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*Research Article*

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

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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

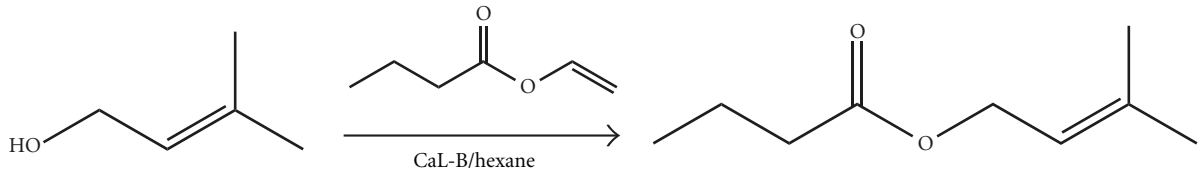
*2.1. Insects.* Virgin adult bugs were obtained from a laboratory colony reared on *E. tereticornis* (adapted from [10]). Males, females, and nymphs were kept together in mesh-covered cages (35 × 50 × 70 cm) in a greenhouse, on *E. tereticornis* potted plants, and with periodical introduction of field 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 $\mu$L 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 $\mu$L) 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 $\mu$m), 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 $\mu$L) 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 $\mu$L, on filter paper, with two *E. tereticornis* leaves); (c) 3-methylbut-2-enyl butanoate versus hexane (1 $\mu$g in 5 $\mu$L, 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, $CDCl_3$): $\delta$(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, $CDCl_3$): $\delta$(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 $\alpha$-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: $P = 0.004$, $\chi^2$ 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 ($P = 0.22$, $\chi^2$ 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 ($P = 0.005$, $\chi^2$ 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 ($P = 0.02$, $\chi^2$ test, $N = 21$; $P = 0.09$, Wilcoxon test, $N = 21$; one-week-old males: $P = 0.66$, $\chi^2$ 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 $\mu$g of the male-specific compound absorbed in filter paper resulted in more males choosing the arm with the stimulus than the control arm ($P < 0.0001$, $\chi^2$ 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: $P < 0.0001$, $\chi^2$ test, $N = 42$; $P < 0.02$, Wilcoxon test, $N = 42$; one-week-old males: $P < 0.001$, $\chi^2$ test, $N = 46$; $P = 0.002$, Wilcoxon test, $N = 46$) (Figure 3(c)).

