SEX ATTRACTANT PHEROMONE FROM THE NEOTROPICAL RED-SHOULDERED STINK BUG, *Thyanta perditor* (F.)

MARIA C. B. MORAES,¹ JOCELYN G. MILLAR,² RAUL A. LAUMANN,¹ EDISON R. SUJII,¹ CARMEN S. S. PIRES,¹ and MIGUEL BORGES^{1,*}

¹Embrapa Recursos Genéticos e Biotecnología, 02372, CEP 70849-970, Brasília, DF, Brazil ²Department of Entomology, University of California, Riverside, CA 92521, USA

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Abstract—Olfactometer bioassays showed that odors from mature *Thyanta perditor* males attracted females but not males. Furthermore, odors from females did not attract either sex, indicating that like other phytophagous pentatomid bugs, the males produce a sex pheromone. Attraction appeared to peak in late afternoon to evening. The headspace volatiles collected from male and female *T. perditor* were analyzed by GC-MS and HPLC. A male-specific compound, methyl (2E,4Z,6Z)-decatrienoate (2E,4Z,6Z-10:COOMe), was identified along with a number of other compounds found in extracts from both sexes. Bioassays carried out with 2E,4Z,6Z-10:COOMe showed it was as attractive to females as the crude extract of male volatiles, suggesting that the male-produced sex pheromone consists of 2E,4Z,6Z-10:COOMe as a single component. Consecutive volatiles collections from males showed that 2E,4Z,6Z-10:COOMe began appearing in extracts from males about 9 d after the final molt, as the males became sexually mature.

Key Words—Sex pheromone, methyl (2*E*,4*Z*,6*Z*)-decatrienoate, defensive compounds.

INTRODUCTION

Soybean, *Glycine max* (L.) Merrill, an important food and forage crop, is susceptible to attack by a variety of phytophagous Hemiptera (Turnipseed and Kogan, 1976; Kogan and Turnipseed, 1987; Jackai et al., 1990; Sediyama et al.,

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^{*} To whom correspondence should be addressed. E-mail: mborges@cenargen.embrapa.br

1993). Soybean is a relatively new crop in the tropics, but the endemic insect fauna have adapted rapidly to feeding on this plant (Panizzi and Corrêa-Ferreira, 1997). In Brazil, more than 30 species of hemipterans are associated with the soybean crop, with several reaching significant pest status (Panizzi and Corrêa-Ferreira, 1997). The most damaging include *Nezara viridula* (L.), *Euschistus heros* (F.), and *Piezodorus guildinii* (Westwood) (Panizzi and Slansky, 1985; Panizzi and Rossi, 1991), whereas others such as *Acrosternum aseadum* (Rolston) and *Thyanta perditor* (F.) are of lesser importance (Panizzi and Slansky, 1985). More than 4×10^6 l of insecticides are used annually to control stink bugs on soybean in Brazil (Corrêa-Ferreira and Moscardi, 1996). Alternatives, such as the exploitation of semiochemicals in the monitoring and control of these pests, might contribute to a more ecologically benign approach to their management.

Male-produced sex or aggregation pheromones have been identified for several species in the sovbean stink bug complex in the last two decades (Aldrich et al., 1987, 1991, 1993, 1994; Baker et al., 1987; Borges et al., 1987, 1998a,b; Borges and Aldrich, 1994; Borges, 1995; Zhang et al., 2003). Two nearctic Thyanta species, T. pallidovirens and T. custator accerra (Millar, 1997; McBrien et al., 2002), use methyl (2E, 4Z, 6Z)-decatrienoate (2E,4Z,6Z-10:COOMe) and the three sesquiterpenes $(+)-\alpha$ -curcumene, (-)-zingiberene, and (-)- β -sesquiphellandrene as a male-produced sex pheromone blend. The congeneric neotropical species, T. perditor, is part of the stink bug complex attacking soybeans in Brazil, as well as other crops such as wheat and sorghum (Gomez, 1980; Grazia et al., 1982; Busoli et al., 1984; Panizzi and Corrêa-Ferreira, 1997). As part of our ongoing studies of semiochemicals of economically important stink bugs in Brazil, we initiated an investigation of the pheromone of this species. Our specific objectives were (1) to determine whether the stink bug T. perditor produces a sex pheromone, similar to those of its two nearctic congeners, T. custator accerra and T. pallidovirens, and if so, (2) to identify the chemical structure(s) of the biologically active compound(s). During these investigations, we also identified several defensive compounds from adult bugs of both sexes.

