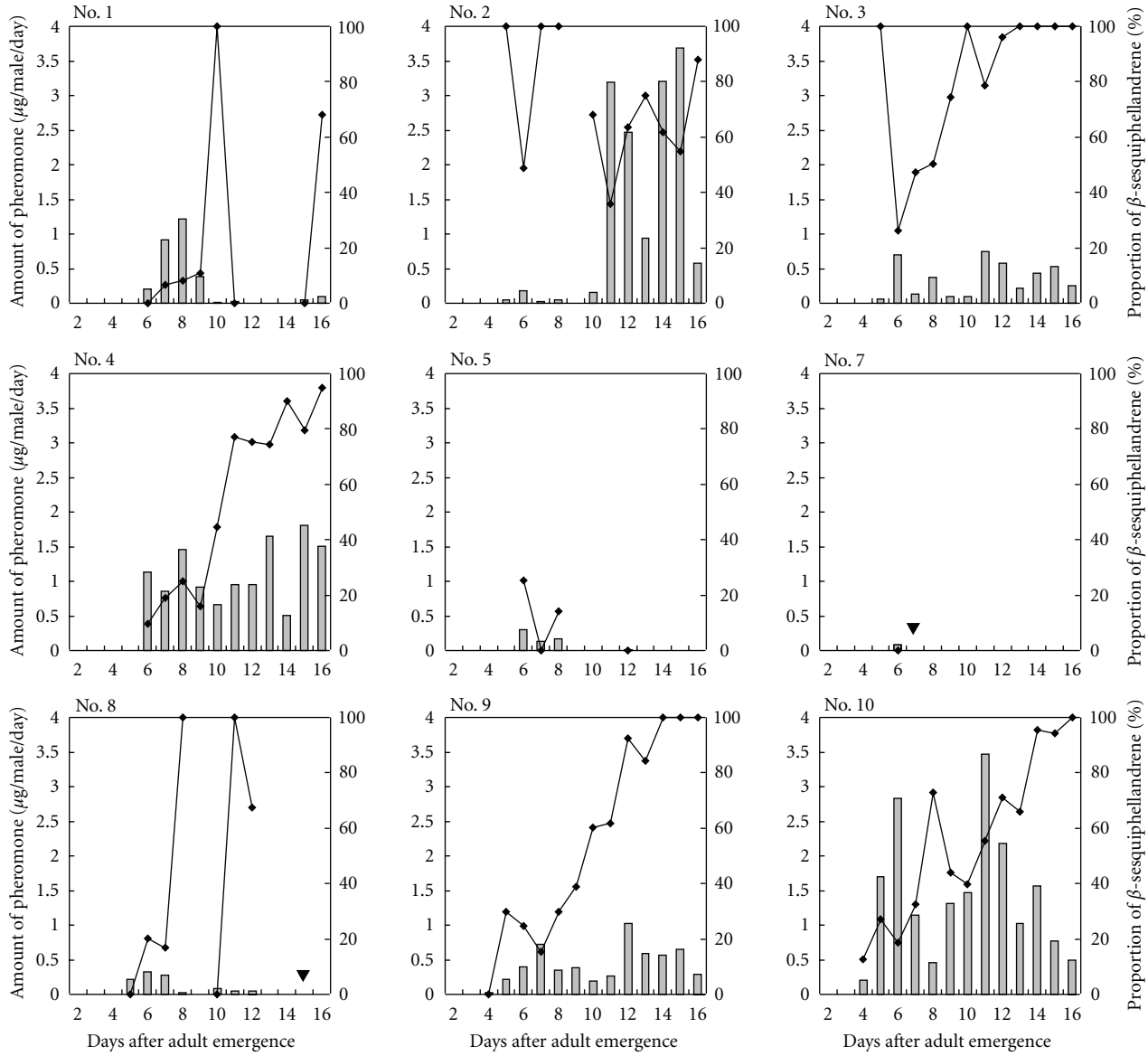

Figure 1: Daily changes in pheromone emission and the proportion of β-sesquiphellandrene from individual males of *Piezodorus hybneri*. The bar shows the total amount of pheromone (μg/male/day). The line chart indicates the proportion of β-sesquiphellandrene (%) among the total pheromone components. Inverted triangle indicates the day when the bugs died. Because male No. 6 did not emit any pheromone, there was no data available in this figure.

4. Discussion

Repeated collection of volatiles with a glass beaker from individual males over a period of 15 consecutive days showed that the pheromone component ratio varied with male age. The proportion of Sesq tended to increase with age and reached about 80% by days 12 to 16. This trend in variable pheromone blend ratios agreed with our previous results obtained from extracts of males [6]. The high correlation between the quantities of pheromone in emissions and those in body extracts, on day 16, indicates that pheromone emission parallels pheromone production in this species.

In some heteropteran species, variations of the pheromone blend ratio among individuals or with physiological condition have been reported. In *N. viridula*, the pheromone blend ratio varied among individuals within a population [7, 9], although individuals' ratios remained constant [9]. Large variability of pheromone component ratios in the bean bug *Riptortus pedestris* (Heteroptera: Alydidae) was also reported [10]. Recently, Moraes et al. [11] reported that food conditions affected the pheromone ratios in the Neotropical brown stink bug *Euschistus heros* (Heteroptera: Pentatomidae). We report here that large changes in the pheromone blend of *P. hybneri* males occur during the first 2 weeks of adulthood. This is the first study documenting age-related shifts in the pheromone blend of Heteroptera.

The pheromone blend ratio is generally calculated from the amounts of components emitted at a specific age.

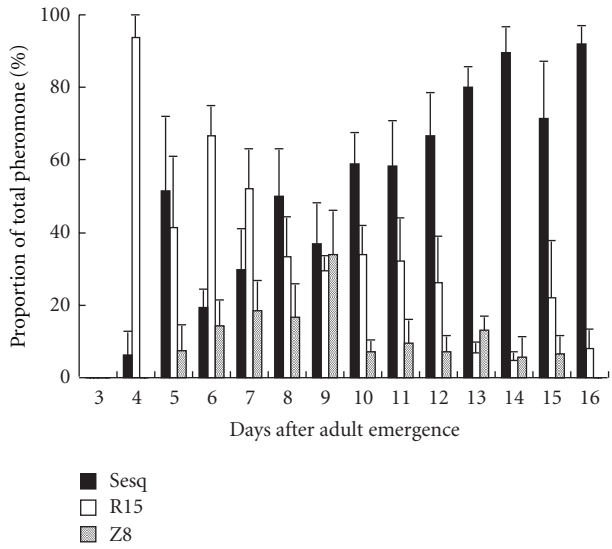

Figure 2: Proportions of each pheromone component of *Piezodorus hybneri* males in relation to adult age. Sesq: β-sesquiphellandrene; R15: (*R*)-15-hexadecanolide; Z8: methyl (*Z*)-8-hexadecenoate. Vertical lines represent SE.

However, this approach is based on the premise that the ratio remains constant throughout adult life. Leal et al. [4] reported that the pheromone blend ratio of *P. hybneri* was Sesq : R15 : Z8 = 10 : 4 : 1. However, our results show that the ratio was not constant and was affected by male age. This may be the case in other species, and thus it should be taken into consideration when pheromone blend ratios are determined.

Pheromone quantity could be also affected by the male physical conditions. Pheromone production of *P. hybneri* is paralleled to the development of male sexual maturity [6], and young *P. hybneri* males cannot produce or emit a large amount of pheromone. In addition, our results show a large variation in pheromone quantity among individuals, and the poor pheromone emitters died early. These results suggest that only vital and sexually mature males can produce sufficient pheromone.

The biological function or attractiveness of each pheromone components or its blends for *P. hybneri* is largely unknown. In fields, each *P. hybneri* pheromone component alone lacked the attractiveness, while three-component mixture was attractive to *P. hybneri* [5]. Leal et al. [4] showed that the full three-component mixture was attractive more than the any binary mixtures in laboratory conditions, whereas the attractiveness of the different blend ratios has never been investigated. In order to clarify the biological functions of *P. hybneri* pheromone blend or its components, it is necessary to compare the attractiveness among different pheromone blends or male ages.

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Research Article

Case Study: Trap Crop with Pheromone Traps for Suppressing *Euschistus servus* (Heteroptera: Pentatomidae) in Cotton

P. G. Tillman[1] and T. E. Cottrell[2]

[1] *USDA, ARS, Crop Protection and Management Research Laboratory, P.O. Box 748, Tifton, GA 31793, USA*
[2] *USDA, ARS, Southeastern Fruit & Tree Nut Research Laboratory, 21 Dunbar Road, Byron, GA 31008, USA*

Correspondence should be addressed to P. G. Tillman, glynn.tillman@ars.usda.gov

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The brown stink bug, *Euschistus servus* (Say), can disperse from source habitats, including corn, *Zea mays* L., and peanut, *Arachis hypogaea* L., into cotton, *Gossypium hirsutum* L. Therefore, a 2-year on-farm experiment was conducted to determine the effectiveness of a sorghum (*Sorghum bicolor* (L.) Moench spp. *bicolor*) trap crop, with or without *Euschistus* spp. pheromone traps, to suppress dispersal of this pest to cotton. In 2004, density of *E. servus* was lower in cotton fields with sorghum trap crops (with or without pheromone traps) compared to control cotton fields. Similarly, in 2006, density of *E. servus* was lower in cotton fields with sorghum trap crops and pheromone traps compared to control cotton fields. Thus, the combination of the sorghum trap crop and pheromone traps effectively suppressed dispersal of *E. servus* into cotton. Inclusion of pheromone traps with trap crops potentially offers additional benefits, including: (1) reducing the density of *E. servus* adults in a trap crop, especially females, to possibly decrease the local population over time and reduce the overwintering population, (2) reducing dispersal of *E. servus* adults from the trap crop into cotton, and (3) potentially attracting more dispersing *E. servus* adults into a trap crop during a period of time when preferred food is not prevalent in the landscape.

