

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Semiochemicals associated with the reproductive behavior of
Cyclocephala paraguayensis Arrow**

Arodí Prado Favaris

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

**Piracicaba
2021**

Arodí Prado Favaris
Bachelor of Biological Sciences

**Semiochemicals associated with the reproductive behavior of *Cyclocephala*
paraguayensis Arrow**

versão revisada de acordo com a resolução CoPGr 6018 de 2011.

Advisor:
Prof. Dr. **JOSÉ MAURÍCIO SIMÕES**
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DEDICATION

*To my parents, Helio, Sandra, my sister Adna,
and to my husband, João Marcos*

ACKNOWLEDGMENTS

This thesis is far from a lonely accomplishment.

I thank God for being present in every moment of this work. The day I “accidentally” found my beetles on bottle-gourd flowers triggered a new perspective for my research I have never thought of before.

I thank my parents, who have been made uncountable efforts for our family. Because of them, I am becoming a Christian scientist. About my sister Adna, she always was a kind of inspiration for me since I was a kid. My husband João Marcos and his all lovely patience and encouragement during my studies were also very important to conduct and finish this work.

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EPIGRAPH

*Man's hand assaults the flinty rock
and lays bare the roots of the mountains.
He tunnels through the rock;
his eyes see all its treasures.
He searches the sources of the rivers
and brings hidden things to light.
But where can wisdom be found?
Where does understanding dwell?
Man does not comprehend its worth;
it cannot be found in the land of the living.
The deep says, 'It is not in me';
the sea says, 'It is not with me.'
It cannot be bought with the finest gold,
nor can its price be weighed in silver.
Where then does wisdom come from?
Where does understanding dwell?
God understands the way to it
and He alone knows where it dwells,
for He views the ends of the earth
and sees everything under the heavens.
When He established the force of the wind
and measured out the waters,
when he made a decree for the rain
and a path for the thunderstorm,
then He looked at wisdom and appraised it;
He confirmed it and tested it.
And He said to man,
'The fear of the Lord – that is wisdom,
and to shun evil is understanding'.*

(Job 28: 9-15; 20; 23-28)

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RESUMO

Semioquímicos associados ao comportamento reprodutivo de *Cyclocephala paraguayensis* Arrow

A comunicação química entre as flores e besouros Cyclocephalini é um forte componente de suas interações ecológicas. Os odores florais atraem os besouros Cyclocephalini às flores, onde encontram oportunidade de cópula, recursos alimentares e abrigo. Uma vez que a fragrância floral tem reunido tanto machos quanto fêmeas nas flores, sugere-se que o feromônio sexual de longa distância não seja crucial para a reprodução dos besouros Cyclocephalini. Neste trabalho, *Cyclocephala paraguayensis* Arrow é relatada visitando flores de *Lagenaria siceraria* (Cucurbitaceae) e *Brugmansia suaveolens* (Solanaceae). Os odores florais foram identificados e os compostos nerolidol de ambas as plantas, bem como o benzoato de metila da *B. suaveolens* foram demonstrados como atraentes potenciais à *C. paraguayensis*. Além disso, o nerolidol, mesmo como um constituinte minoritário do odor floral de *B. suaveolens*, quando combinado com o benzoato de metila, contribuiu ao efeito sinérgico na atração de *C. paraguayensis*. Embora os dois compostos florais têm sido eficientes na atração dos coespecíficos de *C. paraguayensis*, os voláteis emitidos por machos diferiram qualitativamente das fêmeas, sugerindo um possível mecanismo para a comunicação química intraespecífica. Os machos liberaram um proeminente composto, preliminarmente identificado como ácido pulegânico. Embora essa identificação ainda necessita de uma confirmação biológica, os resultados impulsionaram direções futuras para a hipótese da produção do feromônio sexual.

Palavras-chave: Ecologia química; Dynastinae; Besouros antófilos

ABSTRACT**Semiochemicals associated with the reproductive behavior of *Cyclocephala paraguayensis* Arrow**

Chemical communication between flowers and Cyclocephalini beetles is a strong component of their ecological interactions. The floral odors attract Cyclocephalini beetles to the flowers, where they find mating opportunity, food resources, and shelter. Given the floral scent has gathered both male and female adults on flowers, it has been suggested the long-distance sex pheromone is not crucial for the reproduction of Cyclocephalini beetles. In this work, we report *Cyclocephala paraguayensis* Arrow visiting flowers of *Lagenaria siceraria* (Cucurbitaceae) and *Brugmansia suaveolens* (Solanaceae). Floral scents were identified, and the compounds nerolidol from both plants, as well as methyl benzoate from *B. suaveolens*, were demonstrated to be potential attractants to *C. paraguayensis*. Besides, nerolidol even as a minor constituent in *B. suaveolens* floral odor, when combined with methyl benzoate, contributed to a synergic effect on the attraction of *C. paraguayensis*. Although the two floral compounds being efficient on the attraction of *C. paraguayensis* conspecifics, the volatiles emitted by males qualitatively differed from the female ones, suggesting a possible intraspecific mechanism for chemical communication. Males released a prominent compound, preliminarily identified as a puleganic acid. Although this identification still needs biological confirmation, our findings raised future directions for the hypothesis of sex pheromone production.

Keywords: Chemical ecology; Dynastinae; Anthophilous beetles.

GENERAL INTRODUCTION

The chemical compounds which mediate ecological interactions for organisms' communication in the environment are known as semiochemicals (Law and Regnier 1971; Lewis et al. 1976). The semiochemicals have been classified into pheromones and allelochemicals, for intraspecific and interspecific communication, respectively (Dicke and Sabelis 1988).

A considerable number of chemical ecology-based studies have been conducted with the Dynastinae subfamily (Coleoptera, Melolonthidae), with a predominance description of pheromones in the Oryctini tribe and allelochemicals in the Cyclocephalini tribe (Vuts et al. 2014).

The Cyclocephalini tribe is a prominent group of rhinoceros beetles distributed in the Neotropical region, including species with ecological and economic importance (Moore and Jameson 2013; Moore et al. 2018a). *Cyclocephala* Dejean is the largest genus with approximately 350 species, from which 127 are distributed in Brazil (Ratcliffe and Cave 2009; Moore et al. 2018a).

Cyclocephalini beetles are commonly observed as floral visitors, and many species are crucial pollinators for plant reproduction (Schatz 1990; Moore and Jameson 2013). Pollination mutualism frequently involves flowers of early-diverging angiosperm, which commonly have a floral chamber suggested as a rendezvous site for mating, besides protecting beetles against predators and offering food resources (Beach 1982; Bernhardt 2000).

Cyclocephalini beetles can act as both a floral pollinator and predator, consuming the entire reproductive parts of flowers but maintaining ovary integrity (Bernhardt 2000). However, eudicot plants' visitors have shown only predation behavior, suggesting an opportunistic rather than a mutualistic association (Moore et al. 2018a). *Cyclocephala* is thus far the only genus known to visit eudicot flowers (Moore and Jameson 2013), and the chemical ecology involved in this interaction is still unknown.

The aggregation behavior of *Cyclocephala* beetles on flowers has been explained by floral volatile organic compounds (VOCs). The qualitative chemical profile of floral VOCs has shown little complexity, dominated by uncommon and non-related compounds (Maia et al. 2013, 2018), often released at considerable amounts

characterizing the odor pungency during flower anthesis (Bernhardt 2000; Moore et al. 2018a).

The attractiveness of floral VOCs to both male and female adults of *Cyclocephala* species suggests the adults do not rely on sexual pheromones for mating (Schatz 1990; Dieringer et al. 1999; Gibernau et al. 1999). To date, only two *Cyclocephala* species were addressed being attracted by their conspecific (Potter 1980; Haynes et al. 1992; Haynes and Potter 1995). Nevertheless, as any structure elucidation of such possible pheromone was addressed, the intraspecific chemical communication mediated by long-distance volatiles in *Cyclocephala* beetles is still uncertain.

This work approaches both inter-and intra-specific chemical communication of *Cyclocephala paraguayensis* Arrow. This species occurs from Central America to northern Argentina (Endrödi 1985; Moore et al. 2018b). In Brazil, *C. paraguayensis* is widely distributed throughout the country, being the most abundant among *Cyclocephala* species, mainly in the Amazon biome (Gonçalves et al. 2020).

Despite *C. paraguayensis* notorious occurrence, there are sparse data about its biology and behavior. *C. paraguayensis* life cycle takes about 170 days, well-developed in an entirely saprophagous diet, suggesting the species is not rhizophagous in the wild (Albuquerque et al. 2014). Like other Cyclocephaline beetles, *C. paraguayensis* has already been observed visiting flowers forming aggregations (Gottsberger 1986; Munin et al. 2008; Favaris et al. 2020).

Recently, *C. paraguayensis* was registered visiting *Lagenaria siceraria* (Cucurbitaceae) flowers for the first time in Piracicaba, São Paulo state (Favaris et al. 2020). The beetles were also found inside *Brugmansia suaveolens* (Solanaceae) flowers nearby *L. siceraria*. Given the biological relevance of floral allelochemicals for Cyclocephalini beetles, the floral VOCs of *L. siceraria* and *B. suaveolens* were identified regarding their attraction to *C. paraguayensis*. Moreover, the volatiles of males and females of *C. paraguayensis* were investigated for the pheromone-mediated communication issue.

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1. (3S,6E)-NEROLIDOL-MEDIATED RENDEZVOUS OF *Cyclocephala paraguayensis* IN BOTTLE GOURD FLOWERS

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Abstract

Cyclocephalini beetles of the genus *Cyclocephala* (Coleoptera: Melolonthidae: Dynastinae) use flowers of some plants as food, shelter and mating sites. However, little is known about floral scent chemistry involved in this interaction. Here we show that a sesquiterpene alcohol mediates the attraction of *Cyclocephala paraguayensis* Arrow, on bottle gourd flowers, *Lagenaria siceraria* (Molina) Standley (Cucurbitaceae). Both males and females started to aggregate on the flowers at twilight; after that mating began and remained for the entire night. The GC-FID/EAD analysis of the *L. siceraria* floral scent collected in the field revealed that only the major constituent of the airborne volatiles elicited electroantennography responses on male and female antennae of *C. paraguayensis*. This compound was identified as (3S,6E)-nerolidol, which was tested in two field trapping trials in Brazil. In the first bioassay, traps baited with nerolidol (mix of isomers) attracted significantly more adult *C. paraguayensis* than controls. In the second bioassay, the attraction of beetles to the mix and the flower-produced isomer was statistically equivalent, but it was significantly greater than controls. Analysis from the gut content of adult *C. paraguayensis* showed the presence of pollen, suggesting that they also use bottle gourd flowers for their nourishment. Taken together, these results suggest that (3S,6E)-nerolidol plays an essential role in the reproductive behavior of *C. paraguayensis* by eliciting aggregation, mating, and feeding.

Keywords: *Lagenaria siceraria*; Cucurbitaceae; Floral attractants

1.1 Introduction

Floral scents have been reported as important assets for the visitation of beetles of the tribe Cyclocephalini (Melolonthidae: Dynastinae) (Pellmyr and Thien 1986; Schatz 1990; Moore and Jameson 2013; Moore et al. 2018). The interaction of beetles and flowers may result in mutualistic pollination typically by the cantharophilous floral syndrome of basal angiosperms, whose flowers are usually bisexual, protogynous, very scented, and thermogenic (Pellmyr and Thien 1986; Thien et al. 2000). The Cyclocephalini beetles are primarily attracted by strong odors released by flowers as chemical cues to indicate a rendezvous site for food, shelter, and mating (Pellmyr and Thien 1986; Gottsberger 1990; Gottsberger and Webber 2018).

The floral volatile organic compounds (VOCs) in the plant families of Araceae, Annonaceae, Arecaceae, and Magnoliaceae, have been demonstrated to be comprised of pyrazines, ketones, esters, and methoxystyrenes (Dötterl et al. 2012; Gottsberger et al. 2012; Maia et al. 2012, 2013, 2018b; Pereira et al. 2014). The qualitative chemical profile of these VOCs has shown little complexity, dominated by uncommon and non-related compounds that are sufficient to ensure the attraction of beetles, which display an efficient olfactory recognition that evokes a specific plant-pollinator interaction (Maia et al. 2013, 2018a).

By contrast, VOCs produced by eudicot flowers that are visited by Cyclocephalini beetles of the genus *Cyclocephala* have never been identified. It has been suggested that some cyclocephalines of the genus *Cyclocephala* are opportunistic eudicot flower visitors, causing at times damage on flower tissues due to feeding that compromise the reproductive capability of the plant (Moore and Jameson 2013). However, this supposed unspecific attraction is still poorly understood. It has been hypothesized that detection of floral odors of eudicot plants by *Cyclocephala* beetles could be an acquired preference from larval feeding habitat, in the case of roots have similar taste of flowers, preconditioning insects to the adult stage (Gottsberger 1986). Alternately, *Cyclocephala* beetles might visit eudicot plants because flowers, which could be releasing compounds commonly found in insect volatiles, as preconized by the hypothesis of pre-existing sensory bias (Schiestl 2010; Schiestl and Dötterl 2012). That means the beetles have developed olfactory

sensibility to recognize floral scents which are based on chemicals already produced by the insects for intraspecific communication.

Cyclocephala paraguayensis Arrow is a Neotropical species that is frequently found on eudicot plants (Gottsberger 1986; Munin et al. 2008). Here, we observed this beetle visiting flowers of *Lagenaria siceraria* (Molina) Standl (Cucurbitaceae), which incidentally represents a new host flower record for the beetle family Melolonthidae. This plant is originated from Africa and nowadays is worldwide spread (Kistler et al. 2014); it produces white flowers with separate sexes in the same plant (monoecious) but can assume andromonoecious form when hermaphrodite flowers are present (Singh et al. 1996). Anthesis occurs throughout the night, at which time the lepidopteran pollinators, specially hawkmoths, visit the flowers (Morimoto et al. 2004).

We surmised that the volatile components of bottle gourd flowers mediate aggregative behavior of *C. paraguayensis* for feeding and mating. We studied the floral scent chemistry of *L. siceraria* flowers and showed that (3*S*,6*E*)-nerolidol plays an important role in the reproductive behavior of *C. paraguayensis* by eliciting aggregation, mating, and feeding.

1.2 Material and Methods

Sexual and feeding behavior of insects

The behavior of the Cyclocephalini beetle *C. paraguayensis* was observed in bottle gourd flowers of *L. siceraria*, growing area owned by the University of São Paulo, Piracicaba, Brazil (22°42'47.1" S, 47°37'32.7" W). We initially recorded the arrival time of the first beetles on flowers of *L. siceraria*, so the observations in the subsequent days started 30 min earlier. Flowers with insects were marked, and the following parameters were evaluated every 30 min from 7:00 p.m. to 11:00 p.m.: the sex of visited flowers; the total number of insects landed per flower; and the number of mating couples per flower. We repeated these observations for 4 d in the same *L. siceraria* area.

