Journal of Chemical Ecology

Pheromone chemistry of the citrus borer, Diploschema rotundicolle (Coleoptera: Cerambycidae)

--Manuscript Draft--

Manuscript Number:	
Full Title:	Pheromone chemistry of the citrus borer, Diploschema rotundicolle (Coleoptera: Cerambycidae)
Article Type:	Original Research
Keywords:	Citrus borer; Longhorn beetles; Attractants, Pheromone traps, Neotropical Cerambycinae; Retrachydes thoracicus
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Funding Information:	Citrus companies San Miguel Global and Agrisur-Urudor — Mr Andrés González
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Abstract:	The citrus borer, Diploschema rotundicolle , is a Neotropical longhorn beetle that has become a serious citrus pest in southern South America. Management strategies for this insect rely on trimming off damaged shoots, which is expensive and inefficient. We studied the chemical communication system in D. rotundicolle in search for attractants for monitoring or control. GC-MS and chiral-GC analyses of volatile extracts from field-collected adults showed that males produce (R)-3-hydroxy-2-hexanone, irregularly accompanied by minor amounts of 2,3-hexanediol (all four diastereomers) and 2,3-hexanedione. Males emit the compounds only at night, when the adults are active. GC-EAD analyses of natural and synthetic compounds showed that both male and female antennae respond to the natural enantiomer (R)-3-hydroxy-2-hexanone, suggesting that it may function as an aggregation-sex pheromone as seen in many cerambycines. The non-natural (S) enantiomer as well as the minor component 2,3-hexanediol did not trigger antennal responses. Field tests with the racemic 3-hydroxy-2-hexanone, enantiomerically pure (R)-3-hydroxy-2-hexanone, as well as a mixture of racemic 3-hydroxy-2-hexanone and 2,3-hexanediol, showed in all cases low capture levels of D. rotundicolle . However, increasing the elevation of the trap and the emission rate of dispensers enhanced field captures in traps baited with racemic hydroxyketone. Incidental catches of another native cerambycine, Retachydes thoracicus , in traps lured with 3-hydroxy-2-hexanone are also reported. This is the first report of

	pheromone chemistry in the genus *Diploschema* and in the tribe Torneutini, reaffirming the pheromone parsimony well established for the Cerambycinae. Potential factors explaining the weak attraction of *D. rotundicolle* in the field are discussed.

April 14, 2019

Prof. Gary W. Felton
Department of Entomology
Pennsylvania State University
University Park, PA, USA

Dear Prof. Felton:

We are submitting the manuscript entitled "Pheromone chemistry of the citrus borer, *Diploschema rotundicolle* (Coleoptera: Cerambycidae)", for its consideration as a full paper in the Journal of Chemical Ecology.

Our study reports for the first time the pheromone chemistry of this longhorn beetle that has become a citrus pest in southern South America. We found that *D. rotundicolle* represents another example of the parsimonious male aggregation—sex pheromones typical of the Cerambycinae subfamily. To our knowledge, however, this is the first report of pheromone chemistry in the tribe Torneutini, a large Neotropical tribe.

Our study includes the analysis of volatiles in GC-MS, chiral GC and GC-EAD, with interesting results regarding enantiomeric specificity of the antennal response. Field assays are also reported for three seasons. We also report a strong incidental response of another native Cerambycine, *Retrachydes thoracicus*, a species with yet unknown pheromone chemistry.

Overall, our study adds valuable information to further develop attractants for monitoring and potentially controlling this insect. It also contributes to the scant knowledge of chemical communication systems in native longhorn beetles from South America.
We hope everything is in order with our on-line submission process.

Best regards,

Andrés González Ritzel and María Eugenia Amorós
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Manuscript
PHEROMONE CHEMISTRY OF THE CITRUS BORER, Diploschema rotundicolle (COLEOPTERA: CERAMBYCIDAE)
Submission type: original paper

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Please note that, while not directly involved in the study, Profs. Hanks and Millar have provided general advice regarding our work with cerambycid pheromones, and they are aware of our results from previous conversations. This is stated in the acknowledgements.

Journal of Chemical Ecology

PHEROMONE CHEMISTRY OF THE CITRUS BORER, *Diploschema rotundicolle* (COLEOPTERA: CERAMBYCIDAE)

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Abstract - The citrus borer, *Diploschema rotundicolle*, is a Neotropical longhorn beetle that has become a serious citrus pest in southern South America. Management strategies for this insect rely on trimming off damaged shoots, which is expensive and inefficient. We studied the chemical communication system in *D. rotundicolle* in search for attractants for monitoring or control. GC-MS and chiral-GC analyses of volatile extracts from field-collected adults showed that males produce (*R*)-3-hydroxy-2-hexanone, irregularly accompanied by minor amounts of 2,3-hexanediol (all four diastereomers) and 2,3-hexanedione. Males emit the compounds only at night, when the adults are active. GC-EAD analyses of natural and synthetic compounds showed that both male and female antennae respond to the natural enantiomer (*R*)-3-hydroxy-2-hexanone, suggesting that it may function as an aggregation-sex pheromone as seen in many cerambycines. The non-natural (S) enantiomer as well as the minor component 2,3-hexanediol did not trigger antennal responses. Field tests with the racemic 3-hydroxy-2-hexanone, enantiomerically pure (*R*)-3-hydroxy-2-hexanone, as well as a mixture of racemic 3-hydroxy-2-hexanone and 2,3-hexanediol, showed in all cases low capture levels of *D. rotundicolle*. However, increasing the elevation of the trap and the emission rate of dispensers enhanced field captures in traps baited with racemic hydroxyketone. Incidental catches of another native cerambycine, *Retachydes thoracicus*, in traps lured with 3-hydroxy-2-hexanone are also reported. This is the first report of pheromone chemistry in the genus *Diploschema* and in the tribe Torneutini, reaffirming the pheromone parsimony well established for the Cerambycinae. Potential factors explaining the weak attraction of *D. rotundicolle* in the field are discussed.

Key Words - Citrus borer; Longhorn beetles; Attractants, Pheromone traps, Neotropical Cerambycinae; *Retrachydes thoracicus*

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Acknowledgments

We would like to thank the citrus companies San Miguel Global and Agrisur-Urudor for granting financial support and access to their groves. Additional financial support from CSIC (Comisión Sectorial de Investigación Científica from Universidad de la República) and PEDECIBA (Programa para el Desarrollo de las Ciencias Básicas, Uruguay) are also acknowledged. We would also like to thank Alejandro Borges, Marcelo Saavedra, Yamandú Pochintesta, Martín Lanfranco, Gabriela Asplanato, Carmen Rossini, Diana Valle, Anna Paula Burgueño, Federico Rodrigo, María Laura Umpiérrez, Valentina Ocampo and Paula Altesor for various contributions. Chemical standards provided by Jocelyn G. Millar and valuable advice by Lawrence Hanks are much appreciated.

