not show any positive behavioral response to the female odor in an olfactometer. From this behavioral observation and male antennal searching behavior, we had assumed that a pheromone Z9-16:Ald acts as a contact chemical. However, we confirmed that the male antennae responded to the pheromone as a volatile in this study. In the contact chemoreceptive sensillum, a mechanoreceptor cell is often associated with the chemoreceptor cells (ZACHARUK, 1980), and STÄDLER (1984) proposed that close-range olfaction is included in contact chemoreception. Present observation supports the notion that olfaction is associated with contact chemoreception. It will be necessary to elucidate the interaction between mechanosensory and chemosensory mechanisms in perception of the pheromone.

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REFERENCES

- ARN, H., M. TÓTH and E. PRIESNER (1986) *List of Sex Pheromones of Lepidoptera and Related Attractants*, OLIB-SROP, Paris, 123 p.
- KAINOH, Y. (1986) *Appl. Entomol. Zool.* **21**: 1-7.
- KAINOH, Y. and Y. TAMAKI (1982) *Appl. Entomol. Zool.* **17**: 194-206.
- KAINOH, Y., T. NEMOTO, K. SHIMIZU, S. TATSUKI, T. KUSANO and Y. KUWAHARA (1991) *Appl. Entomol. Zool.* **26**: 543-549.
- KAMANO, Y., K. SHIMIZU, Y. KAINOH and S. TATSUKI (1989) *Appl. Entomol. Zool.* **24**: 372-378.
- ROELOFS, W. L. (1984) *Techniques in Pheromone Research* (H. E. HUMMEL and T. A. MILLER, eds.). Springer-Verlag, New York, p. 131-159.
- STÄDLER, E. (1984) *Chemical Ecology of Insects* (W. J. BELL and R. T. CARDÉ, eds.). Chapman and Hall, London, p. 3-35.
- ZACHARUK, R. J. (1980) *Annu. Rev. Entomol.* **25**: 27-47.

Ecdysteroids in Developing Eggs of *Antheraea mylitta* (Lepidoptera: Saturniidae)¹

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Antheraea mylitta is a fairly large insect species commercially reared in India for silk production. Since the size of an egg is approximately 3 mm in diameter, it is advantageous to use it for the investigation of hormonal aspects for embryonic development.

The occurrence of ecdysteroids has been reported in the eggs of various insect species. Several kinds of free and conjugated ecdysteroids have been found in insect eggs and distinct changes of the ecdysteroid levels have been observed during embryonic development (for review see HOFFMAN and LAGUEUX, 1985). We investigated free ecdysteroids in the eggs of *A. mylitta* and compared the results with some other insect species previously reported.

MATERIALS AND METHODS

Silkmoth larvae were reared on an artificial diet recently developed by AKAI et al. (1991). Eggs were incubated at 25°C and 80% R.H. Ten eggs were sampled daily, weighed, and stored in the freezer (-40°C).

The samples were homogenized in a mixture of 2 ml hexane, 700 µl methanol and 300 µl water. The homogenate was centrifuged to separate the hexane and methanol-water phases at 3,000 g. After eliminating the hexane phase with Pasteur pipette, 10 ml water was added to the ecdysteroid-

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Table 1. Weight of eggs used in the experiment

Days after oviposition	Weight of 10 eggs (mg)
1	103.5
2	102.8
3	104.8
4	102.5
5	101.2
6	97.9
7	96.6
8	93.1
9	90.9
10	77.7 (larvae)

containing aqueous phase. It was centrifuged at 3,000 *g*, and the supernatant was adsorbed onto a SEP-PAK C-18 cartridge then eluted with methanol. The eluate was evaporated to dryness under nitrogen.

Ecdysteroids in the extracts of *A. mylitta* eggs were quantified each day during embryogenesis by enzyme immunoassay (EIA). In order to identify the ecdysteroid species, the extract from day 1 eggs was separated by high performance liquid chromatography (HPLC) and further analyzed by EIA.

EIA was performed according to the method of PORCHERON et al. (1989) using 20-hydroxyecdysone-acetylcholinesterase derivative as a competitive tracer. The enzymatic activity of the bound tracer was measured by using the method of ELLMAN et al. (1961). The antibodies used recognized mainly 20-hydroxyecdysone (20-OH ecdyson) and ecdysone; they cross-reacted at 100 percent with both 20-OH ecdyson and ecdyson (PORCHERON et al., 1989).

