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Identification of the Sex Pheromone of Female *Eurata patagiata*[1]

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Abstract. *Eurata patagiata* Burmeister is a diurnal moth of the Arctiinae subfamily of Eribidae. The chemical nature of pheromones of the family has not been well studied. Calling behavior was observed. Using several chemical techniques (gas chromatography-EI-mass-spectrometry, retention indices, micro-reactions, and synthesis) and bioassays with an olfactometer and in the field, (*Z*, *Z*, *Z*)-9,12,15-octadecatrienal (linolenal) and (*Z*, *Z*, *Z*)-9,12,15-octadecatrienol (linolenol) were identified as the main sex pheromone compounds in extracts from the female glands. Behavioral assays showed that more than 90% of male moths were attracted to a mixture of the pheromones rather than to hexane (check). However, the percentage decreased when only one compound was assayed. Preliminary field experiments demonstrated that males were captured in pitfall traps with a mixture of the pheromone compounds.

Introduction

Lepidopteran sex pheromones have been identified from females of more than 670 species (Ando et al. 2004, El-Sayed 2017). Emission of sex pheromone triggers sequential mating behavioral responses between the sexes. Most pheromones can be classified into two types based on the presence (Type-I) or absence (Type-II) of a terminal functional group (Millar 2000, Ando et al. 2004). Type-I pheromones consist of unsaturated C10-C18 primary fatty alcohols and their derivatives, while unbranched C17-C23 polyenyl hydrocarbons and their epoxides are Type-II sex pheromones of female moths (Ando et al. 2008). Recently, additional Type 0 and Type III pheromones based on structural and biosynthetic features were proposed to extend the classification (Löfstedt et al. 2016). Type 0 pheromones correspond to short-chain secondary alcohols and ketones (C7-C9), while Type III pheromones are methyl branched compounds including saturated and unsaturated hydrocarbons, as well as functional hydrocarbons.

Erebid female pheromones or putative ones have been described for approximately 30 species, in which Type I and Type II were reported as pheromones, among them linoleic acid and linolenic acid-derived aldehydes (Millar 2000, Ando et al. 2008, Fujii et al. 2010). In addition to the well-known compounds,

[1]Lepidoptera: Erebidae: Arctiinae

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many miscellaneous Type-III compounds have been identified: 6-methyl, 14-methyl, and 6,14-dimethyloctadecan-2-one were identified from *Lyclene dharma* (Moore 1879) (Adachi et al. 2010), and 5-methylheptadecan-7-ol (a methyl-branched alcohol) was identified as the female sex pheromone of *Miltochrista calamina* (Butler 1877) (Yamakawa et al. 2011). Moreover, 7-propionyloxyheptadecane and 8-propionyloxyheptadecane were identified as the main pheromone compounds of *Barsine expressa* (Inoue 1988) (Fujii et al. 2013). However, information on sex pheromones of Erebidae moths is limited and the chemical ecology is poorly known.

Eurata patagiata Burmeister is a moth of the Erebidae family. It was described by Burmeister in 1876 as widespread throughout the Argentinean provinces of Buenos Aires, Córdoba, Entre Ríos, La Rioja, Misiones, Santiago del Estero, and Tucumán. The species is diurnal, and the larvae feed mostly on Asteraceae plants. *E. patagiata* is a secondary pest of crops of lettuce, *Lactuca sativa* L., and chicory, *Cichorium intybus* L., grown by organic farmers at Santiago del Estero. Despite wide distribution and the great diversity of climates the moths survive, their pheromones have not been identified.

To contribute to the knowledge of chemical communication of the Erebidae family, composition of pheromones in glands of female *E. patagiata* was studied. Bioassays were used to demonstrate the biological significance of the identified compounds and the activity of synthetic homologs in field experiments.