Considering the number of males used for the male

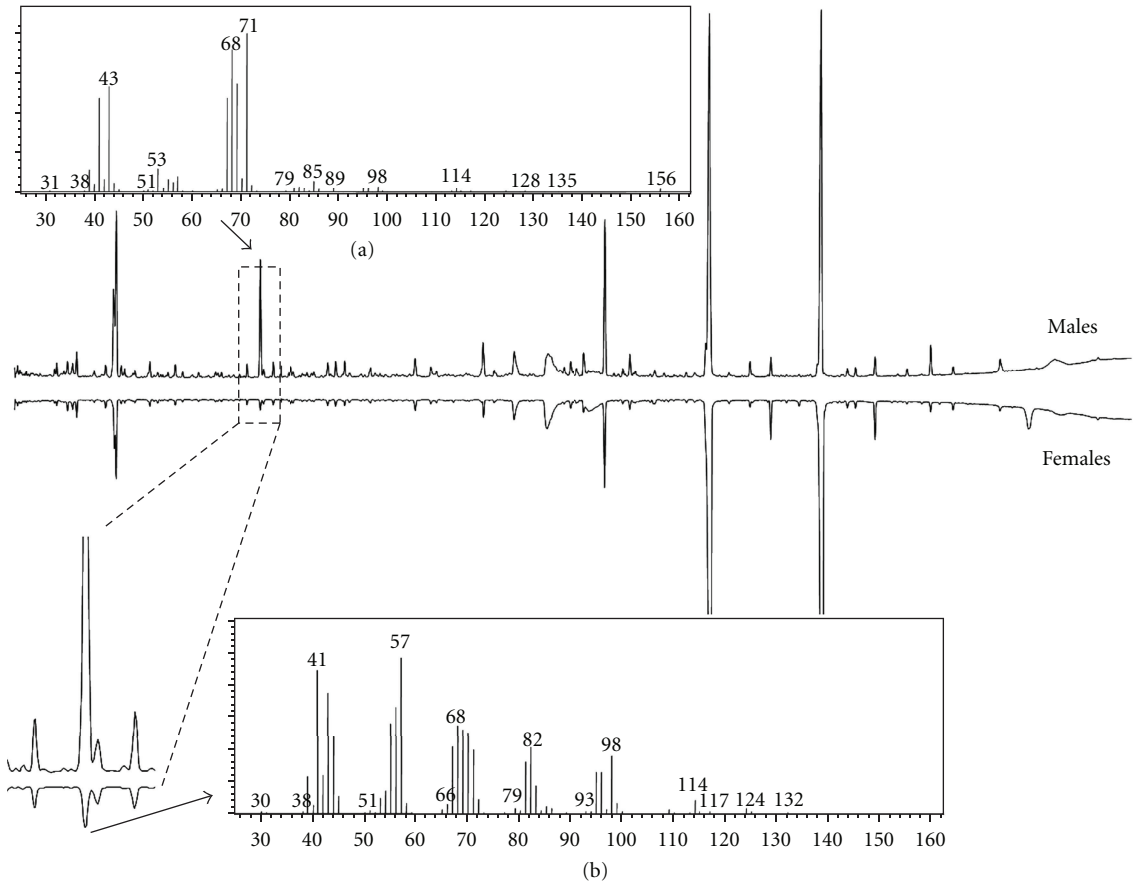



Figure 1: GC-MS traces (TIC) of *T. peregrinus* male (top) and female (bottom) volatile extracts analyzed in a polar column. A male-specific compound was present in male volatile extracts, with a retention time of 10.1 min (mass spectrum shown in insert (a)). A coeluting compound in female volatile extracts 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.

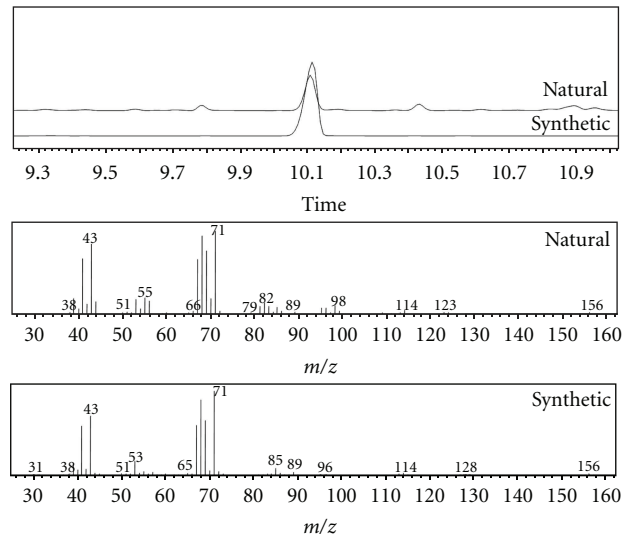



Figure 2: GC-MS traces (TIC) of *T. peregrinus* male volatile extracts (upper trace) and synthetic 3-methylbut-2-enyl butanoate (lower trace). The mass spectra of the natural and synthetic compounds match closely, with small ions in the natural sample corresponding to nonanal, a coeluting compound from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

