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Determination of diel periodicity of sex pheromone release in three species of Lepidoptera by 'closed-loop-stripping' [1]

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Summary. By means of 'closed-loop-stripping' and subsequent GC analyses the diel periodicity of release of (*Z*)-11-hexadecenyl acetate, (*E*)-8-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate, the main constituents of the respective sex pheromone blends of *Mamestra brassicae, Cryptophlebia leucotreta* and *Spodoptera sunia* females, was determined.
Key words. Closed-loop-stripping; sex pheromone release; diel periodicity; *Mamestra brassicae; Cryptophlebia leucotreta; Spodoptera sunia.*

Production of sex pheromones by female moths and the subsequent release during 'calling' depends, among other factors such as age of the females, temperature, neural and hormonal conditions, mainly on the photoperiod experienced by the insects. The mating behavior, of which calling and pheromone release represents an essential part, usually occurs during a discrete period of the day/night cycle, and is often crepuscular or nocturnal. A knowledge of diel periodicity of pheromone release thus becomes an important factor to those interested in pheromone identification in order to maximize the yield of pheromone isolation.

We report here the results of laboratory experiments conducted to determine the effect of the photoperiod on sex attractant release of three different lepidopteran species, *Mamestra brassicae* L. (Noctuidae), *Cryptophlebia leucotreta* Meyr. (Tortricidae) and *Spodoptera sunia* Guenée (Noctuidae) by collecting the airborne volatiles from a 'closed-loop-stripping' [2] system.

Material and methods. M. brassicae females use (*Z*)-11-hexadecenyl acetate (Z-11-16:Ac) as the main component of their sex pheromone [3–7]. The sex attractant of *C. leucotreta* has been reported as a mixture composed mainly of (*Z*)- and (*E*)-8-dodecenyl acetates, depending on the geographical origin of the insects [8–11], and the strain investigated in our laboratory had (*E*)-8-dodecenyl acetate (E-8-12:Ac) as the major component [8]. In *S. sunia,* (*Z*)-9-tetradecenyl acetate (Z-9-14:Ac) represents the main constituent of the pheromone blend [12]. The quantification of the respective main components, Z-11-16:Ac, E-8-12:Ac and Z-9-14:Ac, of the collected volatiles, was performed by gas chromatography (GC).

Insects used in this study were provided by Hoechst AG (Frankfurt) and the Institut für Biologische Schädlingsbekämpfung (BBA Darmstadt). Pupae were sexed and kept at 22 °C in plastic containers lined with moistened filter paper. A reversed 14-h light:10-h dark cycle was maintained throughout the study. Adults were collected daily after eclosion and transferred to plastic boxes containing wicks with 5% sugar solution.

Closed-loop-stripping. The closed-loop-stripping system used for this study is a modification of that of Boland et al. [2]. Air was circulated (1.5 l/min) continuously through the closed system by a membrane pump (Antechnica, Karlsruhe, FRG). The air from the insect container was purged through a small charcoal filter (Brechbühler AG, Schlieren, Switzerland; 1.5 mg charcoal) which retained volatiles (fig. 1, A). The adsorbed volatiles were removed subsequently by solvent extraction. The filter was sealed inside a glass tube as shown in figure 1, C. Both sides of the glass tube ended in ball joints in order to obtain an airtight fit, and provide some flexibility to the system to withstand the vibrations of the pump.

The volume of the insect container was such that up to eight moths could move independently without interfering with one another. The air stream was not sweeping directly across the insects (fig. 1, B). The whole system was kept at an ambient temperature of 25–27 °C and was subjected to a 14:10-h light:dark cycle, which was identical to that maintained in the room where the insects emerged.

Two- to three-day-old female moths, 3–8 individuals per experiment, were placed in the insect container during the dark period and aerated for 10 h. After a 45-min adsorption period the pump was stopped and allowed to cool for 15 min, and the filter was removed for extraction. This procedure was repeated every hour.