Additionally, a sample of 10 males and females of *C. paraguayensis* present within the flowers were taken to verify the presence of pollen in the gut content. Insects were dissected with sharp forceps, and pieces of the midgut and/or the hindgut epithelium were removed and immersed in 0.9% sodium chloride solution. Next, the material was analyzed under a light microscope at 20X magnification. Fresh pollen from *L. siceraria* flowers was prepared in glass slides with a saline solution as reference material.

Sampling floral VOCs

VOCs collection from *L. siceraria* flowers were conducted outdoors in the above-mentioned bottle gourd plantation and at the laboratory through dynamic headspace extraction. In the field, VOCs were collected from four individual male and female *L. siceraria* flowers recognized according to morphological features (Singh et al. 1996). These flowers were enclosed within a 17 × 20 cm polyester oven bags (Wyda®, Sorocaba, São Paulo, Brazil). Collectors consisted of glass pipets packed with 30 mg of the adsorbent polymer Hayesep® Q (80/100 mesh; Supelco, Bellefonte, PA, USA), which were attached to the bag outlets. Charcoal-filtered air was pushed with a portable pump through the bag and collectors at ~200 mL/min. Bottle gourd flowers were aerated from 7:30 p.m. to 08:30 p.m., which corresponded to the active period of the beetles. Trapped VOCs were eluted from collectors using 0.3 mL of distilled hexane into 2 mL amber vials that were stored at -30 °C until analysis. Aerations of empty bags were made in parallel to monitor system contaminants. In

the same fashion, VOCs were collected from one replicate with four detached flowers inside a vertical glass jar (55 cm long × 8 cm id) under laboratory conditions (23 ±2 °C; 60 ±10% RH). Collectors attached to the jar outlets were packed with 150 mg of adsorbent polymer. Charcoal-filtered air was pushed through jars and collectors at ~300 mL/min, and collections were made overnight for up to 11 h. Trapped VOCs were eluted from collectors using 1.5 mL of distilled hexane into 2 mL amber vial. To use these extracts for GC-EAD analysis, the resulting aliquots were then concentrated to 500 µL under a gentle flow of N₂.

GC-EAD recordings

Extracts of VOCs from *L. siceraria* flowers were analyzed by GC-EAD, i.e., gas chromatography (Shimadzu GC-2010 gas chromatograph, Shimadzu Corp., Kyoto, Japan) linked to an electroantennographic detector system (EAD, Ockenfels Syntech, Buchenbach, Germany) using *C. paraguayensis* antenna as the sensing element. The GC was equipped with a low polarity stationary phase column (30 m × 25 µm × 25 mm; Rtx-5; RESTEK, Bellefonte, PA, USA) connected to a stainless steel crosshead split with two deactivated columns for conducting effluent to FID and EAD detectors at the ratio of 1:3. Helium was used as the carrier gas at a linear velocity of 30.3 cm/s, and 98.3 kPa was set for column head pressure. EAD preparations consisted of male or female excised antennae (n=3), and electrodes were made of glass pipette/gold wire filled with a Beadle-Ephrussi Ringer solution (3.75 g NaCl, 0.175 g KCl, 0.14 g CaCl₂·2H₂O, 500 mL H₂O). The base of the antenna was inserted into the indifferent electrode, and the tip of antennal lamellae was connected to the recording electrode for signal acquisition (Universal Probe, signal amplification 10x). The antenna preparation was inserted into a glass duct connected to the EAD deactivated column tip, carrying a humidified air stream at 0.3 mL/min. Two microliter aliquots of laboratory-collected volatile extracts were injected in splitless mode at 250 °C, and the GC oven was set at 40 °C for 5 min and then increased to 250 °C at 10 °C/min. FID and EAD signals were recorded by Syntech GcEad software (version 4.6).

Chemical analysis

The EAD active compound in the VOCs blend was identified by gas chromatography coupled to a mass spectrometer (GC-MS) with a Shimadzu GCQP-2010 Ultra gas chromatograph (Shimadzu Corp., Kyoto, Japan), equipped with a non-polar stationary phase column (30 m × 25 μm × 25 mm; Rxi-1MS; RESTEK, Bellefonte, PA, USA). One microliter of the extract was injected at 250 °C in split mode (1:3). The GC oven temperature was the same for GC-FID/EAD analysis (above). The helium carrier gas was maintained at a flow of 1.3 mL/min in linear velocity at 41.1 cm/s and 70.7 kPa pressure. Both ion source and quadrupole temperatures were set at 250 °C. Mass spectra were recorded in electron impact mode (70 eV) from *m/z* 35-280. Compounds were identified by their retention indexes, mass spectra comparison with Library NIST 11, and injection of authentic standards (Sigma-Aldrich). The target compound was quantified by GC-FID with a Shimadzu GC-2010 gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a non-polar stationary phase column (30 m × 25 μm × 25 mm; Rtx-1; RESTEK, Bellefonte, PA, USA) following the same set-up of GC-FID/EAD analysis. We added 3 μL of a 1 mg/mL solution of octadecane as an internal standard in all samples for quantification.

The absolute configuration of flower-produced nerolidol was determined using an Agilent 6890GC with flame ionization detector equipped with a permethylated β-cyclodextrin stationary phase chiral column (30 m × 25 μm × 25 mm; HP-CHIRAL-20B; J&W Scientific, Folsom, CA, USA). Synthetic nerolidol (a mixture of isomers) and individual geometric isomers were purchased from Sigma-Aldrich to obtain retention time of *cis* and *trans*-nerolidol. A sample of synthetic (3*R*,6*E*)-nerolidol was kindly donated by Dr. Robert Hanus (Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic). All compounds solution in hexane (40 ppm) were injected and detected at 250 °C. Initial oven temperature was 85 °C for 1 min and then increased to 190 °C at 2 °C/min. For both chiral identification and field assays, we used the (*E*)-nerolidol from Sigma-Aldrich, which was previously determined to be comprised of almost 100% (3*S*,6*E*)-nerolidol (Havlíčková et al. 2019), and also confirmed in our GC analysis (Fig 3D).

Field bioassays of synthetic flower volatiles

We conducted three independent field trials. In the first tests, we mimicked the *L. siceraria* flowers by using flower-shaped dummies made from white filter paper, which were impregnated with 100 mg of neat nerolidol (a mixture of isomers, Sigma-Aldrich). Control dummies had no compound. Dummies were attached to wood sticks (25 cm long), which were inserted into the ground, so the dummies were at 23 cm height. Nerolidol-treated and control dummies were randomly distributed in three pairs, with each pair containing one dummy of one treatment. Dummies were spaced 3 m apart, and each pair of dummies was placed ~ 5 m from the other. Bottle gourd flower dummies were checked for visiting beetles from 06:00 p.m. to 09:00 p.m. Soon after landing, Cyclocephalini beetles were removed from the dummies to avoid potential intraspecific attraction. This bioassay was carried out in the same *L. siceraria* groove in Piracicaba, SP, Brazil (see above) for 2 d (March 12-13, 2018), with dummies replacement made for each replicate.

The second field tests were conducted in the same area in Piracicaba and a pasture area owned by the State University of Mato Grosso do Sul, Cassilândia, MS, Brazil (~ 800 km from Piracicaba; 19°05'35.8" S, 51°48'52.5" W). We used custom-made, cross-vane intercept traps (black polystyrene panels: 18 × 24 cm), with 0.5 L plastic jars attached to the bottom of the trap. Both control and treatment traps were loaded with 200 mL of a solution of water and dish detergent to kill the captured Cyclocephalini beetles. Traps were hung from L-shaped PVC pipe frames (1.5 m long) that were supported by 1 m reinforcing steel bars hammered into the ground. The lures were pieces of filter paper (2 × 6 cm) loaded with 50 mg of neat nerolidol (a mixture of isomers). Control lures were filter papers with no compound. Lures were hung in the central opening slot of the traps with a diaper pin 1 h prior to the sunset. Treated and control traps were randomly distributed. Seven even pairs of traps were deployed in Piracicaba and 10 pairs in Cassilândia. Traps were spaced 20 m apart, and pairs of traps were set ~30 m from each other. Traps were serviced for captured beetles in the morning. For every replicate date, the lures were replaced, and the treatment traps were switched in position to avoid positional effects. Overall, the bioassay was repeated for 5 d in Piracicaba (October 17-22, 2018) and for 1 d in Cassilândia (October 1, 2018).

The third field test was conducted in same area in Cassilândia (October 1-2, 2019), which tested the following treatments: (i) 5 mg of neat (3*S*,6*E*)-nerolidol (*trans*-nerolidol, Sigma-Aldrich); (ii) 20 mg of neat nerolidol (a mixture of isomers), considering 5 mg per isomer; and (iii) control (filter paper with no compound). Treatments were assigned randomly to traps within five blocks. Evaluations were carried out every morning for two days. Lures were replaced 1 h prior to the sunset, and treatment traps were changed one position within blocks to control for positional effects.

Captured cyclocephalini specimens were sent to Dr. Paschoal Coelho Grossi (Federal Rural University of Pernambuco - UFRPE) for taxonomy. A voucher specimen of Cyclocephalini beetle, *C. paraguayensis*, was deposited in the collection of the Museum of Entomology (#6945), Department of Entomology and Acarology (University of São Paulo, Piracicaba, Brazil). Collection of beetles in the field were conducted under SISBIO permit #60104 and #67772 from the Brazilian Ministry of the Environment. This work was submitted to Genetic Heritage Management Council by SisGen within project registration #AFC4FA2.

Statistical Analysis

Data from experiments 1 and 2 were analyzed with the two-tailed Exact Binomial test to verify whether the ratio of beetles attracted to nerolidol and control differed significantly from the expected 1:1 ratio at 5% probability (McDonald 2014). The test was performed from a spreadsheet provided by <http://www.biostathandbook.com/exactgof.html> (accessed January 17, 2019).

Differences between treatment means from experiment 3 were analyzed using the nonparametric Friedman's Test (PROC FREQ, option CMH; SAS Institute, 2011), because data did not pass the assumptions of ANOVA (Sokal and Rohlf 1995). Replicates were defined by block and collection dates. Replicates with no capture of *C. paraguayensis* in any trap were excluded from analyses. Once an overall significance was detected by the Friedman's test, pairs of means were compared using the REGWQ multiple range test, which controls for type I experiment-wise error rate (SAS Institute, 2011). For all bioassays, the sex ratio of attracted adults of *C. paraguayensis* was compared to a nominal proportion of 0.5 with 95% Clopper-Pearson Exact Confidence Intervals (Newcombe 1998).

1.3 Results

Sexual and feeding behavior of insects

The visitation behavior of *C. paraguayensis* on *L. siceraria* flowers was observed in field conditions at night for four hours (7:00 p.m. to 11:00 p.m.). Beetles activity started by the twilight (~7:30 p.m.), when males of *C. paraguayensis* were the first to land on *L. siceraria* flowers ($n = 36$; two-tailed Exact Binomial test; $P = 0.011$). At 8:00 p.m., the number of visiting males and females was 18.50 ± 5.97 (mean \pm SD), increasing until 11:00 p.m. to 26.75 ± 7.46 . Paired males and females of *C. paraguayensis* (2.25 ± 3.2) were present on bottle gourd flowers from 8:00 p.m. until the end of observation period. Both sexes of *L. siceraria* flowers were visited. Seventy-five percent of bottle gourd flowers harbored more than one mating couple, thus, suggesting that aggregation on bottle gourd flowers created mating opportunities for the beetles.

These Cyclocephalini beetles usually remained inside the floral receptacles until the next morning, when they were sheltered (Fig 1A-B). Bottle gourd flowers presented gnawing marks or even major damages resulted from beetles feeding (Fig 1E-F). Of note, pollens of these flowers were found in the gut of beetles (80% females; 50% males, $n = 10$) when dissected (Fig 1G-H).



Figure 1. Sexual and feeding behavior of Cyclocephalini beetle, *Cyclocephala paraguayensis*, in bottle gourd flowers, *Lagenaria siceraria*. A) aggregating and mating on flowers at night; B) *C. paraguayensis* inside senescent flowers in the next morning of beetle's arrival; C) non-damaged *L. siceraria* flower in the field; D) stereoscopic cut view of a non-damaged *L. siceraria* flower; E) *L. siceraria* flower damaged by *C. paraguayensis* in the field; F) stereoscopic cut view of a damaged flower showing beetle gnawing and excrement signal as a result of feeding activity; G) fresh pollen grain of *L. siceraria*; H) pollen grains of *L. siceraria* extracted from *C. paraguayensis* gut (black triangles indicate the pollen grains).

Floral scent identification

Chemical profile was similar between male and female of bottle gourd flowers. Gas chromatography linked to electroantennographic detection (GC-EAD) analysis of airborne volatiles collected from *L. siceraria* showed the presence of a prominent, antennally active peak at 20.5 min (Fig 2).

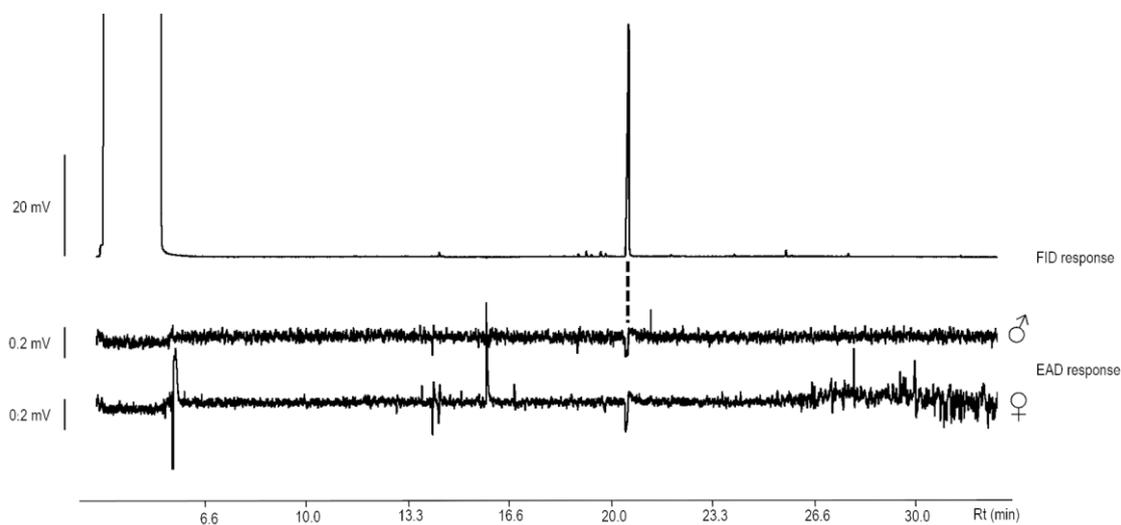


Figure 2. GC-EAD analysis of floral scents extract of *Lagenaria siceraria* by using male and female antenna of Cyclocephalini beetle, *Cyclocephala paraguayensis*, as sensing elements. “FID” flame-ionization detection.