1. Introduction

Agronomic crops across the southeastern US face significant economic losses from stink bugs (Hemiptera: Pentatomidae), mainly the southern green, *Nezara viridula* (L.), the brown, *Euschistus servus* (Say), and the green, *Chinavia hilaris* (Say) [1]. For example, in cotton (*Gossypium hirsutum* L.), eradication of the boll weevil, *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae), along with adoption of *Bt*-crops has decreased use of broad-spectrum insecticides leading to the emergence of stink bugs as major pests [2].

Stink bugs are generalist feeders that exhibit edge-mediated dispersal from early-season crops into subsequent adjacent crops as adults forage for food and sites for oviposition [3–10]. Each year in Georgia, corn (*Zea mays* L.) is one of the earliest agronomic host plants available to stink bugs [11, 12], with peanut (*Arachis hypogaea* L.) and cotton being mid-to-late-season hosts for these pests [13, 14]. Where these three crops are closely associated in farmscapes, *E. servus* disperses from corn into peanut and cotton and from peanut into cotton at the common boundary, or interface, of the source crop and cotton [15, 16].

One strategy for managing dispersing pests is trap cropping where an attractive plant species is used to arrest the pests and reduce their likelihood of entering a crop field [17]. Trap crops have been shown to effectively manage stink bugs in conventional and organic crop production systems [18–22]. Grain sorghum (*Sorghum bicolor* (L.) Moench spp. *Bicolor*) is an important host plant for panicle-feeding stink bugs in Georgia [23], and it can suppress populations of *N. viridula* in farmscapes in Georgia [24].

The pheromone of *Euschistus* spp. is attractive to males, females, and nymphs of *E. servus* and other *Euschistus* spp. [25]. A pyramidal trap [26] was modified [27] to facilitate stink bug capture. When these capture traps contain lures of

Table 1: Planting date (PD) and variety for cotton in sorghum trap crop with pheromone traps (STC/PTs), sorghum trap crop (STC), and control (CO) fields in 2004 and 2006.

Year	Treatment	Rep	Variety	PD
2004	STC/PT	1 & 2	Deltapine 449	5/6
		3	Fibermax 960	5/10
		4	Deltapine 555	5/8
	STC	1	Deltapine 458	5/6
		2	Fibermax 960	4/23
		3 & 4	Deltapine 555	5/6
		5	Deltapine 555	5/15
	CO	1	Deltapine 444	5/31
		2 & 3	Deltapine 555	5/6
		4 & 5	Deltapine 555	5/8
		6	Fibermax 960	5/12
		7	Fibermax 960	4/29
		8	Deltapine 555	5/15
2006	STC	1	Deltapine 555	5/4
		2	Deltapine 555	4/28
		3	Deltapine 555	5/10
	CO	1	Deltapine 555	5/4
		2	Deltapine 555	5/26
		3	Deltapine 555	5/1

a specific stink bug pheromone, they effectively capture *E. servus*, *N. viridula*, and *C. hilaris* [27, 28]. The addition of an insecticidal ear tag improved trap captures of *Euschistus* spp. in pecan orchards by preventing escape of the bugs [29].

Our hypothesis was that sorghum planted in a narrow strip along the length of the interface of a source crop and cotton would attract *E. servus* adults. Additionally, capture traps baited with *Euschistus* spp. pheromone within the sorghum would help reduce dispersal out of sorghum by capturing and killing the pests in the sorghum. Thus, a full-scale field experiment was conducted to determine the effectiveness of sorghum with *Euschistus* spp. pheromone traps to suppress *E. servus* in cotton.

2. Materials and Methods

2.1. Study Sites. Twenty-three commercial cotton fields were sampled during this 2-year study (Table 1). These cotton fields were located in Irwin County GA and ranged from 5 to 15 ha in size. Overall, five cotton varieties were planted (Table 1). Sorghum, variety DK E57, was planted in a 4-row-wide strip along the length of the edge of a cotton field next to a source crop (corn or peanut) on May 5 2004 and on April 14 2007. Growers followed recommended agricultural practices for production of sorghum [30] and cotton [31]. Row width was 0.91 m for each crop, and rows of adjacent crops ran parallel.

2.2. Stink Bug Pheromone Traps. A pheromone trap consisted of a 2.84-liter clear plastic poly-ethylene terephthalate jar (United States Plastic Corp., Lima, OH, USA) on top of a 1.22 m-tall yellow pyramidal base [27]. An insecticidal ear tag (Saber Extra, Coppers Animal Health, Inc., Kansas City, KS, USA) was placed in the plastic jar at the beginning of a test. Active ingredients in the ear tag were lambda-cyhalothrin (10%) and piperonyl butoxide (13%). Rubber septa, each loaded with 40 μL of the *Euschistus* spp. pheromone, methyl (*E,Z*)-2,4-decadienoate (CAS registry no. 4493-42-9) (Degussa AG Fine Chemicals, Marl, Germany), were used as lures [32]. Lures were changed weekly for the duration of a test. Insects from weekly collections were taken to the laboratory for identification.

2.3. 2004 Experiment. Two treatments at the edge of cotton fields were examined for their ability to suppress stink bugs dispersing from an adjacent source crop into cotton: a sorghum trap crop with *Euschistus* spp. pheromone traps (STC/PTs) and a sorghum trap crop only (STC). Control fields had no sorghum or pheromone traps. For STC/PT fields, 21 pheromone traps were placed 12 m apart in sorghum on the row next to the source crop. At the beginning of the study, 17 cotton fields were selected, and each treatment was assigned randomly to various fields (four fields for STC/PT, five fields for STC, and eight fields for control) similar to a completely randomized design. Individual fields were used as replicates because the sorghum trap crops were planted along the full width between the cotton field and source crop.

2.4. 2006 Experiment. Only the STC/PT treatment and a control, both as explained above for the 2004 experiment, were used. At the beginning of the study, six cotton fields were selected, and each treatment was assigned randomly to three fields similar to a completely randomized design. For

the STC/PT treatment, 25–28 pheromone traps (depending on field width) were placed 12 m apart in the first row of sorghum closest to the source crop.

2.5. Insect Sampling. Each year of the study, cotton, sorghum, and pheromone traps were examined for the presence of stink bugs on a weekly basis: from the week of 16 June to the week of 28 July in 2004 and from the week of 28 June to the week of 23 August in 2006. Due to time constraints of sampling these large fields, not all fields were sampled on the same day of the week, but crops and/or pheromone traps within a field were sampled on the same day. For each sorghum sample, the aerial parts of all plants within a 1.83 m length of row were visually checked thoroughly for all stink bugs. For each cotton sample, all plants within a 1.83 m length of row were shaken over a drop cloth and the aerial parts of all plants were visually checked thoroughly for all stink bugs. Voucher specimens are stored in the USDA, ARS, Crop Protection & Management Research Laboratory in Tifton, GA, USA.

For sampling purposes, the edge of a cotton field adjacent to a source crop was referred to as side A, and in a clockwise direction the other 3 sides of a field were referred to as side B, C, and D. In 2004, samples were obtained in each cotton field at two different distances from the field edge along each of the 4 sides of the field. The first edge location was 0–3.66 m from the outside edge of the field, and the second edge location was 3.67–7.31 m from the outside edge of the field. The interior of the field was subdivided into 9 equally sized blocks. During weeks 3–6, samples were collected in each field as follows: 2 samples from each side at the 0–3.66 m location, 2 samples from each side at the 3.67–7.31 m location, and 1 sample from the center of each interior block. During week 7, samples were collected as follows: 2 samples from the center of each interior block and 2 samples from each side at the 3.67–7.31 m location, but at the 0–3.66 m location, 6–12 samples (depending on length of field edge) were collected from each field edge.

In 2006, samples were obtained at 3 distances from the edge on side A (i.e., at rows 1, 2, and 5 from the edge of the cotton field), and from 6 interior locations down the length of the field near to side C (i.e., rows 16, 33, 100, 167, 233, and 300 from the edge of the field on side A). For sides B–D, samples were taken from 2 edge locations, rows 1 and 5 from the edge of the field. The numbers of samples from each field on each date were as follows: 9 from each row on side A, 3 from each row on sides B–D, and 6 from each interior location.

During 2004, the 4-row strip of sorghum was sampled by taking five random samples from rows 1 and 2 and four random samples from rows 3 and 4. In 2006, 9 random samples were obtained from each of the 4 rows.

2.6. Statistical Analysis. For cotton, trap crop treatments in 2004 and 2006 were analyzed using PROC MIXED [33]. For both years of data, preliminary analyses revealed that there was only a significant treatment × week × field location interaction; there were no significant differences among fields. So, when trap crop data were analyzed, the fixed effect was treatment by week by field location, and random effects were replicate within treatment and residual error. Least squares means were separated by least significant difference (LSD) [33] where appropriate. In 2004, two cotton fields (one STC/PT field and one control field) were not included in the data set for sampling week 7 because the grower treated for stink bugs after sampling on week 6. In 2006, one STC cotton field was not included in the data set for sampling during week 9 because it was treated for stink bugs after sampling on week 8. Chi-square analyses were used to compare frequencies of *E. servus*, *N. viridula*, and *C. hilaris* for each trap crop treatment by week in 2004 and 2006 (PROC FREQ, [33]).

For 2004 data, numbers of *E. servus* per sample in the pheromone traps, sorghum with and without pheromone traps, cotton with sorghum trap crops with and without pheromone traps, and control cotton were plotted over time. Least squares means from the above analyses were used for number of *E. servus* per sample for cotton, and only data for side A were used because statistical differences in *E. servus* density were detected among trap crop treatments mainly on this field edge. Means were obtained for number of *E. servus* adults per pheromone trap using PROC MEANS [33]. The numbers of *E. servus* adults per sample per week in the sorghum trap crop, with and without pheromone traps, were compared using *t*-tests; the means were used to plot number of *E. servus* adults per sample in sorghum over time.