Extraction of the filter. A 10-µl droplet CS_2 (CS_2 for IR-spectroscopy) was placed on top of the filter and moved up-and-down about 10 times through the charcoal zone by gentle cooling (ice bath) or heating (hand temperature) with a small Schlenckrohr or bulb attached to the filter tube (fig. 1, D). Afterwards, the droplet was sucked to one end of the coaxial inner tube (fig. 1, C) and taken up with a GC syringe. The

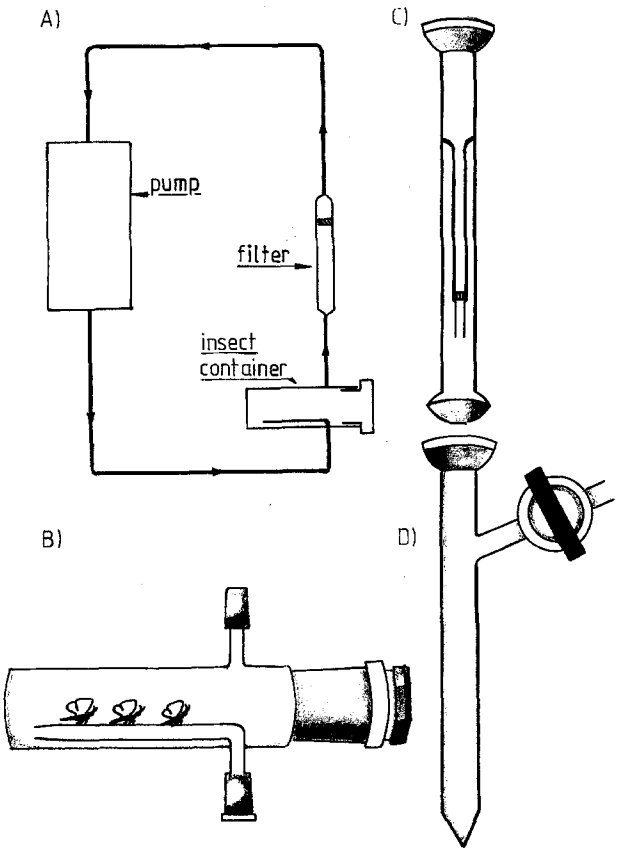

Figure 1. *A* Schematic drawing of the closed-loop-stripping system; *B* Insect container; *C* Glass tube with charcoal filter (1.5 mg); *D* Schlenckrohr for filter extraction.

extraction procedure was carried out three times, and the eluates were combined and concentrated to 1 µl in a stream of nitrogen.

Determination of pheromone isolated. The amount of the main constituent of each pheromone blend, Z-11-16:Ac, E-8-12:Ac and Z-9-14:Ac respectively, adsorbed on the charcoal filter was quantified by gas chromatography performed on a Packard United Technologies 438 A GC with Shimadzu Chromatopac C-R3A data system; 25 m FSCC SP2340, 0.2 mm ID, temp. progr.: 3 min 60 °C, 60–195 °C at 6°/min, split vent closed for 1 min, inj. 220 °C, FID 260 °C, carrier gas N_2, 24 cm/s linear gas velocity. Authentic samples of the three main pheromone-blend components were used as external standards for quantification.

Control experiments. Adsorption of the volatiles on the glass walls of the system reduces the yield of the substances obtained on the charcoal filter. Furthermore, a delayed desorption from the walls may cause an apparent temporal shift of the total profile obtained by plotting the amount of pheromones isolated against the time of the day. In order to minimize discrepancies given by such effects, the following control experiments were carried out: Dodecenyl acetate, tetradecenyl acetate, hexadecenyl acetate and hexadecenol, 100 ng each, were applied onto an electrically heatable aluminum plate (2 × 2 cm) and placed in the insect container. The aluminium plate was heated for 5 min to 120 °C to vaporize most of the test chemicals within a short period of time, while the system was maintained under stripping conditions. After 1 h of aeration, each filter was removed for extraction, and the adsorbed volatiles were quantified by gas chromatography.

The same mixture of the test chemicals was also applied directly onto the charcoal filter. After 15 min the compounds were reextracted and quantified as described above.

