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Investigation of Possible Sex Pheromone Components of Female Loreyi Leafworm, *Acantholeucania loreyi* (Duponchel) (Lepidoptera: Noctuidae) in Taiwan

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H. Y. Ho, R. S. Tsai, E. L. Hsu, Y. S. Chow, and R. Kou (2002) Investigation of possible sex pheromone components of female loreyi leafworm, *Acantholeucania loreyi* (Duponchel) (Lepidoptera: Noctuidae) in Taiwan. *Zoological Studies* **41**(2):188-193. In total, 8 components, i.e., dodecyl acetate (12:Ac), (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac), tetradecyl acetate (14:Ac), (*Z*)-7-tetradecenyl acetate (*Z*7-14:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), hexadecyl acetate (16:Ac), (*Z*)-7-hexadecenyl acetate (*Z*7-16:Ac), and (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac), were extracted and identified from *Acantholeucania loreyi* female tips, with *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac being the 3 major ones. Virgin females initiating calling at 2, 3, 4, or 5 d old, respectively, were analyzed individually for these 3 components via GC. Among different age groups, broad and similar distributions were found both for the titer (X = 121.3 ± 9.4 ng/female, range 66.2~260.5 ng/female) and for the ratio (a combined wash ratio of *Z*7-12:Ac/*Z*9-14:Ac/*Z*11-16:Ac = 14/72/14, range 13-16/70-72/12-17) of the 3 major components. Single components of *Z*7-12:Ac, *Z*7-14:Ac, *Z*9-14:Ac, and *Z*7-16:Ac elicited strong electroantennogram (EAG) responses in 3-d-old virgin males; the other 4 components, 12:Ac, 14:Ac, 16:Ac, and *Z*11-16:Ac, elicited little EAG response. A mixture of *Z*9-14:Ac and *Z*7-12:Ac (in a ratio of 85/15) elicited an EAG response comparable to that elicited by the female extract, and further addition of *Z*11-16:Ac to this mixture (in a ratio of 72/14/14) showed no additive effect on the EAG response. http://www.sinica.edu.tw/zool/zoolstud/41.2/188.pdf

Key words: EAG, *Z*7-12:Ac, *Z*7-14:Ac, *Z*9-14:Ac, *Z*7-16:Ac, *Z*11-16:Ac.

The lorey leafworm, *Acantholeucania loreyi* (= *Mythimna loreyi* = *Leucania loreyi*) (Duponchel) is an insect pest of rice and corn in Taiwan. In Japan, although (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac) and (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) were identified as female sex pheromone components of the same species (Takahashi et al. 1979), the 2 synthetic chemicals elicited much less attraction than did the crude extract. Later a 3rd sex pheromone component, (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac), was identified, and mixtures of *Z*9-14:Ac and *Z*7-12:Ac in ratios of 4:1 and 20:1 showed very high activity in a laboratory bioassay; addition of *Z*11-16:Ac to the above mixture did not increase the activity (Takahashi et al. 1980). In greenhouse tests of trap catches with released *A. loreyi* males, a 9/1 mixture of *Z*9-14:Ac/*Z*7-12:Ac showed the same high attractancy as did a 0.8/8/2 mixture of *Z*7-12:Ac/ *Z*9-14:Ac/*Z*11-16:Ac (Sato et al. 1980). Our previous studies showed that some insect species exhibit geographical variation in sex pheromone blend ratios. Two examples are the smaller tea tortrix moth *Adoxophyes* sp. (Kou et al. 1990) and the Asian corn borer *Ostrinia furnacalis* (Kou et al. 1992). In the present study, the chemical constituents of the sex pheromone gland of the lorey leafworm in Taiwan were investigated, and individual variations of chemical components in the sex pheromone gland were quantified by gas chromatography. Electroantennagram (EAG)

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analysis was conducted to obtain an estimate of the biological activity of each identified component.

