Cuticular Compounds Recognition and Mating Behavior of the Rice Water Weevil *Oryzophagus oryzae* (Coleoptera, Curculionidae)

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Abstract *Oryzophagus oryzae* mating behavior and mate recognition were investigated. Bioassays revealed that couples had intense sexual activity in the first 3 h of photophase and scotophase. Adults mated and re-mated at any time of day in the presence of water; however, in the absence of water, only 10 % of adults mated, meaning that water was important for couples to find each other. In the sequence of pre-mating behaviors bioassay four steps were observed and females were always attracted to males, seeking them, before copulation; after mating, males showed guarding activity of variable duration, allowing remating. Cuticle extractions revealed that both sexes had similar cuticular chemical compounds. They were identified as aldehydes and a ketone, probably because of its aquatic life habits. Bioassays suggested that males recognized females by their cuticle composition.

Keywords Aquatic weevil · copulation · cuticle extraction · mate recognition · pre-mating sequence

Introduction

Curculionidae is one of the largest families of organisms with approximately 44.000 species. Although a few curculionids are aquatic, particularly adult and larval

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Erirhininae (Grimaldi and Engel 2005), there have been no studies concerning the mating behavior and mate recognition of aquatic weevils. These traits may differ from terrestrial weevils that were more investigated so far.

The mating behavior has been studied for a number of terrestrial weevil species, for example, *Anthonomus grandis* (Mayer and Brazzel 1963), *Diaprepes abbreviatus* (Sirot et al. 2007), *Cosmopolites sordidus* (Viana and Vilela 1996), *Euscepes postfasciatus* (Sato and Kohama 2007), and *Hypera postica* (LeCato and Pienkowski 1970). Certain behaviors are commonly described for weevils, such as the postcopulatory mate guarding (Sato and Kohama 2007; Polak and Brown 1995; Vanderbilt et al. 1998), the tapping or stroking mechanism during courtship (Sirot et al. 2007; Vanderbilt et al. 1998) and several matings with the same partner (LeCato and Pienkowski 1970; Wen et al. 2004). Mate recognition is based on visual, olfactory, auditory, or tactile cues (Gillot 2005). In *Diaprepes abbreviates*, *Cylindrocopturus adspersus* and *Aegorhinus superciliosus* (Guerin) both males and females have n-alkanes or monomethyl-branched alkanes as cuticular components (Lapointe et al. 2004; Pomonis and Hakk 1984; Mutis et al. 2009). In *Aegorhinus superciliosus* (Guerin) mating response of males was mediated by cuticular compounds rather than tactile or behavioral signals from females (Mutis et al. 2009).

Oryzophagus oryzae (Costa Lima) is an aquatic weevil and the main pest of irrigated rice crops in Brazil because larvae destroy roots of rice plants (Carbonari et al. 2000). In Rio Grande do Sul State, *O. oryzae* remain in diapause from April-October on Gramineae and Cyperaceae plants close to rice crops (Pugliese 1955). According to Prando (1999) adults feed on parenchyma cells of leaves; females oviposit one egg at a time inside the base of the leaf; the first instar larva remains feeding inside the plant until it opens a hole, moves down and starts feeding on the roots; and pupae have a connection to rice roots from where they obtain an oxygen supply. Larvae are aquatic but they do not have appendices and adults have a plastron, and are excellent swimmers (Martins et al. 2012).

Different control methods are used, such as resistant plants (da Silva et al. 2003), seed treatment (Grützmacher et al. 2003), insecticide spray on leaves (Link et al. 2011) and granulated insecticides formulations (predominantly carbofuran, a carbamate) applied in the irrigation water (Paraíba et al. 2007). However, the development of new control and monitoring system is required. Studying the mating behavior of *O. oryzae* may provide evidences for semiochemicals used for attraction and mate recognition that could be used to develop new control strategies in the future. Therefore, the objectives of this study were to investigate the sexual traits of *O. oryzae* and to identify the main compounds present in the cuticle of males and females testing the attraction of males to washed and painted females.