METHODS AND MATERIALS

Insects. T. perditor individuals were obtained from a laboratory colony started from adults collected from soybean fields during 2002/2003 near Embrapa Genetic Resources and Biotechnology Laboratory in Brasilia, DF, Brazil ($15^{\circ}47'$ S, $47^{\circ}55'$ W). They were reared in 5-1 containers on raw peanuts (*Arachis hypogaea*) and soybean (*Glycine max*) seeds, fresh green beans (*Phaseolus vulgaris*), boldo (*Plectranthus barbatus*) stalks, and water, at 26 ±

 1.0° C and $65\% \pm 10\%$ relative humidity under a 14:10 light/dark photoperiod (from 0600 to 2000 hr). The food supply was renewed three times per week. A square of plastic mesh (~40 mesh, 15-cm²) was placed against an inner wall of each container as an oviposition substrate and shelter for the bugs. Egg masses were collected daily and incubated in 9-cm ID plastic Petri dishes until they hatched. When the resulting nymphs molted into second instars, they were transferred to plastic containers and reared as above.

To prevent interactions between the sexes, males were separated from females after cuticular hardening, ca. 24 hr after their imaginal molt. For all experimental bioassays, sexually mature adults, 9 d after the final molt, were used.

Collection of Volatiles. Volatiles were collected from groups of 20 male or female *T. perditor* (N = 13) in 1-1 glass containers (Zhang et al., 1994) by drawing air through 6-14-mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out through two traps (15 cm \times 1.5-cm OD) containing Super Q (200 mg each; Alltech Associates Inc., Deerfield, IL, USA) by vacuum (\sim 1 l/min). Two traps in series were used to check for breakthrough of the volatiles being collected. Collection of volatiles from male and female bugs was started when they were 5 d old and bugs were aerated continuously for 15 d, changing adsorbent traps daily. This provided a continuous series of extracts reflecting the volatiles produced by adult bugs both before and after sexual maturation. Bugs in aeration chambers were fed with fresh green beans (replaced weekly). The adsorbent traps were combined and stored at -30° C until needed for analysis or bioassays.

Additional material for bioassays and analysis were obtained from aerations of groups of approximately 80 sexually mature, virgin individuals of each sex (N = 20) for periods of 15 consecutive d. The resulting daily extracts were concentrated under N₂ to yield a concentration of approximately 0.1 bug day equivalent (B.E.)/µl of solution for bioassays. For quantitative analysis, the volume of the extract was reduced to ~200 µl and an internal standard [40 ng (*E*)-2-decenyl acetate in 40 µl of hexane] was added to the solution.

Analysis and Fractionation of Extracts. The total and fractionated extracts were analyzed by gas chromatography (Perkin-Elmer GC; DB-1 column, 30 m \times 0.25 mm ID, 0.25 µm film; J&W Scientific, Folsom, CA, USA), using a temperature program of 50°C/2 min, 15°C/min to 250°C. Data were collected with Autosystem Software in ASCII format and processed using Origin 5.0 (Microcal Software). Mass spectra were obtained with a Varian Saturn 2000 mass spectrometer interfaced to a Varian 3800 GC (DB-5 column, 30 m \times 0.25 mm ID, 0.25 µm film; J&W Scientific). Crude extracts were also analyzed using a DB-5 column (30 m \times 0.25 mm ID, 0.25 µm film thickness) at the University of California, Riverside, on a Hewlett-Packard 6890 GC interfaced to an H-P

5973 mass selective detector, using electron impact ionization (70 eV). The temperature program was 40° C/1 min, 10° /min to 250° C, injector temperature 250° C, and transfer line 280° C.

An extract of 80 mature virgin males in 1 ml of hexane was fractionated as described by McBrien et al. (2002). A column (0.5 cm ID \times 6.0 cm long) with 500 mg of silica gel (80/100 mesh, Merck, Germany) was conditioned by eluting with 6 ml 25% ether in hexane followed by 6 ml hexane. The extract was loaded onto the column, which was then eluted sequentially with 3 \times 2 ml hexane, and 3 \times 2 ml ether, collecting each aliquot as a separate fraction. Hydrocarbons eluted in the first hexane fraction, whereas more polar esters, alcohols, and other compounds eluted in the first ether fraction.