Results and discussion. The results of the control experiments showed that about 50–70% of the acetates (the amount decreasing from C_{12} to C_{16} compounds) and 65% of the alcohol were recovered within the 1st h of aeration. In the 2nd h, only 5–10% of the total material applied to the aluminium plate could be recovered in the case of C_{12} and C_{14} acetate, and 12–16% of those of hexadecenyl acetate and the hexadecenol. Since these values which are due to material desorbed from the system walls as well as from substances still vaporizing from the cold aluminium plate, obtained for the 2nd h, were relatively low, no significant (at least not more than 10–16%) broadening of the profile of emission of pheromone and no apparent temporal shift of the maximum of emission are to be expected. Furthermore, this also demonstrated, that with the closed-loop-stripping method, at least for the type of chemicals that were tested, a recovery of 50–70% can be obtained.

In a 2nd series of control experiments, the percentage recovery of adsorbed chemicals from the charcoal filter was determined by direct application of the test compounds onto the filter, reextraction and subsequent GC analysis. It was found, that 98% of the adsorbed material can be recovered by solvent extraction.

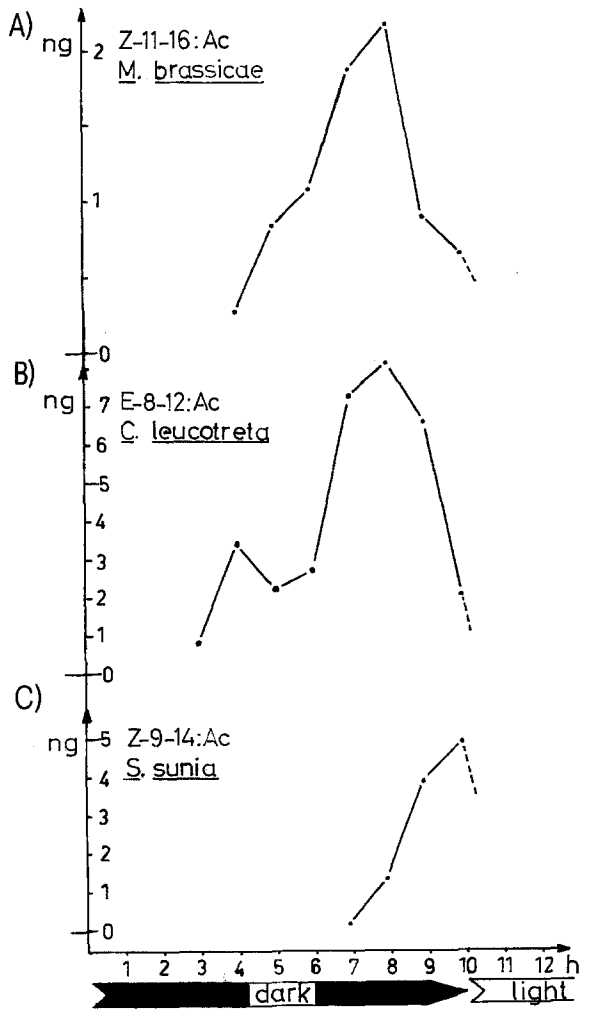

Figure 2. Release of pheromone main components during the scotophase. *A Mamestra brassicae* release of (*Z*)-11-hexadecenyl acetate; *B Cryptophlebia leucotreta* release of (*E*)-8-dodecenyl acetate; *C Spodoptera sunia* release of (*Z*)-9-tetradecenyl acetate.

All the three insect species investigated released their sex attractants during the scotophase only. *M. brassicae* females started calling 3 h after the onset of the scotophase and continued to emit Z-11-16:Ac over a period of 7 h. The maximum amount of Z-11-16:Ac was isolated 7–8 h after the lights were turned off (fig. 2, A). During this maximum period about 4 ng Z-11-16:Ac/h was isolated per female (average of 24 insects). The total for the whole night was about 15 ng. Thus, the absolute amount of Z-11-16:Ac collected by closed-loop-stripping, even if it comprised only 50% of the total emittance, was much more than that obtained by solvent extraction[3, 6] of the glands, and in the same order of that reported for a solid-sampling injection technique[7].

