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ORIGINAL RESEARCH PAPER

Identification and field evaluation of sex pheromones in two hawk moths *Deilephila elpenor lewisii* and *Theretra oldenlandiae oldenlandiae* (Lepidoptera: Sphingidae)

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Abstract The sex pheromones of two species of hawk moth, *Deilephila elpenor lewisii* (Butler) and *Theretra oldenlandiae oldenlandiae* (Fabricius), were analyzed using gas chromatography–electroantennographic detection (GC–EAD) and GC–mass spectrometry (GC–MS). Two and three EAD-active components were found in *D. elpenor lewisii* and *T. oldenlandiae oldenlandiae*, respectively. GC–MS analyses using authentic compounds and extracts derivatized by dimethyl disulfide and 4-methyl-1,2,4-triazoline-3,5-dione identified the two components in *D. elpenor lewisii* as (*E*)-11-hexadecenal (E11–16:Ald) and (10*E*,12*E*)-10,12-hexadecadienal (E10,E12–16:Ald), and the three in *T. oldenlandiae oldenlandiae* as E11–16:Ald, E10,E12–16:Ald, and (10*E*,12*Z*)-10,12-hexadecadienal (E10,Z12–16:Ald). In field-trap tests, no males of either species were attracted to any single components. Male moths of *D. elpenor lewisii* were specifically attracted to a binary blend of E11–16:Ald and E10,E12–16:Ald at a ratio of 85:15, whereas males of *T. oldenlandiae oldenlandiae* were attracted to a ternary blend of E11–16:Ald, E10,Z12–16:Ald and E10,E12–16:Ald at a ratio of 30:40:30. We therefore conclude that the sex pheromone of *D. elpenor lewisii* is a mixture of E11–16:Ald and E10,E12–16:Ald and that of *T. oldenlandiae oldenlandiae* is E11–16:Ald, E10,Z12–16:Ald and E10,E12–16:Ald.

Keywords Sex pheromone · Hawk moth · *Deilephila elpenor lewisii* · *Theretra oldenlandiae oldenlandiae* · Bombykal

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Introduction

Sphingidae, commonly known as hawk moths, is the largest family of Bombycoidea comprising 1,400 species around the world. Species diversity can be facilitated by a species-specific mating system, which is generally mediated by sex pheromones (Linn and Roelofs 1995). However, sex pheromones or sex attractants of hawk moths have been identified in only a few species. Starratt et al. (1979) identified (10*E*,12*Z*)-10,12-hexadecadienal (E10,Z12–16:Ald) (bombykal) from *Manduca sexta* (Linnaeus), and Tumlinson et al. (1994) found (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal (E10,E12,Z14–16:Ald) to be a new minor component essential for male attraction in this species. Although no field-trap tests were done, hexadecenals and hexadecadienals are also reported as sex pheromone candidates in other hawk moth species (Bestmann et al. 1992; Wakamura et al. 1996). Field screenings using synthetic chemicals demonstrated that five species of Sphingidae males are attracted to bombykal family compounds, and these compounds were discovered in pheromone gland extracts of *Sphinx drupiferarum* (J. E. Smith), *Hyles galii* (Rottemburg), and *Amphion floridensis* (B.P. Clark) by gas chromatographic (GC) analyses, although the complete chemical profiles were not described (Reed et al. 1987; Landolt et al. 1989).

As many species of hawk moths such as *M. sexta* and *Agrius convolvuli* (Linnaeus) are important pest insects in

field crops and natural vegetation, information on their sex pheromones is useful for developing pheromone lures for pest management and understanding mechanisms of reproductive isolation and evolution of pheromone systems in hawk moths. *Deilephila elpenor lewisii* (Butler), a subspecies of the elephant hawk moth and the impatience hawk moth *Theretra oldenlandiae oldenlandiae* (Fabricius), are common species and widely distributed in Japan, that damage orchard and vegetable crops including grapes, taro, and rose balsam (Japanese Society of Applied Entomology and Zoology 2006). In the study presented here, we identified candidates of sex pheromone components extracted from virgin females of the two species by using GC coupled with an electroantennographic detector (EAD) and a mass spectrometer (MS). Furthermore, activities of the identified chemicals were evaluated using field-trap experiments to develop effective pheromone lures for males of *D. elpenor lewisii* and *T. oldenlandiae oldenlandiae*.