MATERIALS AND METHODS

Insects. Larvae of *A. loreyi* were reared on an artificial diet (Shorey and Hale 1965) at 26-28 ºC under a 16: 8 (L: D) photoperiod. Sexes were separated at the pupal stage. Pupae were collected daily, and adults were allowed to emerge in screened cages. Moths emerging within 8 h were placed in the same age group. The life span of both female and male adults was 10-12 d.

Preparation of pheromone gland extract. Ovipositors from 100 virgin females 2 to 4 d old were excised during the calling period in the late scotophase and immersed in 5 µl hexane each for 5 min. The extracts were stored at 0 ºC.

Chemical analysis. Gas chromatography (GC) of the extracts was performed on 3 different 30-m x 0.25-mm (ID) capillary columns of Carbowax 20 M, DB-1701, and DB-1 with Shimadzu 14-A GC. Nitrogen was used as the carrier gas at a pressure of 0.5 kg/cm^2, and the column temperature was programmed to rise from 100 to 190 ºC at a rate of 5 ºC/min after which it was held at 190 ºC for 15 min.

Gas chromatographic/mass spectrometric (GC/MS) analysis was conducted using a Finnigan Mat Incos 50 spectrometer (Ho et al. 1996), using a 30-m x 0.25-mm column of Carbowax 20 M phase. The column temperature increased from 120 to 250 ºC at 3 ºC/min and then was held at 250 ºC for 5 min with helium as the carrier gas. Electron impact (EI) mass spectra were collected at 70 eV with the source held at 180 ºC and the transfer line at 280 ºC.

The chemical components were tentatively identified by GC/MS and identifications were confirmed by comparison of the GC retention times and spectra of the unknowns with those of authentic standards. Authentic compounds of 12:Ac, *Z*7-12:Ac, 14:Ac, *Z*7-14:Ac, *Z*9-14:Ac, 16:Ac, *Z*7-16:Ac, and *Z*11-16:Ac were purchased from Sigma Chemical (St. Louis, MO).

Component titer determination. For component titer determination, ovipositor extracts were prepared from virgin females which initiated calling at 2, 3, 4, or 5 d old, respectively. Ovipositors were excised during the calling period in the late scotophase and immersed in 5 µl hexane for 5 min. The extracts were quantified for *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac with external standards.

EAG analysis. The procedure of EAG bioassay experiments was based on Renou (1991) and Yang et al. (1998), with a slight modification. In brief, the tip of the antenna from a 3-d-old virgin male moth was snipped off, and then the cut tip was introduced into the recording electrode. The reference electrode was inserted into the neck. Kaissling saline-filled glass capillary electrodes were used with a connection to a pre-amplifier (NeuroLog NL 102G DC) through chloridized silver wires. The EAG readings were filtered (passband: 0 up to 100 Hz) and monitored on a storage oscilloscope. The amplified signals were digitized and stored in a personal computer with a data acquisition system (Digidata 1200A, Axon, Foster City, CA).

Pheromone and clean-air puffs were delivered via 2 separate series of valves and tubing, and were controlled by an electronic stimulator (modules NL 401 and NL 300, Neurolog System, Digitimer, Welwyn Garden City, CA). Purified moistened air was pumped through the tubing. Each puffer line led to a Pasteur pipette. One line serving as the clean-air puffer flowed continuously over the antenna at a rate of 40 ml/s, and the other serving as the pheromone puffer flowed briefly (50 ms) over the antenna with clean air flowing continuously at 20 ml/s. Choice of puffer line was controlled by a solenoid valve (MAC 35TYP, Auckland, New Zealand). Tested chemicals were dissolved in n-hexane (20 ng/µl solution) and applied (5 µl) directly into a Pasteur pipette, and the solvent was allowed to evaporate for 10 min. Tested chemicals were puffed over the antennal preparation in random order, with approximately 30 s between puffs. Each EAG test was replicated with 8-10 different antennae.

Statistical analysis. Duncan's new multiple-range test (Steel and Torrie 1980) was used to analyze the results.