The correspondent compound was tentatively identified by GC-MS as a nerolidol isomer, which led us to conduct *cis* and *trans* identification followed by chiral analysis. The EAD active compound was then identified as *trans*-nerolidol (i.e., (6*E*)-nerolidol, IR = 1568) by comparing the mass spectra and retention indexes of the natural product and the authentic standards (Fig 3A-F). Lastly, chiral analysis using nerolidol standards led to the full characterization of the natural product as (3*S*,6*E*)-nerolidol (Fig 3G).

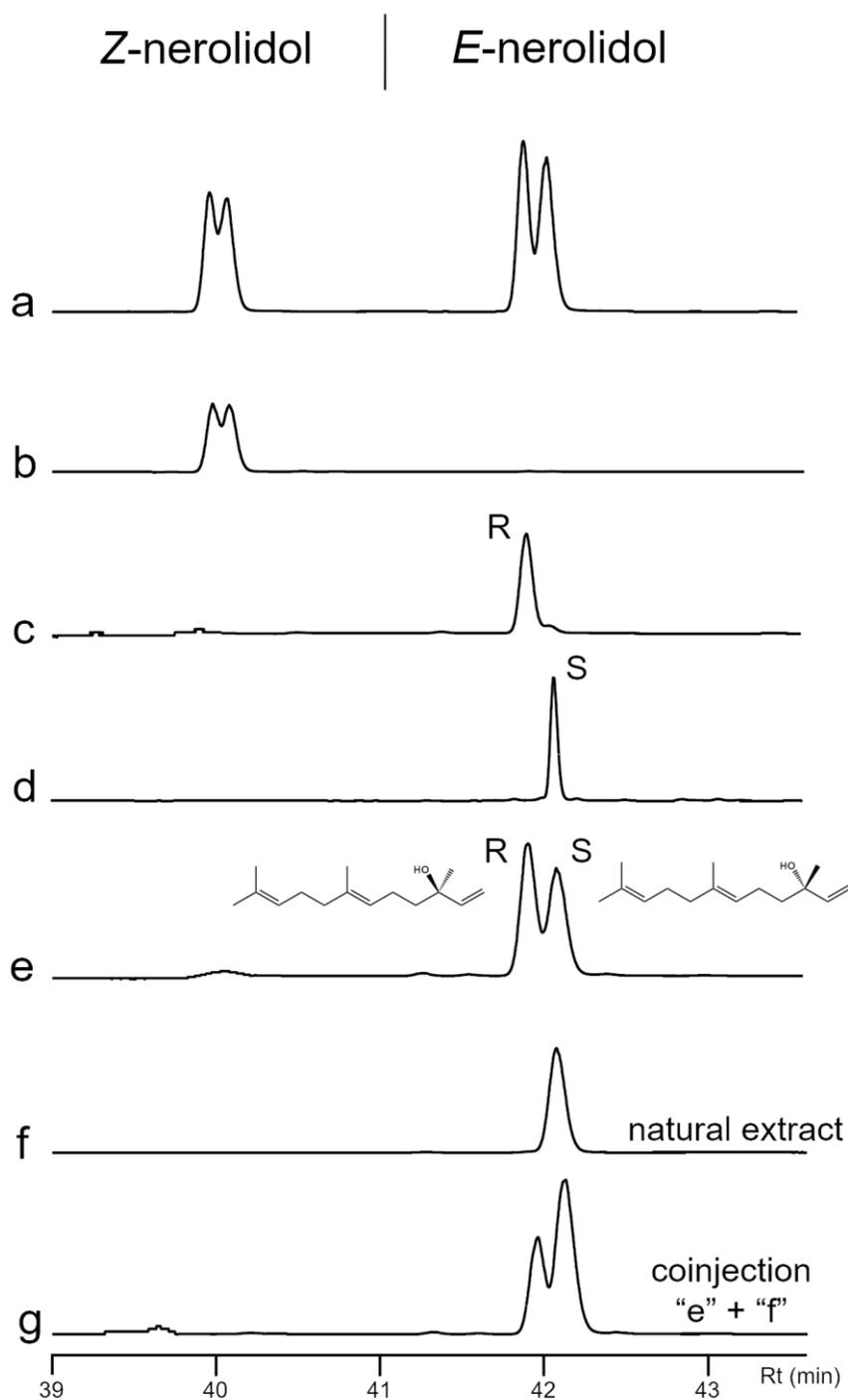


Figure 3. Analysis of bottle gourd flowers, *Lagenaria siceraria*, extract, and synthetic isomers of nerolidol by gas chromatography equipped with a chiral column. a) *Z* and *E* (left to right) isomers of nerolidol (a mix of isomers); b) *Z*-nerolidol isomers; c) laboratory synthesized (3*R*,6*E*)-nerolidol; d) (*E*)-nerolidol from Sigma-Aldrich; e) mix of 40 ng from each (3*R*,6*E*)-nerolidol and (3*S*,6*E*)-nerolidol; f) natural extract from VOCs of *L. siceraria* flowers; g) coinjection of “e” and “f” chromatogram extracts.

Quantification of nerolidol from male and female flowers showed that this VOC comprises 97% of the volatile bouquet and was released at the rate of $\sim 1.3 \mu\text{g/h}$ under field conditions.

Field evaluation of synthetic nerolidol

In our first field trial, a total of six adults of Cyclocephalini beetle, *C. paraguayensis*, landed on nerolidol-treated dummies, whereas no beetle was observed on control dummies (two-tailed Exact Binomial test; $P = 0.031$).

In the second field experiment, we evaluated the response of adults of *C. paraguayensis* to nerolidol-baited traps in two different locations: Cassilândia, State of Mato Grosso do Sul and Piracicaba, State of São Paulo. In both locations, traps baited with nerolidol (a mix of isomers) captured significantly more beetles than control traps. In Cassilândia, 280 adults of *C. paraguayensis* were captured by nerolidol-baited traps during the tests, with no beetles captured in control traps (two-tailed Exact Binomial test; $P < 0.001$; Fig 4A). Although total captures in Piracicaba ($n = 32$) were lower than in Cassilândia, nerolidol-baited traps captured significantly more beetles than control traps (two-tailed Exact Binomial test; $P < 0.001$). Interestingly, the sex ratio of captured beetles did not differ significantly from the 1:1 ratio in both Cassilândia (51.4% females; 95% Clopper-Pearson exact confidence interval: 0.454-0.574; $P = 0.632$) and Piracicaba (41.4% females; 95% Clopper-Pearson exact confidence interval: 0.235-0.610; $P = 0.353$).

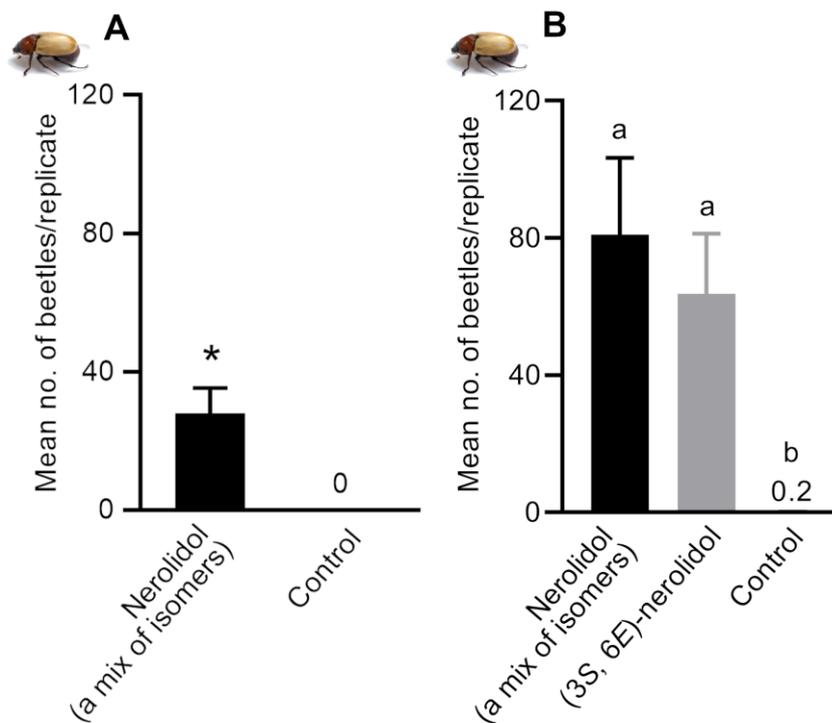


Figure 4. Mean number of Cyclocephalini beetle, *Cyclocephala paraguayensis*, caught per replicate in field experiments conducted in Cassilândia, MS, Brazil. Treatments: Nerolidol (a mix of isomers) = mixture of four isomers of nerolidol; (3S,6E)-nerolidol = synthetic nerolidol, which is identical to the stereoisomer of nerolidol produced by *L. siceraria* flowers. A) Mean number of adults of *C. paraguayensis* caught per replicate of nerolidol-baited traps and controls in the field trial of 2018. *Treatment is significantly different at $P < 0.05$ according to Exact binomial test goodness-of-fit; B) Mean number of adults of *C. paraguayensis* caught by traps containing synthetic nerolidol isomers in the field trial of 2019. Treatments had a significant effect on attraction of Cyclocephalini beetles (Friedman's $Q_{2,30} = 20.002$, $P < 0.001$; $n = 10$ replicates). Means followed by the same letter are not significantly different (REGWQ test, $P > 0.05$).

In the third field tests, we compared captures of *C. paraguayensis* in traps baited with nerolidol (a mix of isomers), (3S,6E)-nerolidol, and control traps. Throughout these experiments, a total of 1,146 beetles were captured in nerolidol traps (i.e., traps baited with either enantiopure nerolidol, or a mix of isomers). By contrast, only two individuals (males) were caught in control traps. Although treatment and control were significantly different ($Q_{2,30} = 20.002$; $P < 0.001$; Fig 4B), there was no statistical difference in catches by the mix of isomers or (3S,6E)-nerolidol (Fig 4B). We, therefore, concluded that the other isomers were neither attractants nor deterrents. Of note, the sex ratio of the captured beetles in the two nerolidol treatments were somewhat female-biased (mix of isomers = 54.4% female, 95% Clopper-Pearson exact confidence interval: 0.509-0.578; $P = 0.012$; and (3S,6E)-nerolidol = 58.4% female, 0.544-0.622; $P < 0.0001$). However, as we did not

measure the sex ratio of the field populations while conducting bioassays, it is not possible to assert that traps captured significantly more female than male beetles.

1.4 Discussion

Our results show that (3*S*,6*E*)-nerolidol mediates aggregation of the Cyclocephalini beetle, *C. paraguayensis*, on bottle gourd flowers, *L. siceraria*. We showed that this sesquiterpene alcohol represents the major VOC emitted by the flowers of *L. siceraria*, and field tests demonstrated that nerolidol-baited traps captured significantly more beetles than control traps. Thus, the most prominent compound in bottle gourd floral scent is sufficient for the attraction of *C. paraguayensis*, which corroborates similar scenarios found in chemical ecology studies involving other anthophilous Cyclocephalini beetles (Dötterl et al. 2012; Gottsberger et al. 2012; Maia et al. 2012, 2013).

Surprisingly, field tests showed captures in traps baited with racemic nerolidol (a mix of four isomers) did not differ significantly from those in traps having the enantiopure nerolidol as the lure. Thus, the three other non-natural isomers of nerolidol are neither attractants nor deterrents.

In the second field experiment, the variation on the beetle catches between Piracicaba and Cassilândia might have been due to the differences in abundance of *C. paraguayensis* in these two locations. Piracicaba comprises the biome Atlantic Rain Forest, which has been observed to harbor small and scattered populations of *C. paraguayensis*. Conversely, Cassilândia is located in the biome Cerrado, which appears to sustain a higher population of this species (Gonçalves et al. 2020).

We, therefore, concluded that *C. paraguayensis* rendezvous in *L. siceraria* is mediated by nerolidol, which promotes mating opportunities. There is no evidence in the literature indicating that chemical communication in *C. paraguayensis* involves sex pheromones. If it does, a long-range aggregation-sex pheromone (sensu Cardé 2014) is probably unnecessary for sexual communication, as nerolidol mediates aggregation of both sexes. It is conceivable, however, that once *C. paraguayensis* beetles are brought together on flowers, species and/or sex recognition may take place by morphological traits (Moore 2012), and/or by a hitherto unknown short-range, contact semiochemicals as cuticular hydrocarbons (Ortiz-Domínguez et al. 2006).

In this study, we showed that *C. paraguayensis* also uses bottle gourd flowers for nourishment given that pollen grains were found in the gut epithelium of males

and females. The energy-rich pollen grains may increase the fitness of these *Cyclocephalini* beetles for mating and oviposition (Pellmyr and Thien 1986).

Our findings do not provide evidence for a mutualistic insect-plant relationship, as previously suggested for other anthophilous cyclocephalines (Gottsberger et al. 2012; Maia et al. 2012). The floral biology traits of cucurbits seem to be more suitable for pollination by bees (Bomfim et al. 2016), and flowers are particularly successful pollinated by hawkmoths (Morimoto et al. 2004). This fact corroborates with the presence of nerolidol in the floral scents of bottle gourd, which is a compound commonly related to sphingophilous flowers (Knudsen and Tollsten 1993) and thereby accounted to belong to “white-floral image” in moth-pollinated taxa (Kaiser 1991). To date, *C. paraguayensis* has been registered only visiting flowers of *Kielmeyera variabilis* Mart. & Zucc. (Calophyllaceae) and *Bauhinia curvula* Benth. (Fabaceae) (Gottsberger 1986; Munin et al. 2008), which are not cantharophilous species. Thus, it is unlikely that the relationship of *C. paraguayensis* with these flowers is specific. Besides, floral preferences by *Cyclocephala* species may reflect in gregarious distribution; once *C. paraguayensis* beetles are widely distributed in Brazil (Gonçalves et al. 2020), we surmise they exploit broader odor components than other *Cyclocephala* species with mutualistic floral interaction. Possibly, *C. paraguayensis* represents a case of beetle acquiring preference for certain floral odors by its feeding habit during the larval stage; and/or it approaches these flowers when few or no cantharophilous species are in anthesis (Gottsberger 1986).