A thermally unstable compound was previously found in the pheromone blend of the nearctic species *T. pallidovirens* (Millar, 1997), and so a crude extract from aeration of male *T. perditor* was analyzed by high-pressure liquid chromatography (HPLC) (Shimadzu) using a reverse-phase column (Lichrosorb RP-18, 250 × 4.6 mm, 5 μ m particle size) eluted with 1:1:1 CH₃CN:CH₃OH:H₂O. The eluent was monitored at 300 nm with a diode array detector.

To determine the number of double bonds in the thermally unstable malespecific compound, a 20- μ l aliquot of the crude extract in hexane was reduced in an H₂ atmosphere with 10% Pd on activated charcoal catalyst in a conical vial, stirring for 1 hr. The resulting mixture was filtered through a 1-cm plug of cellite, rinsing with pentane. The filtrate was analyzed by GC-MS.

Some of the insect-produced components from both sexes of *T. perditor* were tentatively identified by comparison of their GC retention times and mass spectral data with library data (NIST-Wiley database, 2000) with those of authentic standards. The only exception was α -ocimene, for which a standard was not available. All standards were obtained from Aldrich Chemical Co., with the exception of undecane (Matheson, Coleman, and Bell, Cincinatti, OH, USA), and tridecane and pentadecane (K&K Laboratories, Cleveland, OH, USA). The identification of the *T. perditor* male-specific ester was confirmed by comparison with authentic standards of 2*E*,4*Z*,6*Z*-10:COOMe and its isomers, available from previous studies on *T. pallidovirens* (Millar, 1997; McBrien et al., 2002).

Olfactometer Bioassays. Bioassays were carried out using a Y-shaped olfactometer, modified from Collaza et al. (1999) and consisting of an acrylic block ($27.5 \times 21.2 \times 1.5$ cm) in which a 2.0-cm-wide Y-shaped slot had been milled. The two arms of the Y were 10.3 cm long with a 45° angle between them. A 4.0-cm-diameter circle was cut out at the end of the 11.5-cm-long third leg of the olfactometer as a release chamber for the bug to be tested. A hole (0.5 cm ID) was drilled through the end of the slab into each arm to allow attachment of air lines. The end of the release chamber was perforated with two

holes, one of 0.5-cm diameter as an air outlet, and another in a rectangular shape $(1.7 \times 1.2 \text{ cm})$ to introduce test insects into the release chamber. The olfactometer block was placed on top of a translucent glass plate, and the upper surface was covered with transparent glass, with the glass plates held in place with large paper clips. Charcoal-filtered, humidified air was drawn through the system at a flow rate of 800 ml/min (regulated by flow meters, Accura Flow Products Div., Advanced Controls Engineering Corp., Hatboro, PA, USA). The olfactometer was surrounded with a black curtain to exclude external light and was illuminated from below by infrared lamps (homogenous emission of light at 950 nm provided by a panel of 108 light-emitting diodes) and from above with four 40-W fluorescent lamps. The temperature in the bioassay room was maintained at 26.0 \pm 1.0°C. The insect behavior was monitored from above

using a monochrome CCD video camera (Sony SPT M324CE) fitted with a 12.5–75 mm/F1.8 zoom lens interfaced to a personal computer. A video frame grabber digitized analog video signals from the camera, and "Xbug" software (Colazza et al., 1999) was used to process the data.

A single *T. perditor* was gently introduced into the release chamber of the Y tube with the aid of a artist's paint brush (Camel Hair, number 1) and allowed to acclimatize for a short period (ca. 3 min). Then it's pattern of movement (response) was recorded for 15 min/replicate. The first choice of the insect was recorded, i.e., the arm of the olfactometer into which a bug walked up at least 5 cm past the Y junction and remained there for at least 100 sec. The positions of control and treatment arms were alternated every five replicates. The chambers with the treatments consisted of two 10-ml syringes, one each for treatment and control, and were connected to the upwind side of the treatment and control arms, respectively. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried after each five replicates.

Determination of the Pheromone-Producing Sex. The attraction of mature adults of both sexes to odors emitted by five live males or females was tested against a clean air control. The live insects used as an odor source were changed each 10 replicates.