INTRODUCTION

The identification of cerambycid beetle pheromones has experienced remarkable progress in the past fifteen years (reviewed by Hanks and Millar (2016)). A remarkable finding that has emerged from the study of longhorn beetle pheromones is the parsimony of pheromone components, with the same or similar compounds shared by species across genera, tribes, and even subfamilies (Hanks and Millar 2016). Among the most widely studied sub-families, the Cerambycinae are characterized by male-produced aggregation-sex pheromones that attract both sexes (Hanks and Millar 2016). Chemically, short chain α-hydroxyketone type pheromones are highly conserved within the Cerambycinae, and have been reported for numerous species from different regions of the world (Hanks and Millar 2016), including species native to the Neotropical region (Silva et al. 2018; Silva et al. 2017). (*R*)-3-Hydroxy-2-hexanone seems to be the major and in some cases the only component of the sex-aggregation pheromones in many sympatric and synchronic species of this subfamily (Mitchell et al. 2013).

Traps lured with semiochemicals, in particular pheromones, are becoming a valuable tool for the detection and monitoring of invasive cerambycid species (Fan et al. 2018). Even at low densities, during the early stages of establishment, pheromone traps can be sensitive and reliable enough to detect target species (Hansen et al. 2015). The potential of cerambycid pheromones as tools for surveillance has been explored to a greater extent than their potential for pest control. Still, a number of studies have explored this possibility by testing cerambycid pheromones, both sex and aggregation pheromones, in control strategies such as massive trapping or mating disruption (Maki et al. 2011; Sanchez-Husillos et al. 2015, Barbour, 2019 #103). The use of cerambycid pheromones for pest control may be more realistic for fruit crops than for forest pests, particularly in the production of fresh fruit for direct consumption, which is severely restricted in the use of insecticides.

Diploschema rotundicolle (Audinet-Serville, 1834) (Coleoptera: Cerambycidae) is a South American citrus pest distributed through the center-south regions of Brazil, Argentina and Uruguay (Machado and Filho 1999). The adults are elongated (25-40 mm x 8-10 mm) and characterized by a light-brown elytra with a continuous dark-brown border, dark-brown head, pronotum, antennae and legs (Faria et al. 1987). The adults are nocturnal, the female oviposits in the apex of branches upon young flush and leaf axils (Machado and Berti Filho 2006). After egg eclosion, the larvae perforate the epidermis and once in woody tissue they dig longitudinal galleries heading to thicker branches, usually reaching the principal trunk (Machado et al. 1991). By the end of the larval stage, the larvae prepare a pupal chamber with an exit opening for the adult to exit (Faria et al. 1987). The life cycle varies from 8-10

months in sub-tropical areas (Sao Paulo, Brazil) (Faria et al. 1987) up to 20-22 months in temperate regions (Link and Corrêa Costa 1994).

Until recently *D. rotundicolle* was considered a secondary citrus pest for citrus in the region (Bentancourt and Scatoni 1999), but in recent years populations have dramatically increased in focalized areas, particularly in lemon orchards (unpublished data). Serious infestation levels result in poor yields, tree weakening and indirect damages due to invaders of empty galleries (Machado and Filho 1999). Woodborer control is extremely complicated since the larvae are protected inside the wood (Shanley et al. 2009). Insecticides are also much restricted in citrus crops destined to fresh fruit consumption. Therefore, the current management strategy for this insect relies exclusively on cultural control, which consists in the pruning of twigs with evidence of oviposition damage, a strategy has proven expensive and ineffective.

The objective of this study was to characterize the chemical communication system in *D. rotundicolle*, aiming at the development of pheromone-based management strategies. We used air entrainment, GC-MS, chiral GC and GC-EAD methods to analyze volatile compounds from field-collected adults. We also conducted field experiments to study the effect of chirality, minor compounds, trap location and dispenser emission rate, in the trapping of *D. rotundicolle* adults. This study also contributes to the growing but scant knowledge of pheromone chemistry in neotropical Cerambycidae.

METHODS AND MATERIALS

Insects. D. rotundicolle adults were collected in the field during the austral summers of 2017-2018 (season I), 2018-2019 (season II) and 2019-2020 (season III), in a citrus orchard located in San José, Uruguay (34°42′1″S 56°43′37″W). To capture live adults upon emergence, mesh covers were set up around highly infested trunks chosen from visual observation of abundant sawdust at their base. Wrapped trees were checked daily from early summer (mid-December) throughout the adult emergence period spanning from late January through April. Daily checking prevented the desiccation of emerged beetles and provided young adults for volatile collections. Live adults (mostly males) were also collected underneath the bark of *Eucalyptus globulus* windbreaks surrounding the citrus plots. Presumably, the loose bark of this eucalypt species provides shelter to the adults during the day. All captured beetles were maintained individually in small transparent plastic cages (12 cm diameter, 14 cm height) under laboratory conditions (22 ± 1 ºC, 66 ± 9% RH, 14:10 L:D).

Volatile collection and analysis. Insect volatiles were collected in an aeration system composed of a cylindrical glass chamber (23 cm long, 5 cm diameter), PTFE tubing (6 mm i.d.) and a flow of charcoal-filtered humidified air (0.5 L/min) obtained from a PTFE diaphragm pump (KNF, Germany). Volatile compounds were adsorbed in glass Pasteur pipettes containing 50 mg of HaySep-Q 80/100 mesh (HayeSep® Q, Sigma-Aldrich). Adsorbed volatiles were eluted with 1 mL hexane and 100 µL of internal standard solution (IS, geraniol 1mg/mL), then stored at -4 °C. Immediately before GC-MS analysis the samples were concentrated to 100 µL under a gentle flow of N_2.

Volatile collections were performed according to insect availability, under controlled environmental conditions (22 ± 2℃, 55 ± 10% RH, 14:10 L:D). To compare volatiles from males and females, aerations were performed simultaneously for 24 h from individual males and females placed in separate chambers. To determine the diel cycle of male emission, volatiles were collected in two time periods, daytime (9-17 h) and nighttime (17-9 h).

GC-MS analyses were performed in a QP-2010 Shimadzu GC-MS equipped with an apolar column (AT-5MS, 30m x 0.25mm, 0.25 µm, Alltech, USA) operated with a constant carrier gas flow of 1 mL/min (He). The injector was set at 100 ºC to avoid thermal decomposition of volatile compounds, a problem that has been reported for cerambycid pheromones based on α-hydroxy ketones (Schröder et al. 1994). The oven temperature was programmed from an initial temperature of 40 ºC (1 min), then raised to 90 ºC at 5 ºC/min and to 250 ºC at 10 ºC/min. The interphase was heated to 250 ºC and the injection (1 µL) was performed either in split or splitless modes depending on the objective of the analysis. Mass spectra were obtained from *m/z* 28 to *m/z* 350 in the scan mode (70 eV). Chiral GC analyses were performed in a Shimadzu 2010 GC equipped with a chiral column (MEGA-DEX DAC-Beta, 25m x 0.25mm, 0.25 µm, MEGA, Italy) and a FID detector, operated with a constant carrier gas flow of 1 mL/min (H_2). The oven temperature was programmed from 60 ºC (5 min) to 120 ºC at 1 ºC/min (5 min), then to 180 ºC at 5 ºC/min (5 min). The injector and detector temperatures were 200 and 230 °C, respectively, and the injection (1 µL) was in the splitless mode.