Materials and methods

Insects

A female adult of *D. elpenor lewisii* was caught from Kokufu-town, Tottori-city (35.5°N, 134.3°E) in July 2010 and offspring larvae were reared with *Impatiens textori* (Miquel) at 25 ± 2 °C and natural photo regime until pupation. Larvae of *T. oldenlandiae oldenlandiae* were obtained from Tsukuba-city (36.1°N, 140.1°E) in August 2010 and fed on *Cayratia japonica* (Thunberg) at 25 ± 1 °C and 60–70 % relative humidity (RH) under a reversed photo regime of L15:D9 until they emerged. The pupae of both species were sexed based on morphological differences in the genital area and allowed to emerge under the same conditions described above.

Chemicals and extracts

(*E*)-11-Hexadecenal (E11–16:Ald) and (*Z*)-11-hexadecenal were supplied by ShinEtsu Chemical Co., Ltd. (Tokyo, Japan). Four geometric isomers of 10,12-hexadecadienals were supplied from a stock library of our laboratory. The isomeric purity of all compounds was confirmed to be ≥97 % by GC (column: HP-5MS). Pheromone extraction in *D. elpenor lewisii* was conducted with 11 calling females for 3 h after light-off and in *T. oldenlandiae oldenlandiae* with eight calling females for 7 h after light-off. Female abdominal tips, including the pheromone glands of 3- 4-day-old virgin females were cut with ophthalmology scissors and extracted to a small tapered glass vial containing redistilled *n*-hexane for 20 min. After passing through a glass filter, the crude extracts were concentrated with a gentle nitrogen stream and stored at −20 °C until use. Pooled extracts were subjected to chemical analysis and bioassay.

Chemical analysis

Female pheromone gland extracts were analyzed with GC coupled to an GC–EAD. HP-5890 series II GS fitted with HP-5MS capillary column (30 m × 0.32 mm ID, film thickness 0.25 μm; Agilent Technologies, USA) and helium as a carrier gas (37 cm/s). EAD responses of male moths were recorded with Syntech IDAC-2 (Syntech, The Netherlands). GC oven temperature was programmed at 130 °C for 2 min, then at a rate of 5 °C/min to 250 °C and held at this temperature for 10 min. Effluents from the column were split at a ratio of 1:1 between a flame ionization detector (FID) and EAD. Humidified air at 21 °C delivered the GC effluent to the antennal preparation bridged between Ag–AgCl electrodes with electric conductible gel.

GC–MS analysis was conducted using a JEOL MS-600H MS (JEOL Ltd., Japan) coupled with HP-6890N GC (Agilent) with a DB-5MS capillary column (25 m × 0.25 mm ID, film thickness 0.25 μm, Agilent) in electro ionization mode (70 eV). Helium was used as the carrier gas at a liner velocity of 42 cm/s. GC oven temperature was maintained at 100 °C for 1 min, programmed at a rate of 10 °C/min to 320 °C for 7 min. Temperature of the injector, interface, and ion source was 280, 280, and 190 °C, respectively. The structures of EAD-active compounds in the extracts were deduced by analysis of the mass spectra of native and dimethyl disulfide (DMDS)- or 4-methyl-1,2,4-triazoline-3,5-dione (MTAD)-derivatized extracts (Buser et al. 1983; Young et al. 1990).

GC analyses were performed on Shimadzu GC-17A (Shimadzu Co., Ltd., Japan) and Agilent 6890 (Agilent) GS fitted with a nonpolar HP-5MS column and a polar DB-23 column (30 m × 0.25 mm ID, film thickness 0.15 μm; Agilent), respectively. Oven temperatures were controlled as follows: HP-5MS: 130 °C for 2 min, programmed at a rate of 5 °C/min to 250 °C for 10 min; DB-23: 100 °C for 2 min, programmed at a rate of 3 °C/min to 250 °C for 10 min. In both columns, injector and detector temperatures were 250 °C and the carrier gas was helium (32 cm/s). Retention indices (RI) of EAD-active components in the extracts and authentic chemicals were determined by comparisons with retention times of standard hydrocarbons (C_{14}–C_{28}). Ratios of EAD-active components in the extracts were obtained from the GC peak area of each compound.