For 2006 data, numbers of *E. servus* per sample in pheromone traps, sorghum with pheromone traps, cotton with sorghum trap crops with pheromone traps, and control cotton were plotted over time. Means were obtained for number of *E. servus* adults per sample for sorghum and pheromone traps using PROC MEANS [33]. Least squares means from above analyses were used for number of *E. servus* per sample for cotton, and only data for side A, rows 1 and 2, were used because statistical differences in *E. servus* density were detected between trap crop treatments at these field locations.

3. Results and Discussion

3.1. Stink Bug Species Composition. Eight species of stink bugs species, that is, *E. servus*, *N. viridula*, *Oebalus pugnax pugnax* (F.), *Euschistus quadrator* (Rolston), *Euschistus ictericus* (L.), *C. hilaris*, *Euschistus tristigmus* (Say), and *Piezodorus guildinii* (Westwood), were found in sorghum over both years of this on-farm study in Georgia. These stink bugs species were also captured in *Euschistus* spp. pheromone traps (Table 2). As expected, more *Euschistus* spp., especially *E. servus*, were captured in traps baited with the *Euschistus* spp. pheromone than any other stink bug species. *N. viridula* was the predominant species in sorghum, whereas *C. hilaris* was rarely found in sorghum or captured in the pheromone traps. *Thyanta custator custator* (F.) was captured in the pheromone traps but was not found in sorghum even though this species has been collected from other sorghum plots (first author, unpublished data). *E. servus* nymphs were rarely captured in the pheromone traps; only 0.4% of all *E. servus* in these traps were nymphs. Also, for both years of the study,

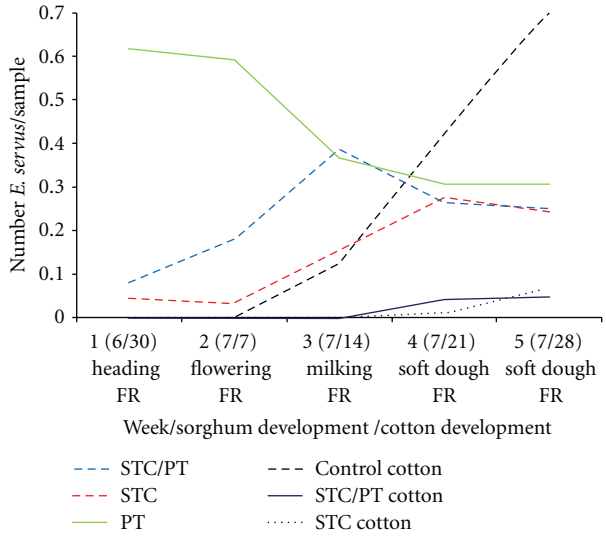

Figure 1: Mean number of *E. servus* per sample in sorghum trap crop with pheromone traps (STC/PTs), sorghum trap crop without pheromone traps (STC), and pheromone traps (PTs), and least squares means for number of *E. servus* in STC/PT cotton, STC cotton, and control cotton in 2004. FR: cotton with fruit. Number of stink bugs in pheromone traps was divided by 10. Only data on side A were used for cotton. Date refers to middle of sampling week.

a range of 60–70% of the *E. servus* killed in these pheromone traps were females. Incorporating pheromone traps in a sorghum trap crop may decrease nymphal development of *E. servus* in the trap crop by reducing oviposition.

3.2. 2004 Experiment. For the first two weeks of the study, the number of *E. servus* per pheromone trap was relatively high, but density of the pest remained relatively low in sorghum with or without pheromone traps (Figure 1). These results indicate that *E. servus* was attracted to pheromone traps and uninterested in feeding on sorghum in the heading and flowering stages. Once sorghum finished flowering and reached the milking stage, *E. servus* began feeding on developing seeds in sorghum heads. Similarly, in an earlier study *E. servus* was observed on sorghum heads soon after completion of flowering [23], and in an additional study, the milking stage of sorghum heads was the most preferred stage for feeding by *E. servus* [22].

The number of *E. servus* adults was statistically higher in sorghum with pheromone traps compared to sorghum without these traps on weeks 2 and 3 (Table 3) indicating that the pheromone traps attracted more dispersing *E. servus* adults into the trap cropping system during a period of time when preferred food was not prevalent in sorghum. As *E. servus* density increased in sorghum, the number of *E. servus* dropped in pheromone traps (Figure 1). A similar response was observed for *E. servus* in peanut-cotton farmscapes as fruit became available on cotton [28]. There are at least two possible explanations for this observed response: (1) once *E. servus* has dispersed into sorghum and fruit are available in the crop, these insects may begin to become more interested in feeding than in responding to the aggregation pheromone, or (2) the attractiveness of the pheromone in the capture traps may decrease as pheromone from *E. servus* males aggregating on sorghum heads disperses throughout sorghum. These results also suggest that the number of *E. servus* in pheromone traps can be more a reflection of dispersal activity of *E. servus* into a crop rather than the density of the pest in a specific crop. Similarly, for the consperse stink bug, *Euschistus conspersus* Uhler, migration of sexually mature females from overwintering sites results in an elevated pheromone (*Euschistus* spp.) trap response relative to the surrounding field population early in the growing season before fruit are available in processing tomatoes, *Lycopersicon esculentum* Miller [34–36]. Also, the synthetic pheromone of the Neotropical brown stink bug, *Euschistus heros* (F.), is attractive to this pest in the field [37], and pheromone-baited traps are more efficient than field sampling mainly during the colonization of soybean [38].

Table 2: Phytophagous stink bugs in *Euschistus* spp. pheromone traps and in sorghum trap crops.

Species	% in pheromone traps	% in sorghum trap crops
E. servus	69.1	10.9
N. viridula	10.6	71.5
O. p. pugnax	8.2	13.2
E. quadrator	7.4	2.8
E. ictericus	2.8	0.1
C. hilaris	1.1	1.3
E. tristigmus	0.6	0.1
P. guildinii	0.1	0.1
T. c. custator	0.1	0

For this trap crop experiment, factorial analyses revealed a significant treatment × week × field location effect ($F = 1.76$; df $= 83, 406$; $P = 0.0002$) for number of *E. servus* per 1.83 m of row in cotton (Table 4). Density of *E. servus* adults per sample on side A was lower in cotton fields with sorghum trap crops (with or without pheromone traps) compared to control cotton fields on weeks 4 and 5. Indeed, for control cotton, density of *E. servus* was significantly higher on side A on weeks 4 and 5 compared to all other locations for those two weeks indicating that there was an edge effect in distribution of stink bugs in these farmscapes. There was no significant difference in density of *E. servus* adults between cotton fields with a sorghum trap crop, with or without pheromone traps, at this location on these two weeks. So, even though sorghum trap crops with pheromone traps may attract more *E. servus* than trap crops without pheromone traps, this increase in attraction does not translate into higher densities of the pest moving into cotton. A total of 4042 and 1406 *E. servus* adults were captured and killed in pheromone traps in these farmscapes in 2004 and 2006, respectively. Evidently, the pheromone traps effectively capture *E. servus*, but not all of them at any point in time, probably because they continue to disperse into the trap crop.

E. servus was first observed in control cotton with fruit on week 3 (Figure 1). In control cotton, *E. servus* density

Table 3: Number (mean ± SE) of *E. servus* adults per 1.83 m of row in sorghum trap crops with pheromone traps (STC/PTs) and sorghum trap crops alone (STC) in 2004.

Week	STC/PT	STC	$\|t\|$	df	P
1	0.0833 ± 0.0467	0.0444 ± 0.0311	0.69	63	0.4908
2	0.1806 ± 0.0636	0.0333 ± 0.019	2.43	160	0.0163
3	0.3889 ± 0.0852	0.1556 ± 0.0497	2.47	160	0.0144
4	0.2639 ± 0.0559	0.2778 ± 0.057	0.17	160	0.8642
5	0.25 ± 0.0585	0.2444 ± 0.0507	0.07	160	0.9427

Table 4: Least squares means for number of all *E. servus* per 1.83 m of row in different locations in cotton for sorghum trap crop with pheromone traps (STC/PTs), sorghum trap crop (STC), and control (CO) fields in 2004.

Location	Week	STC/PT	STC	CO
side A[a]	2	0 1,a,A	0 1,a,A	0 3,a,A
	3	0 1,a,A	0 1,a,A	0.125 3,a,A
	4	0.041 1,b,A	0.0103 1,b,A	0.4292 2,a,A
	5	0.0476 1,b,A	0.0677 1,b,B	0.7015 1,a,A
side B	2	0.125 1,a,A	0 1,a,A	0 1,a,A
	3	0 1,a,A	0.15 1,a,A	0 1,a,A
	4	0.0478 1,a,A	0.2 1,a,A	0.1651 1,a,B
	5	0.0329 1,a,A	0.0254 1,a,B	0.0095 1,a,B
side C	2	0 1,a,A	0 1,a,A	0 1,a,A
	3	0.0625 1,a,A	0.05 1,a,A	0.0003 1,a,A
	4	0 1,a,A	0.05 1,a,A	0.0818 1,a,B
	5	0 1,a,A	0.2494 1,a,AB	0.1224 1,a,B
side D	2	0 1,a,A	0 2,a,A	0 1,a,A
	3	0.125 1,a,A	0.05 2,a,A	0 1,a,A
	4	0 1,a,A	0 2,a,A	0 1,a,B
	5	0 1,b,A	0.3315 1,a,A	0.0836 1,ab,B
block 1	2	0 1,a,A	0 1,a,A	0.0833 1,a,A
	3	0.0833 1,a,A	0.0667 1,a,A	0 1,a,A
	4	0.1346 1,a,A	0.0667 1,a,A	0 1,a,B
	5	0.2444 1,a,A	0.1504 1,a,AB	0.0095 1,a,B
block 2	2	0.0833 1,a,A	0 1,a,A	0 1,a,A
	3	0 1,a,A	0.0667 1,a,A	0.0833 1,a,A
	4	0.0774 1,a,A	0.0667 1,a,A	0 1,a,B
	5	0.0774 1,a,A	0.1028 1,a,B	0.1206 1,a,B
block 3	2	0 1,a,A	0 1,a,A	0 1,a,A
	3	0.0833 1,a,A	0 1,a,A	0.0833 1,a,A
	4	0 1,a,A	0.0667 1,a,A	0 1,a,B
	5	0.2444 1,a,A	0.0551 1,a,B	0.0095 1,a,B

Least squares means within a column followed by the same number are not significantly different between weeks within a location for a single treatment. Least squares means within a row followed by the same lowercase letter are not significantly different between trap crop treatments within a location for a single week. Least squares means within a column followed by the same uppercase letter are not significantly different between locations within a treatment for a single week. (PROC MIXED, LSD, $P > 0.05$, for all *E. servus*, $n = 1550$, SE = 0.0938, df = 1040).

