

Behavioral and physiological response of male *Callisphyrus apicicornis* (Coleoptera: Cerambycidae) to virgin con-specific females' extracts

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ABSTRACT

Callisphyrus apicicornis (Fairmaire & Germain) (Coleoptera: Cerambycidae: Necydalinae) is a native, xylophagous, and polyphagous longhorned beetle, currently having the status of an increasing pest in some fruit orchards in Chile which needs a control strategy. However, no efficient methods have been developed against *C. apicicornis*. Since pheromone-based strategies are promissory against these types of pests, it is necessary to understand the chemical communication within this species in order to collect and identify the pheromonal compounds as the first step to develop pheromone-based control tactics. The objectives of this work were to study behavioral and electrophysiological responses of males to con-specific females and its extracts, to seek out evidence of chemical communication in the intraspecific relationship of *C. apicicornis*. In field, we collected 112 males using 8 baited traps with alive females in two separate experiments. However, neither males nor headspace (HS) extracts were attractive for either sex in field, no males were captured in those treatments (16 traps). On the other hand, in laboratory bioassays males showed significant electroantennographic responses to females' volatiles extracts (1.46 mV) and gland extracts (1.15 mV). Likewise, behavioral bioassay showed significant olfactometric preference for females' volatiles and gland extracts. Our results strongly suggest a female-produced sex attractant occurs within this species, so further studies should attempt to identify and quantify the chemicals compounds with this role.

Key words: Agricultural pest, electroantennography, insect behavior, olfactometry, sex pheromone.

INTRODUCTION

Callisphyrus apicicornis (Fairmaire & Germain) (Coleoptera: Cerambycidae: Necydalinae) is a large (3-4 cm long) xylophagous Chilean native longhorned beetle with at least 20 native and exotic host plants. *Callisphyrus apicicornis* is an occasional pest in several fruit tree crops (e.g. apple, Rosaceae *Malus domestica* (Suckow) Borkh. and raspberries *Rubus idaeus* L.), with an increasing importance in orchards relatively new for the country's agriculture such as blackberries (*Rubus glaucus* Benth.; Rosaceae), hazelnuts (*Corylus avellana* L.; Betulaceae), and blueberries (*Vaccinium corymbosum* L.; Ericaceae) (Barriga et al., 1993; Artigas, 1994; Klein and Waterhouse, 2007; Curkovic, 2008; Grau, 2009). The eggs are laid individually on the branches and the larvae emerge through the chorion base directly into the wood (Curkovic, 2008). The larvae bore and feed only in living wood, including roots, functional branches, and trunks, affecting severely

the tree structure and even killing the host (Barriga et al., 1993; Artigas, 1994). The larvae and pupae are protected under the bark (Curkovic, 2008), and no feasible chemical or biological control practices are yet available. Cultural control by pruning of affected branches may allow the elimination of some individuals, but it requires early detection of plant symptoms and/or insect signs, which are not always evident (Curkovic, 2008). Therefore, new environmentally friendly practical strategies for *C. apicicornis* monitoring and management are needed. A relatively recent approach, proposed and evaluated against other Cerambycid pests, is the manipulation of adult sexual behavior, mediated by either sex or aggregation pheromones. The identification of such compounds might lead to the development of either monitoring tools or control tactics as mass trapping and mating disruption (Sanchez-Husillos et al., 2015; Sweeney et al., 2017). This approach requires studies of behavior and chemical ecology of these organisms (Harris and Foster, 1995). In an earlier work, the sequences of calling behavior of females and the searching behavior of males were described, suggesting the existence of a female-produced sex-pheromone (Curkovic and Ferrera, 2012). In this work, we studied the behavioral and electrophysiological responses of males to live females and to extracts from females in the field and in the laboratory, as a step further towards the future identification of the sex pheromone.