Materials and Methods

Insects

O. oryzae adults were obtained from irrigated rice plantations of the "Estação Experimental de Itajaí—Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A." in Itajaí, Santa Catarina State, from October-December, 2009–

2011. Samples were shipped to Laboratório de Semioquímicos—Universidade Federal do Paraná in Curitiba/Paraná and were maintained on rice plants cultivated inside 400 mL plastic cups filled with tap water. Beetles were sexed before each experiment and couples were created arbitrarily. Males of *O. oryzae* are smaller than females and have a concavity ventrally on their abdomen so that it fits females dorsally during mating. For all experiments it was not possible to confirm whether they were virgin or mated.

Time of Day, Duration, and Number of Copulation Events per Couple

Couples were analyzed every 5 min for a 12-hour period (photophase and scotophase) and classified into three possible conditions: separated from each other, mounted, and copulating ($n=30$ couples/photoperiod). The experimental set was a 400 mL plastic cup containing 150 mL of water and one rice plant of approximately 30 days old. Cups were covered by a transparent fabric tied with an elastic ribbon. Mounting and copulation behavior were easily differed based on the position of males in relation to females. During mounting, males were above females in a horizontal position (180°) while during copulation males were above females in an angle of approximately 60°. A mating period occurred when couples were copulating for at least 15 min. Before the analysis, data was grouped in 30 min intervals and the duration, the number of copulation events/couple, and the mean number of mating couples was estimated. Scotophase replicates ($n=30$) were performed inside an acclimated room (24 °C±1) with a reversed photoperiod (12 L: 12D) during daytime (8 am–8 pm, total of 12 h). Photophase replicates ($n=30$) were performed in the same room during daytime (8 am–8 pm, total of 12 h) using a regular photoperiod. First we performed the photophase observations for a period of 3 days, until all replicates were obtained, followed by the scotophase observations that were performed a week later also for a period of 3 days. Weevils were maintained in this room for one week to acclimate. Different couples were used for each replicate.

Mating in the Absence of Water

This experiment was performed in the first 3 h of photophase with the same methodology described above, with the exception of the absence of water ($n=30$). The mean number of mating couples was estimated every 30 min in the absence of water in the first 3 h of photophase and compared with the mean number of mating couples of the previous experiment at the same time. Because insects were observed mating mainly underwater on the plant when water was present, this experiment aimed to evaluate the absence of water as a mating environment and also to check if insects could find each other only by walking.

Sequence of Pre-Mating Behaviors

During the first 3 h of photophase, the sequence of pre-mating behaviors of 20 couples were analyzed. Methodology was the same as cited in the first experiment.

Mating in the Presence of Rival Males

One mating couple previously separated and three rival males were placed inside plastic cups that contained water and a rice plant that was approximately 30 days old. The reaction of the mating male, rival male, and female toward the rival males was observed ($n=10$).

Extraction of Female Cuticle and Male's Response

The chemical compounds present in the cuticle of a female were extracted with 30 μL of doubled distilled hexane for 10 min, followed by a second extraction with 100 μL for 10 min, to ensure that all residual chemical compounds were removed. The first female extract (30 μL) was concentrated with a filtered airstream to 15 μL, the second extract was discarded (Fonseca and Zarbin 2009; Zarbin et al. 1999).

The washed female (mummy), was offered to two males separately, one at a time, on a paper towel. Males' responses were noted. Then, 10 μL of the extract was reapplied on the mummy' body. The same males' responses were noted. In total, 20 repetitions were performed.

The compounds identified in the cuticle extracts of females were also tested individually and together. In a paper towel, a mummy was painted with 10 μL of each extract, containing one or all compounds, and male's responses were noted.