Additionally, *C. paraguayensis* visitation caused damage to *L. siceraria* flowers, although the effects of these damages on the development of fruits remain to be investigated. This destructive feeding behavior has been commonly observed in *Cyclocephala* species visiting eudicot flowers (Moore and Jameson 2013). Further research on the chemical ecology of *Cyclocephala* species associated with eudicot flowers is fundamental to understand this interaction. This includes the role of floral volatiles for insect reproduction and/or pollination activities, as well as the investigation of *Cyclocephala* beetles’ volatiles for intraspecific communication.

Acknowledgments

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2. METHYL BENZOATE and NEROLIDOL ATTRACT THE DYNASTINE BEETLE *Cyclocephala paraguayensis* TO TRUMPET FLOWERS

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Abstract

Here we report the identification and field-testing of floral volatiles of trumpet flowers, *Brugmansia suaveolens* (Willd.) Sweet (Solanaceae), that mediates *Cyclocephala paraguayensis* Arrow's attraction (Melolonthidae: Dynastinae). Analysis of headspace floral volatiles emitted by *B. suaveolens* revealed 19 compounds, from which eucalyptol (57%), methyl benzoate (16%), and myrcene (6%) were present in the highest amounts, whereas *E*-nerolidol — a known floral attractant for this beetle species — was in much lesser amounts (1.8 %). In a first field bioassay, traps baited with methyl benzoate alone or blended with the other two major volatile compounds were more attractive to adult *C. paraguayensis* than traps containing myrcene and eucalyptol alone and controls. In a second field bioassay, traps containing methyl benzoate combined with nerolidol were more attractive to conspecific beetles than either these compounds alone or controls, suggesting a synergistic interaction. The attraction of *C. paraguayensis* to these floral volatiles are in accordance with the extended preference of these beetles for sphingophilous plants, which is critical when cantharophilous flowers are not available. As consequence, this might have contributed to the successful widespread of the species throughout the Brazilian biomes.

Keywords: Coleoptera, Kairomones, Floral volatiles, Sphingophilous plants, Traps.

2.1 Introduction

The beetles in the genus *Cyclocephala* (Coleoptera: Melolonthidae: Dynastinae: Cyclocephalini) possesses ~350 described species distributed from southeastern Canada to Argentina and West Indies (Ratcliffe and Cave 2009). This genus comprises the highest number of anthophilous species within the subfamily Dynastinae, being the only coleopterans to visit eudicot plants' flowers (Moore and Jameson 2013). The adults of *Cyclocephala* are scent-driven to flowers for nourishment (i.e., beetles feed on pollen and plant tissues) and mating (Gottsberger 2016; Gottsberger and Webber 2018; Favaris et al. 2020).

Data reporting plant damage caused by larvae and adults of *Cyclocephala* are conjectural, and it is uncertain whether the feeding on flower tissues indeed compromises plant reproduction (Moore et al. 2018a). Besides, the larvae of these beetles are primary saprophagous (Moore et al. 2018a), and because adults are flower visitors, they might act as pollinators, playing, thus, an essential role in the plant life history (Maia and Schindwein 2006; Gottsberger et al. 2012; Gottsberger 2016).

Cyclocephala paraguayensis Arrow is widely distributed in South America, and it occurs in all five Brazilian macroregions (Moore et al. 2018b). In fact, this is the most abundant *Cyclocephala* species in the Brazilian biomes Amazon, Cerrado, Atlantic Forest, and Caatinga (Gonçalves et al. 2020). This beetle has been recorded on flowers of native and non-native eudicot plants, such as *Kielmeyera variabilis* Mart. & Zucc. (Calophyllaceae) (Gottsberger 1986), *Bauhinia curvula* Benth. (Fabaceae) (Munin et al. 2008), *Lagenaria siceraria* (Molina) Standl. (Cucurbitaceae) (Favaris et al. 2020), and white angel's trumpet flowers, *Brugmansia suaveolens* (Willd.) Sweet (Solanaceae) (A.P.F., personnel observation) represents a new host flower record for *C. paraguayensis*.

Brugmansia suaveolens is native to the Atlantic Forest in eastern Brazil (Hay 2014), and in some countries, it was originally introduced as ornamentals (CABI 2020). Trumpet flowers are night-blooming plants with white to cream-colored, very scented flowers, which are primarily pollinated by nocturnal hawkmoths (Lepidoptera: Sphingidae) (Knudsen and Tollsten 1993; Kite and Leon 1995; Raguso et al. 2003). Like other sphingophilous flowers, the volatile organic compounds (VOCs) of *B. suaveolens* flowers contain terpenes, benzenoids, lactones, and nitrogen-derived

compounds such as oximes (Knudsen and Tollsten 1993; Miyake et al. 1998; Raguso et al. 2003; Anthony et al. 2009). Specifically, its flower scent is often dominated by 1,8-cineole (eucalyptol), followed by *E*-nerolidol (Anthony et al. 2009), methyl benzoate, and α -terpineol (Knudsen and Tollsten 1993).

A first approach on the kairomones exploited by *C. paraguayensis* has shown that conspecific adults of both sexes are attracted by (3*S*,6*E*)-nerolidol, which elicits the beetle aggregation on bottle gourd flowers, *L. siceraria*, for feeding and mating (Favaris et al. 2020). Within this context, we hypothesized that *E*-nerolidol and/or other floral VOCs mediates the attraction of this beetle to trumpet flowers.

Here we evaluated the composition of floral VOCs of *B. suaveolens* and the influence of the most abundant volatiles on the attraction of *C. paraguayensis*. We found that *E*-nerolidol, despite emitted in lesser amounts by trumpet flowers, along with one of the major floral compounds, mediate this dynastine beetle's attraction to *B. suaveolens*.

2.2 Material and Methods

Source of chemicals

Eucalyptol (CAS 470-82-6), methyl benzoate (CAS 93-58-3), myrcene (CAS 123-35-3), and nerolidol (a mix of isomers; CAS 7212-44-4) used for laboratory analyses and field bioassays were purchased from Sigma-Aldrich (Merck KGaA, St. Louis, Missouri, USA).

Collection of floral VOCs

The floral VOCs of *B. suaveolens* were collected from a plant growing in a garden owned by the University of São Paulo, Piracicaba, Brazil (22°42'46.8" S; 47°37'36.2" W). Three flowers were excised from the plant, and peduncles were individually maintained inside glass tubes with cotton moistened with distilled water. Flowers were individually enclosed in polystyrene oven bags (27 cm width x 41 cm height; Qualitá®, Colombo, PR, Brazil), and the two bag vertices were opened for dynamic headspace collection under field conditions. The volatile collector consisted of a glass pipet filled with 100 mg of HayeSep® Q adsorbent (80/100 mesh; Supelco, Bellefonte, PA, USA), which was attached to the bag outlet. The vacuum pump tube was connected to the collector pulling the air at 300 mL/min for 15 minutes. Aeration of empty bags was made in parallel to control system contaminants. Trapped compounds were eluted from collectors using 1 mL of distilled hexane into amber glass vials, which were stored at -30° C until analysis. A voucher specimen of *B. suaveolens* has been deposited in a herbarium of the Department of Biological Sciences (ESALQ/USP), Piracicaba, Brazil.

Chemical analysis

Floral volatile extracts of *B. suaveolens* were initially analyzed by gas chromatography with flame ionization detector (GC-FID; GC-2010, Shimadzu Corp., Kyoto, Japan) equipped with a non-polar stationary phase column (30 m x 25 µm x 25 mm; Rtx-1; RESTEK, Bellefonte, PA, USA). Tetradecane which was absent among floral compounds was added (50 µL of a 10 ng/µL solution) in each sample as an internal standard for quantification. One microliter was injected in splitless mode with injector port at 250 °C, and the GC oven set at 40 °C for 5 min, then 7 °C/min to

250 °C (1 min). The relative amount of each floral compound was determined based on the tetradecane peak area using GC Solution software (version 2.32.00).

Chemical identification was performed in gas chromatography coupled to a mass spectrometer (GC-MS; GCQP-2010 Ultra, Shimadzu Corp., Kyoto, Japan), equipped with a non-polar stationary phase column (30 m × 25 µm × 25 mm; Rxi-1MS; RESTEK, Bellefonte, PA, USA). One microliter of the floral extract was injected in splitless mode using the same parameters set for GC-FID analysis. Quadrupole ion source and transfer line were kept at 250 °C for electron impact analysis at 70 eV (35-300 *m/z*). Compounds were tentatively identified by using the NIST 11 library and non-isothermal Kovats retention indices (Van Den Dool and Kratz 1963). Chemical identity of each compound was confirmed by comparison of their mass spectra, retention time, and retention indices with those of authentic standards. All identified compounds were classified according to their biosynthetic origin (Croteau and Karp 1994).

Field bioassays of synthetic floral compounds

The synthetic floral volatiles of *B. suaveolens* was field-tested in two individual bioassays in a pasture area owned by the State University of Mato Grosso do Sul, Cassilândia, MS, Brazil (~ 800 km from Piracicaba; 19°05'35.8" S, 51°48'52.5" W). This area was chosen because it harbors a high population of *C. paraguayensis*.

For the two bioassays, we used custom-made intercept panel traps (same model used by Favaris et al. 2020). Lures consisted of polyethylene sachets (5 cm width × 7 cm height, 0.08 mm wall thickness; Bompac®, Itajaí, SC, Brazil) containing a dental cotton roll (Coltene Whaledent Inc., Langenau, Germany) loaded with a solution of the floral compound in paraffin oil or neat floral compound. Particularly for the nerolidol, the lure was a filter paper strip (2 cm × 5 cm) impregnated with the neat compound, considering efficiency on beetles trapping demonstrated by Favaris et al. (2020). The amount of each compound per lure (Table 1) was individually calibrated according to their respective release ratio previously determined (see Supplementary Material S1). Lures were hung in the central open slot of traps with a diaper pin 1 h before the sunset (5 p.m.). Treatments were randomly assigned to traps within blocks, and each block had all treatments. Traps and blocks were spaced 20 m apart. Traps were serviced the following morning, and captured beetles were taken to the laboratory for count and sex determination. Lures

were replaced daily, at which time the treatments were changed to one position within blocks to control for positional effects.

In the first bioassay, we evaluated the attraction of *C. paraguayensis* to the three major floral compounds of *B. suaveolens*, i.e., eucalyptol (Euc), methyl benzoate (Mebe), and myrcene (Myr). The treatments were: 1) Blend 1, i.e., Euc+Mebe+Myr at equal release ratio; 2) Blend 2, i.e., Euc+Mebe+Myr at same release ratio found in the volatile extracts from trumpet flowers 3) Mebe; 4) Euc; 5) Myr; and 6) Control (paraffin oil). For treatment details, see Table 1. The six treatment traps were assigned within three blocks, and the bioassay was conducted during four consecutive days (04-07 October 2019).

In the second bioassay, we verified if there was a synergistic interaction of Mebe with nerolidol (Nero) on the attraction of *C. paraguayensis*. Mebe was the best attractant in the first bioassay, whereas the nerolidol, found in smaller amounts in floral scents of *B. suaveolens*, has previously been reported to be highly attractive to the conspecific beetles (Favaris et al. 2020). The treatments were: 1) Mebe + Nero; 2) Nero; 3) Mebe; and 4) Control (paraffin oil). All lures were prepared according to the release rate proportion found in natural floral extracts (see details in Table 1). Treatment traps were randomly assigned within five blocks for 11 evaluation dates (December 5-8 and 12-15, 2019; and October 1, 2, 4, 2020) and four blocks for two evaluation dates (October 13 and 14, 2020).

Table 1. Amount and release ratio of the synthetic floral compounds of *Brugmansia suaveolens* used per treatment lure during the two field bioassays with the dynastine beetle *Cyclocephala paraguayensis*. Eucalyptol, methyl benzoate, and myrcene were added to dental cotton rolls inside polyethylene sachets, whereas nerolidol was added to filter paper strips.

Bioassay	Treatment	Compound(s)	Release ratio (mg/h)	Volume/lure (Concentration)
1	Blend 1	Eucalyptol	10	40 µL (neat)
		Methyl benzoate	10	0.5 mL (240 mg/mL)
		Myrcene	10	0.5 mL (400 mg/mL)
	Blend 2	Eucalyptol	10	40 µL (neat)
		Methyl benzoate	3	0.5 mL (60 mg/mL)
		Myrcene	1	0.5 mL (40 mg/mL)
	Mebe	Methyl benzoate	10	0.5 mL (240 mg/mL)
Euc	Eucalyptol	10	40 µL (neat)	
Myr	Myrcene	10	0.5 mL (400 mg/mL)	
	Control	-	-	0.5 mL of paraffin oil
2	Mebe+Nero	Methyl benzoate	10	0.5 mL (240 mg/mL)
		Nerolidol (mix of isomers)	1.5	20 µL (neat)
	Nero	Nerolidol (mix of isomers)	1.5	20 µL (neat)
	Mebe	Methyl benzoate	10	0.5 mL (240 mg/mL)
	Control	-	-	0.5 mL of paraffin oil

The field bioassays were conducted under SISBIO permit #60104 and #67772 from the Brazilian Ministry of the Environment. This work was submitted to the Genetic Heritage Management Council by SisGen within project registration #AFC4FA2. Voucher specimens have been deposited in the Department of Entomology and Acarology (USP/ESALQ) collection, Piracicaba, Brazil.

Statistical analysis

In all field bioassays, we considered count data evolving random effect of blocks repeated across times. Thus, the treatment effect (fixed effect) on beetle

catches was analyzed using generalized linear mixed models (GLMM) with Poisson distribution (Bolker et al. 2009). The statistical modeling was carried out using the package 'MASS' (function 'glmer') in R Statistics software (R Development Core Team 2019). The model's overdispersion was tested applying the function 'overdisp_fun' (Nelson 2017) and corrected by the negative binomial distribution (function 'glmer.nb'). The best adjustment of the model was chosen by "Akaike's Information Criterion" (AIC) comparison to verify the estimated model's quality. Quantile residuals were generated from 1,000 simulations of the models used with DHARMA package version 0.3.3.0 (Hartig 2020). The difference between the treatment means of GLMM estimated values (link function: log) was analyzed by ANOVA and Tuckey's test at $P < 0.05$ with Bonferroni correction (package 'emmeans', R Development Core Team 2019). For all bioassays, the sex ratio of attracted adults of *C. paraguayensis* was compared to a nominal proportion of 0.5 with 95% Clopper-Pearson Exact Confidence Intervals (Newcombe 1998).