Materials and Methods

Laboratory colonies of *E. patagiata* were established from larvae collected in May 2014 at Santiago del Estero, Argentina (27°52'21.4"S 64°14'34.8"W). Larvae were reared until pupation on leaves of *Porophyllum ruderale* (Jaqc.) Cass., locally known as Quirquiña. Males and females were individually separated into 200-ml plastic cups containing a piece of cotton moistened with sucrose solution (2% v/v). Insects were kept at 26 ± 2°C and a photoperiod of 12:12 light:dark hours.

Calling and mating activities of virgin female and male moths (1- or 2-day-old) were observed. For observations of calling, females were individually confined with a supply of water in 90-ml plastic cups. Four male and female moths were released into a cage, and the occurrence of mating was observed for 1 hour (N = 10). Pairs that mated were promptly removed from the cages, and the moths were studied for an entire 24-hour cycle.

To extract sex pheromones, the abdominal tip of 2-day-old virgin female *E. patagiata*, including the pheromone gland, were cut during calling and immersed in tri-distilled hexane (10 µl/tip per 20 minutes), and organic layers were placed into 1.5-ml vials (Thermo Scientific, Buenos Aires, Argentina). The pheromone extracts were stored in sealed flasks at -20°C in a freezer until analysis.

Extracts were analyzed by using a Thermo Scientific Focus gas chromatograph coupled with DSQII electron ionization mass detector. The gas chromatograph was operated in the splitless mode. A TR-5MS (30 m x 0.25 mm x 0.25 µm) (Thermo Fisher Scientific) capillary column was used with the following analytical conditions: the initial column temperature was 50°C for 5 minutes, increased at a rate of 7°C minute^{-1} to a final temperature of 250°C, and held for 10 minutes. Helium was used as a carrier in a constant flow of 1 ml per minute. A chromatograph Konik 3000 series equipped with a ZB-FFAP (30 m x 0.25 mm x 0.25 µm) capillary column (Phenomenex) coupled to a flame ionization detector

also was used. The column oven was kept at 50°C for 4 minutes, increased by 7°C minute^{-1} to 250°C, and maintained for 10 minutes. Nitrogen was used as a carrier. The use of two types of columns allowed comparison of the retention indices of the pheromone and chemical standard in two phases to assess that both molecules were the same.

The concentration of pheromones was determined by using an internal standard method with synthetic samples of linolenal, linolenol, and heneicosane as reference compounds. The reactant methyl linolenate (assay grade, 99% purity) was purchased from Sigma-Aldrich, Buenos Aires, Argentina.

Pheromone synthesis was by the methodology of Henrick (1977) (Fig. 1). Reduction of linolenic acid methyl ester with lithium aluminum hydride ($LiAlH_4$, Sigma-Aldrich) in dry diethyl ether at room temperature produced a 90% yield of corresponding linolenol. The resulting linolenol was oxidized with pyridinium chlorochromate (Sigma-Aldrich) in dry CH_2Cl_2 at room temperature to produce a 75% yield of linolenal.

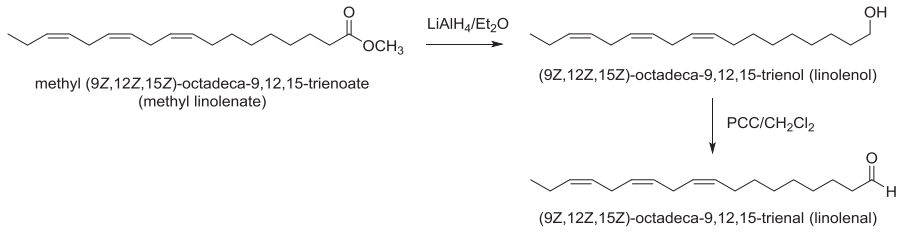

Fig. 1. Pheromones synthesized.