Cuticle Compounds Extraction of Males and Females

Extractions of 15 to 30 individuals for each sex separately were performed for 30 min with 150 μL to 200 μL of distilled hexane, which covered the insects. At least 15 repetitions were performed for each sex. In order to compare the chromatograms of both sexes, extracts were concentrated in the proportion of 1:1 and injected in a GC-2010 (Shimadzu), GCMS-QP 2010 Plus (Shimadzu) and GC-Fourier transform infrared spectroscopy (GC-FTIR) (GC-2010 coupled to a DiscovIR-GC—Shimadzu). Injections were composed of 1 μL of extract using the splitless mode with an injector temperature of 250 °C. Column oven temperature was maintained at 50 °C for 1 min and raised to 250 °C at a rate of 7 °C min^{-1}, where it was maintained for 10 min. GC and GCMS were equipped with a RTX-5 column (30 m×0.25 mm i.d. and 0.25 mm film thickness; Restek, Bellefonte, Pennsylvania, USA) using helium as a carrier gas at a column head pressure of 170 kPa. All analyses used the same parameters. Quantification of the compounds present in the extracts was performed by comparing the area of an internal standard (C13—tridecane) with the relevant peaks. To support the first analysis, 1 μL of the 5 μL female cuticle extract that remained from the female washing cuticle experiment was also injected and analyzed. In the infrared analysis, GC was operated in splitless mode and was equipped with a DB-5 (0.25 mm, 0.25 mm×30 m) (J&W Scientific, Folsom, California, EUA) capillary column and helium carrier gas. Column oven was maintained at 50 °C for 1 min and was subsequently increased to 250 °C at 7 °C min^{-1}. A liquid nitrogen cooled photoconductive mercury cadmium telluride (MCT) detector was used with a FT-IR resolution of 8 cm^{-1}.

Synthesis and Identification of Cuticle Compounds

Cuticle compounds identification was based on mass spectra (MS) analyses and co-injections with synthetic standards in two different columns (DB-5 and EC-1). Hexadecanal and octadecanal were synthetized by oxidation of the corresponding alcohols using pyridinium chlorochromate (PCC). Eicosanal was synthetized by reducing its corresponding ester using lithium aluminium hydride ($LiAlH_4$), and subsequently oxidizing it with PCC (Fonseca et al. 2010). Nonadecan-2-one was synthetized with the addition of the Grignard reagent to the octadecanal generating the secondary alcohol; then, an oxidation reaction was performed using the Jones reagent originating the ketone. The authentic standards, hexadecanol and octadecanol, were purchased from Acros Organics (Geel, Turnhout, Belgium) and methyl stearate was purchased from Aldrich Chemical Company (Milwaukee, WI, USA).

Statistical Analysis

The R software, version 2.13 (R Development Core Team 2011), was used in all analyses. A GLM ANOVA model (Quasi-Poisson) at 5 % significance level was used to compare means of mating in photophase and scotophase, number of matings in the absence and presence of water, and number of matings per couple. A GLM ANOVA model (Gaussian) at 5 % significance level assessed the duration of mating. A chi-square test with expected equal proportions analyzed mating versus not mating in 30 min intervals for both photophase and scotophase. To compare the main cuticular compounds from males and females a normality test (Shapiro) was performed followed by the nonparametric Wilcox test.

Results

Time of Day, Duration, Number of Matings Per Couple and Mating in the Absence of Water

Adults mated during day and night (Fig. 1) and the main peak mating period occurred during the 2nd hour of photophase when 87 % of the couples mated. There were some clear differences in the mean number of couples mating between individual time periods in photophase and scotophase, however when photoperiods were compared, they were not significantly different (p-value=0.222; F=1.533) and no distinct pattern was observed. Thus, the number of matings per couple (2 to 15 remating) and their length (5–115 min) were not statistically different (respectively, p-value=0.255; F=1.321; GLM—Quasi-Poisson; p-value=0.424; F=0.648; GLM—Gaussian). The total mean length of mating period was approximately 310 min in scotophase and 279 min in photophase. Between copulation events males remained mounted on females (guarding activity) for periods that varied from 5 min to 160 min in photophase and from 5 min to 250 min in scotophase. After the first mating, 50 % of couples in photophase and 36 % in scotophase separated from each other for a period of 5 to 365 min and 5 to 270 min, respectively.
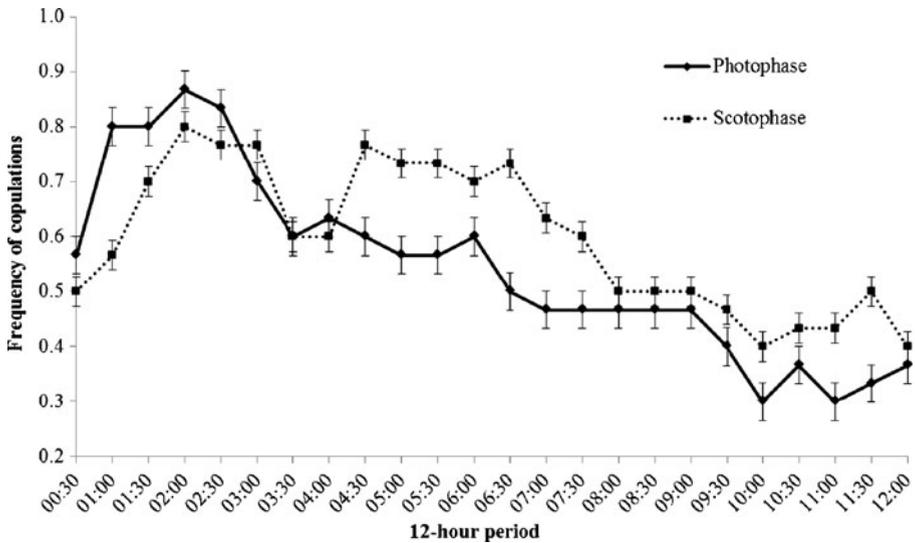
Fig. 1 Frequency of copulations (with standard error bars) estimated in every 30 min throughout the 1st–12th hour of photophase and scotophase