HPLC analysis was performed on a Radial Nova Pak C18 (5 mm × 10 cm) column by isocratic elution with 40% methanol at a flow rate of 1 ml/min. The absorbance was monitored at 254 nm.

RESULTS AND DISCUSSION

Eggs began to develop immediately after being laid and most of the larvae hatched on day 10. The weight change of 10 eggs along the embryogenesis is shown in Table 1.

Quantities of total ecdysteroids in the extracts were measured along the development (Fig. 1). Ecdysteroids detected in large quantities (7.6 ng eq./egg) on day 1, rapidly decreased to 0.6 ng

eq./egg on day 3. A sharp increase and decrease took place from day 4 to day 6 (the highest level was 6.1 ng eq./egg on day 5). The level then gradually decreased during the last 3 days of embryonic development and only 0.1 ng eq. remained in a newly hatched larva on day 10.

HPLC analysis was conducted using day 1 eggs because of their high titers of ecdysteroids. Each fraction separated by HPLC was further analyzed by EIA as shown in Fig. 2. The ecdysteroids contained in day 1 eggs were identified as 20-OH ecdysone and ecdysone according to their retention time in HPLC. The 20-OH ecdysone:ecdysone ratio was approximately 11:1. Other immunoreactive fractions (24–25 min) were detected and the identification of these compounds is in progress.

It was found that 20-OH ecdysone and ecdysone were the main ecdysteroids in newly-laid eggs of *A. mylitta*. High titers of various ecdysteroids have been found in newly-laid eggs of *Bombyx mori* (MIZUNO and OHNISHI, 1975; OHNISHI et al., 1977), *Manduca sexta* (THOMPSON et al., 1984), *Galleria mellonella* (HSIAO and HSIAO, 1979), *Locusta migratoria* (LAGUEUX et al., 1979) and *Schistocerca gregaria* (DINAN and REES, 1981; SCALIA et al., 1987). In these insects, in addition to large amounts of free ecdysteroids, still larger amounts of polar, conjugated ecdysteroids were found in the eggs which were suggested to be synthesized in the pupal or adult ovary and transferred into the eggs.

In contrast, low quantities of ecdysteroids have been found in newly-laid eggs of two phasmids, *Carausius morosus* (FOURNIER and RADALLAH, 1988) and *Clitumnus extradentatus* (CAVALLIN and FOURNIER, 1981), blowfly, *Calliphora erythrocephala* (BORDES-ALLÉAUME and SAMI, 1987) and cockroach, *Nauphoeta cinerea* (IMBODEN and LANZREIN, 1982). We did not investigate the conjugated ecdysteroids and could not exclude the possibility that other less polar ecdysteroids were also present in this silkworm. However it is noteworthy that relatively high quantities of free ecdysteroids are present in *A. mylitta* eggs during early stages of embryonic development.

The fluctuation in the titer of free ecdysteroids in *A. mylitta* resembled that of *S. gregaria* (SCALIA et al., 1987). After a decrease in ecdysteroid levels from newly-laid eggs, a major peak occurred in the middle of embryonic development which subsequently diminished to quite a low level in the hatching larva. A single major peak in the middle

Fig. 1. Change of ecdysteroid titre in 10 eggs during embryogenesis. Ecdysteroid was measured by direct EIA.

Fig. 2. Separation of ecdysteroids in day 1 eggs by reverse-phase HPLC and measurement of the ecdysteroid titre in each fraction by EIA.

of embryogenesis was also observed in isolated embryos of *Sarcophaga bullata* (WENTWORTH and ROBERTS, 1984) and eggs of *C. erythrocephala* (BORDES-ALLÉAUME and SAMI, 1987), respectively, while in *Pieris brassicae* the ecdysteroid titers appeared to increase until hatching (BEYDON et al., 1989). More than two peaks were detected in *B. mori* (GHARIB and DE REGGI, 1983), *L. migratoria* (LAGUEUX et al., 1979), *N. cinerea* (IMBODEN and LANZREIN, 1982) and *C. extradentatus* (CAVALLIN and FOURNIER, 1981), and it was suggested that each peak was correlated with the onset of the deposition of a cuticle.