2.3 Results

Identification of floral VOCs of *B. suaveolens*

Nineteen compounds were found in the volatile headspace extracts from *B. suaveolens* flowers distributed into isoprenoid and benzenoid classes (Table 2). The isoprenoids were divided into three subclasses (monoterpenes, oxygenated monoterpenoids, and oxygenated sesquiterpenoids), whereas the benzenoid was represented solely by the aromatic ester methyl benzoate. The most abundant floral VOCs were eucalyptol (~57%), followed by methyl benzoate (~16%) and myrcene (~6%). The remaining compounds represented less than ~3% of volatiles.

Table 2. List of headspace volatile compounds collected from *Brugmansia suaveolens* flowers. Compounds were ordered in classes and subclasses according to the GC-retention time in the non-polar column Rtx-1. Rt = retention time (minutes); KI = Kovats retention index; BP= base peak.

Floral volatiles (class and subclasses)	Rt (min)	KI	Relative abundance (ng/ μ L) mean \pm SD	Relative percentage of total emission of volatiles %
ISOPRENOIDS				
<i>Monoterpenes:</i>				
α -thujene	11.17	926	22.91 \pm 11.47	1.63
α -pinene	11.35	932	25.11 \pm 11.75	1.79
Sabinene	12.34	968	53.49 \pm 26.91	3.81
β -pinene	12.43	971	23.15 \pm 11.18	1.65
Myrcene	12.83	985	87.38 \pm 42.52	6.22
Limonene	13.97	1029	0.60 \pm 0.28	0.04
(Z)- β -ocimene	14.24	1040	33.16 \pm 24.48	2.36
(E)- β -ocimene	14.50	1051	0.54 \pm 0.17	0.04
(Z)-sabinene hydrate	14.60	1055	4.14 \pm 2.73	0.30
α -terpinolene	15.22	1080	2.18 \pm 1.06	0.16
δ -terpineol	16.79	1149	3.10 \pm 2.17	0.22
<i>Oxygenated monoterpenoids:</i>				
1,8-cineole (Eucalyptol)	13.80	1023	805.29 \pm 423.76	57.35
Linalool	15.35	1086	23.10 \pm 22.36	1.65
α -terpineol	17.35	1174	38.60 \pm 26.94	2.75
Nerol	18.66	1237	25.02 \pm 12.83	1.78
Geranial	18.84	1246	0.90 \pm 0.52	0.06
<i>Oxygenated sesquiterpenoid:</i>				
Dihydro- β -ionone	22.24	1422	1.80 \pm 1.26	0.13
(E)-nerolidol	24.47	1550	25.62 \pm 17.87	1.82
BENZENOID				
<i>Aromatic ester:</i>				
Methyl benzoate	15.01	1072	228.04 \pm 167.52	16.24

Field bioassays of synthetic floral compounds

A total of 426 adult males and females of *C. paraguayensis* was caught during the first field bioassay, and there was a significant treatment effect on the attraction of beetles (GLMM, $n = 12$, $\chi^2 = 85.81$, d.f. = 5, $P < 0.001$). In this case, the beetles were significantly more attracted to traps containing methyl benzoate alone or in blends with eucalyptol and myrcene (Fig 1). The sex-ratio of beetles attracted by the Blend 1 was female-biased (60.49% females; 95% Clopper-Pearson exact confidence interval: 0.525-0.680; $P < 0.01$), whereas the sex ratio of attracted beetles did not differ from the 1:1 ratio in either Blend 2 (50.38% females; 95% Clopper-Pearson exact confidence interval: 0.415-0.592; $P = 1.00$) or methyl benzoate (51.56% females; 95% Clopper-Pearson exact confidence interval: 0.425-0.604; $P = 0.791$).

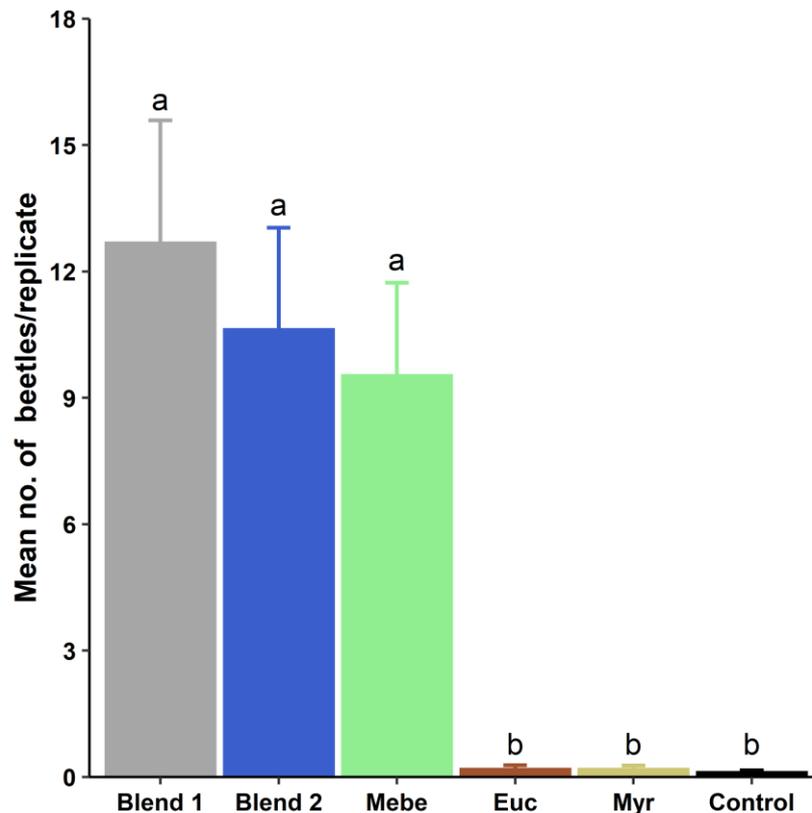


Figure 1. Mean (\pm SE) number of adult *Cyclocephala paraguayensis* (sexes combined) caught with traps baited with synthetic floral volatiles of *Brugmansia suaveolens* during the field bioassay 1. Treatment abbreviations: Blend 1= blend of methyl benzoate, eucalyptol and myrcene at equal release ratio (10 mg/h); Blend 2 = blend of methyl benzoate, eucalyptol and myrcene at same ratio emitted by *B. suaveolens* flowers (i.e., 10, 3 and 1 mg/h, respectively); Mebe = methyl benzoate; Euc = eucalyptol; Myr = myrcene; and Control = Solvent (neat paraffin oil). Means with different letters are statistically different according to Tukey's test ($P < 0.05$).

In the second field bioassay, a total of 842 adult males and females of *C. paraguayensis* was caught, and there was a significant treatment effect on the attraction of beetles (GLMM, $n = 63$, $\chi^2 = 160.62$, $d.f. = 3$, $P < 0.001$). In this case, traps containing the blend methyl benzoate + nerolidol attracted significantly more beetles than the individual compounds or controls (Fig. 2). The sex-ratio of beetles attracted by the blend methyl benzoate + nerolidol was female-biased (57.87% females; 95% Clopper-Pearson exact confidence interval: 0.537-0.619; $P < 0.001$), whereas the sex-ratio of attracted beetles did not differ from 1:1 in either nerolidol (49.79% females; 95% Clopper-Pearson exact confidence interval: 0.433-0.562; $P = 1$) or methyl benzoate (75% females; 95% Clopper-Pearson exact confidence interval: 0.476-0.927; $P = 0.07$).

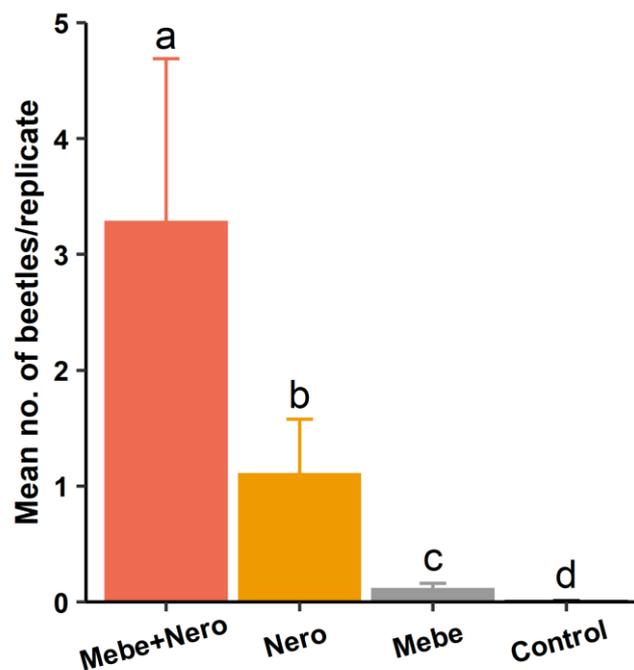


Figure 2. Mean (\pm SE) number of adult *Cyclocephala paraguayensis* (sexes combined) caught by traps baited with synthetic floral volatiles of *Brugmansia suaveolens* during the field bioassay 2. Treatment abbreviations: Mebe = methyl benzoate; Nero = a mix of isomers of nerolidol; Control = Solvent (neat paraffin oil). Means with different letters are statistically different according to Tukey's test ($P < 0.05$).

2.4 Discussion

Our findings offer further evidence that floral VOCs are critical components in the reproductive behavior of the dynastine beetle *C. paraguayensis*. We showed that conspecific beetles were attracted by one of the major floral VOCs of *B. suaveolens*, the methyl benzoate. The other major floral VOCs (eucalyptol and myrcene) were neither attractive as one component nor influenced the beetles' attraction when they were blended with methyl benzoate. Moreover, the nerolidol, despite present in much lesser amounts in volatiles from trumpet flowers, combined with methyl benzoate was more attractive to *C. paraguayensis* than traps baited with compounds separately.

Methyl benzoate *per se* was attractive to adult *C. paraguayensis*, confirming previous findings that single compounds at high release amounts may drive the flight of Cyclocephalini beetles to their hosts (Dötterl et al. 2012; Gottsberger et al. 2012). Moreover, this compound is widespread in floral scents from several plants (Knudsen et al. 2006), including representatives from the families Araceae, Annonaceae, Arecaceae, and Nymphaeaceae which are known to be visited by Cyclocephalini beetles (Moore and Jameson 2013).

Nevertheless, minor components may have a crucial role on insects' behavior, influencing their host preferences (Clavijo McCormick et al. 2014). Here we demonstrated that the attraction of *C. paraguayensis* to methyl benzoate was dramatically increased in the presence of nerolidol in comparison to these compounds alone, suggesting a synergistic interaction. Previous work has shown that (3*S*,6*E*)-nerolidol comprises the dominant floral VOC of the bottle gourd flowers, *L. siceraria*, which renders adult *C. paraguayensis* of both sexes to aggregate on the plants for feeding and mating. Despite the absolute configuration of nerolidol in trumpet flowers remain to be determined, this compound, even as a mix of isomers, synergized the beetles' attraction to the methyl benzoate.

The shared nerolidol by flowers of *L. siceraria* (Favaris et al. 2020) and *B. suaveolens* (present study) might explain the preference of *C. paraguayensis* by sphingophilous plants. Oxygenated sesquiterpenes like nerolidol have been reported as primary compounds emitted by hawkmoth-pollinated flowers, i.e. *Hillia parasitica* Jacq. (Rubiaceae), *Escobedia grandiflora* (L.f.) Kuntze (Orobanchaceae), *Hedychium coronarium* J. Koenig (Zingiberaceae), and *Coussarea* spp. (Rubiaceae) (Knudsen and Tollsten 1993). Although methyl benzoate has not been gathered on chemical

groups from sphingophilous flowers, this compound has been frequently found in moth-pollinated plants (Knudsen and Tollsten 1993; Raguso et al. 2003; Peter et al. 2009), and it seems to be essential for flight orientation of some lepidopteran species (Schiestl 2015).

Additionally, the extended olfactory preference of *Cyclocephala* beetles for sphingophilous flowers would likely enhance beetles' visitation of several plant taxa, contributing to their wide distribution throughout Brazilian biomes. In fact, the exploitation of sphingophilous flowers by other *Cyclocephala* species has been reported (Gottsberger 1986; Silva and Sazima 1995; Raguso et al. 2003), indicating that this phenomenon may be critical for the beetles' life history when cantharophilous flowers under anthesis are scarce (Gottsberger 1986).

Notwithstanding the flower-visiting beetles are primarily guided by floral VOCs, the visual stimuli might also work synergistically with the olfactory system (Pellmyr and Thien 1986), even under dark conditions (Oike et al. 2017). Therefore, research with experimentally-controlled visual and olfactory stimuli might be worthy to better understand the interaction of *C. paraguayensis* with sphingophilous flowers.

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Supplementary Material

Analysis of volatile dispenser for field trials

The lure dispensers loaded with compounds of *B. suaveolans* flowers for *C. paraguayensis* field trials – methyl benzoate (Mebe), eucalyptol (Euc), and myrcene (Myr) were evaluated by differential weighing to inspect releasing rate proportions with time.

Compounds were individually dissolved in paraffin oil at 40 mg/mL and 20 mg (0.5 mL of solution) were impregnated in a cotton dental wick (Coltene Whaledent Inc., Langenau, Germany) inside a polyethylene sachet (5 cm width × 7 cm height, Arroba Embalagens, Piracicaba, Brazil). Neat eucalyptol was also impregnated on cotton (0.04 mL, ~40 mg) inside the sachet for releasing rate adjustments. Neat nerolidol (20 mg) was impregnated in filter paper strips (2 × 6 cm). Lures with paraffin oil alone (0.5 mL) were evaluated as a control.

After lures preparation, the initial weight of sachets (time 0) was taken and then they were maintained inside the fume hood (~23 °C). Weight measurements were taken hourly for 6 hours from 4 replicates/lure.

Releasing rate data were statistically modeled for predicting lure volatilization over time. We applied the mixed linear model, considering the time as a fixed factor, and each sachet as the aleatory factor, in the conditions of normality and homogeneity of data distribution; otherwise, we applied the generalized linear mixed model with a gamma distribution (link function “log”) in software R 3.6 (R Development Core Team 2019), packages lme4 (Bates et al. 2015).

Results

Although compounds as paraffin oil solution were impregnated at the same amount (20 mg), they exhibited different release rates over time (mg/h). From all tested compounds, control ($P=0.99$), myrcene ($P=0.85$), and nerolidol ($P=0.59$) exhibited a constant release rate (Fig S1).