Electroantennogram studies. GC-EAD analyses were performed in a HP 5890 Series II gas chromatograph equipped with an EC-WAX column (30 m, 0.25 mm i.d., 0.25 µm; Alltech Econo-Cap™, USA), operated with a constant carrier gas (H_2) flow of 2 ml/min. The column effluents were split (1:1) in a vitreous silica outlet splitter (SGE, Austin, TX, USA), adding N_2 as make-up gas (30 ml/min) prior to the splitter. The split effluents were directed through inert capillary column pieces (50 cm, 0.25 mm i.d.) to the FID and EAD detectors. The column directed towards the antenna passed through an interphase tube (Synthech, Germany) heated to 240 °C, then discharged into a glass tube

(1 cm diameter) with a current of humidified, charcoal-filtered air (150 ml per min), which delivered the volatiles to the antennal preparation located 4 cm downstream. The antenna was excised from the insect and attached to glass electrodes filled with saline solution (NaCl 7.5g/L, KCl 0.4 g/L, $CaCl_2$ 0.2 g/L, $NaHCO_3$ 0.2g/L), with the antennal tip removed to ensure better contact. The glass electrodes were connected to an EAG pre-amplified probe and further to a high impedance amplifier (IDAC 2) (Synthech, Germany) to receive simultaneous signals from the FID and EAD detectors. Data were analyzed using GC-EAD 2014 software (v.1.2.5).

To evaluate the response of male and female antenna to natural male volatiles, the GC conditions were as previously described for GC-MS analyses. To evaluate the antennal response to the stereoisomers of 3-hydroxy-2-hexanone, an isotherm (90 °C) method was used and 1 µL of each stimulus was sequentially injected in the split mode at 1-minute intervals, with continuous acquisition of FID and antennal response. The compounds were injected as follows: synthetic (*R*)-3-hydroxy-2-hexanone, synthetic (*S*)-3-hydroxy-2-hexanone, natural male volatiles, synthetic racemic 3-hydroxy-2-hexanone. Finally, to evaluate the antennal response to 2,3-hexanediol, the oven was programmed with an initial temperature of 70 ºC (3 min), then raised to 90 ºC (5 ºC/min) and to 250 ºC (10ºC/min). The injector temperature was maintained at 100 °C, and the injection was of 1 µL in split mode. The test solution contained synthetic racemic 3-hydroxy-2-hexanone and synthetic generic 2,3-hexanediol (all four stereoisomers).

Field tests. The attraction of *D. rotundicolle* adults to the synthetic pheromone compounds and blends was evaluated in a highly infested lemon (*Citrus aurantifolia*) grove located in Kiyú, San José, Uruguay, during three consecutive seasons (I, II and III). The trapping devices consisted in homemade cross-vane traps (74 cm height, corrugated black cartonplast) attached to buckets half filled with soapy water. The trap panels were coated with Fluon® (Insect-A-Slip, PTFE DISP30, BioQuip Products, Inc) to improve trapping efficiency (Graham et al. 2010). In seasons I and II the traps were suspended from tree branches so that the buckets were 60 cm above ground. In season III two trap heights were evaluated. Low traps were hung as in the previous seasons, while elevated traps were hung from water pipes so that the buckets were 1.8 m above ground.

In seasons I and II, the lures consisted in double polyethylene sachets (press-seal bags, 5 × 7 cm) with one 4-cm cotton wick loaded with an isopropanol solution (1 mL) of the tested stimuli. In season I the major pheromone compound was evaluated both as racemic mixture and pure enantiomer. The lures were loaded with 50 mg of racemic 3-hydroxy-2-hexanone or 25 mg of (*R*)-3-hydroxy-2-hexanone. In season II the goal was to test the addition of 2,3-hexanediol, so the lures were loaded

with 50 mg of racemic 3-hydroxy-2-hexanone or 50 mg of racemic 3-hydroxy-2-hexanone plus 25 mg of generic 2,3-hexanediol (mixture of stereoisomers) in separate sachets. In both seasons control lures were loaded with 1 ml isopropanol. In season III only racemic 3-hydroxy-2-hexanone was used and the lures were modified to test different emission rates. Two different dispensers were used: low-rate emission dispensers consisted in an Eppendorf tube (1 mL) with a perforated cap (1 mm) and a 1-cm cotton wick inside. The cotton was loaded with 50 mg of neat racemic 3-hydroxy-2-hexanone. The high-rate emission dispensers were single polyethylene sachets with cotton wicks as used in the previous seasons, loaded with a high dose (500 mg) of neat racemic 3-hydroxy-2-hexanone. The use of solvent-less lures allowed for a more precise control over the emission rates of 3-hydroxy-2-hexanone. These were evaluated under laboratory conditions resulting in 4 mg/day and 40 mg/day for the low and high emission rate dispensers, respectively (data not shown). These emission rates were in turn 10 and 100 times higher than the highest emission rate observed for a single male (see Results). Treatments in season III were hence arranged so that low-height and elevated traps were lured with *i)* low-rate dispensers, *ii)* high-rate dispensers, *iii)* control (dispenser materials). In addition, to compare captures across seasons, an additional low-trap treatment was included with lures identical to those used in the previous seasons, *i.e.* 50 mg racemic 3-hydroxy-2-hexanone in 1 mL of isopropanol.

The traps were deployed within the 1-ha citrus plots (but see below), 6 m apart from the plot border and with a separation of at least 20 m between traps. Trap arrangement followed a randomized block design, with each replicate containing all treatments. Ten replicates were set up in seasons I and II, and five in season III. In season II five replicates were setup within the citrus plots and five were arranged along the eucalyptus windbreaks surrounding the plots. Lure replacement and trap service was done every 15 days in seasons I and II, and weekly in season III. The treatments were assigned to the traps randomly on the day of set up, then rotated in every lure replacement to control for location effects. Other cerambycid beetles trapped were recorded opportunistically.