MATERIALS AND METHODS

Insect rearing

Branches with symptoms of *Callisphyris apicicornis* infestation (Curkovic, 2008) were collected from known hosts: raspberry (*Rubus idaeus* L.), Andean blackberry (*R. glaucus* Benth.), quince (*Cydonia oblonga* Mill.; Rosaceae), and pineapple guava (*Acca sellowiana* [O. Berg] Burret; Myrtaceae) in several localities in central Chile, near Santiago (33°26' S, 70°40' W), Rancagua (34°10' S, 70°44' W), and Linares (35°47' S, 71°35' W), from August through October, during the 2011-2012 and 2012-2013 seasons. They were kept in Flanders cages (as described by Curkovic and Ferrera, 2012) in the laboratory at $\sim 20 \pm 5$ °C, $50 \pm 5\%$ RH, and a natural photoregime. Cages were revised daily at $\sim 08:00-09:00$ h, Monday through Friday, to collect freshly emerged (virgin) adults. In 2011, 95 adults emerged from the wood mostly in October, including 56 males and 39 females (male biased sex ratio close to 1.4), and their life span was determined to be 20.4 ± 8.8 and 26.3 ± 9.5 d for males and females, respectively. Several of those adults were later used in field trials and indoor experiments. *Callisphyris apicicornis* individuals were identified (Artigas, 1994) and sexed (Curkovic and Muñoz, 2011). To ensure virginity, immediately after emergence the beetles were held individually in 500 mL transparent plastic vials covered with a metallic mesh lid, and provided with 5% sugar solution via a cotton wick. Insects between 1 and 15 d-old were used for the bioassays. Only active individuals, moving around and/or quickly escaping after a brush contact, were used in the experiments.

Insect volatile collections and gland extracts

Callisphyris apicicornis virgin females were used for collection of naturally released volatiles with the headspace (HS) technique. Volatiles were trapped by enclosing five individuals in a 900 mL Pyrex glass chamber (9 cm internal diameter [id] and 14.5 cm high) for a 24 h period. Curkovic and Ferrera (2012) identified conditions for putative pheromone release (“calling”) from females in the field and those were recreated as much as possible in this work inside the HS chambers. Volatiles were collected on Porapak Q (80-100 mesh, Waters Associates, Milford, Massachusetts, USA) traps. The entrainment was done using a positive/negative pressure air system as described by Agelopoulos et al. (1999). A purified airstream (5-Å molecular sieves and charcoal) at 1.2 L min^{-1} was drawn through the glass chamber to the Porapak traps inserted into the upper outlet. Volatiles were eluted with 1 mL of redistilled hexane (Chromatographic grade, Optima Scientific, Green Bay, Wisconsin, USA), concentrated then to 100 μL (= 1 HS female equivalent or HSFE) and stored in a freezer at -15 °C until use in the tests. Additionally, calling females (Curkovic and Ferrera, 2012) were frozen at -15 °C for 1.5 min, then the tip of the abdomen was gently pressed, and the terminal everted segments (where the pheromone gland is supposed to be placed) were excised with dissection scissors. The putative pheromone gland was extracted and macerated, one at a time, at room temperature with 2 mL hexane, subsequently the samples were filtered (Whatman Nr 1 filter paper) and concentrated to 100 μL (= 1 female gland equivalent or FGE), and stored, as described above.

Insect extracts-based bait for *C. apicicornis*

The HS extracts, and live males and females, were evaluated as potential lures in a severely infested blackberry orchard in Linares, central Chile. Cone Lindgren funnel traps (Plásticos Los Cerrillos, Santiago, Chile) were used, with four replicates. Low density polyethylene press seal bags (Baguette model 14770, 5.1 × 7.6 cm, 0.05 mm wall thickness, Cousin Corp., Largo, Florida, USA), a type of bags used in field trials as pheromone dispensers for cerambycids in previous reports (e.g. Barbour et al., 2011), were loaded with 50 µL solvent (control) or HS extracts (= 1/2 HSFE). Bags were glued to the inner wall of the top cone. Alive insects (1 trap⁻¹) were placed inside the top cone, provided with a sugar solution via a cotton wick, and confined in the cone with the opening covered with metallic mesh. Traps were hung at ca. 1.8 m height and distanced at least at 30 m between them. A randomized block design was used given an infestation gradient observed within the orchard. Two field trials were conducted in 2011 (Table 1). In both trials, traps were revised 7 d after installation. *Callisphyris apicicornis* catches were identified and sexed. Captures trap⁻¹ d⁻¹ (CTD) was determined for every field trial.