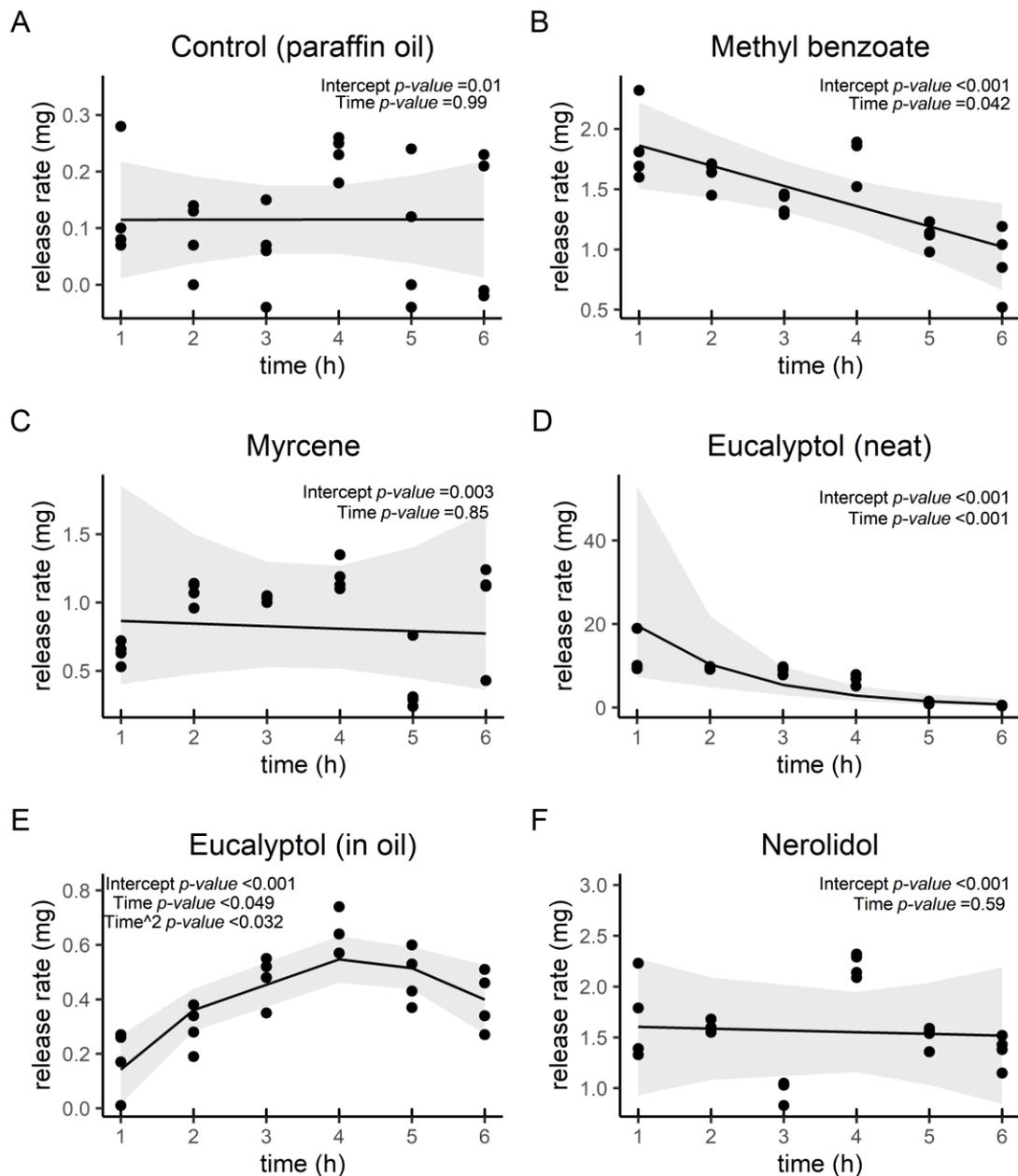


Figure S1. Release rate (mg/h) of major compounds from *Brugmansia suaveolens* flowers used in trapping field experiments. Compounds were evaluated as paraffin oil solution (eucalyptol, methyl benzoate, and myrcene), and without dilution (neat eucalyptol in cotton dental wick+polyethylene sachets and nerolidol in a filter paper strip).

Eucalyptol in paraffin oil was released at low amounts (less than 1mg/h; Fig S1E). Thus, in the first field test, we used neat eucalyptol (~40 mg) to obtain ~10mg/h, which was achieved even release rate varied with time ($P < 0.001$; Fig S1D).

Methyl benzoate release rate was almost constant ($P = 0.042$; Fig S1B). Then, we considered 20 mg being released at ~1-2 mg/h to estimate, in a direct proportion,

30 mg and 120 mg might be releasing respectively ~2-3 mg/h (for “Blend 2” treatment), and ~10 mg/h (for “Blend 1” and “Mebe” treatments).

Myrcene (20 mg) had a constant release rate ($P=0.85$; Fig S1C) of ~1 mg/h. Thus, 20 mg was used for the “Blend 2” treatment, and 200 mg for the “Blend 1” and “Myr” to achieve ~10mg/h.

Nerolidol (20 mg) was released at ~1.5 mg/h, which was used in the second field test, where the natural proportion of compounds found in floral extracts was maintained.

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3. CHEMICAL PROFILE OF VOLATILES EMITTED BY MALE AND FEMALE OF *Cyclocephala paraguayensis*

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Abstract

Sex pheromones have been reported in *Cyclocephala* species, but their identification is still unknown. Recent studies approaching the chemical ecology of Cyclocephalini beetles suggest floral attractants are sufficient to ensure *Cyclocephala* partner encountering. Here we investigated whether *Cyclocephala paraguayensis* produces a sex pheromone for mating, even though floral attractants promote adults aggregation. Headspace volatiles from males and females adults of *C. paraguayensis* showed sex-specific compounds released by both sexes. Female-specific compounds were identified as anisole and phenol, which did not attract conspecifics in field trapping experiments. Sex-specific constituents from males were unveiled at a prominent concentration in extracts, and initial chemical identification has shown puleganic acid as a candidate chemical structure. Further advances on chemical elucidation are needed to confirm whether puleganic acid has been released by males, as well as its biological function for the species.

Keywords: Cyclocephalini beetles; Intraspecific communication; Semiochemicals.

3.1 Introduction

The phytophagous beetles from the Melolonthidae family are known to produce pheromones for sex communication. The first sex pheromone was identified from *Costelytra zealandica* White (Henzell and Lowe 1970). Posteriorly, demands for controlling agricultural and turf pests were an important factor in propelling pheromone identification in Melolonthidae beetles, mainly in Rutelinae, Melolonthinae, and Dynastinae subfamilies (Leal 1998). Thus, a variety of molecules was identified, comprising derivatives from lactones, terpenoids, amino acids (Leal et al. 1992, 1993a; Leal 1998, 1999), and unusual structures, such as 1,3-dimethyl-2,4-(1H,3H)-quinazolinedione, an alkaloid with anti-inflammatory and analgesic properties for mammals (Leal et al. 1997).

Unlike lepidopteran insects, the major component of the sex pheromone from Melolonthidae beetles has been ensured conspecific attraction; however, minor compounds might be decisive for sex communication (Leal et al. 1993b). Melolonthidae beetles may also share their pheromone composition; nevertheless, the beetles' temporal occurrence or their geographical distribution maintains species boundaries, mitigating cross-attraction chances (Leal et al. 1994).

The knowledge on pheromone production by species in the genus *Cyclocephala* Latreille (Dynastinae, Cyclocephalini) is still scarce but intriguing. There is behavioral evidence of sex pheromone production by females of *Cyclocephala lurida* Bland and *Cyclocephala borealis* Arrow, which share the same airborne sex attractants (Potter 1980). Besides, males of *C. lurida* and *C. borealis* are more attracted to grubs and pupae than female adults, suggesting pheromone is also present in immature stages (Haynes et al. 1992; Haynes and Potter 1995). Although natural extracts were also efficient in trapping experiments for *C. lurida* and *C. borealis*, the pheromone identification has never been addressed.

Cyclocephalini beetles are commonly scent-oriented floral visitors. Thus, in the last decade, floral attractants have been identified for *Cyclocephala* beetles, mainly comprising a single and major compound from the floral scent bouquet (Maia et al. 2013, 2018; Moore et al. 2018). The capability of floral volatiles in aggregating both sexes of Cyclocephalini beetles on flowers has raised the hypothesis of long-distance pheromones being unnecessary for mating activity (Schatz 1990; Dieringer et al. 1999). Possibly, pollinators of the genus *Cyclocephala*, which are closely

associated with flowers, do not rely on pheromones for reproduction (Gibernau et al. 1999). Nevertheless, the intraspecific chemical ecology of the Cyclocephalini beetles remains to be investigated regarding the sex pheromone production and its association with pollinator and non-pollinator species.

In this study, volatiles from males and female adults of *Cyclocephala paraguayensis* Arrow were investigated. To date, *C. paraguayensis* has been reported to aggregate on eudicot flowers which appears to be a non-apparent pollination activity (Gottsberger 1986; Munin et al. 2008; Favaris et al. 2020). Moreover, floral attractants, i.e. *E*-nerolidol (Favaris et al. 2020) and methyl benzoate (see results Chapter 2), have attracted conspecific *C. paraguayensis* on flowers, promoting mating opportunities. Based on behavioral aspects displayed in the field, our main goal was to investigate the volatiles from adult *C. paraguayensis*, comparing male and female airborne compounds, and selecting sex pheromone candidates for intraspecific communication.

3.2 Material and Methods

Observation of *Cyclocephala paraguayensis* behavior in the field

Aspects of *C. paraguayensis* behavior in the field were observed for considering a sex pheromone production hypothesis. The observations were conducted at a pasture area owned by the State University of Mato Grosso do Sul (Cassilândia-MS; 19°05'35.8" S, 51°48'52.5" W).

In the first observation, we inspected ~20-30 native plants and collected the pioneer beetle of *C. paraguayensis* landing on them (n=15) at the beginning of flight activity (~30 min after sunset). The beetles from each plant were placed in individual plastic containers and taken to the laboratory for sex identification.

In the second observation, beetles aggregated after four hours from the sunset on 16 native plants were collected in plastic recipients to calculate the sex ratio of adult *C. paraguayensis*.

Data of total male and female of *C. paraguayensis* from the two observations were analyzed by a "two-sided" exact binomial test ($P < 0.05$) using R statistics software (R Development Core Team 2019).

Volatile collection of insects

Adults of *C. paraguayensis* were collected in an experimental field in the Department of Acarology and Entomology at the University of São Paulo, Piracicaba, Brazil (22°42'47.1" S, 47°37'32.7" W). Males and females were taken to the laboratory and sexed by the thickened anterior tarsi of males (Endrödi 1985).

Adult beetles of the same sex were aerated in groups of 8-12 individuals (N=3) in cylindrical glass chambers (21.5 cm length x 4 cm internal diameter) containing a paper towel sheet as support (Kimberly-Clark Professional Scott®, SP, Brazil). Additionally, aeration chambers were covered with aluminum foil allowing only a slight entrance of light. Charcoal-filtered air was pushed in at 0.3 L/min through the inlet chamber. In the air outlet, we connected a glass volatile collector with 30 mg of the adsorbent polymer Hayesep® Q 80/100 mesh (Supelco, Bellefonte, PA, USA). Headspace collection was conducted during 10 h of the photophase (from 08h00 a.m. to 06h00 p.m.); the same collection chamber was also aerated during 14 h of the scotophase (from 06h00 p.m. to 08h00 a.m.). A chamber with only a paper towel sheet was also aerated to inspect system contaminants. Volatiles were recovered

from collectors with 300 μ L of distilled hexane. Samples were stored at -30 °C until analysis.

Chemical analysis

Volatile samples were analyzed on a gas chromatograph (GC) coupled with a flame ionization detector (GC-FID; GC-2010, Shimadzu Corp., Kyoto, Japan) and equipped with a non-polar stationary phase column (30 m \times 25 μ m \times 25 mm; Rtx-1; RESTEK, Bellefonte, PA, USA). Helium was used as the carrier gas at a linear velocity of 35.1 cm/s, and 113.7 kPa was set for column head pressure. One microliter of each extract was injected in splitless mode at 250 °C. Column oven was initially set at 35 °C for 1 min, increasing at 5 °C/min to 40 °C held for 5 min, and finally increasing at 10 °C/min to 250 °C, held for 20 min. The area of selected peaks was taken by GC Solution version 2.32.00 to calculate the proportion between compounds.

The candidate compounds were analyzed on GC coupled to a mass spectrometer (GC-MS; Shimadzu GCQP-2010 Ultra, Shimadzu Corp., Kyoto, Japan) and equipped with a non-polar stationary phase column (30 m \times 25 μ m \times 25 mm; Rxi-1MS; RESTEK, Bellefonte, PA, USA). One microliter of the extracts was injected at 250 °C in splitless mode. The GC oven temperature was the same for GC-FID analysis (above). The helium carrier gas was maintained at a flow of 1.33 mL/min in linear velocity at 41.4 cm/s and 70.7 kPa pressure. Ion source and quadrupole temperatures were set at 250 °C. Mass spectra were recorded in electron impact mode (70 eV) from m/z 35-260. Compounds were identified by their Kováts retention indexes (Van Den Dool and Kratz 1963), mass spectra comparison with Library NIST 11, and injection of authentic standards (Sigma-Aldrich).

Male extracts of *C. paraguayensis* were analyzed in additional procedures for chemical identification of the male-specific compound. We injected male extracts onto the GC-FID with the same temperature setup described above and equipped with a semi-standard non-polar column (30 m \times 25 μ m \times 25 mm; HP-5; Agilent J&W, Santa Clara, CA, USA). After that, the GC was equipped with a polar column (30 m \times 25 μ m \times 25 mm; Rtx-WAX; RESTEK, Bellefonte, PA, USA) for another injection of male extracts. A standard of alkanes was also injected onto the two columns for retention indexes calculation.

Male headspace samples were also analyzed by proton nuclear magnetic resonance (^1H NMR) spectroscopy using CDCl_3 (Chloroform-d; Sigma-Aldrich) as the solvent for polymer desorption. NMR spectra (^1H , ^{13}C , DEPT-135, COSY, HMBC, and HSQC) were obtained from an Avance NEO Ascend (600 MHz, Bruker) and Avance-III 400 Nanobay spectrometer. Chemical shifts were registered in δ and coupling constants (J) calculated in Hertz (Hz). Tetramethylsilane (TMS) was used as internal reference ($\delta\text{H} = \delta\text{C} = 0$).