Chemicals. Racemic 3-hydroxy-2-hexanone for lures was purchased from ChemTica Internacional, S.A. and Bedoukian Inc. (*R*)-3-Hydroxy-2-hexanone, (*S*)-3-hydroxy-2-hexanone and 2,3-hexanediol were synthesized according to (Heguaburu et al. 2017). The four diastereomers of 2,3-hexanediol were obtained from carbonyl reduction of (*R*)- and (*S*)-3-hydroxy-2-hexanone, following column separation of diastereomers and NMR assignment of absolute configuration at C2. (*R*)- and (*S*)-3-hydroxy-2-hexanone standards were also kindly provided by Prof. Jocelyn Millar (UC Davis, USA).

Statistical analysis. Day and night pheromone emission were expressed as amount of 3-hydroxy-2-hexanone relative to the internal standard and subjected to a *Wilcoxon test* for paired samples. Beetle trap captures (*i.e.* total catches per block/replicate throughout the season) in seasons I and II were subjected to a generalized linear mixed model (*GLMM*) with Poisson distribution and "block" as a random factor. Treatment means were compared using *Tukey's HSD* test ($\alpha = 0.05$). Due to limitations of *GLM* models, any treatment with zero catches was not included in the analysis. In season II, trap location (within plot vs. windbreak) was considered as a fixed factor. In season III, due to low beetle captures, differences among treatments for *D. rotundicolle* and *R. thoracicus* captures were analyzed with the *Friedman's* test followed by the *Conover* post-hoc test ($\alpha = 0.05$). Male and female comparisons across seasons were done with *Chi-square* tests using the pooled number of beetles caught per season in all treatments and blocks. The tests were run with R statistical software (0.99.892 version – © 2009-2016 RStudio, Inc.) (RStudioTeam 2015) and Infostat statistical software (Di Rienzo et al. 2011).

RESULTS

Volatile collection and analysis. Aeration samples of *D. rotundicolle* males and females were obtained and analyzed throughout seasons I and II. Male volatile samples consistently showed a major compound that accounted for 96.8% [92.8-98.8] (median [interquartile range], N = 10) of the GC relative area. No volatiles samples from females showed this compound or any other distinctive volatile (Fig. 1a). The retention time and mass spectrum of the male-specific compound clearly matched those of a synthetic standard of 3-hydroxy-2-hexanone (Fig. S1). Chiral GC analysis with enantiomerically pure synthetic standards showed that the natural enantiomer emitted by the males is (*R*)-3-hydroxy-2-hexanone (Fig. 1b). Quantification of 3-hydroxy-2-hexanone by peak area comparison with an internal standard showed that males emit highly variable amounts, ranging from 0.5 to 391 μg in 24 h (8.8 μg [0.9-54.5], median [interquartile range], N = 17).

Males also produce some minor compounds that were not consistently observed even in different samples from a single male. When present, minor compounds accounted for 2.8% [1.2-5.3] (median [interquartile range], N = 10) of the GC relative area. The more abundant minor compounds showed matching retention times and mass spectra with synthetic standards of diastereomers of 2,3-hexanediol (Fig. S1). Chiral GC comparisons with standards of possible stereoisomers of 2,3-hexanediol showed that all four are present in the natural samples, with the 3*R* diastereomers more abundant than the 3*S* (Fig. 1c). Another minor compound with shorter retention time was also

observed in male aerations, again inconsistently. The mass spectra of this compound suggest that it is 2,3-hexanedione (Fig. S1).

The study of diel pheromone emission pattern showed that males emit (*R*)-3-hydroxy-2-hexanone almost exclusively during the evening and night, between 17 and 9 h (Wilcoxon, $P = 0.002165$) (Fig. 2). Samples collected overnight contained 68.6 μg/male [4.8-201.3] (median [interquartile range], N = 6), whereas diurnal collections showed only traces of the compound 1.5 μg/male [0.8-1.8] (median [interquartile range], N = 6).

Electroantennogram studies. Coupled GC-EAD analyses were performed to evaluate the response of *D. rotundicolle* female and male antennae to volatile extracts and synthetic compounds. The antennae showed low signal to noise ratios, allowing for the obtention of clear data only from a limited number of insects. Nevertheless, well-defined responses to natural (*R*)-3-hydroxy-2-hexanone from male volatile extracts were obtained from both male and female antennae (Fig 3a). The antennae do not seem to respond to the non-natural (*S*)-3-hydroxy-2-hexanone. This was shown by subsequent applications of four samples to the same antennae, namely synthetic R and S enantiomers of 3-hydroxy-2-hexanone, male volatiles extracts and racemic 3-hydroxy-2-hexanone. By working under isothermal conditions and injecting the samples with 1-min differences, the same antennae was stimulated with compounds eluting 1 min apart. Male antennae responded to the synthetic and natural R enantiomer, as well as to the racemic 3-hydroxy-2-hexanone, but not to the S enantiomer even though it was applied between the other stimuli (Fig. 3b). The corresponding experiments with female antennae did not show conclusive results, although the weak responses found suggest that the response pattern is the same in males and females (data not shown). Finally, 2,3-hexanediol (mixture of diastereomers) did not trigger any response from male (N = 5) or female (N = 6) antennae, even though the same antennae clearly responded to racemic 3-hydroxy-2-hexanone that was co-injected with the diol (Fig. 3c).

Field tests. In general, considering the high infestation levels of the field site, low captures of *D. rotundicolle* adults have been observed. In season I, a total of 29 beetles were found in the traps, with no significant differences among traps lured with racemic 3-hydroxy-2-hexanone, (*R*)-3-hydroxy-2-hexanone and control traps (*GLMM, Tukey's HSD*: $P>0.5$ for all contrasts) (Fig 4a). Interestingly, incidental catches of another cerambycine species was observed in numbers that surpassed that of the target species. The species was identified from local collections as *Retrachydes thoracicus* (Olivier, 1790), a well-known species native to the neotropics (Monné 2018). A total of 56 beetles were captured in season I, and in this case the captures were clearly different among pheromone and control traps, with all 56 beetles captured in pheromone-baited traps and zero catches in the control. No

significant differences were observed between the two pheromone treatments (rac. vs. R) (*GLMM, Tukey's HSD*: P= 0.424) (Fig. 4a).

In season II, a total of 35 *D. rotundicolle* adults were captured, 8 in the citrus plot traps and 27 in the windbreak traps. The data was pooled and analyzed altogether, showing significant differences between catches in the control and the racemic 3-hydroxy-2-hexanone (*GLMM, Tukey's HSD*: P=0.0240). Captures in control traps and traps baited with the mixture of racemic 3-hydroxy-2-hexanone and 2,3-hexanediol were not significantly different, but showed a clear tendency in favor of traps lured with the mixture (*GLMM, Tukey's HSD*: P=0.0669). Finally, traps baited with the hydroxyketone alone or in combination with the diol showed no differences (GLMM, Tukey's HSD: P=0.8495) (Fig. 4b). Similar to the previous season, 56 *R. thoracicus* beetles were trapped in season II, 55 of them in pheromone-baited traps. No significant differences in *R. thoracicus* captures were found between traps lured with racemic 3-hydroxy-2-hexanone or with the hydroxyketone:diol blend (GLMM, Tukey's HSD: P=0.58230). When compared to the control traps, both captured significantly more beetles (P< 0.01) (Fig. 4b).