Chemical derivatizations were used to characterize the presence of functional groups in the isolated molecule (Attygalle 1998). An aliquot of 50 µl of crude hexane extract from the pheromone glands of females was placed into a screw-capped glass vial, and 0.5 mg of 10% Pd/C (Sigma-Aldrich) was added. A balloon filled with hydrogen was attached to the vial, and, after flushing, the mixture was stirred for 8 hours at room temperature. The mixture was filtered through a cotton plug in a Pasteur pipette and analyzed by gas chromatograph-mass spectrometry (Favaro et al. 2012).

To 50 µl of crude hexane extract in a screw-capped glass vial, 1 mg of lithium and aluminum tetrahydride (Sigma-Aldrich) was added. After stirring for 3 hours at room temperature, 50 µl of water was added to the mixture. The mixture was filtered over anhydrous magnesium sulfate and analyzed by gas chromatography-mass spectrometry.

To 50 µl of crude hexane extract in a screw-capped glass vial, 100 µl of a dichloromethane suspension of PCC (1 mg/ml) was added. After stirring for 3 hours at room temperature, the mixture was filtered through silica gel and analyzed by gas chromatography-mass spectrometry.

The behavioral response of 2-day-old males to *E. patagiata* pheromones was studied in a binary-choice Y-tube glass olfactometer (4-cm diameter, 40 cm long, with 20-cm-long arms) operated at 2.5-liter minute^{-1} flow of humidified and charcoal-filtered air (Rodriguez et al. 2016). Odor sources were placed at the base of the arms of the olfactometer and consisted of a 2 x 2-cm piece of filter paper

coated with synthetic pheromone or hexane as a check. Tested were: 1) synthetic standard linolenal (1 ng. ml^{-1}) versus hexane, 2) synthetic standard linolenol (1 ng ml^{-1}) versus hexane, and 3) mixture of linolenal and linolenol (1 ng ml^{-1} of each compound) versus hexane. A single male moth was placed at the base of the main olfactometer tube, and its behavior was observed for 20 minutes (N = 40). A positive response was defined as an insect walking against the airflow and more than 5 cm toward an odor source in an arm and remaining there for more than 2 minutes. No response was defined when the insect did not leave the main tube. Each insect was counted as one data point, and each was tested only once. The odor source was replaced after each test. Insects that did not choose either of the arms were excluded from statistical analysis. The olfactometer was moved (turned) after every test to cancel positional effects. Data on frequencies of choices were analyzed using a Chi-square test (contingency tables) implemented in the statistical InfoStat (2012) package. The experiment was done at 26 ± 2°C during the last 2 hours of photoperiod (the hours were recognized as the most mating activity observed in laboratory conditions).

Experiments also were done in the field. One milligram of a mixture of two pheromone components (two-component lure) dissolved in 100 µl of hexane was applied to a red rubber septum (8 mm OD, Aldrich Chemical Co.) not coated with any antioxidants or stabilizers. After vaporization of the solvent, the septum was placed into a trap made of a 3-liter soda bottle with two windows (5 x 5 cm) at the top of the bottle. The trap contained 200 ml of a mixture of water and detergent to retain the moths captured. For a check, one 2-day-old virgin female was placed inside tulle in the trap.

Each lure was replicated three times, and four extra traps with a septum without chemicals were used as a check. Traps were set 50 cm above the ground to emulate the average height of the flowers that females usually visit. Traps were arranged in a randomized complete block design. The distance between traps within a block was approximately 50 m, and the distance between blocks was approximately 100 m. Moths captured were recorded and removed daily.

The number of males captured in traps was transformed by (x + 0.5)1/2 to normalize the variance before analysis (Zhang and Polavarapu 2003). Treatment means were compared by one-way analysis of variance (ANOVA) followed by Tukey's test for significance at a = 0.05.

Results and Discussion

Because *E. patagiata* is diurnal, calling behavior was studied during the photophase. Mating was observed mostly between the last 2 hours of photophase. For this reason, calling behavior was analyzed in this time range. Females used a rhythmic pumping of their ovipositors to release pheromone in discrete puffs. The curious pheromone release behavior was reported in other erebid females (Conner et al. 1985, Zhang and Polavarapu 2003).