Field experiment

Field bioassays were conducted in an experimental area of the State University of Mato Grosso do Sul, Cassilândia, MS, Brazil ($19^\circ05'35.8''$ S, $51^\circ48'52.5''$ W). Only the female-specific compounds (phenol and anisole) were used in the traps because the male-specific compound's identity was not fully characterized before field tests. Custom-made, cross-vane intercept traps (black polystyrene panels: 18×24 cm) with 0.5 L plastic jars attached to the bottom of the trap were used for the beetle's capture. The compounds were dissolved in isopropyl alcohol, and 500 μL were loaded in a cotton dental wick inside a polyethylene sachet. Treatments were: (i) 1:1 blend of phenol+anisole (20 mg of each compound); (ii) natural blend of phenol (38 mg)+anisole (2 mg); (iii) phenol (40 mg), (iv) anisole (40 mg), and (v) control (neat isopropyl alcohol).

Five traps were positioned 20 m apart in each of four blocks and were hung at 1.5 m from the ground. Treatment and control lures were randomly distributed inside the blocks and installed in the central opening of the trap 1 h previous to *C. paraguayensis* flight activity ($\sim 05\text{h}00$ p.m., MS time zone). The bottom plastic jars of the traps were filled with ~ 200 mL of water with dish detergent. Beetle's catches were evaluated the following day. The experiment was conducted for two days (October 02-03, 2019), totalizing eight replicates for each treatment. Data were evaluated by descriptive statistical analysis.

3.3 Results

Cyclocephala paraguayensis behavior

Males and females of *C. paraguayensis* showed aggregations and mating behavior during their flight activity in natural conditions (Fig 1).



Figure 1. Aggregation and mating behavior of *Cyclocephala paraguayensis* beetles on an unidentified native plant.

Male was the pioneer sex landing on plants (80% males; two-sided exact binomial test: $P < 0.05$). Usually, beetles were flying altogether and immediately forming aggregations on plants, with several couples exhibiting mating behavior.

After aggregations, 261 adults of *C. paraguayensis* were collected from buddings, and males were present at a higher proportion than females (67.43% males; two-sided exact binomial test: $P < 0.001$).

Selection of candidate compounds

The compounds from male and female *C. paraguayensis* headspace collection were compared by GC-FID analysis. We found one female-specific compound identified as anisole (Rt = 10.5 min; KI = 895; non-polar column), and one unidentified male-specific compound (Rt = 18.4 min; KI = 1256; non-polar column). Both compounds were considered as pheromone candidates of *C. paraguayensis*. Additionally, a common compound emitted by both sexes – phenol (Rt = 12.5 min; KI = 967; non-polar column) – was also identified (Fig. 2).

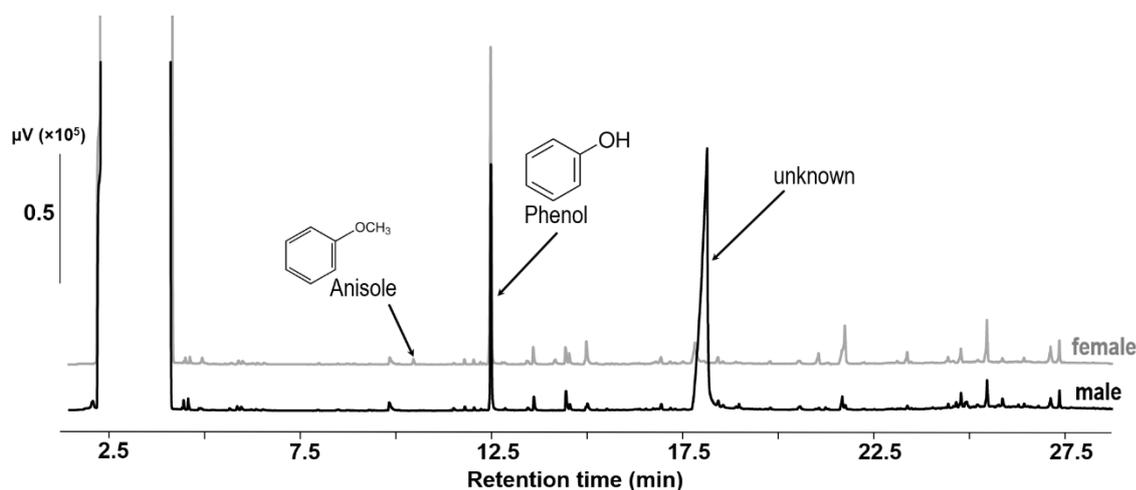


Figure 2. Chromatographic profiles of male and female headspace collection of *Cyclocephala paraguayensis*. Anisole = female-specific compound; Phenol = common compound between sexes; “Unknown” = male-specific compound.

In female extracts, phenol was the major peak which comprised ~ 51% of the chemical profile. The sex-specific compound, anisole, corresponded to 6% of the phenol peak area. Thus, this proportion was considered the natural proportion for lures used in field experiments. In male extracts, the sex-specific compound was the major peak, comprising ~ 91% of the total peak area, with phenol corresponding to ~ 5.7%.

Chemical analysis of male-specific compound

The male-specific compound (“unknown” peak; Fig. 1) was not identified before the field experiment. Nevertheless, some advances in the compound analysis have suggested a putative chemical structure.

We obtained two additional retention indexes for the candidate compound: KI=1285 (semi-standard non-polar column; HP-5) and KI= 2164 (polar column; Rtx-WAX). The difference between the KI values obtained from Rtx-WAX and HP-5 columns (~ 879) is close to the observed for carboxylic acids, suggesting this organic function in the structure. This phenomenon occurs due to the stronger interaction of polar structures with polar stationary phases (Linstrom 1997).

GC-MS analysis of the compound exhibited the following prominent ions (m/z): 41, 55, 69, 81, 87, 95, 110, 127, 137, 152, 170 (Fig. 3). The MS fragmentation pattern suggested the structure to be a $C_{10}H_{18}O_2$ monoterpene, with two degrees of unsaturation, with a possible M^+ at m/z 170. The MS spectrum was very similar to the spectrum from citronellic acid, except for the fragments between m/z 80 and 90, corroborating a carboxylic acid suggestion.

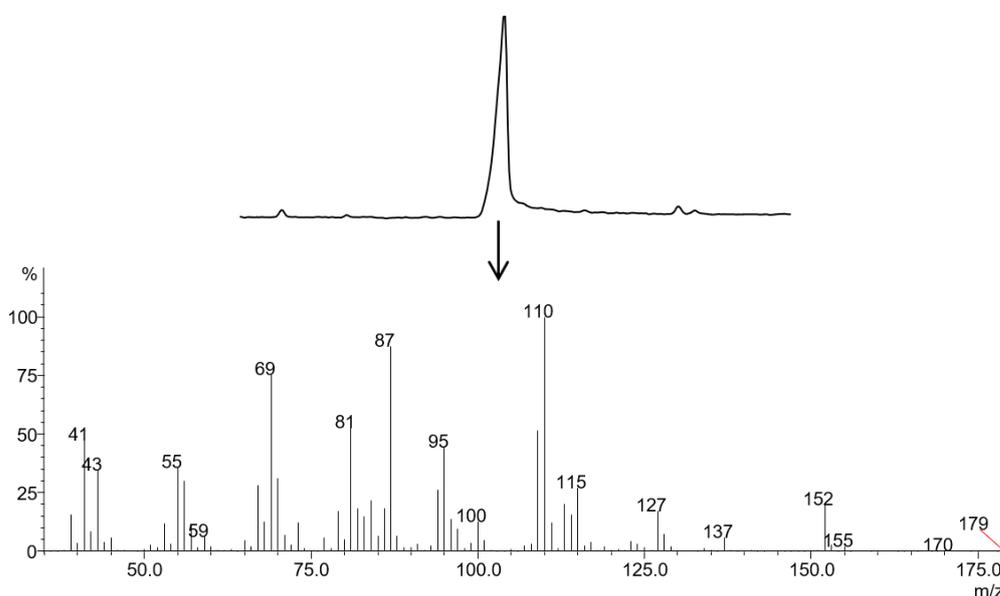


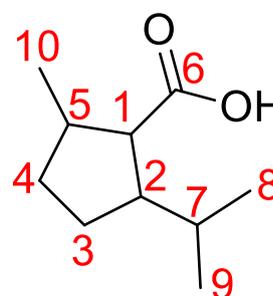
Figure 3. Mass spectra of the selected compound as candidate pheromone from male headspace extract of *Cyclocephala paraguayensis*.

Data showed in Tables 1 and 2, and in the spectra (Supplementary Material; Fig S1-S6) allowed the elucidation of the male-specific compound's chemical structure as pulegamic acid, an unprecedented animal's natural product.

The ^{13}C NMR spectrum showed a total of 10 signals, confirming the proposed C_{10} structure. It should be highlighted that the signal at $\delta 179.2$ (C6), showed no correlations in HSCQ but correlated with H1, H2, and H5 in HMBC, suggesting to be related to a carboxylic acid. HMBC correlations of signals 53.4, 51.1, 38.5, 33.6, and 29.8 were the keys to determine that the structure would be cyclic (Table 1).

Table 1. ^{13}C NMR chemical shifts assignments and HSQC and HMBC correlations for natural pulegamic acid.

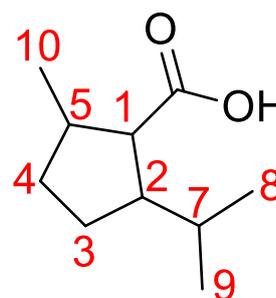
δ (ppm) - ^{13}C	C Atom	Type	HSQC correlations	HMBC correlations
179.2	6	C	-	H1; H2; H5(w)
53.4	1	CH	H1	H2; H4a(w); H4b(w); H10
51.1	2	CH	H2	H3a(w); H3b(w); H4b; H8; H9
38.5	5	CH	H5	H2; H4a; H10
33.6	4	CH_2	H4a; H4b	H3a; H10
29.8	3	CH_2	H3a; H3b	H1; H2; H4; H7
29.5	7	CH	H7	H2(w); H8; H9
22.4	8 (or 9)	CH_3	H8 (or H9)	H2; H7; H9
22.0	10	CH_3	H10	H1; H4a(w); H4b(w)
21.8	9 (or 8)	CH_3	H9 (or H8)	H2; H7; H8



^1H NMR spectra showed three duplets at δ 0.90, 0.95, and 1.06 (3H each), which were assigned to methyl groups 8, 9, and 10, respectively, based on their coupling constants and COSY correlations (Table 2). COSY correlations observed for hydrogens H1, H2, H3, H4, and H5 were the keys to reinforce the cyclopropyl ring's presence and the connectivity of the substituents.

Table 2. ^1H NMR chemical shifts, multiplicity and integration assignments and COSY correlations for natural pulegamic acid.

δ (ppm) - ^1H atom	multiplicity	J (Hz)	integration	COSY correlations	
0.90	8 (or 9)	d	6.6	3	H7; H9
0.95	9 (or 8)	d	6.6	3	H7; H8
1.06	10	d	7.0	3	H4a; H5
1.14-1.22	4a	m		1	H4b; H10
1.50-1.64*	3a	m		n/a	H2; H4b
1.64-1.71	7	m		1	H2; H8; H9
1.78-1.85	2, 3b	m		2	H1; H3; H7
2.06	4b	dtd	12.9; 8.2; 1.6	1	H3a; H4a; H5; H9; H10
2.36	5	dq	7.0; 3.8	1	H1; H4b; H10
2.52	1	dd	8.0; 3.8	1	H2; H5



* signal superimposed by the residual water signal.

The chemical synthesis of pulegamic acid is underway. It would be useful to unequivocally confirm the proposed structure and help determine its biological role and allow the start of the studies regarding the stereochemistry of the natural compound.

Field experiment

The number of *C. paraguayensis* adults caught by all traps/treatments in two days of experiments totalized eight insects (Table 3).

Table 3. Total number of males and females adults of *Cyclocephala paraguayensis* caught by traps in field experiment using anisole (female-specific volatile) and phenol (common volatile between sexes) as lures.

Treatments	Total number of beetles caught by traps		
	Male	Female	Mean (\pm SD)
Phenol (20 mg) +Anisole (20 mg)	0	1	0.125 \pm 0.25
Phenol (38 mg) + Anisole (2 mg)	1	1	0.25 \pm 0.34
Phenol (40 mg)	3	0	0.37 \pm 0.54
Anisole (40 mg)	1	0	0.125 \pm 0.25
Control (isopropyl alcohol)	0	1	0.125 \pm 0.25

Results of beetles caught by each trap from Table 1 were not representative, considering experiments carried out at the same site and period (see Fig. 1; Chapter 2 of this thesis).

3.4 Discussion

Cyclocephala paraguayensis adults exhibited aggregation behavior during their flight activity. Previous studies have reported *C. paraguayensis* aggregating on flowers, like other Cyclocephalini beetles (Favaris et al. 2020); here, however, *C. paraguayensis* encountering occurred on plants in the vegetative stage as a mating site. In our observations, males preceded aggregations on plants, suggesting they might release an aggregation-sex pheromone sensu Cardé (2014). Interestingly, in our previous work, males were also pioneers on *L. siceraria* flowers (Favaris et al., 2020); however, the involvement of a pheromone in the chemical communication of *C. paraguayensis* remained uncertain once floral-scent has been shown to be an efficient chemical attractant for mating pairs.

The pioneer sex landing on plants might also be influenced by biological aspects like the sex ratio of *C. paraguayensis*. A sampling of *C. paraguayensis* by light traps has indicated that the sex ratio is female-biased (Albuquerque et al. 2016). However, our manual collection of adults on plants showed a male-biased abundance. Methods for insect sampling in the field may vary according to species; light traps, for example, have been demonstrated to be a non-accurate method to estimate sex ratios (Cronin et al. 2000). The sex proportion of adults flying in the field might also be male-biased once females are monogamous, remaining in the soil after mating for oviposition (Arakaki et al. 2004). Our observations of *C. paraguayensis* behavior in the field indicate indirect evidence of sex pheromone production; thus, we conducted laboratory volatile collection from male and female adults.

The characterization of volatiles emitted by males and females of *C. paraguayensis* showed both sexes produced specific compounds that we considered pheromone candidates. The female-specific compound – anisole – was already reported as a sex pheromone in Melolonthidae beetles from the genus *Holotrichia* (Leal et al. 1996; Ward et al. 2002), as well as phenol (Henzell and Lowe 1970; Ruther et al. 2002; Zarbin et al. 2007), that was found in both sexes of *C. paraguayensis*, being a major constituent only in female extracts. Neither anisole nor phenol and their combination incited attraction to *C. paraguayensis*. Once the female-specific compound has been reported as a sex attractant, we still consider repeating field experiments with improvements of compounds dispensers. Phenol is not a sex-

specific compound in *C. paraguayensis* but might be an attractant, considering previous reports, besides possible male and female participation for sex pheromone production (Ruther et al. 2001).

