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L-LEUCINE METHYL ESTER: THE FEMALE-PRODUCED SEX PHEROMONE OF THE SCARAB BEETLE, *Phyllophaga lanceolata*

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Abstract—The female-produced sex pheromone of the scarab beetle *Phyllophaga lanceolata* was identified as the methyl ester of an essential amino acid, L-leucine. During field testing, 239 male *P. lanceolata* were caught in traps baited with L-leucine methyl ester. L-Isoleucine and L-valine methyl esters, similar in structure to L-leucine methyl ester and previously identified as female-produced sex pheromone compounds employed by other *Phyllophaga* species, were also tested. Addition of L-valine or L-isoleucine methyl esters to the L-leucine methyl ester in 1:1 ratios completely inhibited attraction of *P. lanceolata* males. Males of *P. squamipilosa* were also captured using L-leucine methyl ester. This is the first record of *P. squamipilosa* from Kansas.

Key Words—L-Valine, L-isoleucine, L-leucine methyl ester, sex pheromone, electroantennogram, *Tostegoptera, squamipilosa*, Scarabaeidae, Melolonthinae.

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INTRODUCTION

The genus *Phyllophaga* (*sensu lato*) (Coleoptera: Scarabaeidae: Melolonthinae), encompasses about 200 species in the United States north of Mexico (Woodruff and Beck, 1989). Only two species are found in the subgenus *Tostegoptera*, *P*. (*Tostegoptera*) *lanceolata* and *P*. (*Tostegoptera*) *squamipilosa* Saylor. Whereas *P. lanceolata* was described in 1824, *P. squamipilosa* was not described until 1936 by L.W. Saylor, who discovered the species in a series of *P. lanceolata* from Texas (Saylor, 1936). Nothing is known of the life history or biology of *P. squamipilosa*.

P. lanceolata is unusual among the *Phyllophaga* in that it is diurnal, and the females are flightless (Travis, 1939; Reinhard, 1940). Travis (1939) reported that on days that are "hot and sunny," the greatest number of beetles "are visible between 8 and 10 o'clock in the morning." He further reports that "On many occasions, males were noted feeding, apparently unconcerned, on the same leaf or plant with a female until a female extruded her genitalia. Immediately, males within a radius of 15 to 20 yards flew toward her."

P. lanceolata is distributed throughout the Great Plains including Colorado, Iowa, Kansas, Missouri, Nebraska, Nevada, New Mexico, Oklahoma, South Dakota, Texas, and Wyoming. It has also been reported from the province of Ontario, Canada (Luginbill and Painter, 1953). It has been an important pest species in both the larval and adult stages on wheat, cotton, corn (Hayes, 1919), and sunflowers (Rogers, 1978). Reinhard (1940) listed a series of truck crops on which *P. lanceolata* fed including spinach, lettuce, carrot, cowpea, rape, turnip, mustard, onion, beans, and Irish potato. We present here the identification of the female-produced sex pheromone of *P. lanceolata* and the results of field trapping tests.

METHODS AND MATERIALS

Pheromone Collections. Third-instar larvae of *P. lanceolata* were dug from infested cattle range on a ranch in Kiowa County, Kansas, in late April 2000. Larvae were individually housed in ~30-ml plastic cups in a 3:1 mix of greenhouse sand and screened peat moss raised to about 12% moisture. The cups were housed in a controlled environment room maintained at 25°C during the 16-hr photophase and 20°C during the 8-hr scotophase. After pupation and adult emergence, females were placed in observation cages. When females were observed calling (abdominal pheromone gland everted, see Figure 1) (Leal et al., 1993) during the photophase, they were removed from the cages, and the glands were excised and soaked in 200 μl of dichloromethane. After 20 min, the glands were removed and the extract concentrated under a nitrogen stream to a volume of about 20 μl.