Field tests

Attractions of *D. elpenor lewisii* and *T. oldenlandiae oldenlandiae* to synthetic lures were examined on the campus

of Tottori University (35.5°N, 134.2°E) and in Kokufu-town field (35.5°N, 134.3°E) in Tottori-city, Tottori Prefecture, during May and June 2011. For *D. elpenor lewisii*, 500 μg of two isomers of 11–16:Ald, two isomers of 10, 12–16:Ald and a binary mixture of E11–16:Ald and E10,E12–16:Ald (85:15) were used for lures. A ternary mixture of E11–16:Ald,E10,Z12–16:Ald,E10,E12–16:Ald (30:40:30) was used for *T. oldenlandiae oldenlandiae* as well as single candidate chemicals at a total of 500 μg per trap. Cross-attractions on the binary and ternary mixture were also assayed. The synthetic lures were protected from isomerization and oxidation with 5 % butylated hydroxy toluene (BHT), ladened on gray halo-butyl isoprene rubber septa (West Corp., Singapore) and placed in the center of a sticky board trap (SE-trap®, 30 cm × 27 cm bottom plate with a roof; Sankei Chemical Co., Ltd., Japan). Traps were placed 1.5 m above the ground and at least 10 m from each other. Numbers of captured males in each trap were counted every few days. After each count, the traps were rotated to eliminate any positional effects. Numbers of males captured per week (x) were transformed $\sqrt{(x + 0.5)}$ prior to one-way analysis of variance (ANOVA), followed by a Tukey–Kramer's honestly significant difference (HSD) test.

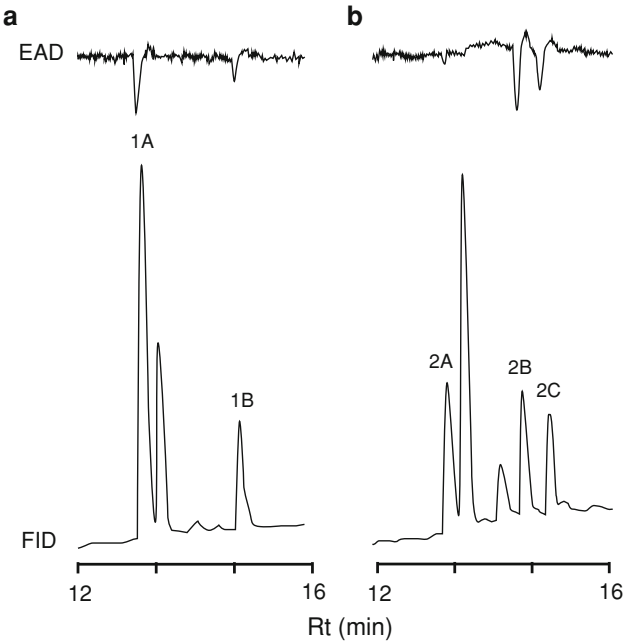

Fig. 1 Gas chromatography–electroantennographic detection (GC–EAD) analysis of female pheromone extracts of **a** *Deilephila elpenor lewisii* and **b** *Theretra oldenlandiae oldenlandiae*

Results

Analysis of pheromone components

The male antennae of *D. elpenor lewisii* showed conspicuous responses to two components **1A** and **1B** in the female pheromone extracts by GC–EAD analysis (Fig. 1a), whereas EAG responses of *T. oldenlandiae oldenlandiae* were observed to three components **2A**, **2B**, and **2C** (Fig. 1b). Amounts of EAD-active component are approximately 300 ng/female (**1A**:**1B** = 85:15 in a ratio) and 200 ng/female (**2A**:**2B**:**2C** = 30:40:30 in a ratio) on *D. elpenor lewisii* and *T. oldenlandiae oldenlandiae*, respectively.

GC–MS analysis showed components **1A** and **2A** produced almost identical diagnostic ion peaks, molecular ion peaks at *m/z* 238 (M^+, **1A**, 9 %; **2A**, 8 %), *m/z* 220 ($[M - 18]^+$, **1A**, 18 %; **2A**, 17 %), *m/z* 55 ($[C_4H_7]^+$, base peak for each component), suggesting the component is hexadecenal ($C_{16}H_{30}O$). Analysis of the DMDS-derivatized extracts of both species indicated a double bond at the 11-position because of three diagnostic ion peaks: molecular ion at *m/z* 332 (22 %), *m/z* 215 ($[C_{12}H_{23}OS]^+$, base peak), and *m/z* 117 ($[C_6H_{13}S]^+$, 54 %), indicating an unsaturated double bond in the C_{11} position. Comparisons of RI of compounds **1A** and **2A** and authentic compounds including E11–16:Ald with the DB-23 column indicate the EAD-active components **1A** and **2A** are E11–16:Ald (Table 1).