In the mating experiment, comparing matings with or without water, more matings were detected in the presence of water ($F=479.86$; p-value$=3.121^{-12}$*). In the absence of water only one couple mated.

Sequence of Pre-Mating Behaviors

Of the 20 couples observed, 10 couples were considered for analysis. Other couples did not copulate, because in most cases males were rejected. A sequence of four steps was detected before couples actually mated (Fig. 2). Female first approached the male (*step a*); after she touched or was close to him, he approached, mounted her (*step b*) and attempted to copulate. The first contact between females and males occurred in the water in all replicates. Female refused the first mating attempt in 90 % of replicates. After refusal, the male walked over her back, tapped her head with his forelegs (*step c*) and a new mating attempt was made. This step followed by a new mating attempt was repeated from 0 to 8 times (mean of 4.3). When female decided to mate, she moved her abdomen to the side and accepted the male (*step d*). No other mating behavior was observed during copulation. After copulation couples remained mounted until the next mating occurred.

Mating in the Presence of Rival Males

Couples reacted against the interest of rival males during or after mating occurred. Rival males walked up the couples in 90 % of repetitions and 30 % positioned themselves in the middle of the couple in an attempt to stop the mating. The only response of the mating male was to remain mounted, guarding the female. In 40 % of replicates, females carrying the males on their back walked away from rival males.
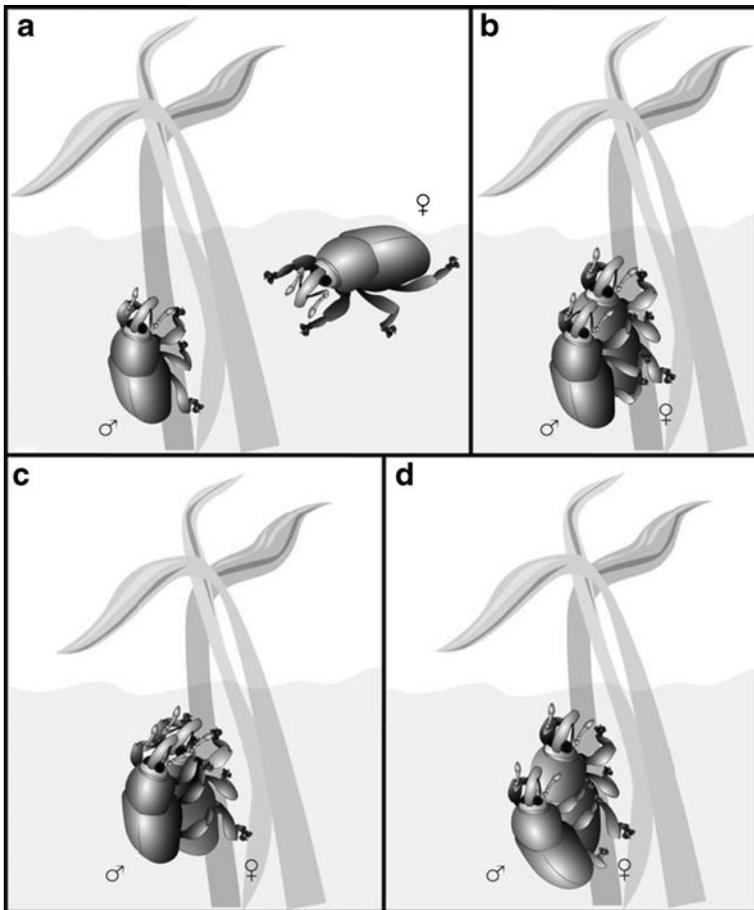
Fig. 2 Pre-mating sequence of *Oryzophagus oryzae*. Sequence of four steps before mating (**a–d**): **a** female going in male's direction; **b** male mounting the female; **c** male walking over the female's back and taping her with his forelegs (tapping or stroking mechanism); **d** female accepting the male and mating