Determination of Period of Maximum Activity. Bioassays were carried out to determine the period of maximum pheromone-mediated activity, using five live male bugs as a pheromone source, with individual, sexually mature, female bugs as the test subjects. The males were replaced every 10 replicates. For this experiment, the following periods were designated: morning (M, 0800–1100 hr), early afternoon (EA, 1200–1500 hr), late afternoon (LA, 1600–1800 hr), and evening (E, 1900–2100 hr). A minimum of 74 individuals were tested during each time period and the number attracted to the pheromone source was recorded.

Bioassays with Insect Extracts and Synthetic Compounds. The Y-tube bioassay was used to compare the biological activity of crude male extracts,

fractions of extracts, and synthesized standards, with the solution of test stimulus [one bug day equivalent (BE) of extract or fraction] being spotted on a strip of filter paper (1.5×0.5 cm). Controls consisted of filter papers treated with hexane. Only sexually mature virgin females (>9 d old) were tested in these bioassays, which were carried out during the late afternoon and evening period.

Thirty bioassays were carried out with a crude extract obtained from a 24hr aeration of 20 male bugs. The extract contained approximately 4 μ g of 2*E*,4*Z*,6*Z*-10:COOMe. Hexane and ether fractions of an extract from males were tested vs. a hexane control (*N* = 16), and 20 and 14 replicates of the ether and hexane fractions, respectively, were assayed vs. the crude male extract. The ether fraction was also compared to the hexane fraction (*N* = 34). Finally, bioassays tested the responses of *T. perditor* females to synthetic 2*E*,4*Z*,6*Z*-10:COOMe vs. a hexane control (*N* = 45).

Statistical Analyses. Data from experiments comparing females' responses during different times of the day were analyzed using Kruskal–Wallis ANOVA and Dunn's test. Data on the number of responses of males and females to different treatments (live insects, aeration extracts and fractions thereof, and synthetic compounds, vs. controls) were analyzed using chi-square tests. Statistical analyses were carried out using MathCAD 2001.

RESULTS

Bioassays with Live Insects. Female bugs were attracted to odors from live mature males ($\chi^2 = 18$, N = 32, P < 0.001) but not to those of mature females ($\chi^2 = 0.25$, N = 20, P = 0.62) (Figure 1). Males were not attracted to odors of either sex (male: $\chi^2 = 0.29$, N = 20, P = 0.58; female $\chi^2 = 1.32$, N = 20, P = 0.25) (Figure 1). The attraction of females to males was highest in the afternoon/evening (H = 8.60, df = 3, P = 0.035; Dunn's test, P < 0.05) (Figure 2), indicating that females were most responsive and/or that production of pheromone by males was highest during this period.

Chemical Analyses of Insect Extracts. Comparisons of extracts from both sexes by GC and GC-MS analyses showed one large male-specific compound that was only seen in sexually mature individuals and was produced at a rate of about 200 ng/d, in addition to several compounds that were common to both sexes (Figure 3). Most of the components in the extracts were identified as α -pinene (1), β -pinene (2), α -ocimene (3), (*E*)-2-octenal (4), (*E*)-2-octen-1-ol (5), undecane (6), nonanal (7), dodecane (8), (*E*)-2-decenal (9), tridecane (10), and pentadecane (12). Most of these compounds are typical components of stink bug defensive secretions (e.g., Borges and Aldrich, 1992).



FIG. 1. Proportion of mature *T. perditor* males and females responding in a Y-olfactometer to different treatments vs. solvent controls: (A) Females' response to odors of live males. (B) Females' response to odors of live females. (C) Males' response to odors of live males. (D) Males' response to odors of live females. ***P < 0.001; ns, not significantly different (chi-square test).



FIG. 2. Response of mature *T. perditor* females to odors of live males during different periods of the day in laboratory bioassays. The photophase in the laboratory was divided into four periods: "morning" (M), from 0800 to 1100 hr; "early afternoon" (EA), from 1200 to 1500 hr; "late afternoon" (LA), from 1600 to 1800 hr; and "evening" (E), from 1900 to 2100 hr. Response bars followed by the same letters are not statistically different (Kruskal–Wallis ANOVA and Dunn's test, P < 0.05).