Table 1 Retention indices of electroantennographic detection (EAD)-active components and synthetic compounds on gas chromatograph (GC) columns with different polarities

Compounds	Relation index (RI)	
	HP-5MS	DB-23
Compound **1A**	1813	2246
Compound **1B**	1879	2441
Compound **2A**	1813	2246
Compound **2B**	1863	2429
Compound **2C**	1879	2440
E11–16:Ald	1812	2448
Z11–16:Ald	1812	2266
Z10,E12–16:Ald	1854	2411
E10,Z12–16:Ald	1863	2429
Z10,Z12–16:Ald	1873	2435
E10,E12–16:Ald	1879	2440

Compounds **1B**, **2B**, and **2C** gave similar diagnostic ion peaks, molecular ion peaks at *m/z* 236 (M^+, **1B**, 36 %; **2B**, 29 %; **2C**, 28 %), *m/z* 67 ($[C_5H_7]^+$, base peak on each component), *m/z* 96 ($[C_7H_{12}]^+$, **1B**, 33 %; **2B**, 32 %; **2C**, 39 %) and *m/z* 109 ($[C_8H_{13}]^+$, **1B**, 26 %; **2B**, 27 %; **2C**, 26 %), suggesting a hexadecadienal ($C_{16}H_{28}O$). In addition, two characteristic ions (*m/z* 96 and *m/z* 109) and relatively high intensity of molecular ions (*m/z* 236)

suggested a conjugated double bond at ω4 and ω6 positions, 10 and 12 positions in the hexadecadienals (Ando et al. 1988). This was confirmed by further GC–MS analysis of MTAD-derivatized female extracts of both species. MTAD-adducts corresponding to compounds **1B**, **2B** or **2C** showed—in addition to a molecular ion peak at *m/z* 349 (18 %)—two other typical diagnostic ion peaks at *m/z* 306 ($M–C_3H_7$, 45 %) and *m/z* 208 ($M–C_9H_{17}O$, 91 %), indicating a conjugated 10,12 dienyl structure of the aldehyde. As shown in Table 1, the RI of **2B** was identical to that of E10,Z12–16:Ald; the RI of **1B** and **2C** were identical or almost identical to that of (10*E*,12*E*)-10,12–16:Ald (E10,E12–16:Ald) on both columns used.

Field-trap experiment

Pheromone activity of six combinations including E11–16:Ald, E10,Z12–16:Ald, and E10,E12–16:Ald against *D. elpenor lewisii* and *T. oldenlandiae oldenlandiae* were assessed in field-trap tests. *D. elpenor lewisii* males were remarkably attracted to a binary blend of E11–16:Ald and E10,E12–16:Ald at a ratio of 85:15, but not to each component alone (ANOVA, $df = 5$, $F = 32.7$, $P < 0.001$). *T. oldenlandiae oldenlandiae* males were not attracted to any single component lure, but remarkably responded to a ternary combination of E11–16:Ald, E10,Z12–16:Ald, and E10,E12–16:Ald at a ratio of 30:40:30 (ANOVA, $df = 5$, $F = 5.2$, $P < 0.001$). Additionally, these two species were attracted exclusively to their own pheromone blend (Table 2).

Table 2 Field-trap catches to lures baited with synthetic candidate compounds

C_{16} compounds (μg)				Total male catches (mean per week ± SEM)[a]	
Monoene		Diene			
E11	Z11	EZ	EE	*D. elpenor elpenor* ($N = 7$)	*T. oldenlandiae oldenlandiae* ($N = 8$)
425	–	–	75	51 (7.57 ± 1.60)a	0b
150	–	200	150	0b	31 (3.88 ± 2.09)a
500	–	–	–	0b	0b
–	500	–	–	0b	0b
–	–	500	–	0b	0b
–	–	–	500	0b	0b

SEM standard error of mean, *E11* E11–16:Ald, *Z11* Z11–16:Ald, *EZ* E10,Z12–16:Ald, *EE* E10,E12–16:Ald

[a] Mean values followed by the same letter within the same column are not significantly different at $p < 0.05$ by Tukey–Kramer's honestly significant difference (HSD) test. Catches were transformed $\sqrt{(x + 0.5)}$ prior to the test