Extraction of Female Cuticle and Male Response

Males were not attracted sexually to hexane-washed females. Only after females were painted with their cuticle extract the recognition was reestablished, and 70 % of males mounted on females. Male responses were described in Fig. 3.

Cuticle Compounds Extraction of Males and Females

Extracts revealed the presence of four main compounds apparently with quantitative differences in males and females (Fig. 4, Table 1). They were identified as aldehydes and ketones (hexadecanal, octadecanal, eicosanal, and nonadeca-2-one). Although females presented higher amounts of all compounds, they were not statistically different from males (hexadecanal: $W=27$, p-value=0.8048; octadecanal: $W=34$, p-value=0.2593; nonadeca-2-one: $W=40$, p-value=0.05303; eicosanal: $W=24$, p-value=1).

WASHED FEMALE:

PAINTED FEMALE:
CUTICLE EXTRACT
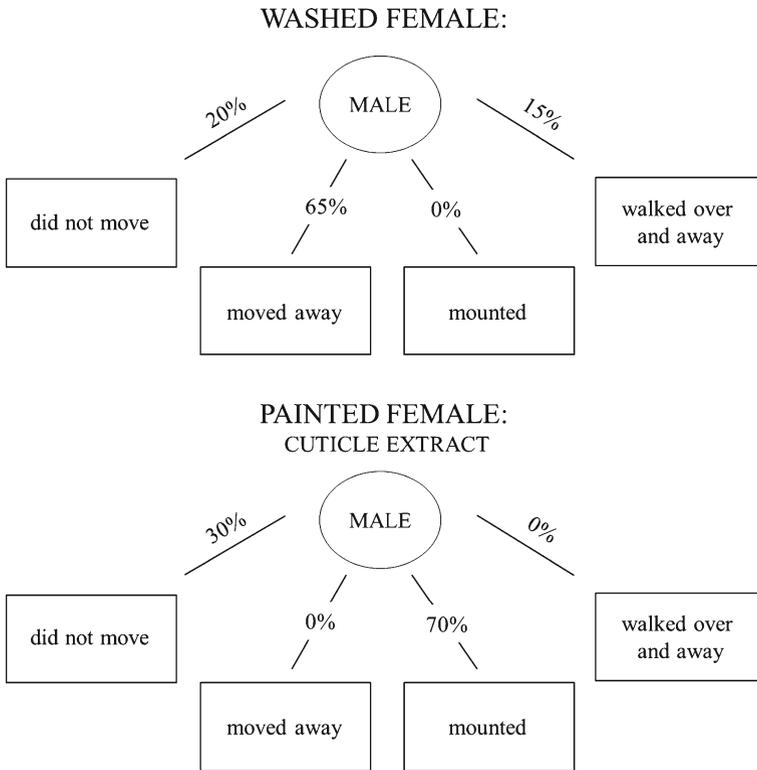
Fig. 3 Responses (%) of *Oryzophagus oryzae* males to washed females and females painted with their cuticle extract

Despite statistical analyses, we tested males' responses to females painted with each one of the compounds and a mixture of all four compounds. However, males did not respond by mounting.