The sex pheromone extracts of *E. patagiata* females revealed two main compounds by gas chromatography-mass spectrometry (Fig. 2). The chemical structures of the pheromones were identified based on fragmentation patterns in gas chromatography-mass spectrometry, retention index, micro-reactions, and comparison with a known standard.

Analysis by gas chromatography-EI-mass-spectrometry suggested the identity of two components: compound 1 (retention time: 31.5 minutes; KI: 2,045;

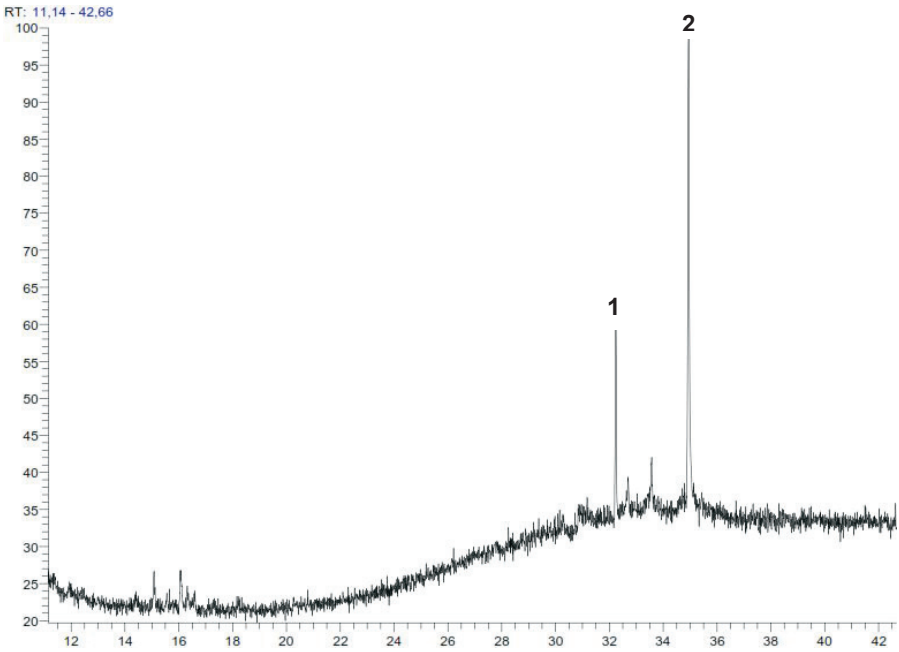

Fig. 2. Gas chromatogram of volatile extracts from pheromone glands of 10 *Eurata patagiata* females, showing the main compounds. Capillary column TR-5MS (30 m x 0.25 mm x 0.25 µm).

m/z (%) = 262 (2), 121 (10), 108 (24), 79 (100), 67 (90), 55 (65), and 42 (96)) and compound 2 (retention time: 34.05 minutes; KI: 2,058; *m/z* (%) = 264 (2), 121 (10), 108 (26), 79 (100), 67 (82), 55 (78), and 41 (79)) as (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienal (linolenal) and (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienol (linolenol), respectively.

After hydrogenation of gland extract, the two compounds disappeared and two new peaks were observed. One corresponded to octadecan-1-ol (KI: 2062), and typical loss of water was observed in its mass spectrum. The other compound was identified as octadecanal (KI: 2,025). The results proved that molecules 1 and 2 each had three saturations and were related to C18 alcohol and C18 aldehyde.

To establish the presence of a terminal aldehyde group in the molecule, the pheromone extract was reduced with lithium aluminum hydride. The peak of compound 1 disappeared with a concomitant area increase in the peak of compound 2. When gland extract was oxidized with PCC, the opposite was observed, peak 2 disappeared and the area of peak 1 increased. Both the reduction and oxidation reactions demonstrated that the only difference between compounds 1 and 2 was the oxidation state. Linolenal and linolenol were synthesized, and the pure compounds were co-injected with the natural extracts. Both peak areas alternatively increased, indicating that synthetic and natural compounds co-eluted.