Season III resulted in the overall lowest captures of both beetle species, possibly due to a very dry summer. A population decrease in *D. rotundicolle* was evident from the notorious decrease in oviposition damage in the citrus plants, the absence of adult emergence from meshed trees, and the lack of catches in control traps, all factors that clearly contrasted with the previous seasons. Nevertheless, some relevant observations can be made from the results. A total of 14 *D. rotundicolle* adults were trapped, all of them in traps baited with racemic 3-hydroxy-2-hexanone. Moreover, all but one beetle were captured in the higher traps, and most of them (8) in the traps with high-rate emission dispenser (*Friedman Chi-squared*, P=0.0053) (Fig. 4c). In this third season, *R. thoracicus* catches were similarly low with 13 adults trapped, all of them, as observed in previous seasons, in 3-hydroxy-2-hexanone baited traps (*Friedman Chi-squared*, P=0.0037). In this case, no clear pattern was observed between different trap heights (7 catches in high traps and 6 in low traps) nor emission ratios (8 in high rate and 5 in low rate) (Fig. 4c).

Of note, throughout all three seasons significantly more females than males were captured for both beetle species (χ^2 $P < 0.05$ for all comparisons) (Fig. 5). Moreover, this pattern was observed in all trap treatments, including the control traps (Fig. S2).

DISCUSSION

This is the first report on the pheromone chemistry of the South American citrus borer, *D. rotundicolle*. Similar to several cerambycine species (Hanks and Millar 2016), males of *D. rotundicolle* emit (*R*)-3-hydroxy-2-hexanone, along with minor compounds that are not consistently emitted. To our knowledge this is the first report of a species within the tribe Torneutini to share this conserved pheromone chemistry, further expanding the taxonomic distribution of this chemical *motif* within the Cerambycinae. The males emit (*R*)-3-hydroxy-2-hexanone mostly at night, when they are behaviorally active. Emitted amounts in the laboratory proved to be highly variable among males, most commonly around 10-50 μg per day, but reaching almost 400 μg in a single night.

Our electroantennogram results show that both male and female antennae respond to the male-specific compound, suggesting that (*R*)-3-hydroxy-2-hexanone functions as a male pheromone involved in communication with both sexes. As shown for several related species (Hanks and Millar 2016), the compound most likely serves as an aggregation-sex pheromone. Our GC-EAD results also showed, clearly in the case of males, that the antenna does not respond to the non-natural (*S*)-3-hydroxy-2-hexanone. The fact that the insects do not perceive the "wrong" enantiomer is consistent with several studies showing that the non-natural enantiomer does not affect field captures. Furthermore, our results showed similar amplitudes in the antennal responses to (*R*)-3-hydroxy-2-hexanone before and after stimulation with the S-enantiomer, indicating that the non-natural enantiomer does not inhibit pheromone detection. This is relevant from an applied perspective, since the racemic 3-hydroxy-2-hexanone is commercially available and cheaper to synthetize.

Field attraction of *D. rotundicolle* adults to traps baited with 3-hydroxy-2-hexanone, either racemic or enantiomerically pure, was not as strong as expected. Despite working in a crop field with high infestation levels, total catches did not exceed a few tens of adult beetles in three field seasons. Judging from the damage observed particularly in the first two seasons, and the fresh sawdust underneath almost every tree in the field, these catches likely represent an insignificant fraction of the population. An indication of the high abundance of the insect during the first two seasons was the unexpected captures in control traps. Indeed, captures in pheromone-baited traps in the first season were no different than by-chance captures in control traps, and significantly but slightly higher in the second season. Clearly some other factor was missing that could be related to the chemistry of the bait, the emission rates of lures, the structure of the trap, or factors related to the biology of the insect. Regardless of the low captures, the first field season strongly suggested that the pure enantiomer and the racemic mixture would not behave differently as attractants in the field, which was expected from our GC-EAD results.

The lack of minor compounds was clearly a potential explanation for the low capture levels. It has been shown that minor compounds are important in determining pheromone specificity in cerambycines that share the main pheromone component (Mitchell et al. 2015). In our analysis of *D. rotundicolle* male volatiles we identified 2,3-hexanedione and 2,3-hexanediol, both known compounds from other cerambycine species. These compounds were found inconsistently, with some male volatile samples showing only the main hydroxyketone alone. 2,3-Hexanedione has been commonly found in cerambycines but has not shown any biological activity so far (Hanks and Millar 2016). We therefore focused on 2,3-hexanediol, which was more abundant and has shown pheromonal activity in other studies. Our GC-EAD and field results, however, suggest that the diol does not play an ecologically relevant role for *D. rotundicolle*. Male and female antennae that clearly detected the hydroxyketone showed no response to the co-injected diol in GC-EAD, and the addition of the diol to the hydroxyketone in lures did not increase attraction in the field. Interestingly, our chiral GC analysis showed the natural diol is present in all four diastereomers, whereas the hydroxyketone in *D. rotundicolle* is enantiomerically pure. Combined, these results led us to hypothesize that for some species, the diol may just be the precursor of a final oxidation step that favors the 3R diastereomers, while in other species it has acquired communication value.

Trap architecture or placement may also be factors causing low captures of *D. rotundicolle* despite high population levels. While minor design details may be overlooked in home-made traps, lubricant-treated cross-vane traps have shown to be adequate devices for trapping cerambycines (Allison et al. 2014). Unexpected support for our trap design came from the incidental catches of *Retrachydes thoracicus* in our field experiments. *R. thoracicus* is a native cerambycine species reported in Argentina, Brazil, Paraguay, Bolivia and Uruguay (Bentancourt and Scatoni 1999). It is a polyphagous species associated with several woody hosts, among which are citrus, eucalyptus and casuarina trees, all present in our experimental site. Relatively large numbers of this beetle, similar in size to *D. rotundicolle*, were captured almost exclusively in the pheromone-baited traps (1 out of 125 in a control trap) in all three seasons of study. *R. thoracicus* is not regarded as a pest in citrus and they were not found emerging from mesh-covered citrus trees, so their population density is probably far lower than that of *D. rotundicolle* in our experimental site. We conclude that captured *R. thoracicus* adults were actually attracted from the surroundings towards the pheromone traps, thus representing an unintended positive control for the capturing and retaining capacity of our traps. Our results strongly suggest that 3-hydroxy-2-hexanone plays an important role in the chemical communication of *R. thoracicus*. Being sympatric species, *R. thoracicus* and *D. rotundicolle* would not overlap or cross-attract each other because the former is active during the day (Bentancourt and

Scatoni 1999), whereas *D. rotundicolle* is reported to have nocturnal habits, both for oviposition (Machado and Filho 1999) as for pheromone production (this study).