GC-EAD Analysis. GC-EAD instrumentation used in this study was the same as described previously (Nojima et al., 2003), except for using an acrylic holder

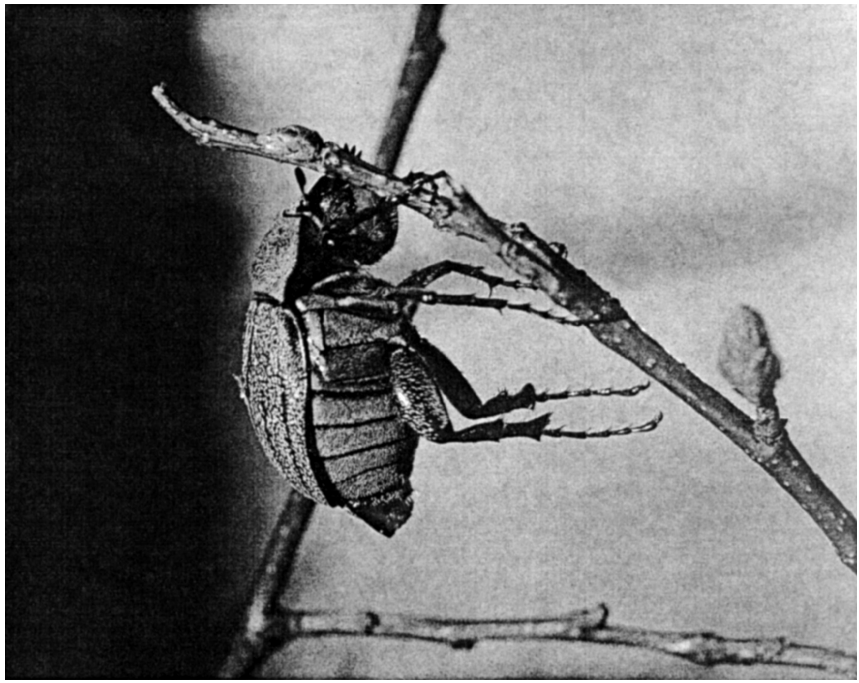


FIG. 1. Female *P. lanceolata* in calling posture with sex pheromone gland everted.

designed for a beetle's antenna. A nonpolar SPB-1 capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, PA) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Alltech, Deerfield, IL) was used for GC-EAD analyses. Nitrogen was used as carrier gas (flow 2.0 ml/min). The GC was programmed for 40°C for 5 min, then 15°C/min to 250°C and held for 10 min. Injector and detector temperatures were 260°C. A β-DEX 120 chiral capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, PA) was used for resolution of enantiomers, with N_2 carrier gas (flow 2.0 ml/min). The GC program was 40°C for 5 min, 4°C/min to 130°C, then 10°C/min to 220°C and held for 20 min. Injector and detector temperatures were 260°C. Six individual virgin female gland extracts were subjected to GC-EAD analysis, and antennae from four different males were used for EAD recordings.

Chemical Analysis. GC-MS analysis was performed on a Shimadzu QP 5050A equipped with a nonpolar DB-1ms capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness; J&W Scientific, Folsom, CA) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25-μm film thickness;

Alltech, Deerfield, IL) in EI mode (70 eV) and the same conditions as those of GC-EAD analyses, but with helium as the carrier gas at constant flow (1.0 ml/min). The EAD active compound was tentatively identified by mass spectral matches to library spectra. The identity was confirmed by comparing GC retention times, mass spectra, and EAD activity with those of an authentic sample.

Chemicals. L-Leucine, L-valine, and L-isoleucine methyl esters were obtained as hydrochloric acid salts (Aldrich, Milwaukee, WI). Free amino acid methyl esters were regenerated by dissolving the salts in an aqueous alkali solution and extracting the mixture with three portions of ether. Ether layers were combined, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The resulting methyl esters were used without further purification. D-Leucine methyl ester was prepared by methyl esterification of D-Leucine (Aldrich, Milwaukee, WI) according to Anand and Vimal (1998).

Preparation and Field Evaluation of Synthetic Lures. Lures were formulated by dissolving the neat compounds in hexane (20 μg/μl), dispensing appropriate amounts into 5-mm rubber septa (Thomas Scientific, Swedesboro, NJ), and allowing the hexane to evaporate in a fume hood. Lures were deployed in the field in lab-constructed cross-vane traps.

The six treatments tested included the methyl esters of L-leucine, L-isoleucine, and L-valine, each tested alone at 4 mg/septum, L-leucine + L-isoleucine (1:1) at 4 mg each/septum, L-leucine + L-valine (1:1) at 4 mg each/septum, and a solvent only control.