In none of the four aeration experiments conducted with *M. brassicae* were we able to detect (*Z*)-11-hexadecenol (Z-11-16:OH) among the substances trapped in the charcoal filter, although it has been found by a solid-sampling technique[7] to be present in the sex gland membrane of *M. brassicae* females. This can be taken as evidence, that Z-11-16:OH is only a biosynthesis precursor[13], and it is not released to the surroundings by calling females. Furthermore, Z-11-16:OH is known to act as a strong inhibitor for attraction of *M. brassicae* males[6].

In an investigation of the effect of temperature on the calling activity, Subchev has observed that the mean time for onset of calling is about 7.5 h into the scotophase, and the mean duration of calling is about 2 h at both 15 and 25 °C[14]. The results are in good agreement with our maximum for Z-11-16:Ac recovered in the 7th and 8th h of scotophase (fig. 2, A), when the insects were maintained at 25–27 °C in the laboratory.

Thirty-seven *C. leucotreta* were used in five experiments. The profile of emittance was broad, similar to that of *M. brassicae*, starting at the 3rd h of scotophase and lasting till the end. Maximum amount was isolated at the 8th h. In four experiments out of the five, we found a small, but distinct second maximum period of E-8-12:Ac emission, preceding the main one, around the 4th h of the scotophase. It is yet not clear if this is a result of a biological effect, or whether it happened by chance. This should be a matter of further investigations.

During the maximum emission period of *C. leucotreta* about 8 ng of E-8-12:Ac/insect (N = 37) was isolated. The total amount of acetate collected over the whole dark period was about 33 ng/insect. This again is much more than the amount that has been obtained by solvent extraction[9] by a corresponding sex gland analysis. In another experiment involving 8 female insects, the amount of (*E*)-8-dodecenol (E-8-12:OH) isolated was also determined. The detection of this alcohol in the volatiles released into the air, in contrast to the example mentioned above for *M. brassicae*, implies, that this alkenol, which has also been found by solid injection[8], is a real pheromone constituent for *C. leucotreta*.

In an independent experiment, we also determined the amounts of other pheromone-related minor constituents isolated during the period of maximum E-8-12:Ac emission. The presence of the following was recorded (by comparing their GC retention times with those of authentic samples and by GC/MS analysis): (*Z*)-8-dodecenyl acetate (Z-8-12:Ac), (*E*)-8-dodecenol (E-8-12:OH), (*Z*)-8-dodecenol (Z-8-12:OH), decyl acetate (10:Ac), dodecyl acetate (12:Ac), and tetradecyl acetate (14:Ac). We found a ratio of 100:22 for the two isomeric dodecenyl acetates. A ratio of 100:13 has been found by solid-sampling analyses[8]. The other minor substances, already detected in female abdominal tips[8] were present in a ratio of 14:2:1:19:3 in the order mentioned above.

In contrast to the other two species, *S. sunia* females showed effective calling only during the last two h of the scotophase. Altogether, 10.2 ng Z-9-14:Ac were trapped per insect (N = 37) per dark period. This was one third of the substance found in the sex gland by a solid sampling technique[12]. In two experiments, involving 7 and 8 insects each, the amount of the alcohol (*Z*)-9-tetradecenol (Z-9-14:OH) isolated was determined (with synthetic Z-9-14:OH as an external GC standard). The profile of emittance was similar to that obtained for Z-9-14:Ac, and the amount was found to be one tenth of that of the acetate. This is in agreement with the published results[12].

In this study we could show that the closed-loop-stripping procedure is a suitable technique for biological investigations, similar to those presented here, as well as for pheromone isolation in general. The number of insects required is relatively small, and the amount of material collected is often greater than that obtained by conventional solvent extraction techniques. A major advantage of the method is that it yields only volatile substances, thus the usual time-consuming purification procedures to remove nonvolatile tissue impurities can be avoided.

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