RESULTS

Chemical analysis. GC analysis showed 8 peaks in the *A. loreyi* gland extract (Fig. 1). The mass spectra of these 8 components of female tips all gave a fragmentation ion at *m*/*z* 61, indicating that these 8 components are all acetates. The molecular ions of these compounds were not observed on the EI mass spectra. The biggest fragmentation ion of these compounds is the ion of molecular ion which had lost the CH_3COOH group, i.e., M^+-60. With the fragmentation ions at *m*/*z*

168, 196, and 224 of peaks 1, 3, and 6, these 3 components were identified as 12:Ac, 14:Ac, and 16:Ac. The 2nd peak has a fragmentation ion of *m*/*z* 166 in place of 168, indicating a mono-unsaturated derivative of 12:Ac. Both the 4th and 5th peaks gave a fragmentation ion of *m*/*z* 194, indicating isomers of mono-unsaturated 14:Ac. Both the 7th and 8th peaks gave a fragmentation ion of *m*/*z* 222, indicating isomers of mono-unsaturated 16:Ac. Previously reported sex pheromone components for *A. loreyi* in Japan include *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac (Takahashi et al. 1979; Takahashi et al. 1980). These 3 compounds and other related compounds were prepared so that their mass spectra and GC retention times could be compared. After comparing the GC retention times and spectra of these components with authentic compounds, the 8 peaks were identified to be 12:Ac, *Z*7-12:Ac, 14:Ac, *Z*7-14:Ac, *Z*9-14:Ac, 16:Ac, *Z*7-16:Ac, and *Z*11-16:Ac, as shown in table 1, with a relative ratio of 6/7/4/4/64/2/4/9 of the pooled extract.

EAG analysis. The results of EAG analyses are shown in figure 2. Among all tested single compounds, *Z*7-12:Ac, *Z*9-14:Ac, *Z*7-14:Ac, and *Z*7-16:Ac elicited strong EAG responses in 3-d-old virgin males, although these EAG responses were a little lower than that elicited by the extract of 1 female equivalent. Other single compounds, 12:Ac, 14:Ac, 16:Ac, and *Z*11-16:Ac, elicited no significant EAG response (compared with the control). A mixture of *Z*9-14:Ac and *Z*7-12:Ac (in a ratio of 85/15) elicited an EAG response comparable to that elicited by the female extract; further addition of *Z*11-16:Ac to this binary mixture (in a ratio of *Z*9-14:Ac/*Z*7-12:Ac/*Z*11-16:Ac = 72/14/14) showed no significant difference between the binary and ternary blends.

Component titer determination. Since *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac were the most abundant components in the extracts of female tips, these 3 components were selected for titer measurement in individual virgin females. The average component titer of individual females initiating calling at different ages is listed in table 2. The average titer ranges of *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac were 7.1~26.9, 45.8~134.4, and 6.7~31.1 ng/female, respectively. Though there is no significant difference in the average amount, the total amount of the 3 major components did