Olfactometric bioassays (OM)

To evaluate the response of male *C. apicicornis* to both, live females and extracts, a behavioral bioassay was carried out using a Y-tube glass as olfactometer. Each arm of the Y-tube (21 cm long with 4 cm id) were connected to an odor chamber (9 cm id and 14.5 cm high) containing 10 µL each extract (i.e. 1/10 of either HSFE or FGE) loaded onto a Whatman Nr 1 filter paper strip. In our bioassays, insect progress inside the Y-tube was facilitated by placing a piece of plastic mesh (replaced in every test) that allowed the male to walk forward (they were not able to do so directly on the glass). This arena somewhat matches the final approach of a *C. apicicornis* male in the field, which usually occurs by walking on the branch where the female is calling (Curkovic and Muñoz, 2011). The olfactometer was connected to a vacuum pump to pull the airflow through the arms at 1500 mL min⁻¹. One male at a time was introduced into the base of the Y-tube, allowing free movement thereafter. The common arm section of the olfactometer was considered a decision zone, and the arms of Y-tube were designated as control (hexane) and stimulus (*C. apicicornis* extracts or aerations) zones, respectively. Tests were conducted between 09:00 h and 17:00 h, using virgin males, under lab conditions (natural photo-regime and fluorescent illumination, 22 ± 0.5 °C). The position of odor chambers in each arm was reversed after each replication to minimize directional bias. Five minutes before starting the experiment, the test specimen of *C. apicicornis* was placed in the olfactometer entry (common arm) for acclimation (with no extracts/solvent in the odor chamber yet). Each insect was used only once a day, with 10 replicates per treatment in a completely randomized experimental design. To characterize the insect preference toward stimuli, the insect was allowed to walk freely in the olfactometer for 15 min and the time spent in each olfactometer arm was recorded (Ceballos et al., 2015; Mutyambai et al., 2015). The olfactometer was cleaned with neutral soap, rinsed with acetone, and dried at 60 °C for 30 min each time a new treatment (either females or extracts) was tested.

Electroantennographic (EAG) recordings

The electrophysiological responses of *C. apicicornis* males to female emitted volatile compounds were studied with an electroantennograph (Syntech, Hilversum, The Netherlands). Stronger physiological signals were observed using the whole live insect, while when excised antennae or heads were used, responses were much lower, 0.05 mV in average.

Table 1. *Callisphyris apicicornis* male captures in two separate field trails (FT) setup in an infested blackberry orchard in Linares, central Chile (n = 4 traps [t]/treatment (lures)).

Treatments (lures)	FT1 ¹				FT2			
	t1	t2	t3	t4	t1	t2	t3	t4
Live females	1	2	14	13	7	0	38	37
Live males ²	0	0	0	0	-	-	-	-
Headspace (HS) ♀ extract ³	-	-	-	-	0	0	0	0
Control	0	0	0	0	0	0	0	0

¹FT1 was set on 17 October, and FT2 on 25 October 2011.

²Males were tested only during the first FT.

³HS extracts were tested only during FT2.