Discussion

GC–EAD-active components in female extracts of *D. elpenor lewisii* were E11–16:Ald and E10,E12–16:Ald. No males of *D. elpenor lewisii* were attracted to E11–16:Ald or E10,E12–16:Ald presented separately, but a mixture of the two elicited a substantial response, demonstrating that the binary blend is essential for male attraction. Similar results were also observed in *T. oldenlandiae oldenlandiae*, where three components, E11–16:Ald, E10,Z12–16:Ald, and E10,E12–16:Ald, were discovered in female extracts, and males were attracted to a ternary blend of the three but not to each single component in field tests. These results indicate that the sex pheromones of these two species consist of 10,12-hexadienals (bombykal family) as well as those of the other hawk moths previously studied. It remains to be examined whether all the three components are necessary to attract *T. oldenlandiae oldenlandiae* males, because activities of binary mixtures were not examined in this study. However, *T. oldenlandiae oldenlandiae* males have never been captured by our previous field-trap tests using binary blends of E10,Z12–16:Ald and E10,E12–16:Ald (unpublished data). Thus, at least, E11–16:Ald would be an essential component, mixed with 10,12-hexadienals, to attract *T. oldenlandiae oldenlandiae* and *D. elpenor lewisii*. Our GC–EAD surveys on Sphingidae pheromones detected E11–16:Ald as a candidate component in female pheromone gland extracts from five of 12 hawk moths examined (Uehara et al. 2011). This suggests that monoenyl aldehydes and bombykal family compounds may be key components in sphingid pheromones.

Bombykal family sex pheromone components were previously identified in *M. sexta* (Tumlinson et al. 1994) and *D. elpenor* (Bestmann et al. 1992). In addition, Reed et al. (1987) and Landolt et al. (1989) reported that single or binary combinations of 10,12-hexadecadienals attracted male moths to field traps in pheromone surveys of six other species of hawk moths. These results imply that bombykal family pheromone components are widespread in the sex pheromone systems of hawk moth species. Bombykal family compounds are also found in pheromones of other moth families, including Bombycidae (Daimon et al. 2012), Crambidae (Raina et al. 1986; Klun et al. 1986; Honda et al. 1994), Saturniidae (McElfresh and Millar 1999a, b; McElfresh et al. 2001), and Noctuidae (Cork et al. 1988).

Species specificity of insect pheromones is not only the result of component structural diversity with differences in number of carbon atoms; the number, position, and configuration of unsaturation, and functionality, but also in the multiplicity of components of pheromone systems (Tamaki 1977). In the pheromone systems of hawk moths, a single-component system was reported in *A. convolvuli* (11*E*,13*E*)-

11, 13-hexadecadienal (E11,E13–16:Ald) (Wakamura et al. 1996) and a two-component system in *D. elpenor* (Bestmann et al. 1992). In this study, we also confirmed a two-component system in *D. elpenor lewisii*. In addition, we demonstrated for the first time a possible three-component pheromone system in Sphingidae, in *T. oldenlandiae oldenlandiae*. These results indicate hawk moths drive pheromone divergence by an increase/decrease of number of components as well as structural modifications.

Differentiation in sex pheromone systems facilitates reproductive isolation of insect subspecies. Lepidopteran subspecies may discriminate each other by different pheromone systems with minor modifications in a common basic motif of compositions and additional compounds or modified ratios (Ando et al. 1997; McElfresh and Millar 1999a; Kakizaki and Sugie 2002). *D. elpenor* moths are widely distributed in the Palaearctic region represented by subspecies of *D. elpenor elpenor* and *D. elpenor lewissi* (Pittaway 1993). E11–16:Ald and E10,E12–16:Ald were reported as pheromone candidates from European populations of *D. elpenor*, with no detailed taxonomical status (Bestmann et al. 1992). More detailed information on sex pheromone systems is needed for biogeographical understanding of reproductive isolation in *D. elpenor* subspecies.

We also found a new ternary pheromone system in *T. oldenlandiae oldenlandiae*, which is composed of E11–16:Ald, E10,E12–16:Ald and E10,Z12–16:Ald, as the first report of a three-component system from hawk moths. In addition to E11,E13–16:Ald from *A. convolvuli* (Wakamura et al. 1996), our ongoing pheromone survey suggests the possibility of a nonbombykal pheromone component in other hawk moth species (Uehara et al. 2011). These results provide a better understanding of sphingid sex pheromone systems that are seldom discussed. At the same time, our findings on sex pheromone components of *D. elpenor lewissi* and *T. oldenlandiae oldenlandiae* will also contribute to the development of sex pheromone lures for population monitoring as well as management of these species.

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