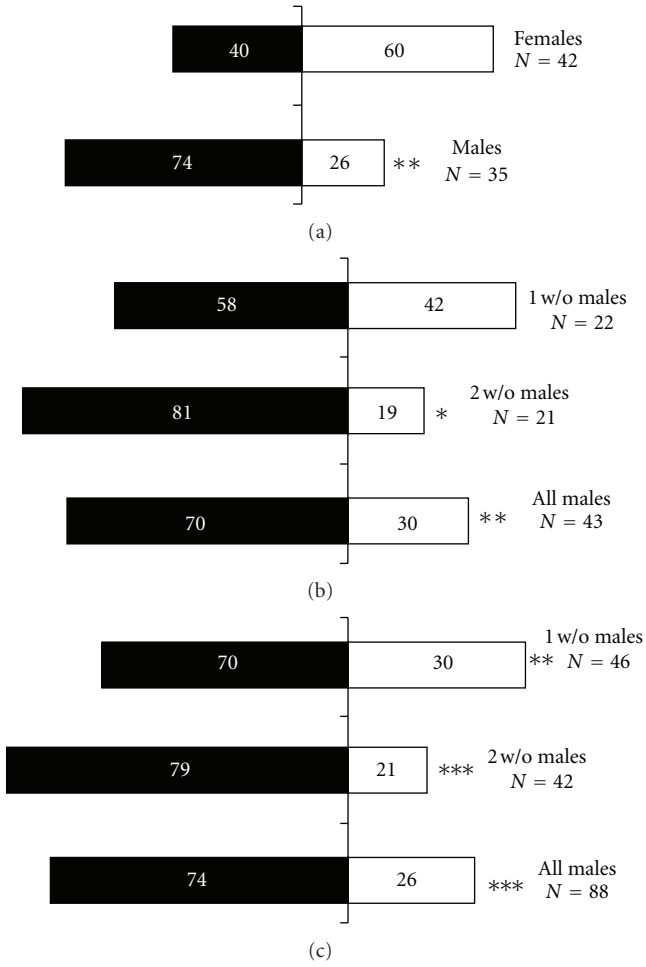



Figure 3: First arm choice of *T. peregrinus* adults in Y-tube olfactometer tests. Bars show the percent (numbers within bars) of insects choosing the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live males versus control. (b) Response 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 $\mu$L of the tested extract was 0.25 $\mu$g. 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 $\mu$g/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

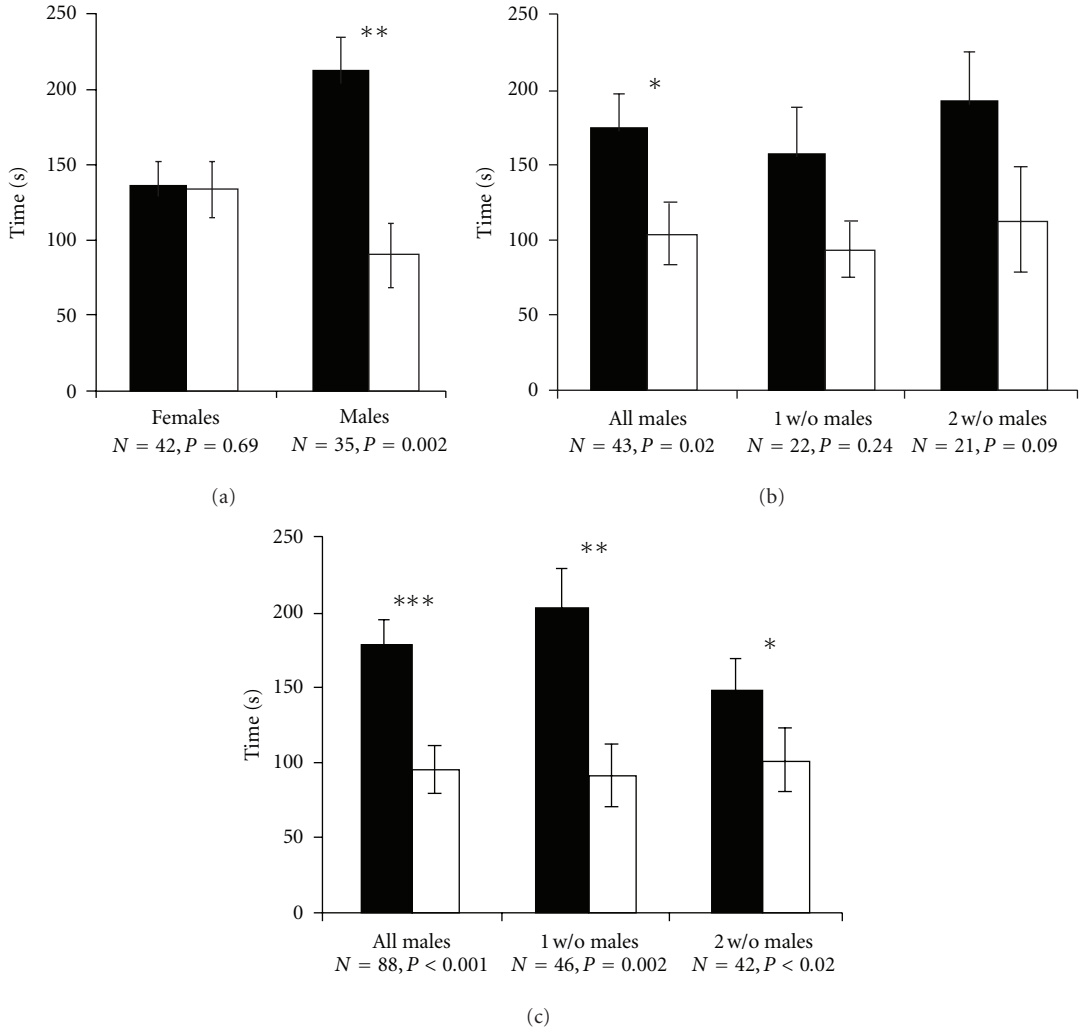



Figure 4: Time of permanence of *T. peregrinus* adults in the stimulus and control arms of the Y-tube olfactometer. Bars show the cumulative time (in 10 min.) that the insects spent in the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live 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.

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