[a]Cotton edge along the common boundary of a cotton field and a field of another crop that was a source of stink bugs.

was significantly higher on week 4 compared to week 3 and higher on week 5 compared to week 4 on Side A (Table 4). Thus, *E. servus* density increased in control cotton from week 3 to 5 as depicted in Figure 1. During this time, the pest probably dispersed from the source crops into control cotton as previously reported in other cotton farmscapes in Georgia [15, 16]. *E. servus* first occurred in cotton with sorghum trap crops (with or without pheromone traps) on week 4. For both trap crop treatments, density of *E. servus* in cotton was similar on weeks 4 and 5 (Table 4). As *E. servus* density increased in control cotton, it remained relatively low in the two treatments with sorghum trap crops (Table 4, Figure 1). Thus, both trap cropping systems effectively arrested *E. servus* reducing dispersal of this pest into cotton. Edge effects occur on other field edges, as expected, and not just at the interface of the source crop and cotton. For the sorghum

Table 5: Frequency (%) of *E. servus*, *N. viridula*, and *C. hilaris* in cotton by week for sorghum trap crop with pheromone traps (STC/PTs), sorghum trap crop (STC), and control (CO) fields in 2004 and sorghum trap crop (STC) and control (CO) fields in 2006.

			E. servus		*N. viridula*		*C. hilaris*				
Year	Wk	Trt	*n*	%	*n*	%	*n*	%	χ^2	df	*P*
2004	3	Control	7	15.91	37	84.09	0	0			
		STC/PT	5	20.83	8	33.33	11	45.83			
		STC	8	53.33	5	33.33	2	13.33			
		Total	**20**	**20.83**	**8**	**33.33**	**11**	**45.83**	**36.4**	**4**	**0.0001**
	4	Control	11	8.09	121	88.97	2	2.94			
		STC/PT	5	11.36	38	86.36	1	2.27			
		STC	8	11.43	57	81.43	5	7.14			
		Total	**24**	**9.6**	**216**	**86.4**	**10**	**4.0**	**3.5**	**4**	**0.4852**
	5	Control	22	5.45	373	92.33	9	2.23			
		STC/PT	13	6.4	182	87.89	8	3.94			
		STC	41	11.68	287	81.77	23	6.55			
		Total	**76**	**7.93**	**842**	**87.89**	**40**	**4.18**	**20.9**	**4**	**0.0003**
2006	6	Control	8	18.18	29	65.91	7	15.91			
		STC/PT	1	14.29	0	0	6	85.71			
		Total	**9**	**17.65**	**29**	**56.86**	**13**	**25.49**	**16.2**	**2**	**0.0003**
	7	Control	19	32.2	37	62.71	3	5.08			
		STC/PT	2	3.08	26	40.0	37	56.92			
		Total	**21**	**16.94**	**63**	**50.81**	**40**	**32.26**	**44.4**	**2**	**0.0001**
	8	Control	43	32.82	76	58.02	12	9.16			
		STC/PT	3	2.42	34	27.42	87	70.16			
		Total	**46**	**18.04**	**110**	**43.14**	**99**	**38.82**	**107.5**	**2**	**0.0001**

trap crop alone treatment, *E. servus* density was significantly higher on side D on week 5 than on side A and B and block 2 and 3 (Table 4).

Three species of stink bugs, *E. servus, N. viridula*, and *C. hilaris*, were observed feeding on cotton fruit in both years (Table 5). In 2004, *E. servus* in cotton comprised 5–53% of the stink bug species over the three treatments. *N. viridula* was the predominant stink species in cotton except during week 3 when *C. hilaris* was predominant (with trap crops and stink bug capture traps). Also, during this same week in STC cotton, *E. servus* was the predominant species on cotton, and frequency of this pest was higher in these cotton fields than in STC/PT and control fields. On week 5, frequency of *E. servus* in cotton with sorghum trap crops alone was twice that for cotton with the STC/PT and control treatments. Apparently, incorporating the pheromone traps with the sorghum trap crop reduced dispersal of *E. servus* from the trap crop at least two out of three weeks.

3.3. 2006 Experiment. While sorghum was flowering, *E. servus* was captured in pheromone traps, but pest density was relatively low in sorghum (Figure 2). There was a slight drop in the number of *E. servus* per pheromone trap as the pest peaked during the milking stage of sorghum. Apparently, *E. servus* was drawn into the pheromone traps and then was arrested on sorghum when it was available as food. *E. servus* first appeared when small fruits were available in control cotton fields during week 3. Trap capture of *E. servus* increased from week 6 to week 7, and *E. servus* density increased in control cotton on week 7 and 8. This major influx of *E. servus* into pheromone-baited traps and control cotton indicates a significant dispersal of adults from the source crop as has been previously reported for peanut-cotton farmscapes in Georgia [15]. The pest occurred in cotton with sorghum trap crops and pheromone traps for the first time on week 7 indicating that the trap cropping system had effectively stopped *E. servus* from dispersing into cotton for 4 weeks, from weeks 3 through 6.

For this trap crop experiment, factorial analyses revealed a significant treatment × week × field location effect ($F = 4.68$; df = 71, 779; $P = 0.0001$) for numbers of *E. servus* per 1.83 m of row in cotton (Table 6). There was an edge effect in distribution of stink bugs in control cotton; density of *E. servus* was significantly higher on row 1 on side A compared to all other locations except row 2 on week 7 and on rows 1 and 2 on side A compared to all other locations on week 8. In control cotton, *E. servus* density was significantly higher on week 7 compared to week 6 and higher on week 8 compared to week 7 so density of the pest increased in cotton over time probably due to continual dispersal of *E. servus* from the source crop into the adjacent cotton rows as has been observed in other peanut-cotton farmscapes in Georgia [15]. The trap cropping system, though, effectively suppressed this pest in these farmscapes, for density of *E. servus* per sample was higher in control cotton fields compared to cotton fields with sorghum trap crops with pheromone traps on rows 1 and 2 of side A on weeks 7 and 8.

Table 6: Least squares means for number of all *E. servus* per 1.83 m of row in different locations in cotton for sorghum trap crop with pheromone traps (STC/PT) and control (CO) fields in 2006.

Side	Row	Week	STC/PT	CO
A[a]	1	6	0 1,a,A	0.037 3,a,A
	2		0 1,a,A	0.0926 1,a,A
	5		0.0185 1,a,A	0.0185 1,a,A
	16		0 1,a,A	0 1,a,A
	33		0 1,a,A	0 1,a,A
	100		0 1,a,A	0 1,a,A
	167		0 1,a,A	0 1,a,A
	233		0.0006 1,a,A	0 1,a,A
	300		0.0006 1,a,A	0 1,a,A
B			0 1,a,A	0 1,a,A
C			0 1,a,A	0 1,a,A
D			0 1,a,A	0 1,a,A
A	1	7	0.0142 1,b,A	0.1667 2,a,A
	2		0.0003 1,b,A	0.1111 1,a,AB
	5		0.0003 1,a,A	0.0370 1,a,B
	16		0.0003 1,a,A	0 1,a,B
	33		0.0003 1,a,A	0 1,a,B
	100		0.0003 1,a,A	0 1,a,B
	167		0.0419 1,a,A	0.0556 1,a,B
	233		0.0008 1,a,A	0 1,a,B
	300		0.0008 1,a,A	0 1,a,B
B			0.0003 1,a,A	0 1,a,B
C			0.0003 1,a,A	0 1,a,B
D			0.0003 1,a,A	0.0185 1,a,B
A	1	8	0 1,b,A	0.4259 1,a,A
	2		0.0550 1,b,A	0.1852 1,a,B
	5		0 1,a,A	0.037 1,a,C
	16		0 1,a,A	0.0556 1,a,C
	33		0 1,a,A	0 1,a,C
	100		0 1,a,A	0 1,a,C
	167		0 1,a,A	0 1,a,C
	233		0.0002 1,a,A	0 1,a,C
	300		0.0002 1,a,A	0 1,a,C
B			0 1,a,A	0.0741 1,a,C
C			0.0273 1,a,A	0.0185 1,a,C
D			0 1,a,A	0.037 1,a,C

Least squares means within a column followed by the same number are not significantly different between weeks within a location for a single treatment. Least squares means within a row followed by the same lowercase letter are not significantly different between trap crop treatments within a location for a single week. Least squares means within a column followed by the same uppercase letter are not significantly different between locations within a treatment for a single week. (PROC MIXED, LSD, $P > 0.05$, for all *E. servus*, $n = 2539$, SE = 0.0342, df = 1811).

[a]Cotton edge along the common boundary of a cotton field and a field which was a source of stink bugs.

In 2006, *N. viridula* was the predominant stink bug species in control cotton, and *C. hilaris* was the predominant species in cotton with sorghum trap crops (Table 5). On weeks 7 and 8, the frequency of *E. servus* was higher in control cotton than that in cotton with sorghum trap crops with pheromone traps. Apparently, incorporating the pheromone traps with the sorghum trap crop reduced the dispersal of *E. servus* into cotton.