Trap location and dispenser emission rates were investigated during our third field season. Despite significant lower catches in this season, the results show that higher traps and higher emission rates have a positive effect in the attraction of *D. rotundicolle* to 3-hydroxy-2-hexanone. Trap height is a factor that has proven relevant for capturing cerambycids (Graham et al. 2012; Schmeelk et al. 2016). While the citrus crop does not present a large extent of vertical gradient, the higher traps in our experiment performed clearly better (13 out of 14 *D. rotundicolle* were captured in high traps). Whether this is a result of higher traps being more exposed or due to an actual flight stratification of the beetles cannot be concluded. Of note, in the case of *R. thoracicus* higher traps did not show a better performance, suggesting that trap exposure alone is not an obvious explanation.

Dispenser emission rates also produced a significant effect in the captures of *D. rotundicolle*. It is known in cerambycids that male-produced aggregation-sex pheromones are often produced in large amounts, ranging up to tens or hundreds of micrograms per hour. Hence, lures may need to approximately match or exceed these rates to be effective, releasing at least several milligrams of pheromone per day (Millar and Hanks 2018). Because the lures we used in the first two seasons contained solvent, our efforts to measure release rates did not produce reliable results. The use of solvent-free dispensers in the third season allowed us to measure release rate by weight loss. We used release rates of 4 and 40 mg per day, representing 10 and 100 times more than the highest amount of pheromone emitted by a male in the laboratory (*ca*. 400 μg/day). Traps with high-emission rate dispensers captured about twice as many adults of *D. rotundicolle*, but again, the low overall captures prevent a definite conclusion.

In summary, we here show that males of the South American citrus borer, *Diploschema rotundicolle*, emit (*R*)-3-hydroxy-2-hexanone, to which both male and female antenna respond. The antennae respond to the racemic mixture as well, but not to the non-natural enantiomer. Males also emit all four diastereomers of 2,3-hexanediol, which do not trigger antennal responses or enhance field captures. Traps baited with racemic 3-hydroxy-2-hexanone showed low captures levels, although these were enhanced by higher emission rate dispensers and elevated trap height. Unintended but abundant catches of *Retrachydes thoracicus* in pheromone-baited traps are also reported. For both species, significantly more females were captured across treatments and seasons, suggesting that females are the more mobile sex.

Effective trapping of *D. rotundicolle* adults would be a significant contribution to pest management in the citrus sector in southern South America. While other emerging pests and diseases pose

important threats to citrus crops in the region, focalized population explosions of *D. rotundicolle* are becoming more common. Although (*R*)-3-hydroxy-2-hexanone must play a relevant role in the chemical communication of the insect, some cerambycids are moderately or not attracted at all to their pheromones alone, requiring for instance the presence of host plant volatiles (Hanks et al. 2018). This and other factors will be the focus of our future work to optimize pheromone traps for *D. rotundicolle*.

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Figure legends

Fig. 1 Chemical analysis of D. rotundicolle volatile extracts: A) Representative total ion chromatograms of individual male (upper trace) and female (lower trace) volatile extracts after 24 h of aeration (see Figure S1 for mass spectra). B) Chiral GC analysis of male volatile extracts compared to racemic and enantiomerically pure (3R/3S)-hydroxy-2-hexanone. C) Chiral GC analysis of 2,3-hexanediol isomers in male volatile extracts (lower trace), synthetic generic diol (upper trace) and the co-injection of natural and synthetic diols (middle trace). The four diastereomers were assigned based on isolated synthetic standards.

Fig. 2 Diel pheromone emission of 3-hydroxy-2-hexanone (HK) by *D. rotundicolle* males. Night: 17-9 h; day 9-17 h; scotophase: 20-6 h. An asterisk indicates a significant difference (*Wilcoxon* $P<0.05$).

Fig. 3 GC-EAD analysis of *D. rotundicolle* male volatile extracts and synthetic standards: A) Male and female antennal response to (R)-3-hydroxy-2-hexanone (R HK) in natural aeration samples. B) Responses of male antennae to synthetic samples of 3-hydroxy-2-hexanone (R, S and rac HK) and natural male volatiles (natural). The samples were sequentially injected at 1-min under isothermal GC conditions. C) Female and male antennal response to a blend of racemic 3-hydroxy-2-hexanone (rac HK) and generic (four diastereomers) 2,3-hexanediol (diols).

Fig. 4 Boxplots showing field trapping of *Diploschema rotundicolle* (dark grey) and *Retrachydes thoracicus* (light grey) in three consecutive seasons (austral summers of 2018-2020). HK stands for racemic 3-hydroxy-2-hexanone, diol stands for generic 2,3-hexanediol. Different letters indicate significant differences (post-hoc Friedman Conover test, $\alpha = 0.05$). In C: high and low stand for trap height, high rate / low rate stand for dispenser emission rate.

Fig. 5 Field trapping of male and female *Diploschema rotundicolle* (left) and *Retrachydes thoracicus* (right) per season. All treatments combined. Asterisks indicate $P < 0.05$ in the *Chi-square* test.

Figure

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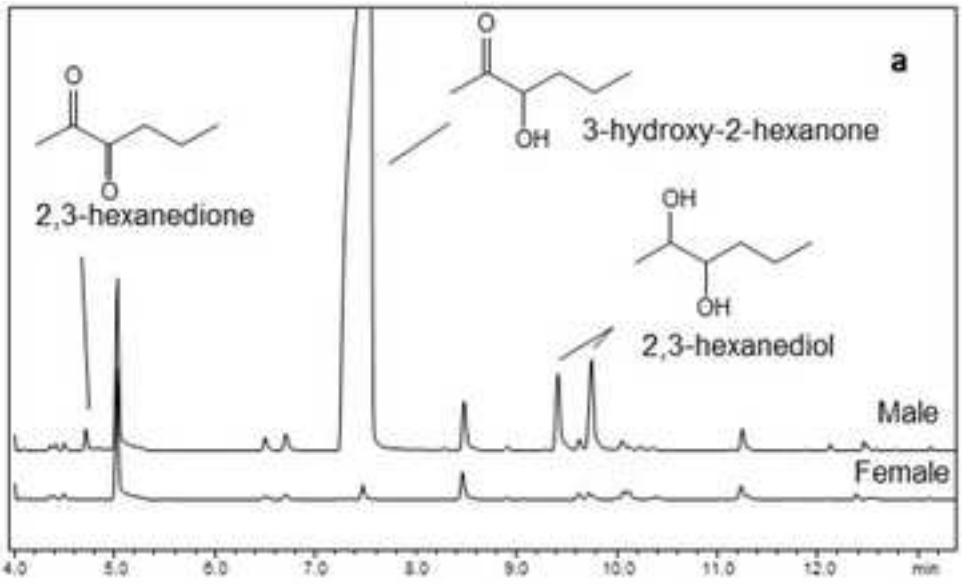