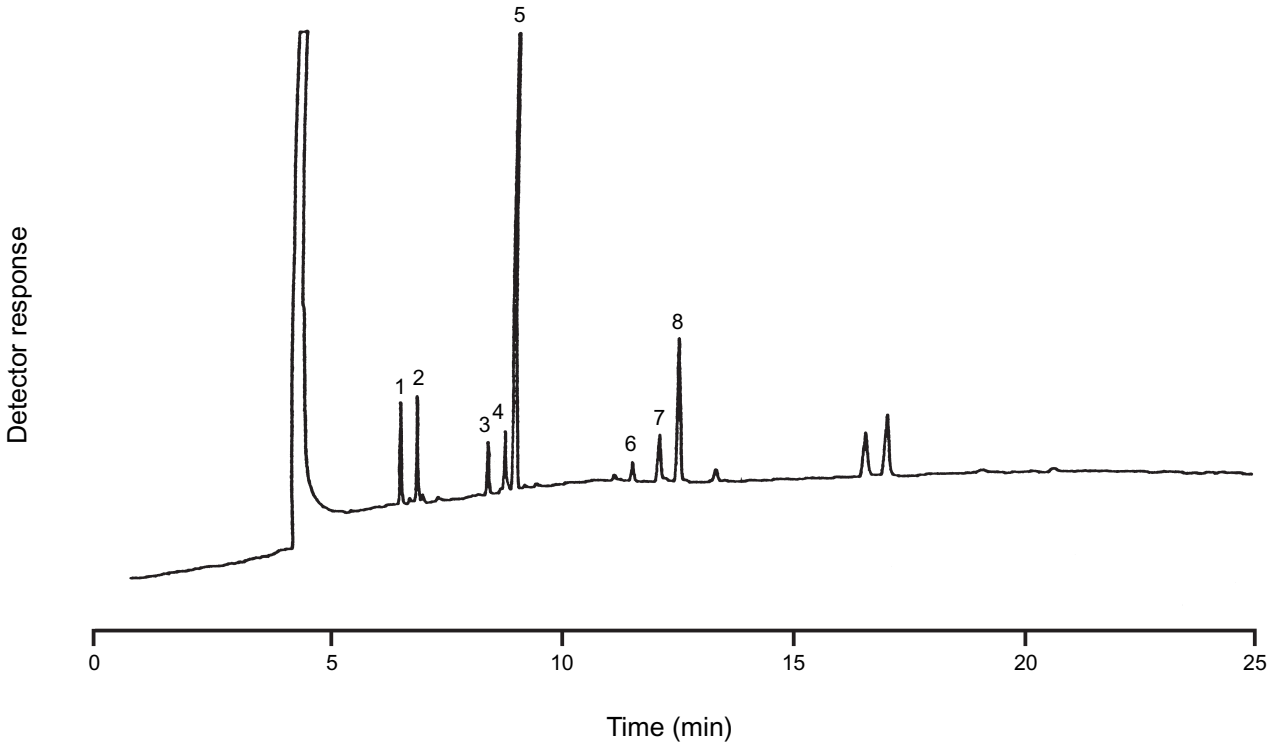

Fig. 1. Gas chromatogram of the combined wash of female crude extract. Gas chromatography was performed on a 30-m X 0.25-mm Carbowax 20 M column. The temperature was programmed to rise from 100 to 190 ºC at 5 ºC/ min. Identifications of peaks: (1) 12:Ac, (2) *Z*7-12:Ac, (3) 14:Ac, (4) *Z*7-14:Ac, (5) *Z*9-14:Ac, (6) 16:Ac, (7) *Z*7-16:Ac, and (8) *Z*11-16:Ac.

show a trend of increasing to a maximum at 3-d-old and decreasing afterwards. The mean ratio of *Z*7-12:Ac/*Z*9-14:Ac/*Z*11-16:Ac did not vary widely among females who initiated calling at different ages (Table 2).