3.4. Effectiveness of Trap Cropping System for Suppression of E. servus . An ideal trap cropping system should include a host plant which is strongly preferred by the pest over the cash crop and should be able to reduce the likelihood of the pest dispersing into the cash crop [17]. In 2004, when sorghum alone was utilized as a trap cropping system, *E. servus* adults strongly preferred sorghum (from the milking stage through the soft dough stage) to cotton (with fruit). Furthermore,

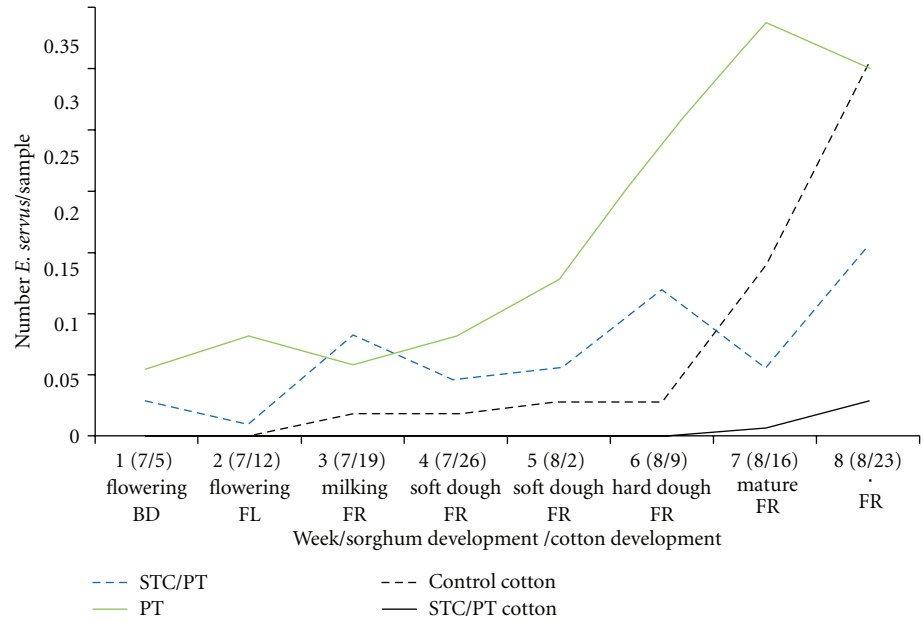

Figure 2: Mean number of *E. servus* per sample in sorghum trap crop with pheromone traps (STC/PTs), pheromone traps (PTs), STC/PT cotton, and cotton control in 2006. BD: cotton with buds; FL: cotton with flowers; FR: cotton with fruit. Number of stink bugs in pheromone traps was divided by 10. Only data for side A, rows 1 and 2, are used for cotton. Date refers to middle of sampling week.

over both years of the study a sorghum trap crop with or without pheromone traps effectively attracted *E. servus* in sorghum reducing dispersal of this pest into cotton.

Strategic placement of the trap cropping system in time and space also apparently was essential to the success of this suppression tactic in these farmscapes. Corn is an early summer source of stink bugs dispersing to peanut and cotton, and peanut is a mid-to-late summer source of stink bugs moving to cotton, especially at the interface of these farmscapes [15, 16]. Thus, in this study, a trap cropping system was established at the interface of a source crop (i.e., corn or peanut) and cotton, and the trap crop was planted in time to provide preferred food to stink bugs dispersing from the source crop into cotton. In farmscapes where stink bugs are active throughout the season, a season-long trap cropping system may be needed to protect cotton. Season-long trapping of stink bugs should reduce stink populations throughout the seasonal succession of host plants, possibly eliminating the need for additional control measures in cotton. A season-long stink bug trap cropping system that includes triticale, hairy vetch, and crimson clover during the spring followed by sunflower, buckwheat, sorghum, and pearl millet during the summer and fall has been developed to effectively manage stink bugs in organically grown soybean [22]. The system is economical to culture and manage provides a range of physical practices, including ratooning (mowing), and a range of maturity dates. All of these could be used alone or together by growers to customize the system for general use.

Stink bugs are well-adapted opportunists that will take advantage of available food resources at crop interfaces with both sorghum and soybean [13] being preferred over cotton. However, some stink bugs will still move into cotton near the preferred trap crop. In the current study, even though sorghum trap crops arrested *E. servus* and subsequently reduced dispersal of this pest into cotton, some *E. servus* apparently dispersed from sorghum into cotton. Indeed, in the 2004 experiment, some *E. servus* moved into cotton even though the sorghum heads were still in the attractive developmental stage. In a preliminary on-farm test, *E. servus* adults were significantly higher in a soybean trap crop than in adjacent fruiting cotton, but adult stink bugs still fed on some cotton fruit in the first two rows adjacent to soybean, over the period of attractiveness of soybean (first author, unpublished data).

Stink bug pheromone traps containing lures with *Euschistus* spp. pheromone (and insecticidal ear tags) have been shown to effectively capture and kill *E. servus* [27, 28], and thus they have great potential to suppress this pest in agricultural landscapes. One of the questions to be considered on how to utilize these traps as a management tool is whether they have the ability as a single tool to suppress *E. servus* in crops. In two separate experiments, establishing *Euschistus* spp. pheromone traps at the interface of peanut-cotton plots did not inhibit dispersal of *E. servus* when cotton fruit became available as a food source [28]. In another small-plot trap cropping experiment in peanut-cotton plots, *Euschistus* spp. pheromone traps captured *E. servus* adults, but *E. servus* density was equally high in cotton in plots with only the pheromone traps and control plots when cotton fruits were present (first author, unpublished data). These results indicate that as the sole management tool, *Euschistus* spp. pheromone traps cannot effectively stop dispersal of *E. servus* from peanut into fruiting cotton. However, in the second experiment, it was also determined that density of *E. servus* in cotton was statistically lower in cotton in plots

with a soybean trap crop with pheromone traps compared to control plots suggesting that pheromone traps are more effective in suppressing *E. servus* when used in combination with a trap crop. Apparently, stink bugs require a source of preferred food to remain in a location. Interestingly, though, *Euschistus* spp. pheromone can attract *E. servus* adults, dispersing from an adjacent crop within the agricultural landscape, into a sorghum trap crop even when sorghum heads have not yet developed to the preferred feeding stage. Perhaps, pheromone traps should be established in sorghum before heads develop seeds and remain in the trap crop throughout the period cotton that is susceptible to economic damage. Initially pheromone traps would attract and kill *E. servus* dispersing from a source crop and then pheromone traps would capture and kill *E. servus* attracted to sorghum.

Even with incorporation of pheromone traps, some *E. servus* still dispersed from sorghum into cotton. During 2004, preferred food was still available in sorghum when stink bugs moved into cotton. In this experiment pheromone traps were placed only on the sorghum row adjacent to a source crop. Perhaps, placing pheromone traps on every row of sorghum would decrease dispersal from sorghum into cotton. During 2006, *E. servus* probably began dispersing from sorghum into cotton because the seeds were no longer in the preferred feeding stage for the pest. Ratooning the sorghum heads or providing multiple plantings of the trap crop could extend the length of time preferred food available to the pest. In a small-plot trap cropping experiment, *E. servus* density was significantly lower in cotton plots with a stink bug barrier (1.83 m tall plastic wall) than in cotton plots with a soybean trap crop (first author, unpublished data). Thus, planting a tall crop such as Sudan grass (*Sorghum bicolor* (L.) Moench spp. *drummondii* (Nees ex Steud.) de We & Harlan) between a trap crop and cotton could possibly further decrease opportunistic movement from the trap crop into the cash crop.

The question remains whether a trap crop with pheromone traps is more effective in suppressing *E. servus* in cotton than a trap crop alone. Even though there was no significant difference in density of *E. servus* in cotton between the two trap crop treatments in 2004, incorporation of pheromone traps in trap crops can provide additional benefits including the following:

(1) reducing the density of *E. servus* adults in a trap crop, especially females, to possibly decrease the local population over time and reduce the overwintering population,

(2) reducing the dispersal of *E. servus* adults from the trap crop into cotton,

(3) potentially attracting more dispersing *E. servus* adults into a trap crop during a period of time when preferred food is not prevalent in the landscape.

A multifunctional habitat with a combination of trap crops to detract stink bugs from feeding and ovipositing on cash crops and pheromone traps with insecticidal ear tags to capture and kill stink bugs has the greatest potential for suppressing stink bugs in cotton. In Georgia, *N. viridula* and *C. hilaris*, along with *E. servus*, can cause economic damage to cotton fruit. Thus, a trap cropping system established to protect cotton from stink bugs must provide host plants preferred for feeding by all three stink bugs. Unfortunately, *C. hilaris* rarely occurs in sorghum, but because they readily feed on soybean (*Glycine max* (L.) Merr.) pods (first author, unpublished data), this plant may be a more suitable trap crop for this pest. However, *N. viridula* is highly attracted to grain sorghum heads in the milk stage through the hard dough stage [39], and *N. viridula* adults prefer sorghum to cotton [24]. Thus, a combination of grain sorghum and soybean could serve as an effective trap cropping system for these three stink bug species. In addition, other host plant species could be added to the trap cropping system to extend its longevity. Even though *N. viridula* prefers sorghum to cotton, some individuals of this pest can disperse into cotton rows adjacent to the trap crop [24]. Under field conditions, *N. viridula* can be trapped with its reported pheromone, and *C. hilaris* is significantly cross-attracted to the *Plautia stali* Scott pheromone [28]. Perhaps lures with these attractants could be included in the pheromone traps to reduce dispersal of these two pests from a trap cropping system designed to arrest all three stink bug species.