A pheromone gland of a *E. patagiata* female produced approximately 525 ± 13 ng of 2 and 170 ± 8 ng of 1, in a ratio of 3:1, respectively (N = 3). The linolenic aldehyde (linolenal) has been identified in the sex pheromone gland of several erebid species including red hairy caterpillar, *Amsacta albistriga* (Walker) (Persoons et al. 1993), Bihar hairy moth, *Diacrisia obliqua* (Walker) (Yadav et al. 2001), saltmarsh caterpillar, *Estigmene acrea* (Drury) (Hill and Roelofs 1981), and fall webworm, *Hyphantria cunea* (Drury) (Hill et al. 1982, El-Sayed et al. 2005, Su et al. 2008). Other compounds for *H. cunea* were identified as (9Z,12Z)-octadecadienal,(3Z,6Z,9S,10R)-9,10-epoxy-3,6-heneico-sadiene, (3Z,6Z,9S,10R)-9,10-epoxy-1,3,6-heneicosatriene,(3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-1,3,6-heneicosa-triene, and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-1,3,6-icosatriene (Hill et al. 1982, Toth et al. 1989, El-Sayed et al. 2005, Su et al. 2008). Something similar happened with *D. oblicua* when linolenal was the main pheromone component with Type II epoxides (Persoons et al. 1993, Yadav et al. 2001). Erebiid species use Type II pheromones for the mating call. Similar behavior was found for *E. patagiata*.

To our knowledge, linolenol (linolenic alcohol) is reported here for the first time in the Erebidae family, being reported as pheromone only in the *Bombus* genus (Zacek et al. 2009). Because lepidopterans are thought not to be able to synthesize either linoleic or linolenic acid *de novo* (Blomquist et al. 1991), this seems to be another case where insects metabolize plant-derived precursors to pheromone components. The fatty acid linolenic acid is reduced to alcohol and oxidized to aldehyde by specific enzymes (Fig. 3). These are well-known biosynthetic steps in other moths (Jurenka and Roelofs 1993).

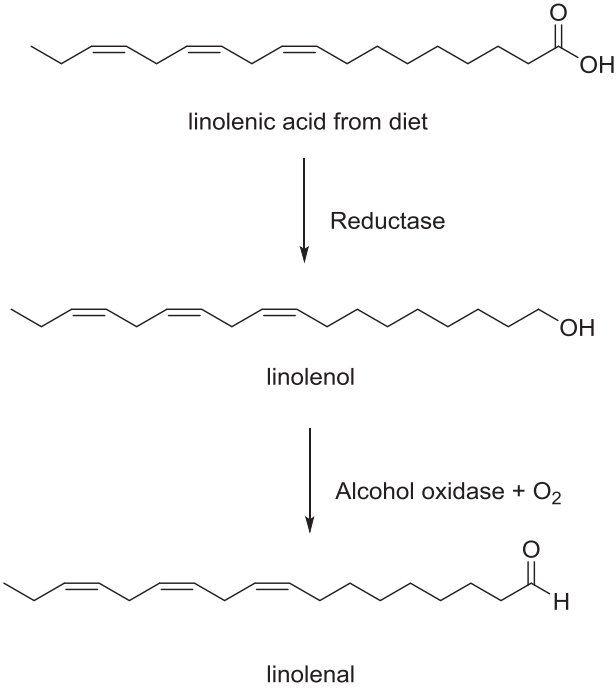

Fig. 3. Biosynthesis of *E. patagiata* pheromones.