Male adults of *C. paraguayensis* released a major sex-specific compound. We proposed puleganic acid as a possible molecule based on analysis of the chemical structure. Puleganic acid has been reported as a minor constituent of catmint oils and a byproduct from the hydrogenation of nepetalactone to produce dihydronepetalactone (DHN), which is an efficient insect repellent (Spero et al. 2008; Sengupta et al. 2018). Although puleganic acid is an undesirable component during DHN formulation (Spero et al. 2008; Sengupta et al. 2018), it has also been registered as arthropod repellent for topical treatments (Scialdone 2007). Insects have been reported to produce DHN as a component of defensive secretions (Cavill and Clark 1967; Cavill et al. 1982), and other terpenes that are chemically related to nepetalactone (Eisner 1964). Puleganic acid, however, has never been addressed as a natural product from insects, neither as an attractant compound. Given the proven activity of nepetalactone and similar structures, the male-specific compound from *C. paraguayensis* adults needs to be chemically confirmed, as well the biological function for the species.

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Supplementary Material

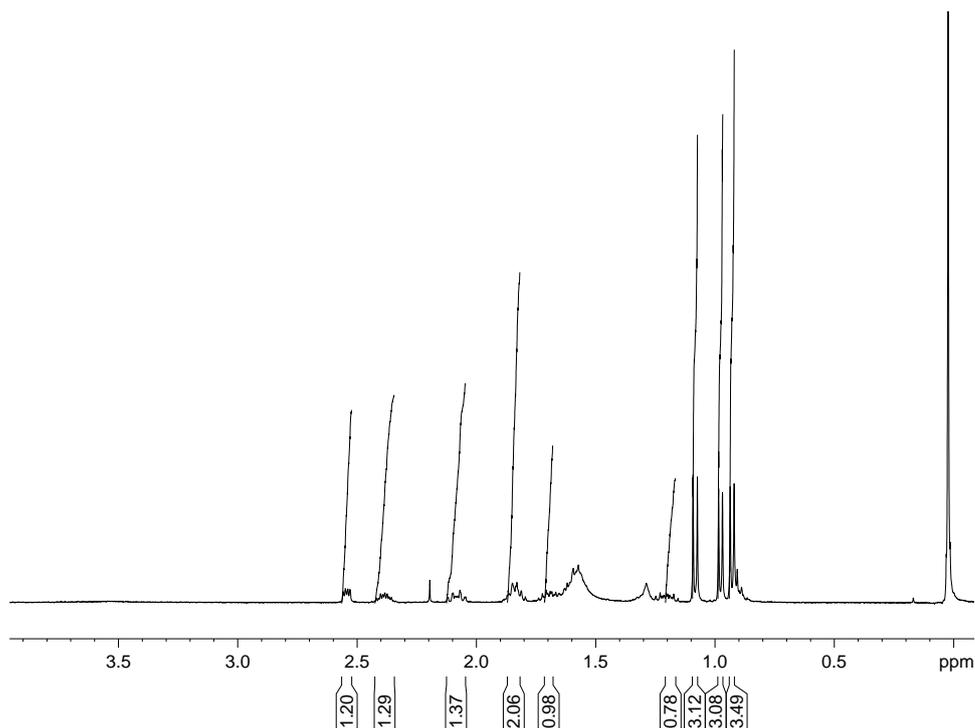


Figure S1. ¹H NMR spectrum (400 MHz, CDCl₃) of male *Cyclocephala paraguayensis* headspace extracts.

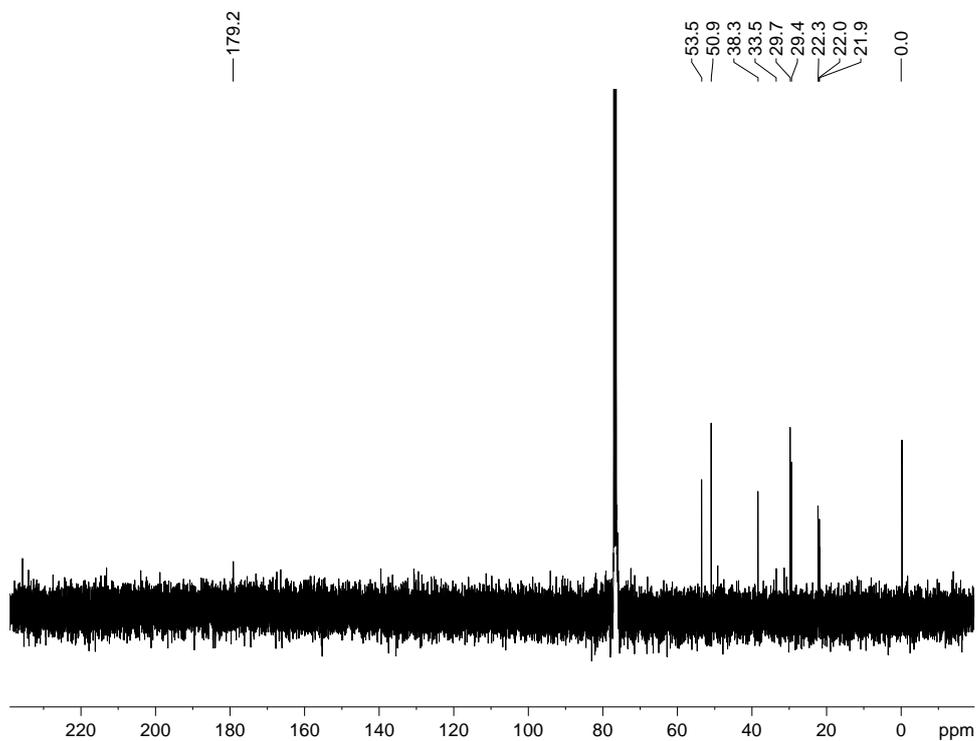


Figure S2. ¹³C NMR spectrum (100 MHz, CDCl₃) of male *Cyclocephala paraguayensis* headspace extracts.

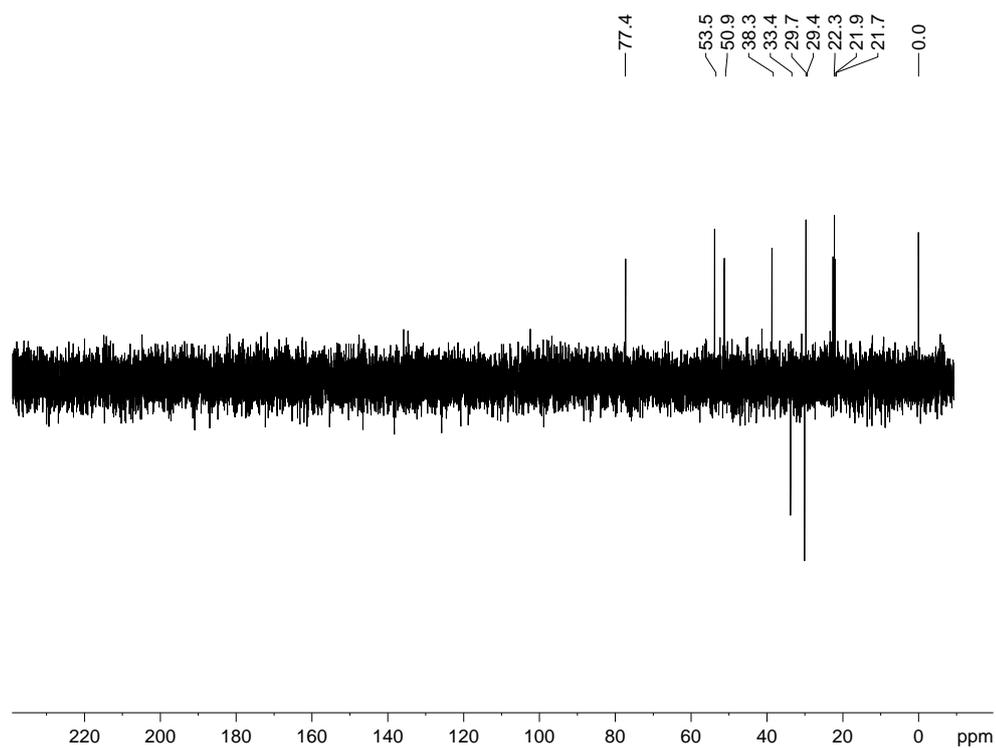


Figure S3. DEPT-135 NMR spectrum (100 MHz, CDCl_3) of male *Cyclocephala paraguayensis* headspace extracts.

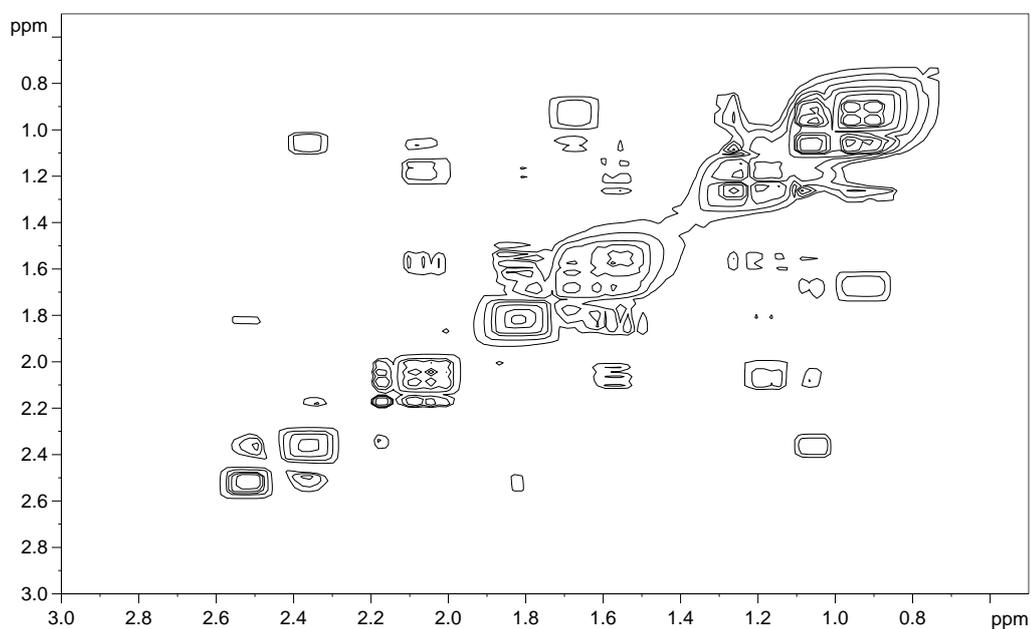


Figure S4. COSY correlation map (400 MHz, CDCl_3) of male *Cyclocephala paraguayensis* headspace extracts.

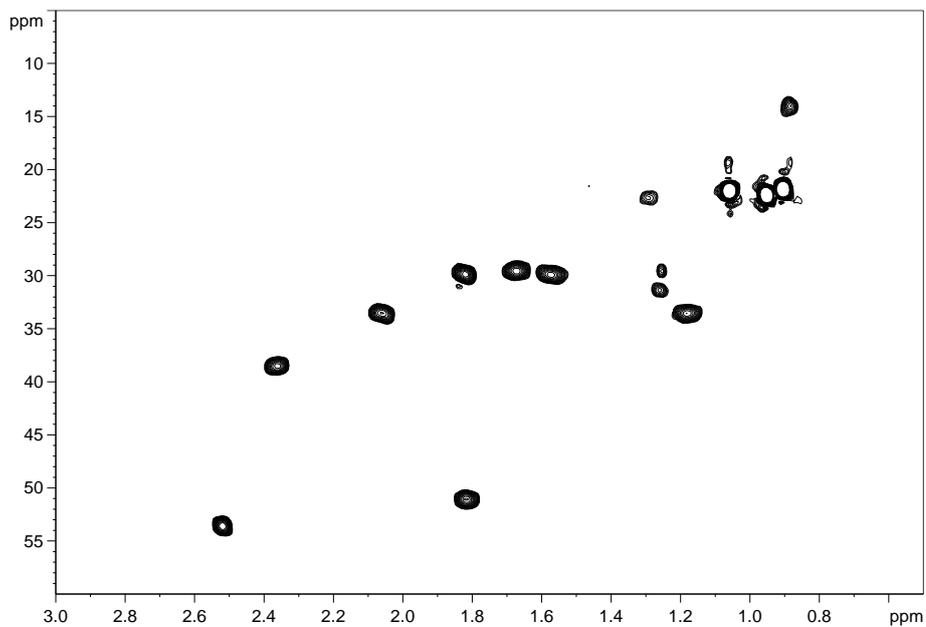


Figure S5. HSQC correlation map (600 MHz, CDCl₃) of male *Cyclocephala paraguayensis* headspace extracts.

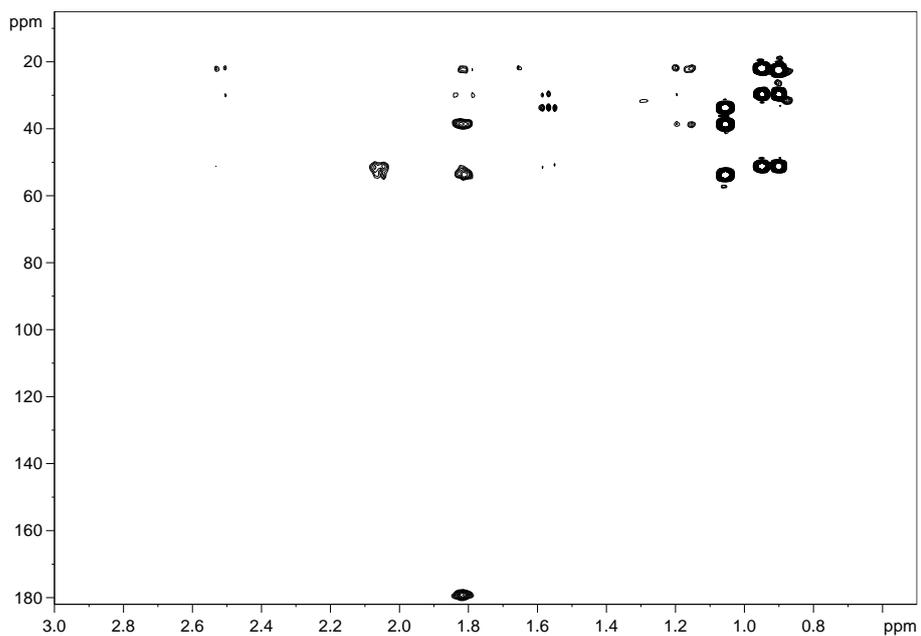


Figure S6. HMBC correlation map (600 MHz, CDCl₃) of male *Cyclocephala paraguayensis* headspace extracts.