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Review Article

Structure Determination of a Natural Juvenile Hormone Isolated from a Heteropteran Insect

Toyomi Kotaki,[1] Tetsuro Shinada,[2] and Hideharu Numata[3]

[1] *Division of Insect Sciences, National Institute of Agrobiological Sciences, Ohwashi, Tsukuba, Ibaraki 305-8634, Japan*
[2] *Graduate School of Science, Osaka City University, Osaka 558-8585, Japan*
[3] *Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan*

Correspondence should be addressed to Toyomi Kotaki, kotaki@affrc.go.jp

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Juvenile hormone (JH), which occurs in several forms in different insects, is one of the most important insect hormones. The structure of JH in Heteroptera has not been elucidated until recently, although insects in this suborder have long been used as experimental animals for JH research. Here we review the structure determination of a novel JH in a stink bug, *Plautia stali*, which was named juvenile hormone III skipped bisepoxide [JHSB$_3$: methyl (2*R*,3*S*,10*R*)-2,3;10,11-bisepoxyfarnesoate], based on the arrangement of two epoxides at C2,3 and C10,11 with a skipped double bond at C6,7.

1. Introduction

Juvenile hormone (JH) is an important regulator of many functions in all insects (Figure 1) [1, 2]. It controls various aspects of development including metamorphosis, reproduction, and polyphenism. This hormone was discovered in a blood-sucking bug, *Rhodnius prolixus*, by Wigglesworth in 1934 [3]. He demonstrated a hormone, produced by a gland behind the brain, the corpus allatum (CA), that was responsible for the maintenance of juvenile characters as the insect grew, hence the name juvenile hormone [3]. He also found that the gland became inactive to allow metamorphosis to the adult and active again in the adult to support reproductive functions such as deposition of yolk in eggs and production of secretions of accessory reproductive glands [4, 5]. Since the chemical structure of JH isolated from a moth, *Hyalophora cecropia,* was elucidated in 1967 [6], several forms of JH were also determined [1, 2]. JH III is the most common among various insect orders while JH 0, JH I, JH II, and 4-methyl JH I were found in Lepidoptera. JHB$_3$ was identified as a JH specific to higher Diptera (Cyclorrhapha) [1, 2]. However, the structure of JH in the suborder Heteroptera has remained uncertain in spite of attempts to identify it [7–13]. Recently, we identified the structure of JH in a stink bug, *Plautia stali*, and named it juvenile hormone III skipped bisepoxide (JHSB$_3$) and demonstrated its biological function as the JH in this stink bug [14, 15]. Here we review the process of structure determination of JHSB$_3$ and its biological function as JH in *P. stali*.

2. Heteropteran Insects in JH Research

In his pioneering work, Wigglesworth found a humoral factor controlling metamorphosis and reproduction in *R. prolixus*, which he first referred to as "inhibitory hormone" [3, 5] and later as JH [16]. Since then heteropterans have been employed for evaluation of the biological efficacy of synthetic derivatives of natural JHs and anti-JH compounds, which lead to the discovery of a JH analogue specific to *Pyrrhocoris apterus*, juvabione and anti-JH, precocenes [17–20].

Heteropteran insects are used for studies to elucidate the role of JH in various aspects of insect development. For example, in *Oncopeltus fasciatus*, JH was demonstrated to control reproduction, adult diapauses, and diapause-associated migration [21, 22]. In diapausing adults of *P. apterus*, the activity of CA was inhibited by the brain via nervous connections [23, 24]. The production of methyl farnesoate [25] and JH III [26] by the CA *in vitro* was reported in *Dysdercus fasciatus*, *O. fasciatus,* and *Nezara viridula*.

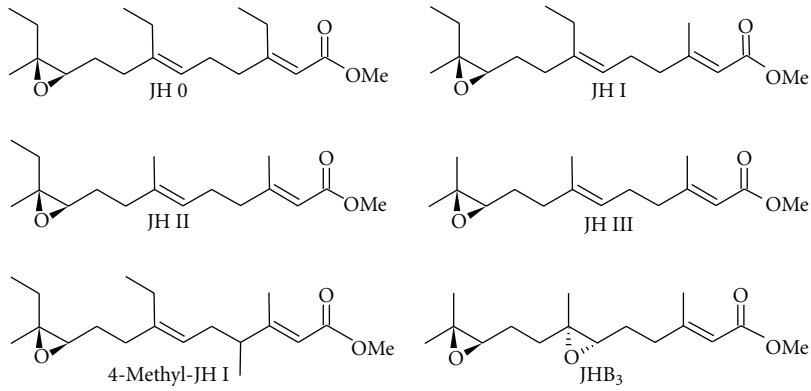

Figure 1: Structure of known JHs.

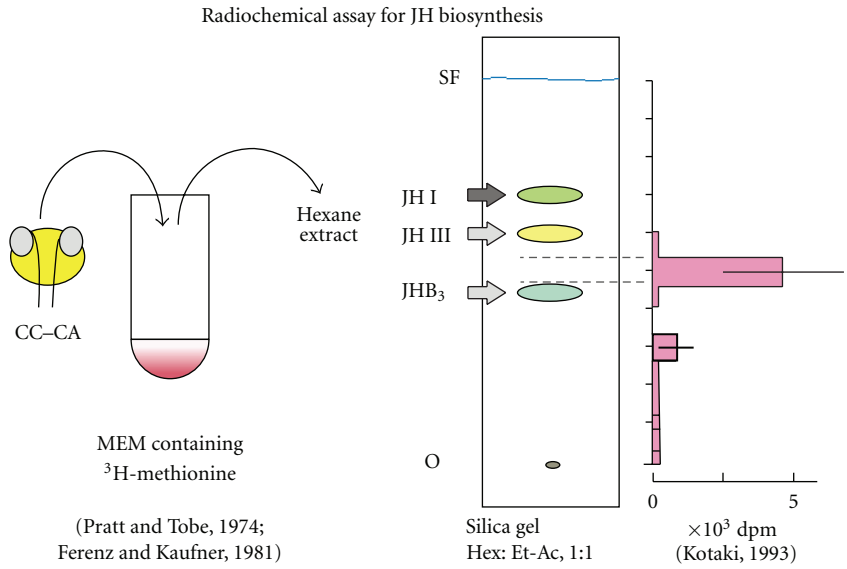

Figure 2: Radiochemical assay for JH biosynthesis. Incubation of CA from *Plautia stali* adults in a medium containing radiolabeled methionine revealed the presence of an unknown product with an R_f value different from those of JH I, JH III, or JHB_3 in TLC analysis.

However, Baker et al. failed to detect any form of known JHs in *O. fasciatus* [12].

3. Evidence for a New JH in Stink Bugs

In 1989, we reported that the CA product was responsible for control of adult diapause in *P. stali* [27]. This was shown by surgical operations such as CA extirpation and implantation. To gain insight into the CA product, the *in vitro* radiochemical assay [28, 29] was adopted to this bug (Figure 2). Assuming that the CA product was a methyl ester as with known JHs, the tritium-labeled CA product would be obtained by the incubation experiments in the presence of *L*-[*methyl*-^{3}H]methionine in the medium. After the incubation period, the product was extracted with hexane and analyzed by thin-layer chromatography (TLC) using a liquid scintillation counter to detect radioactivity of each part of TLC plate. The CA of *P. stali* and three other heteropteran species were subjected to this assay. The R_f values for the CA product of four species were almost identical with each other, whereas they were different from those for JH I, JH III, and JHB_3 [8, 9]. When precursors of JH III, *E,E*-farnesol or farnesoic acid were added to the medium, the biosynthesis of CA product was enhanced. This suggested that the product possessed the same sesquiterpenoid skeleton as did JH III [8]. Because radiolabeled JH III added to the incubation medium was not converted to the "CA product," the possibility that the CA released JH III but the latter was degraded or metabolized to the "CA product" in the incubation medium was ruled out [9].

Thus far, at least 7 species from 4 families of Heteroptera seem to share this product [7, 9, 10, 30]. Although the CA of *O. fasciatus, D. fasciatus,* and *N. viridula* were reported to produce JH III and/or methyl farnesoate *in vitro* [25, 26] and JH I was found in the hemolymph of *R. pedestris* (formerly *R. clavatus*) [31], the production of JH III and JH I in *N. viridula* and *R. pedestris*, respectively, was not confirmed [9].

To assess the JH activity of the CA product, we developed a bioassay method using *P. stali*. The CA product, collected as hexane extracts of medium for CA incubation, was topically

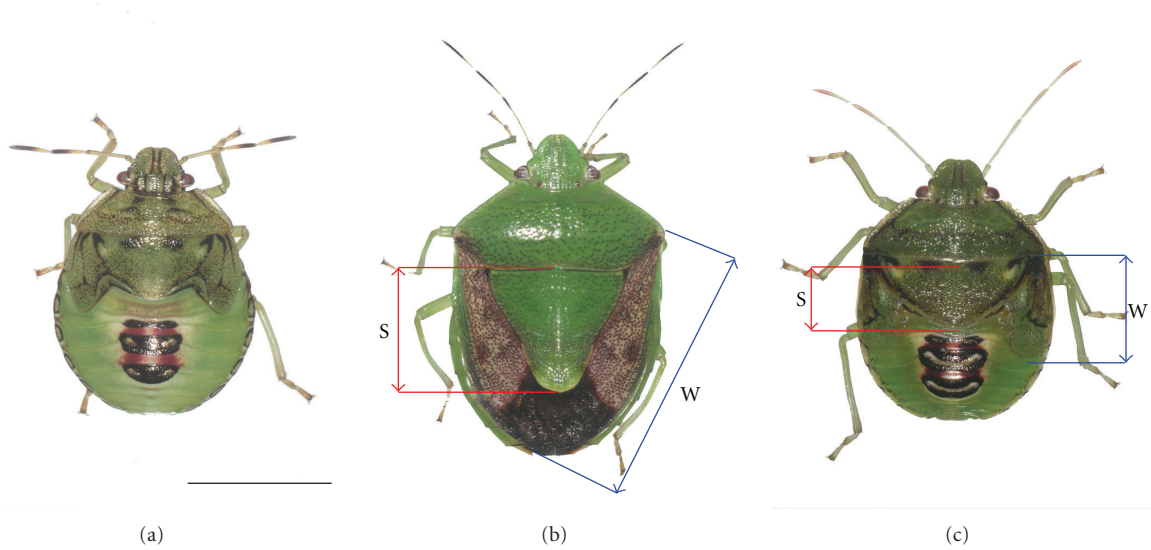

Figure 3: Bioassay for juvenilizing activity in *Plautia stali*. A last instar nymph (a), an adult (b), and a nymph-adult intermediate (c) obtained as a result of application of JH-active sample. Scale bar: 5 mm. Arrows with labels W and S indicate forewing and scutellum lengths and pronotum width, respectively.