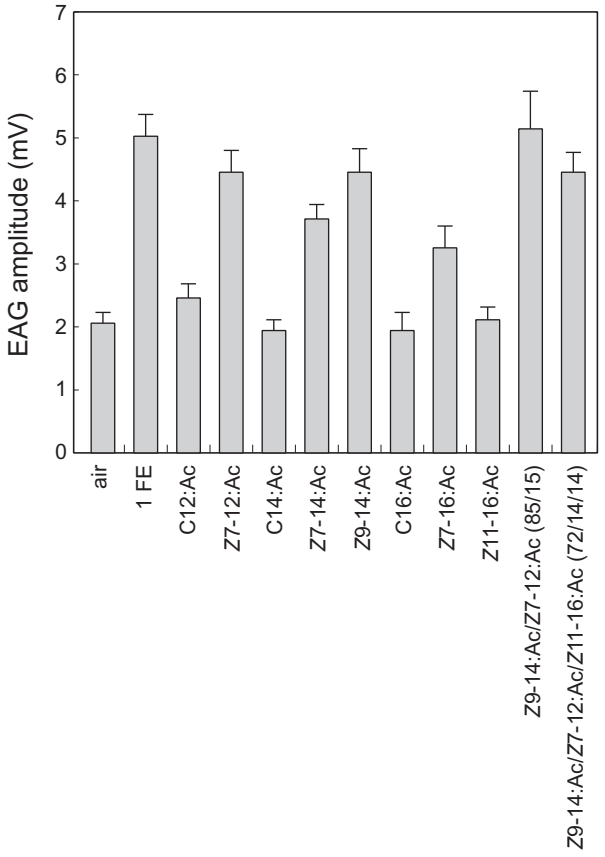

Fig. 2. Profile of electroantennogram amplitudes (mV) of 3-d-old *A*. *loreyi* virgin males in response to a series of 10 pheromone compounds at a concentration of 100 ng. FE: female equivalent. Mean values of 7-9 determinations ± SEM.

DISCUSSION

Compared with previous studies in Japan (Takahashi et al. 1979, Takahashi and Kawaradani 1980), 5 more components were identified from the extract of *A*. *loreyi* female sex pheromone glands, including 3 saturated acetates, 12:Ac, 14:Ac, and 16:Ac, and 2 mono-unsaturated acetates, *Z*7-14:Ac and *Z*7-16:Ac. A weak EAG response of the saturated compounds, 12:Ac, 14:Ac, and 16:Ac, is not surprising, since these compounds have been found in almost every moth species. Nevertheless they often exhibit no activity.

The facts that the EAG response of the mixture of *Z*7-12:Ac and *Z*9-14:Ac is equivalent to that of female extract and that the addition of *Z*11-16:Ac did not increase the EAG response from male antennae indicate that *Z*7-12:Ac and *Z*9-14:Ac are the active components of the gland extract. This result is consistent with field tests by Sato et al. (1980). In their report, a 9/1 mixture of *Z*9-14:Ac/*Z*7-12:Ac showed the same high attractancy as a 0.8/8/2 mixture of *Z*7-12:Ac/*Z*9-14:Ac/*Z*11-16:Ac in the greenhouse test of trap catches with released *L*. *loreyi* males (Sato et al. 1980). In our present study, the mean ratio of *Z*9-14:Ac/*Z*7-12:Ac in the extract is 85/15 (nearly 6/1), which differs from that reported in Japan.

*Z*11-16:Ac is the 2nd major component, but it elicited little EAG response. According to Roelofs (1995), for Δ11 desaturase in combination with a 2-carbon chain-shortening reaction, Δ11-16:CoA, Δ9-14:CoA, and Δ7-12:CoA can be synthesized from 16:CoA. Then the respective acetates are produced. If this is the biosynthetic pathway of sex pheromone production of the Lorey leafworm and *Z*9-14Ac and *Z*7-12:Ac are the target compounds, then *Z*11-16:Ac is the precursor of the major sex pheromone. This might be the reason for the very notable presence of *Z*11-16:Ac in

Table 1. Retention times of *Z*7-12:Ac, *Z*7-14:Ac, *Z*9-14:Ac, *Z*7-16:Ac, *Z*11-16:Ac, 12:Ac, 14:Ac, 16:Ac, and pheromone gland extract of female *A*. *loreyi* on 3 gas chromatographic columns

	Retention time (min)															
Column type	12:Ac	*Z*7-12:Ac	14:Ac	*Z*7-14:Ac	*Z*9-14:Ac	16:Ac	*Z*7-16:Ac	*Z*11-16:Ac	Extract							
Carbowax 20 M	6.59	6.91	8.38	8.83	8.97	11.44	12.07	12.47	6.58	6.92	8.42	8.78	8.99	11.49	12.07	12.48
DB-1701	—	6.90	—	9.77	9.90	—	13.05	13.61	—	7.04	—	9.71	9.90	—	13.05	13.51
DB-1	4.97	4.94	5.70	5.60	5.65	6.97	6.81	6.92	—	4.94	—	—	5.64	—	6.80	6.89