Thus, whole live insects were used and only the tip of the antenna was cut for a better connection to the electrode. The setup of the bioassay was performed using Ag/AgCl wires inside glass capillaries filled with 1 M KCl and 0.1% polyvinylpyrrolidone as electrodes. The indifferent electrode was inserted into the base of insect's head (*scutellum*). Thus, the insect assembly was placed with the antenna facing the air stream receiving effluent from the EAG apparatus (Ceballos et al., 2015). The signal was acquired and processed with an intelligent data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) linked to a PC running the EAG software (GcEad/2012, Syntech, Hilversum, The Netherlands). A sample of 10 μ L of each extract (described above) or hexane (control) were loaded individually onto a paper strip (5 \times 50 mm filter Whatman Nr 1), a 20 s period was allowed for solvent evaporation, and then the strips were inserted into Pasteur pipettes (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The airstream with the stimuli were applied on the antennal preparation for 2 s using an electronic stimulus controller (CS-05, Syntech, Hilversum, The Netherlands) and consecutive recordings were spaced out by 1 min for antennal recovery (Ceballos et al., 2015), with 20 replicates per treatment in a completely randomized experimental design.

Data analysis

In field trials, only female-baited traps produced captures, thus field data were not subjected to statistical analysis. As described in Ceballos et al. (2015), the time spent by insects in the stimulus or the control arm of the olfactometer was used to calculate an olfactometric-preference index (OPI), where $OPI = 2S/(S + C)$; S and C represent the time spent by the insect in the stimulus or control arm, respectively. A maximum attraction is denoted by OPI = 2, inversely an OPI = 0 indicates no-attraction to tested stimulus. To minimize any EAG variation among antennae, mV responses from each treatment were divided by the corresponding solvent amplitude to a relative electroantennographic response (RER) (Barata et al., 2000). To determine differences of either OM or EAG responses to the treatments, data were subjected to an ANOVA and Tukey's test ($P \leq 0.05$) for group separation.

RESULTS AND DISCUSSION

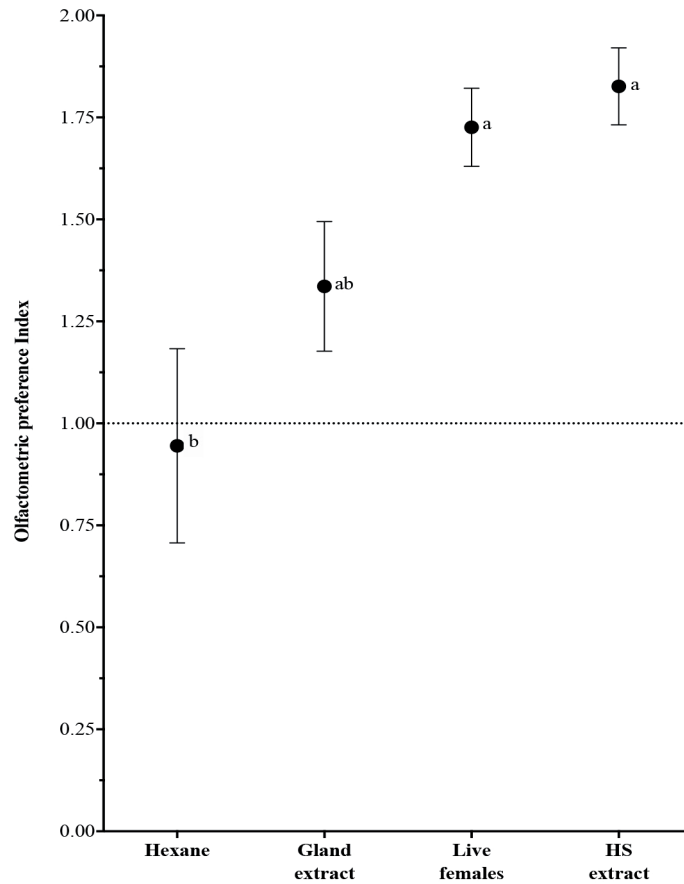
Field trapping of *C. apicicornis*

In the first field trial (FT1), wild *C. apicicornis* males were captured ($n = 30$) only in reared female-baited traps (Table 1). CTD was ca. 1.1 (4 traps, 7 d in the field). In the second field trial (FT2, Table 1), again, only the female-baited traps produced captures, all males ($n = 82$, CTD was 2.9). Overall, no captures (neither males nor females) were obtained with reared male-baited traps, HS extracts, or the solvent alone. Thus, field data support the hypothesis that *C. apicicornis* mating is mediated by a female-produced sex-pheromone, agreeing with a previous behavioral study (Curkovic and Ferrera, 2012), however that study did not include bioassays.