Discussion

Mating of *O. oryzae*

In scotophase, the period of mating had a mean duration of approximately 310 min. This period is much higher than that of the alfalfa weevil, *Hypera postica* Gyllenhal, which has a mean duration of 58 min of mounting (LeCato and Pienkowski 1970). Considering the number of matings per couple, *O. oryzae* presented a mean of 5.37 at photophase and 6.33 at scotophase, and reached a maximum of 10 and 15 matings, respectively, in different couples. The alfalfa weevil mates only a maximum of 10 times (LeCato and Pienkowski 1970). Therefore, couples of *O. oryzae* have intense sexual activity in the initial hours of photophase and scotophase.

In a study with the sweet potato weevil, *Euscepes postfasciatus* (Fairmaire), Sato and Kohama (2007) formulated and tested two hypotheses that
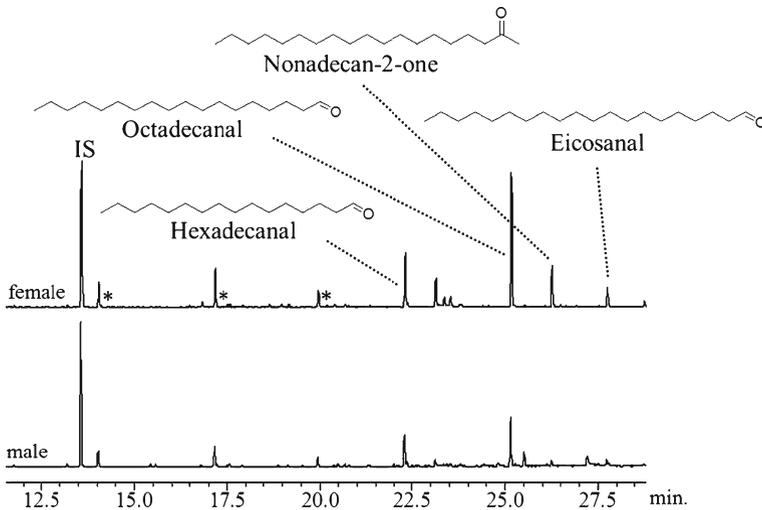
Fig. 4 Comparison of the chromatograms of male and female's cuticular extracts of *Oryzophagus oryzae*. *IS* internal standard; * column bleed or contaminant

investigated how mate guarding could be beneficial for males after mating. Hypotheses were that mate guarding aided to prevent future copulations by rivals (hypothesis 1) and to gain additional copulations (hypothesis 2). Authors concluded that mate guarding in *E. postfasciatus* was better explained by hypothesis 2. For *O. oryzae*, both hypotheses could be accepted. The second hypothesis is supported by the fact that couples remated up to 15 times. The first hypothesis is supported by the fact that rival males dispute females, and guarding activity is sufficient to prevent rival males. Mate guarding is also common in other weevils (Sato and Kohama 2007; Polak and Brown 1995; Vanderbilt et al. 1998).

Adult females of *O. oryzae* mated from 2 to 15 times with the same male. Other weevil species, *Hypera postica* and *Hylobitelus xiaoi* Zhang (LeCato and Pienkowski 1970; Wen et al. 2004), mate during day and night with the same partner. Thus, *O. oryzae* males in all experiments remained mounted on females after the first mating, guarding them for the next mating.

Table 1 Quantification (ng) of the chemical compounds identified in the cuticular extracts of males and females of *Oryzophagus oryzae* (Coleoptera: Curculionidae)

RT	Chemical compounds	Females (ng) Mean SE	Males (ng) Mean SE	
22.309	Hexadecanal	11.08±5.80	8.83±4.45	ns
25.159	Octadecanal	30.73±12.89	21.43±10.91	ns
26.259	Nonadecan-2-one	8.98±4.56	7.11±6.20	ns
27.769	Eicosanal	3.83±1.83	14.61±13.20	ns

RT retention time, *SE* standard error, *ns* not significant

Mating in the Absence of Water

In the south of Brazil, during October, adults fly to irrigated rice plantations where they feed and mate probably stimulated by the beginning of the spring rainfalls. *O. oryzae* swims well and has hydrofuge scales and a plastron for underwater survival (Martins et al. 2012), thus larvae are aquatic and need irrigated plantations to survive. Therefore, rainfall could be an indication that the insects would find enough water to reproduce. According to our experiments, water was also an important factor for couples to find each other, because in the absence of water there was a drastic reduction in the number of matings. In the plastic cups used in the experiment testing the mating without water, female and male weevils walked to locate their mates, while in the presence of water in pre-mating sequence experiment, they always found their mates by swimming.