FIG. 3. Gas chromatograms of volatiles collected from live male (top) and female (bottom, inverted) *T. perditor*: 1, α -pinene; 2, β -pinene; 3, α -ocimene; 4, (*E*)-2-octenal; 5, (*E*)-2-octen-1-ol; 6, undecane; 7, nonanal; 8, dodecane; 9, (*E*)-2-decenal; 10, tridecane; 11, distorted peak from thermal decomposition of methyl (2*E*,4*Z*,6*Z*)-decatrienoate; 12, pentadecane.

The male-specific compound (11) gave a broad, irregularly shaped peak, suggestive of a thermally unstable compound (Figure 3). The mass spectrum of the unknown showed a distinct molecular ion of m/z 180 (M⁺) and fragments at [m/z (abundance)] 149 (12), 133 (5), 120 (20), 105 (70), 91 (100), 79 (60), 74 (16), 65 (16), 39 (28) (Figure 4). The presence of fragments at m/z 105 and 74 suggested McLafferty rearrangement of a methyl ester and the fragments at m/z148 and 149 (M⁺-32, loss of CH₃OH, and M⁺-31, loss of CH₃O, respectively) also indicated a methyl ester (Figure 4). The relatively low intensity of the m/z74 ion suggested that the compound might be unsaturated at C_{α} and C_{β} or C_{γ} and C_{δ} . The molecular weight of 180 amu and the presence of a methyl ester resulted in a possible molecular formula of C₁₁H₁₆O₂, corresponding to three double bond equivalents once the ester carbonyl was accounted for. Experiments were carried out to determine the number, position, and stereochemistry of the double bonds. First, an aliquot of the crude extract was hydrogenated, resulting in the disappearance of the irregularly shaped, male-specific peak, and the appearance of a new peak, readily identified as methyl decanoate $(m/z \ 186$ amu) by comparison with an authentic standard, confirming that the insectproduced compound consisted of an unbranched chain with three carbon-carbon double bonds. HPLC analysis of the crude extract using a diode array detector showed that the unknown peak with m/z 180 amu comprised a single component with a UV absorption maximum at 300 nm, with the position of the absorbance proving that all three double bonds had to be conjugated with the ester carbonyl



FIG. 4. Mass spectra of the naturally occurring ester in the male volatiles (top) and a synthetic standard of methyl (2E,4Z,6Z)-decatrienoate (bottom).

(Millar, 1997), that is, a methyl 2,4,6-decatrienoate isomer. Comparison of the GC retention time and peak shape, and the mass spectrum (Figure 4) of the unknown peak with those of the (2E,4E,6E)-, (2E,4Z,6E)-, (2E,4E,6Z)-, and (2E,4Z,6Z)-isomers of methyl (2,4,6)-decatrienoate resulted in matches only with the last compound. Furthermore, 2E,4Z,6Z-10:COOMe was the only isomer that was thermally unstable, apparently rearranging upon heating via a 1,7 sigmatropic hydrogen shift (Millar, 1997). Thus, the male-produced compound was identified as 2E,4Z,6Z-10:COOMe, identical to the compound produced by the congeneric *T. pallidovirens* (Millar, 1997; McBrien et al., 2002).

Bioassays with Insect Extracts. T. perditor females chose the treatment arm with the crude extract of males significantly more often than the hexane control arm ($\chi^2 = 6.13$, N = 30, P = 0.013) (Figure 5A). Whereas the hexane fraction was no more attractive than the hexane control ($\chi^2 = 0.11$, N = 16, P =0.74) (Figure 5B), the ether fraction containing 2E,4Z,6Z-10:COOMe was ($\chi^2 =$ 3.84, N = 16, P = 0.045) (Figure 5C). The ether fraction was more attractive than the hexane one ($\chi^2 = 5.76$, N = 34, P = 0.016) (Figure 5D) and equivalent to the crude extract ($\chi^2 = 0.053$, N = 20, P = 0.82) (Figure 5E), whereas the crude extract was more attractive than the hexane fraction ($\chi^2 = 0.807$, N = 14, P = 0.005) (Figure 5F). In a final bioassay, synthetic 2E,4Z,6Z-10:COOMe proved to be highly attractive, with 90% of the females choosing the arm with this stimulus ($\chi^2 = 15.51$, N = 45, P < 0.001) (Figure 5G).