Olfactometric bioassays

Male responses within the Y-tube, expressed as OPI values, are presented in Figure 1. There were greater OPI values in males exposed to female volatiles (HS) extract and live females (although with no differences between both treatments), which did not differ from gland extracts, but were significantly greater ($P = 0.0006$) than the values obtained for the solvent. Also, no differences occurred between the gland extracts and the solvent. Individuals making no choice, i.e. those not leaving the decision zone during the test, were not included in the analysis (they represented 3%-10% out of the total tests). Despite that cerambycids usually fly to the source in nature, olfactometers (where specimens cannot fly) have been used to test potential biologically airborne active compounds (as pheromones) or other extracts (Fonseca and Zarbin, 2009; Fonseca et al., 2010), but in most of those cases female responses were evaluated since males are usually the sources for pheromones within the family (Lacey et al., 2007; Mitchell et al., 2017). In particular, no olfactometric studies using live females, gland extracts, or HS samples from females were found (Ray et al. [2011] studied HS samples but using GC-EAD), and just a few reports evaluated these types of samples obtained from males (e.g. Fonseca et al., 2010). Moreover, no reports contrasting OPI values on cerambycids were found, but the index has been used for other Coleoptera as Bruchinae (Chrysomelidae) and Curculionidae to reflect the effect of the stimulus on the insect's behavior (Parra et al., 2009; Ceballos et al., 2015).

Figure 1. Olfactometric preference index (OPI \pm SE) of *Callisphyris apicicornis* males in the olfactometer in response to live females, extracts (headspace [HS] or glands) or the hexane solvent (n = 10 males per treatment).



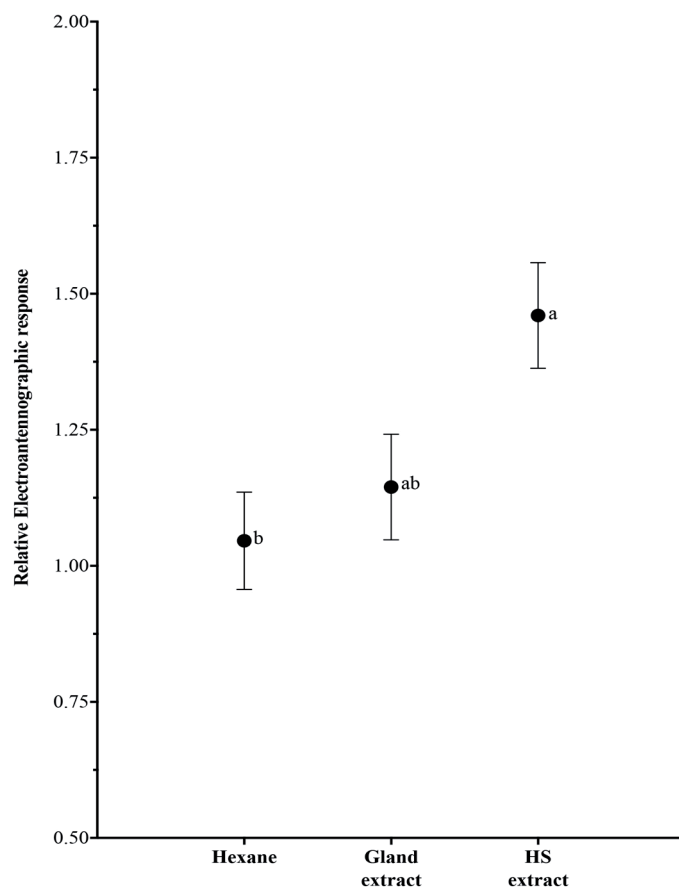
Means sharing a letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