L-Isoleucine and L-valine methyl esters have been reported as sex pheromone components for other *Phyllophaga* species (Zhang et al., 1997; Leal et al., 2003). They were included in the field test because when antennae of *P. lanceolata* males were challenged with these two compounds in GC-EAD assays, they elicited antennal responses similar to those produced by the L-leucine methyl ester (Figure 2B).

Three replicates of the six treatments were deployed June 1, 2001, on cattle range at the ranch from which the larvae had been obtained and where adult activity had been observed in previous years. Each replicate of the six treatments was deployed in a line with traps placed about 15 m apart. Traps were hung on metal stakes such that the bottom of the trap touched the ground. Trap positions within a line were randomized at initial placement and rerandomized each time they were checked. Traps were checked nine times between June 2 and June 16, when the traps were removed from the field. The pooled data were log transformed $(x + 1)$ to insure homogeneity of variance before calculating one sample t confidence intervals (0.95) for those treatments that had catches greater than 0.

RESULTS AND DISCUSSION

Pheromone Identification. GC-EAD analyses of pheromone gland extracts of female *P. lanceolata* using both nonpolar and polar columns consistently revealed

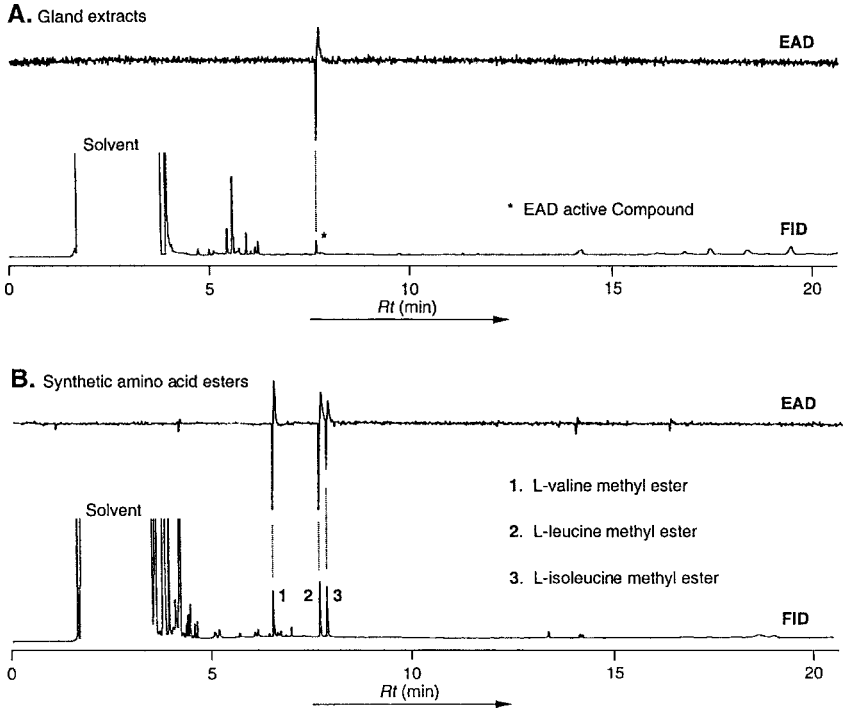


FIG. 2. GC-EAD traces using the antennae of male *P. lanceolata* stimulated with the female pheromone gland extracts (A) and synthetic amino acid methyl esters (B) on a nonpolar capillary column.

one EAD active compound (Figure 2A). The mass spectrum of the compound matched that of leucine methyl ester. The identity was confirmed by comparing GC retention times and MS spectra with those of authentic samples. The chirality of the leucine methyl ester was confirmed by means of a chiral GC-EAD analysis. When run on a chiral column, authentic L-leucine methyl ester had a retention time (32.43 min) and EAD activity similar to that of the natural compound (32.48 min), whereas the D-leucine methyl ester did not (31.98 min).