Fig. 4 shows behavioral responses of *E. patagiata* males in a dual-choice experiment when stimulated with linolenal, linolenol, and mixture in a 1:3 ratio, using hexane as a check in each experiment. The mixture of 1 + 2 and the single compounds 1 or 2 were more attractive than the check. The mixture and compound 2 elicited positive response by approximately 90% of males. Compound 2 generated a better response than did compound 1 against the check.

On the basis of the results of the intra-laboratory bioassay, field trapping was used to determine the pheromone activity of the identified compounds (Fig. 5). Mixture of linolenal and linolenol (1:3) at a dose of 1 mg per lure was tested in the field when *E. patagiata* was found and was compared with the effect of trapped virgin females as a check. The synthetic mixture of the two compounds lured 5.1 ± 0.6 males per trap, and there was a significant difference ($P < 0.5$) in catches in traps containing virgin females that lured 14.5 ± 0.9 males per trap. No moths were trapped in check traps. The difference in effectiveness was ascribed to the difference between the pulsed aerosol plume produced by females and the plume of vapor emitted by passive pheromone release devices such as rubber septa. The latter did not mimic the variation in volatile concentration gradient in a short period of time, perhaps because of a modulation in the communication systems in the moths to make it more specific. Optimum dose of synthetic pheromone for monitoring *E. patagiata* was not determined and should be the focus of studies to understand the role of the compounds in *E. patagiata* species. Contrary to previous reports for *H. cunea*, linolenal was effective alone (Y-tube olfactometer) or mixed with linolenol in the bioassays (Zhang et al. 1996, Su et al. 2008).

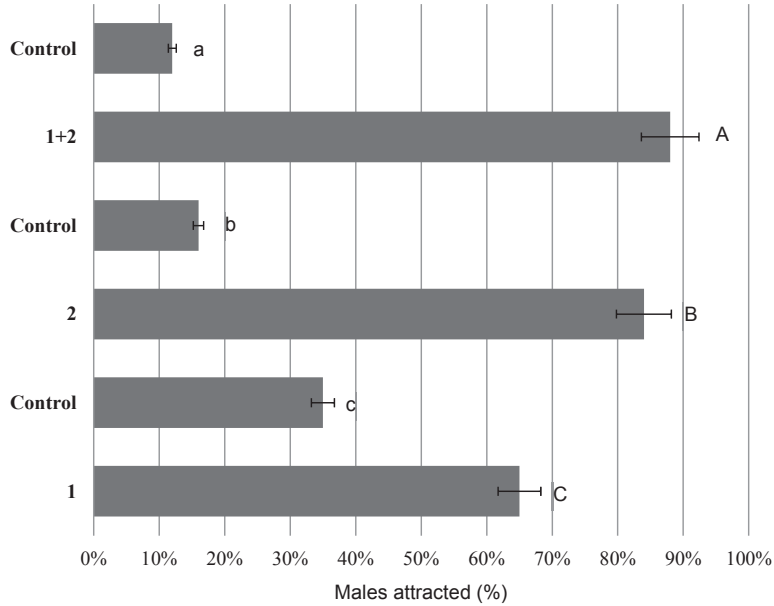

Fig. 4. Preference of *Eurata patagiata* males in a Y-tube olfactometer bioassays for air flow containing synthetic pheromones 1, 2, and a mixture of 1:2, each tested against hexane (check) in three experiments ($N = 33$). Capital letters indicate differences significant from the check (X^2 test, $p < 0.05$).

Fig. 5. Trap with *E. patagiata* males.

In summary, calling behavior of *E. patagiata* females was described. Extracts from the gland at the tip of the abdomen were mostly linolenal and linolenol identified based on mass spectrum, retention indices, micro reactions, and co-injection with synthetic standards. Linolenol was described for the first time as a pheromone component in the erebid family. Results from bioassays identified the compounds that act as female sex pheromones.

Preliminary field trapping showed a mixture of the compounds can be used for monitoring the moth species. In future work, single-compound lures and dose optimization of synthetic pheromones for monitoring *E. patagiata* will be determined.

Acknowledgment

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