applied to last instar nymphs, and following the final ecdysis, relative lengths of forewings and scutellum were determined [8]. Adults have fully developed long forewings and scutellum (Figure 3(b)) while in last instar nymphs and metamorphosis-inhibited insects by JH application, forewings and scutellum were not developed into the adult form yet but remained in buds or partially developed, short form (Figures 3(a) and 3(c)). The more the CA product was applied to test insects, the shorter their wings and scutellum were. This result indicated that the CA product had the activity to inhibit metamorphosis of the test insects, hence JH activity. Therefore, JH in *P. stali* was very likely to be a new JH that is different from any known JH.

4. Structure Determination of Stink Bug JH

To elucidate the structure of stink bug JH, we first examined the molecular weight of CA product using high resolution fast atom bombardment ionization (FAB) mass spectrometry to estimate its compositional formula. The mass of protonated molecule $[M+H]^+$ was estimated to be 283.1885, leading to a compositional formula, $C_{16}H_{26}O_4$, for the CA product. This formula is identical to that for dipteran JHB_3. However, previous studies clearly showed that the R_f values of the CA product and JHB_3 were not the same despite the supposition that the CA product and JHB_3 shared a sesquiterpenoid skeleton in common [8, 9]. Based on these observations, we proposed that the structure of JH in *P. stali* would be a regio- or geometric isomer of JHB_3.

To test this hypothesis, we synthesized a compound mixture consisting of 32 isomers of bisepoxides of methyl farnesoate with 2*E* or 2*Z* double bond, epoxides at C2,3 and at either C6,7 or C10,11 by two steps starting from a mixture of *E*- and *Z*-geranylacetone. The bioassay for JH activity indicated that the mixture was JH active. In a GC-MS analysis using a DB-35MS column the mixture gave several peaks. Cochromatography of the mixture and the CA product indicated that one of peaks of the mixture coincided with the CA product in retention time and mass spectrum.

Using a normal phase chiral column, Chiralpak IA (DAICEL Co., Ltd) on an HPLC system, the bisepoxide mixture was separated into 21 fractions and each of them was subjected to the bioassay for JH activity. Two fractions (retention time: 28 min. and 38 min.) applied topically to last instar nymphs showed a juvenilizing effect at a dose of 0.1 μg/insect. These biologically active fractions were subjected to one and two-dimensional ^{1}H NMR analyses. Data obtained indicated that each fraction consisted of a single stereoisomer of a novel JH structure, methyl *E,E*-2,3;10,11-bisepoxyfarnesoate (Figure 4). Although stereoisomers of this bisepoxide had distinctly different retention time when separated by the chiral HPLC, their ^{1}H NMR data were not distinguishable from one another. Probably the positions of two epoxides were too distant to give different signal patterns. Therefore, we synthesized four possible stereoisomers **1**–**4** in an optically pure form (Figure 4), and these were compared with the natural CA product to determine the relative and absolute structure. Each stereoisomer was obtained starting from *E,E*-farnesol by asymmetric Katsuki-Sharpless epoxidation and Sharpless dihydroxylation reactions in which stereochemical outcomes were reliably controlled by ingeniously designed chiral reagent systems [32]. Bioassay indicated that isomers **1** and **2** showed more JH activity than the other two isomers. The chiral HPLC analysis of these stereoisomers gave four peaks. Elution time for isomers **1** and **2** were almost the same as that for the JH-active fractions obtained by the chiral HPLC separation of the bisepoxide mixture. In consideration of the detection limit of HPLC analysis using a UV detector, the stereoisomers and CA product were subjected to more sensitive GC-MS (chemical ionization, CI with NH_3 as

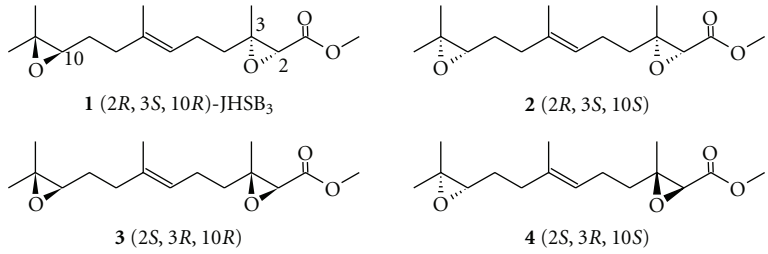

Figure 4: Four stereoisomers of $JHSB_3$.

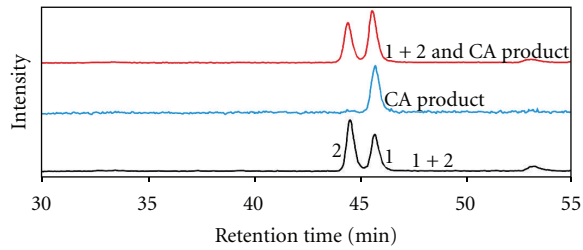

Figure 5: Chiral GC-MS(CI) analysis of synthetic standards of $JHSB_3$ (**1**) and isomer **2** and natural product by the CA of *Plautia stali* using an Rt-βDEXcst column. Vertical axis indicates signal intensity for m/z 300, $[M+NH_4]^+$. Black, blue, and red lines indicate analysis of $JHSB_3$ (**1**) and isomer **2** (10 ng each), CA product, and cochromatography of these two samples, respectively [15].

a reagent gas) using a chiral column, Rt-βDEXcst. With this column, isomers **2**, **3**, and **4** showed almost the same retention time of 44.7 min while isomer **1,** with retention time of 45.7 min, was distinctly separated from the others (Figure 5). The CA product produced one main peak at 45.8 min in this system. The mass spectra for isomer **1** and CA product were identical (Figure 6 insets). Cochromatography of isomer **1** and CA product indicated that the peaks for these two overlapped completely with an increase in peak height in an additive fashion (Figure 5). These results demonstrate that isomer **1**, a novel form of JH, is the natural JH in *P. stali*. We named it juvenile hormone III skipped bisepoxide [$JHSB_3$: methyl (2*R*,3*S*,10*R*)-2,3;10,11-bisepoxyfarnesoate], based on the arrangement of two epoxides at C2,3 and C10,11 with a skipped double bond at C6,7.

5. Biological Function of $JHSB_3$

Although, in the process of $JHSB_3$ structure determination, a juvenilizing JH activity was indicated in $JHSB_3$ and one of its stereoisomers, how active the remaining two stereoisomers at higher doses were was not explored. JH activity of the four isomers were, therefore, compared using last instar nymphs as well as adult females of *P. stali* kept under reproduction-promoting, long-day conditions whose CA were surgically removed. The latter test examined the activity to stimulate reproduction, another function of JH.

Topical application of $JHSB_3$ to last instar nymphs inhibited their metamorphosis in a dose-dependent fashion (Figure 7). Nymphs treated with 0.001 μg of $JHSB_3$ molted to normal-looking adults. With an increase in the dose, the relative lengths of forewings and scutellum decreased. At a dose of 0.1 μg or higher, nymphs molted to intermediates with wings and a scutellum reduced to a similar extent to those of normal last instar nymphs. A diastereomer of $JHSB_3$, isomer **2**, revealed a similar dose-response curve. On the other hand, isomers **3** and **4** were less active than $JHSB_3$ and isomer **2**. A dose of 1 μg of isomers **3** and **4** showed little effect on the metamorphosis of bugs. At a dose of 5 μg, bugs molted to an intermediate, but their wings and scutellum were still slightly longer than those of bugs treated with 0.1 μg or 1 μg of $JHSB_3$. Application of 10*R*-JH III showed a similar dose response to those of isomers **3** and **4**, but even at the highest dose of 10 μg, its effect was not so evident as that of isomers **3** and **4** at 5 μg.

Extirpation of the CA from females reared under long-day conditions inhibited the development of ovaries. More than half (10 of 14) of allatectomized hexane-treated females underwent oosorption when they were dissected 4 days after allatectomy. Topical application of $JHSB_3$ rescued those adults from the inhibitory effect of CA removal in a dose-dependent fashion (Figure 8). Isomer **2** seemed as potent as $JHSB_3$ whereas isomers **3** and **4** showed virtually no stimulatory effect on oocyte development even at the highest dose of 5 μg. These results indicated that $JHSB_3$ and isomer **2** were highly JH active while the other two were about 1,000 times less active. The downward epoxide configuration at C2,3, shared by $JHSB_3$ and isomer **2**, seems important for manifestation of JH activity.