Field Evaluation of Synthetic Lures. Of the 240 male *P. lanceolata* captured in field testing of the compounds, 239 were captured in the L-leucine methyl ester baited traps (Table 1). No female beetles were taken in the traps. We conclude that L-leucine methyl ester is the sex pheromone of *P. lanceolata*. The four treatments that compared the L-isoleucine and L-valine methyl esters, each alone and each in combination with the L-leucine methyl ester, yielded only a single male *P. lanceolata* that was captured in a trap baited with L-valine methyl ester. The confidence intervals calculated for the two treatments that caught beetles indicated the catch

TABLE 1. AVERAGE NUMBER OF MALES CAPTURES/TREATMENT (±SE)

L-Leucine	L-Isoleucine	L-Valine	L-Leucine + L-Isoleucine	L-Leucine + L-Valine	Solvent control
79.7 ± 18.6	0[a]	0.33 ± 0.33[a]	0[a]	0[a]	0[a]

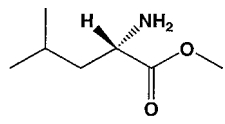
[a] Not significantly different, one sample *t* confidence interval, 95%.

from the traps baited with the L-valine methyl ester (a single beetle) were not significantly different from those traps that captured no beetles (Table 1), whereas the catches from the L-leucine methyl ester baited traps were different. L-Valine and L-isoleucine methyl esters are clearly nonattractive to the males when presented alone and demonstrated antagonistic effects when presented in combination with L-leucine methyl ester, the sex pheromone of *P. lanceolata.*

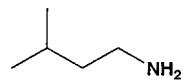
In light of the identification of L-leucine methyl ester as the sex pheromone of *P. lanceolata*, the work of Travis (1939) is of particular note. In that publication, Travis reported results of field tests of a series of 24 volatile compounds assessing whether they would attract *P. lanceolata* adults. One of the compounds tested was isoamylamine. Travis states.

> The last named (isoamylamine) was the only one of these compounds that proved attractive to the males, and none of them seemed to stimulate the females. Isoamylamine produced a male response similar to that made to crushed or to sexually active females. The crushed and receptive females appeared to produce an emanation or odor that was rapidly disseminated by air movement, as male activity was always greater on the leeward side of the female. The same type of response was secured when isoamylamine was released.

A comparison of the structures of L-leucine methyl ester, the authentic pheromone, and isoamylamine (3-methyl-butylamine), (Figure 3) demonstrates that the work of Travis presaged the identification of L-leucine methyl ester as the sex pheromone of *P. lanceolata*. Future field tests will compare the relative attractancy of the methyl ester of L-leucine and isoamylamine to *P. lanceolata* males.



L -leucine methyl ester



isoamylamine

FIG. 3. Structures of L-leucine methyl ester and isoamylamine.

Of additional interest is the capture of *P. squamipilosa* using L-leucine methyl ester. In an unreplicated test in Stevens County in southwestern Kansas in mid-June of 2001 (G.A.S., personal observation), a septum loaded with 4 mg of L-leucine methyl ester was hung (using a piece of wire) in low brush in grassy rangeland habitat. No trap was used. Beetles were captured both crawling on the septum as well as while flying upwind in the pheromone plume. The 16 male beetles captured were subsequently identified as *P. squamipilosa.* This is the first record of *P. squamipilosa* from the state of Kansas. Very little is known regarding the degree of overlap in the ranges of *P. lanceolata* and *P. squamipilosa*, and nothing is known about their interspecific relationships where they occur sympatrically.

Methyl esters of essential amino acids are employed as sex pheromones in the genus *Phyllophaga* (Zhang et al., 1997; Leal et al., 2003) as well as the closely related genus *Holotrichia* (Leal et al., 1992). They are termed essential amino acids because they are generally required for growth and development by insects and other animals and cannot be synthesized *de novo* (McFarlane, 1985). Therefore, prior to utilization of these compounds as sex pheromones (subsequent to methyl esterification) they must be sequestered from plants or produced by endosymbionts. Virgin female *P. anxia* (LeConte) individuals are capable of emitting L-valine and L-isoleucine methyl esters before any adult feeding has taken place (P.S.R., unpublished data). Furthermore, the sex pheromone of *Costelytra zealandica* (White), another melolonthine scarab beetle, identified as phenol (Henzell and Lowe, 1970), was later found to be produced by bacterial endosymbionts (Hoyt and Osborne, 1971). Further work is planned to identify the source of the essential amino acids used by these *Phyllophaga* species to make sex pheromone compounds.

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