FIG. 5. Response of mature *T. perditor* females in the Y-tube olfactometer to different treatments and controls. In all cases, the first treatment mentioned is the open bar, and the second treatment (control) is the solid bar. (A) Crude aeration extract of male-produced volatiles vs. hexane. (B) Hexane fraction of a crude extract vs. hexane. (C) Ether fraction of a crude extract vs. hexane fraction of the extract. (E) Crude aeration extract of male-produced volatiles vs. the ether fraction of the extract. (F) Crude aeration extract of male-produced volatiles vs. the hexane fraction of the extract. (G) 4 µg of synthetic methyl (2*E*,4*Z*,6*Z*)-decatrienoate in hexane vs. hexane (**P* < 0.05, ****P* < 0.001); ns, not significantly different.

DISCUSSION

Bioassays using all combinations of live insects as odor sources and responders showed that females were attracted to odors of male bugs, but that no other combination of odor and test subject produced significant attraction. These results indicated that male *T. perditor* produce a sex pheromone, analogous to the congeneric *T. pallidovirens* and *T. custator accerra* (McBrien et al., 2002).

Maximum attraction of males to females occurred during the afternoon/ evening, similar to the peak response period of the stink bug *E. heros* (Borges et al., 1998b). In contrast, other pentatomids such as *N. viridula* (Borges et al., 1987) and *E. obscurus* (Borges and Aldrich, 1994) appear to exhibit no periodicity of response throughout the photophase. The reason for the differences in activity patterns between these species is unclear, but because they often share hosts and habitats temporal separation may play a role in maintaining interspecific reproductive isolation.

Our results suggest that the sex pheromone of *T. perditor* consists of only 2*E*,4*Z*,6*Z*-10:COOMe, one component of the sex pheromones of the nearctic congeners *T. pallidovirens* and *T. custator accerra,* (McBrien et al., 2002).

2E,4Z,6Z-10:COOMe alone attracted neither of these species and required at least one of the three sesquiterpenes, (+)- α -curcumene, (-)-zingiberene, and (-)- β -sesquiphellandrene, produced by the males (McBrien et al., 2002), but none of these sesquiterpenes were present in aeration extracts from *T. perditor*. The phenomenon of shared pheromone components appears to be common in the family Pentatomidae. For example, Aldrich et al. (1991) reported that methyl (2E,4Z)-decadienoate was a major male-specific component of volatiles produced by several *Euschistus* species. Pheromonal parsimony has also been seen with *N. viridula* and several *Acrosternum* species, all of which produce bisabolene epoxide pheromone components (Aldrich et al., 1993). *P. guildinii* and *E. heros* (Borges et al., 1999) also have methyl-2,6,10-trimethyltridecanoate as a shared component in their pheromones.

The reasons for the comparative simplicity of the pheromone of *T. perditor* relative to its congeners are not immediately apparent and merit further attention. Although they share many host plants, such as soybeans, this may be a relatively recent and, thus, a misleading "ecological" artefact resulting from man's introduction of exotic crop plants into new habitats rather than the evolution of species-specific communication systems. Furthermore, although *T. perditor* is now sympatric with *T. pallidovirens* and *T. curstator accerra* in Florida (Panizzi et al., 2000), it is of tropical origin (Panizzi et al., 2000), whereas the two neartic congeners are restricted to the western and southern United States (Schotzko and O'Keefe, 1990).

The defensive compounds identified in the headspace volatiles from adults of both sexes of *T. perditor*, such as (*E*)-2-octenal, (*E*)-2-decenal, nonanal, tridecane, and undecane, have been reported previously from other stink bug species (Borges and Aldrich, 1992), although (*E*)-2-decenal and nonanal were only observed in the male volatiles. In addition, both genders released some compounds that have been less commonly reported from stink bug airborne collections, such as α -pinene and β -pinene. These compounds are clearly insectproduced and not from the diet, because extracts of exuvia and metathoracic glands of both sexes contain these compounds. The specific functions of these compounds remain to be determined.

In summary, our results have shown that female *T. perditor* are attracted to 2E,4Z,6Z-10:COOMe, a pheromone released by sexually mature males of this species Because female bugs were strongly attracted to synthetic 2E,4Z,6Z-10:COOMe in laboratory bioassays, future efforts will focus on determining whether this compound can be exploited as an effective and selective trap bait for monitoring this stink bug species.

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