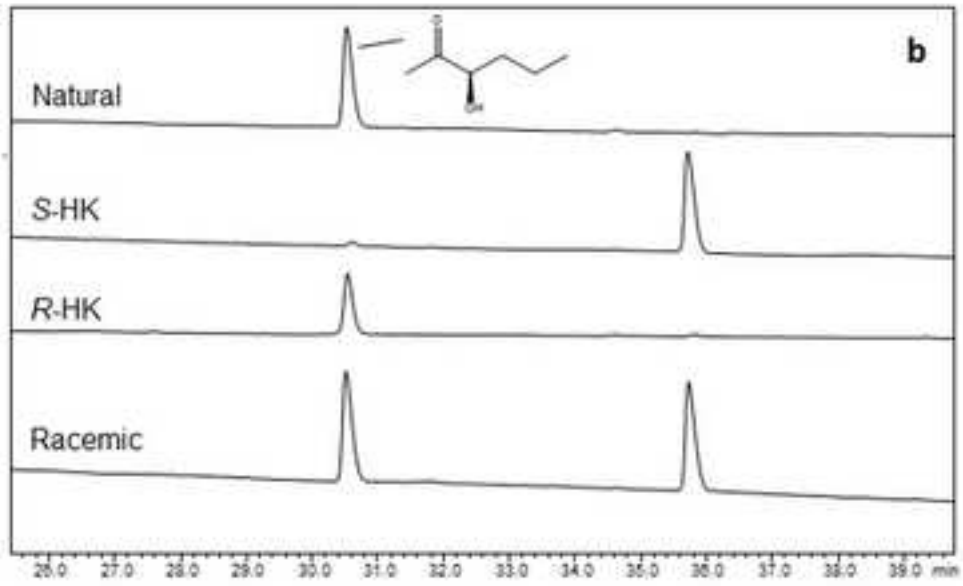

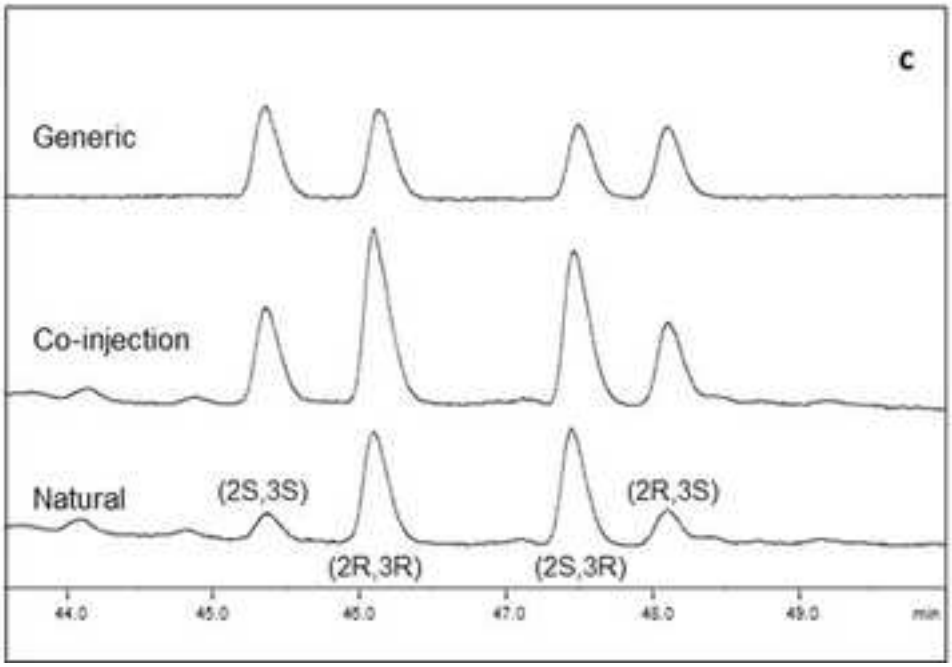

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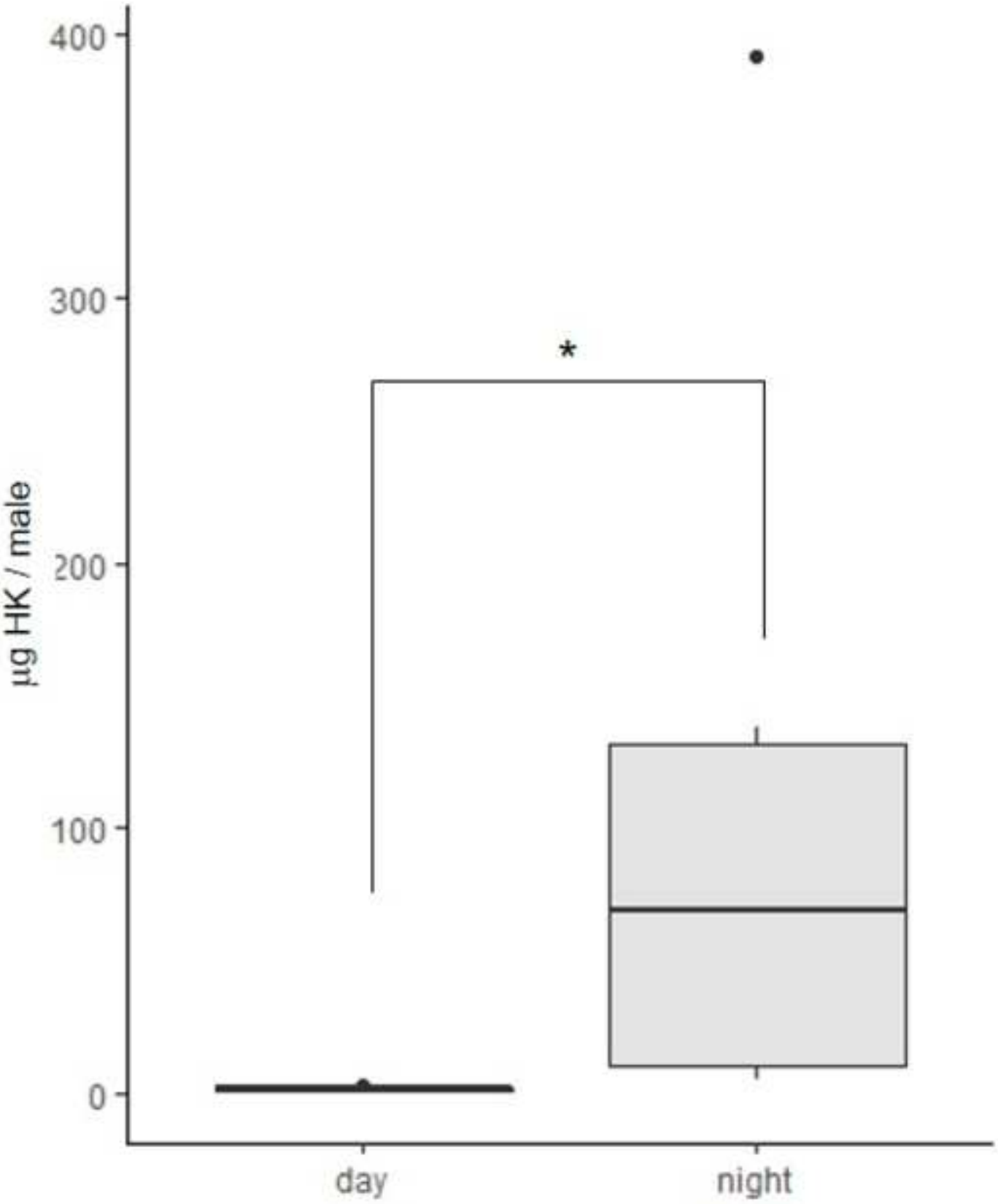