Our results also suggest neither visual nor acoustic signals are used during the approach between sexes, since females in traps were not visible to the approaching male from the outside, and no sounds were perceived during trap set up or in the previous calling study (Curkovic and Ferrera, 2012). *Callisphyris apicicornis* females do stridulate but only when handled, presumably as a defensive response due to stress (as mentioned by Mitchell et al., 2017), and never when calling. In fact, stridulation in Cerambycidae has not been observed to be related to sexual behavior (Finn et al., 1972).

Electroantennographic activity of insect volatiles and extracts

Signals recorded from whole *C. apicicornis* males ranged between 1.42 and 2.54 mV. The calculation of the RER index was considered more appropriate than the raw mV EAG values to contrast treatments, as recommended when specimens potentially present high variability (due to different origins or ages) or are re-used in subsequent tests (Rodríguez, 2013). Our results (Figure 2) demonstrate that female aerations (HS) produced significantly greater ($P = 0.009$) physiological responses than the control (solvent). Female gland extracts did not differ significantly from both the HS and the control, similarly as observed in the olfactometry tests. Regarding electrophysiological tests, no previous data were found for *C. apicicornis* males. The EAG values reported herein were, in general, greater than those reported for other cerambycids (also using whole insects) like *Cerambyx welensii* (Küster), *Phoracantha semipunctata* F., or *Prinobius germari* Dejean exposed to HS extracts, plant volatiles, or general attractants (Barata et al., 2000; Sánchez-Osorio et al., 2007). Only in *Steirastoma breve* (Sulzer) (Liendo et al., 2005) greater signals have been reported (up to 7.5 mV). No reports were found comparing both sources (female glands vs. HS female aerations), using EAG, within Cerambycidae.

Figure 2. Relative electroantennographic response (RER \pm SE) of male *Callisphyris apicicornis* to volatile extracts (either female headspace [HS] extracts or female glands) and the hexane solvent (n = 20 per treatment).



Means sharing a letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

Overall, despite the differences between the types of experiments, results from both field experiments and behavioral and physiological bioassays strongly suggest that *C. apicicornis* females produce a sex pheromone to attract conspecific males. In the field, only females were able to attract wild males, whereas neither males nor female extracts were attractive. On the other hand, indoor experiments (EAG and OM) showed responses to both, live females and their extracts.

In olfactometric tests, males significantly preferred live *C. apicicornis* females and their extracts, while the solvent led to behavioral indifference. Similarly, HS extracts from virgin calling females caused a significant electrophysiological response, producing important signals in the male antennae after exposure, whereas the solvent alone did not. Gland extracts were statistically neutral, being not different from either the solvent or the HS extract. Regarding *C. apicicornis*, other volatiles have been identified recently, but the compounds are not sex pheromones, and act only on odor mimicry (Mitchell et al., 2017).

Interestingly, most male attractants described in Cerambycidae are aggregation pheromones produced by males (Lacey et al., 2007; Mitchell et al., 2017), and there are only a few reports of female sex pheromones (Rodstein et al., 2009; Ray et al., 2012), none within the sub-family Necydalinae where *C. apicicornis* belongs (Monne, 2005). However, additional behavioral evidence suggesting their existence is available in other species in the same sub-family (Krahmer, 1990), meaning that to find the *C. apicicornis* female sex pheromone represents a real challenge for scientists, and research should continue further, including chemical analysis to elucidate both identity and quantities of compounds. It will also be necessary to test extracts (HS or gland's) using GC-EAD to identify biologically active fractions.

CONCLUSIONS

Based on our field and laboratory results, the mating behavior of *Callisphyrus apicicornis* is mediated by female pheromonal compounds, which is a rare case within the Cerambycidae family.

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