Table 2. Average quantity (ng/female) of *Z*7-12:Ac, *Z*9-14:Ac and *Z*11-16:Ac produced by individual *A*. *Loreyi* virgin females that initiated calling at 2, 3, 4, and 5 d old, respectively (*n*= 8 for each age group)

Age group	Mean component titer (ng/female, X ± SEM)				Mean ratio of *Z*7-12:Ac/*Z*9-14:Ac/*Z*11-16:Ac
	*Z*7-12: Ac	*Z*9-14: Ac	*Z*11-16: Ac	total	
2 d old	15.9 ± 3.1	87.2 ± 12.9	21.3 ± 3.7	124.4	13/70/17
3 d old	17.4 ± 1.7	96.3 ± 12.4	19.5 ± 2.0	133.2	13/72/15
4 d old	20.2 ± 2.4	90.1 ± 15.7	14.6 ± 2.8	124.9	16/72/12
5 d old	13.1 ± 1.1	71.8 ± 5.2	11.5 ± 1.2	96.4	14/74/12

female tips, but the presence of only a small EAG response from male antennae.

Although the average titer of each component showed no significant difference among age groups, production of *Z*11-16:Ac was halved from day 2 to day 5, but *Z*9-14:Ac and *Z*7-12:Ac production did not decrease as much. This fact also helps explain *Z*11-16:Ac as the pheromone precursor, since *Z*11-16:Ac is used for the synthesis of *Z*9-14:Ac and *Z*7-12:Ac.

The other 2 mono-unsaturated acetates, *Z*7-14:Ac and *Z*7-16:Ac, which were not found by the Japanese group, elicited strong EAG responses. A good EAG response of a compound does not specifically mean that there is behavioral attraction to the compound. The fact that blends of *Z*7-12:Ac and *Z*9-14:Ac elicit equivalent responses as does the female tip extract indicates that these 2 minor components might not be sex pheromone components. The reason for the strong EAG response from both *Z*7-14:Ac and *Z*7-16:Ac may be because of their similar configuration to the sex pheromone *Z*7-12:Ac, since all 3 compounds have a cis double bond at carbon-7. If these 2 minor mono-unsaturated acetates are not active pheromonal components, the role of these 2 compounds in the gland extract needs to be studied further.

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羅氏夜蛾 *Acantholeucania loreyi* (Duponchel) 雌蟲性費洛蒙之探討

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羅氏夜蛾雌蟲的腹部末端性費洛蒙腺體內，共鑑定出 12:Ac, *Z*7-12:Ac, 14:Ac, *Z*7-14:Ac, *Z*9-14:Ac, 16:Ac, *Z*7-16:Ac 及 *Z*11-16:Ac 等八種成分，其中以 *Z*7-12:Ac, *Z*9-14:Ac 及 *Z*11-16:Ac 為主成分。由 2、3、4 及 5 日齡開始求偶行為的處女雌蟲分析結果顯示，其性費洛蒙腺體內的三個主要成分在不同日齡個體具有廣泛且相似的含量分布 (X = 121.3 ± 9.4 ng/雌蟲，範圍為 66.2~260.5 ng/雌蟲) 及比例 (*Z*7-12:Ac/*Z*9-14:Ac/*Z*11-16:Ac = 14/72/14，範圍為 13-16/70-72/12-17)。*Z*7-12:Ac, *Z*7-14:Ac, *Z*9-14:Ac 及 *Z*7-16:Ac 可引起強烈的 EAG 反應。*Z*9-14:Ac 及 *Z*7-12:Ac (85/15) 混合物能引起相當於雌蟲萃取液的 EAG 反應，而 *Z*11-16:Ac 的加入並無引起 EAG 的加成反應。

關鍵詞：EAG，*Z*7-12:Ac，*Z*7-14:Ac，*Z*9-14:Ac，*Z*7-16:Ac，*Z*11-16:Ac。

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