Pre-Mating Sequence

The first steps of pre-mating sequence (*b*-*c*) (Fig. 2) revealed that males attempted to copulate several times before females accepted them as partners. Females refused the first mating attempt in 90 % of the couples. Males tried to mate from 0 to 8 times with a mean number of 4 times per male. According to Seymour and Sozou (2009), females that do not accept males immediately use this initial rejection to screen the males. "Good" males, with a higher fitness ratio, have longer courtship lengths than "bad" males.

Step *c* of pre-mating sequence (Fig. 2) can be described as a tapping or stroking mechanism (LeCato and Pienkowski 1970). This behavior was also described for the alfalfa weevil *H. postica* (LeCato and Pienkowski 1970), *Diaprepes abbreviatus* (L.) (Sirot et al. 2007) and *Rhinchophorus cruentatus* (Vanderbilt et al. 1998). In *H. postica*, immediately after mounting, males almost invariably pat the pronotum and the head of females with their antenna both before and during actual copulation. Authors describe this mechanism as a possible calming act to prevent struggle or in some way to prepare females for insertion of the aedeagus. In *D. abbreviatus*, stroking is performed either with the antennae touching the female's thorax or elytra or occasionally wiping the hind tarsi alternately on the female's abdominal sternites during copulation. In *O. oryzae*, the tapping always occurred before mating and couples mated several times after tapping; therefore, we hypothesize that this mechanism was used to prevent struggle and to prepare the females for copulation.

Cuticle Extraction of Females and Males' Response

Bioassays revealed that males recognized the female cuticle composition and responded to them by mounting. This response was lost after females were washed with hexane and restored after females were painted with their cuticle extracts. Analyzing males and females' chromatograms of cuticle extracts, the same compounds could be detected. Interestingly, they were not identified as hydrocarbons as it would be expected for terrestrial weevils, but as aldehydes (hexadecanal, octadecanal, and eicosanal) and a ketone (nonadecan-2-one) (Fig. 4, Table 1). After females were painted with these compounds individually and grouped together, the recognition was

not restored. Therefore, in addition to the four identified compounds, more compounds could be involved in the recognition process.

Less polar compounds, generally found as cuticular hydrocarbon compounds in insects, were found in notably low quantities. This difference in the cuticular composition of the rice water weevil may be due to its aquatic activities, because ketones and aldehydes have more compatible polarities with an aquatic environment.

The same cuticle recognition was evidenced in the raspberry weevil, *Aegorhinus superciliosus* (Guerin). Cuticle extractions revealed that although males and females had the same compounds, their concentrations were different, and males recognized the compounds present in the cuticle of females. Cuticular extracts contained a contact pheromone (Mutis et al. 2009).

Final Considerations

Despite its aquatic habits, the rice water weevil performed the same mating behaviors as terrestrial weevils. The mounting behavior, the tapping mechanism and the remating were observed. However, the composition of the cuticle was unique, when compared with terrestrial weevils. Aldehydes and a ketone were identified, and although they were not responsible for the complete reestablishment of cuticular recognition, they could be if grouped together in a specific ratio. Thus, maybe a minor compound present in the extracts could also be responsible for this recognition.

In the field, thousands of weevils can be found in an irrigated rice plantation, which reduces the possibility that males and females would not find each other. Most likely, after diapause, rains stimulate weevils to fly into plantations and mate. However, the potential existence of a male pheromone that attracts females and is carried by water cannot be ignored; such pheromone would explain why females were always attracted to males (Fig. 2) and the reduction of matings in the absence of water. Although *O. oryzae*'s body is covered with hydrophobic scales, the antenna does not have such scales in area covered with trichoid sensilla (Martins et al. 2012), permitting the reception of chemicals directly from the water.

Future studies should focus water extraction methods to examine the existence of semiochemicals in *O. oryzae,* on bioassays investigating additional compounds present in the cuticular composition of females, and on behavioral experiments to clarify if adults are attracted to plant odor.

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