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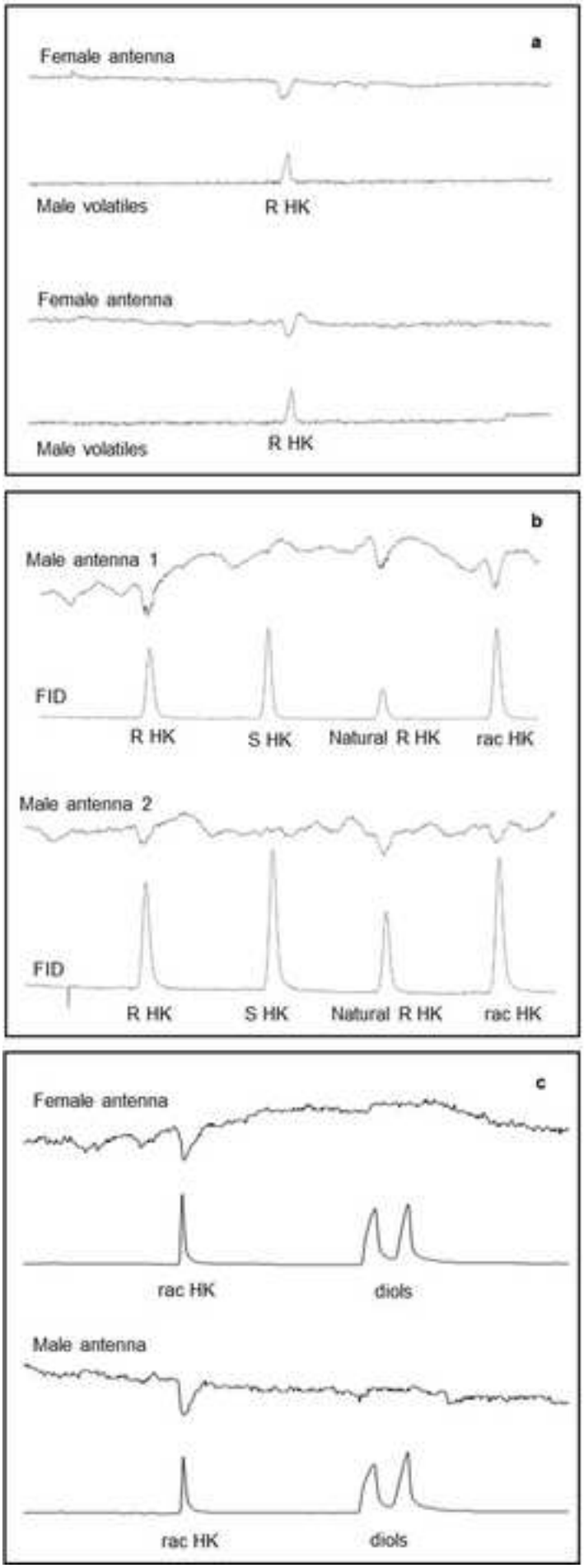

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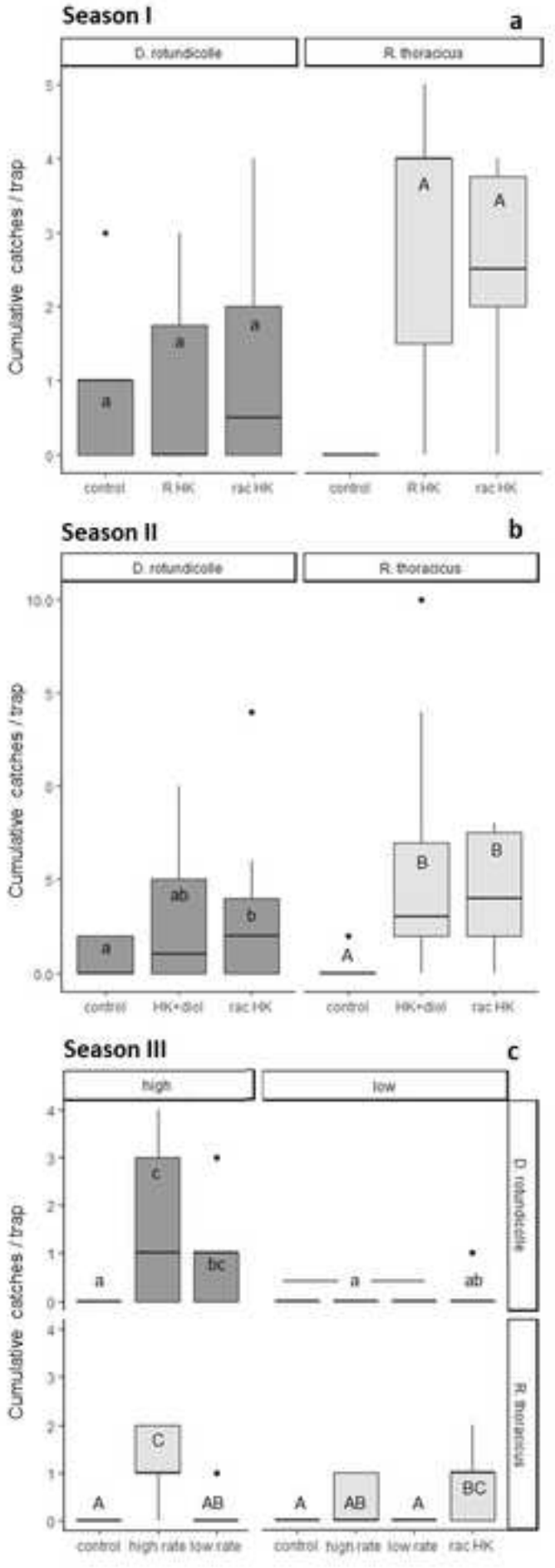

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