$JHSB_3$ structure determination was accomplished by analyzing the product of CA *in vitro*. The biosynthesis of $JHSB_3$ by the CA, however, does not automatically imply its presence in the hemolymph *in vivo*. It must be experimentally verified by detecting $JHSB_3$ in the circulating hemolymph. We collected hemolymph samples from reproductively active and diapause females. According to the previous studies [27, 33], in the former the concentration of JH in the hemolymph is expected to be high while in the latter low. These two samples were analyzed using LC-MS. In the hemolymph sample from reproductively active females, a peak was observed at the same retention time accompanied by the same mass spectrum as synthetic $JHSB_3$ standard. No significant peaks of characteristic fragments corresponding to JH I, II, nor III were observed in the hemolymph samples. This indicated the presence of $JHSB_3$ alone in the hemolymph from reproductively active females, and on the other hand, virtually no peak corresponding to $JHSB_3$ or any other

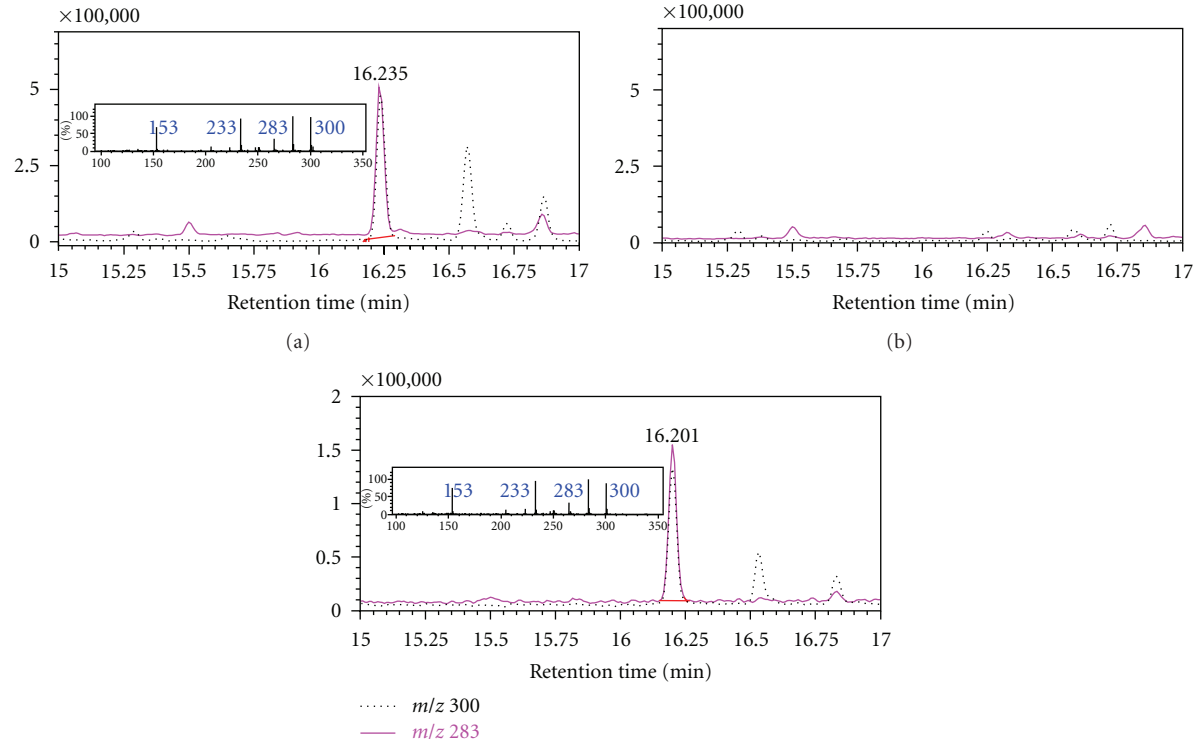

Figure 6: Detection of $JHSB_3$ (**1**) from the hemolymph of *Plautia stali* females. Hemolymph samples collected from reproductively active (a) and diapausing females (b), and 10 ng of synthetic standard of $JHSB_3$ (c) were analyzed on GC-MS using a DB-35MS column. Vertical axis indicates signal intensity for m/z 283, $[M+H]^+$ (solid line) and m/z 300, $[M+NH_4]^+$ (dotted line). Insets in a and c indicate a mass spectrum of the peak at 16.2 min [14].

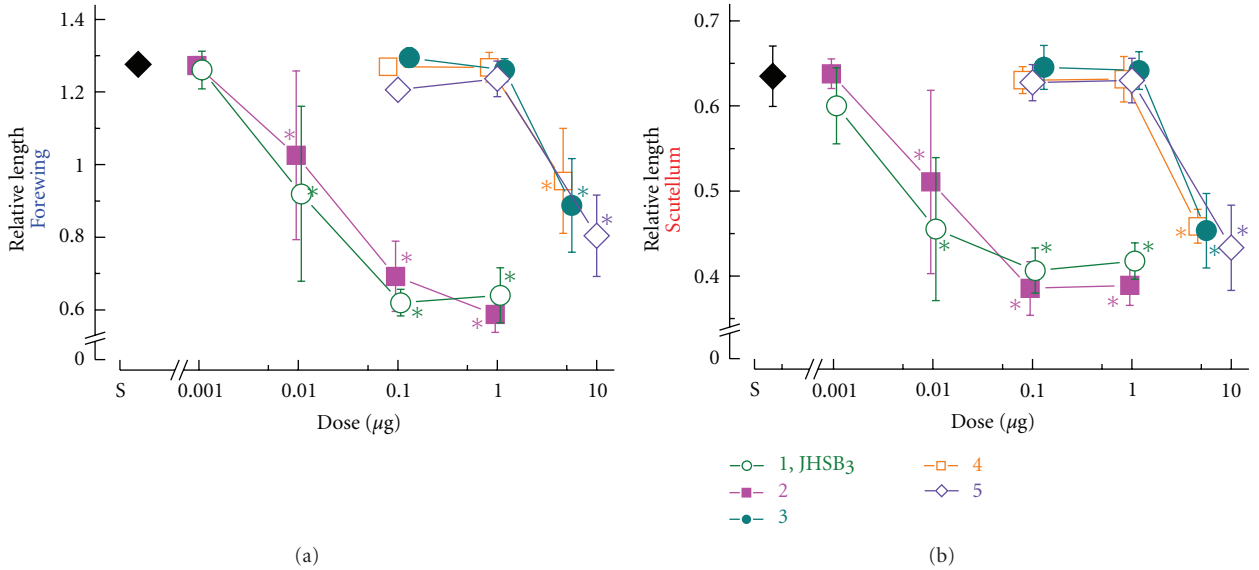

Figure 7: Juvenilizing activity of $JHSB_3$ (**1**), its stereoisomers and 10*R*-JH III on *Plautia stali*. Last instar nymphs were treated with a test compound. Following the final molt, lengths of forewing (a) and scutellum (b) relative to the width of pronotum were determined. S on the horizontal axis indicates solvent control. Each datum point and error bar represents average value ± SD (n = 8–18). Asterisks indicate that the average value was significantly different from that of the solvent control (Steel's test, $P < 0.05$) [14].

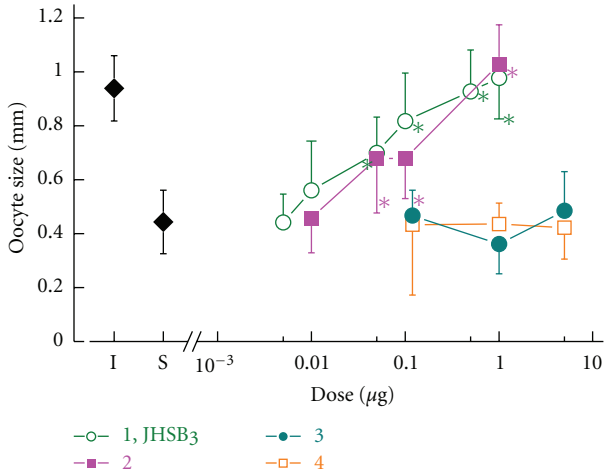

FIGURE 8: Reproduction-stimulating effect of $JHSB_3$ (**1**) and its stereoisomers on allatectomized females of *Plautia stali* reared under long-day conditions. Females were allatectomized and treated with a test compound on day 4 of adult life, and oocyte diameter was determined on day 8. Solid diamonds labeled with I and S on the horizontal axis indicate results of day 8 untreated and solvent-treated adults, respectively. Each datum point and error bar represents average value ± SD (n = 8–12). Asterisks indicate that the average value was significantly different from that of the solvent control (Steel's test, $P < 0.05$) [14].

JHs was detected in the sample from diapause females. $JHSB_3$ was, therefore, the only molecule found in the hemolymph and its concentration was likely to fluctuate as expected by the previous studies.

Dahm et al. [34] pointed out three criteria that have to be fulfilled to chemically identify JH. These points, derived from the classical definition of the hormone, are as follows: (1) production by the CA; (2) titer fluctuation in synchrony with the processes controlled by JH; (3) rescue effect in JH-deprived insects. As indicated above, these three criteria were met for $JHSB_3$ in *P. stali*. $JHSB_3$, therefore, functioned as the JH in this species. It was likely that other heteropteran insects share this new JH in common because the CA of at least seven heteropterans other than *P. stali* also produced the products *in vitro* that behaved similarly to that of *P. stali* on the TLC plate [7, 9, 10, 30].

6. Conclusion

JH in Heteroptera has been a long-lasting enigma in spite of that JH research began with morphological studies in *R. prolixus* [3, 4]. We have successfully determined the structure of novel, Heteroptera-specific JH, $JHSB_3$. Because $JHSB_3$ is the only JH with a C2,3 epoxide, heteropterans using this molecule likely possess an enzyme responsible for conversion of the C2,3 double bond to epoxide. The presence of specific JH suggests underlying specific pathways for not only biosynthesis but also degradation. A $JHSB_3$-specific receptor should also play a role in heteropterans. Our discovery has provided a basis for all these suppositions and will enhance further studies on heteropteran JH. An attempt to elucidate the enzymes involved in $JHSB_3$ biosynthesis is in progress. How far $JHSB_3$ is shared among the suborder Heteroptera or the order Hemiptera is a question to be answered in the future in the viewpoint of insect endocrinology and practical insect control, as well. Structure-activity relation study is underway to gain insight into specificity of $JHSB_3$ receptor and development of $JHSB_3$-based control agents.

Acknowledgments

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