These differences in different insect species probably reflect the sensitivities of the methods employed besides differences in cross-reactivities of the antibodies as well as sampling intervals. Nevertheless, variations seem certainly to exist among insect species.

The definite functions and the source of free ecdysteroids during embryonic development in *A. mylitta* remain unsolved. Histological studies and more detailed chemical analysis of ecdysteroids both free and conjugated are needed for a better understanding of ecdysteroid levels during embryonic development.

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REFERENCES

- AKAI, H., M. SUTO and K. ASAKA (1991) *Wild Silkmths '89-'90* (H. AKAI and M. KIUCHI, eds.). International Society of Wild Silkmths, Tsukuba, p. 121-127.
- BEYDON, P., A. D. PERMANA, J. COLARDEAU, M. MORINIÉRE and R. LAFONT (1989) *Arch. Insect Biochem. Physiol.* **11**: 1-11.
- BORDES-ALLÉAUME, N. and L. SAMI (1987) *Int. J. Invertebr. Reprod. Dev.* **11**: 109-122.
- CAVALLIN, M. and B. FOURNIER (1981) *J. Insect Physiol.* **27**: 527-534.
- DINAN, L. N. and H. H. REES (1981) *J. Insect Physiol.* **27**: 51-58.
- ELLMAN, G. L., K. O. COURTENAY, V. ANDRES and R. M. FEATHERSTONE (1961) *Biochem. Pharmacol.* **7**: 89-95.
- FOURNIER, B. and D. RADALLAH (1988) *Arch. Insect Biochem. Physiol.* **7**: 211-224.
- GHARIB, B. and M. DE REGGI (1983) *J. Insect Physiol.* **29**: 871-876.
- HOFFMAN, J. A. and M. LAGUEUX (1985) In *Comprehensive Insect Physiology and Pharmacology*. Vol. 8 (G. A. KERKUT and L. I. GILBERT, eds.). Pergamon Press, Oxford, p. 205-262.
- HSIAO, T. H. and C. HSIAO (1979) *J. Insect Physiol.* **25**: 45-52.
- IMBODEN, H. and B. LANZREIN (1982) *J. Insect Physiol.* **28**: 37-46.
- LAGUEUX, M., C. HETRU, F. GOLTZENE, C. KAPPLER and J. A. HOFFMANN (1979) *J. Insect Physiol.* **25**: 709-723.
- MIZUNO, T. and E. OHNISHI (1975) *Dev. Growth Differ.* **17**: 219-225.
- OHNISHI, E., T. MIZUNO, F. CHATANI, N. IKEKAWA and S. SAKURAI (1977) *Science* **197**: 66-67.
- PORCHERON, P., M. MORINIÉRE, J. GRASSI and P. PRADELLES (1989) *Insect Biochem.* **19**: 117-122.
- SCALIA, S., A. SBRENNIA-MICCIARELLI, G. SBRENNIA and E. D. MORGAN (1987) *Insect Biochem.* **17**: 227-236.
- THOMPSON, M. J., J. A. SVOBODA and G. F. WEIRICH (1984) *Steroids* **43**: 333-341.
- WENTWORTH, S. L. and B. ROBERTS (1984) *J. Insect Physiol.* **30**: 157-163.

Behavioral and Electrophysiological
Evidence of Sex Pheromone(s) in
Anomala schonfeldti OHAUS
(Coleoptera: Scarabaeidae)¹

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The use of sex pheromone in the pest management of scarab beetles has been demonstrated to be a safe alternative to conventional pesticides, as highlighted by the examples of the Japanese beetle *Popillia japonica* and the soybean beetle *Anomala rufocuprea*. As in other parts of Japan, in Chiba Prefecture there has been a high demand for new methods of pest management and users have been strongly advised to minimize the applications of pesticides. Thus, identification of these sex pheromones in other scarabs are of utmost importance to investigate their feasibility for minimizing or replacing pesticides. Identification of scarab beetle's semiochemicals has been restricted to a few cases (HENZELL and LOWE, 1970; TUMLINSON et al., 1977 and TAMAKI et al., 1985; LEAL, 1991; LEAL et al., 1992b) due chiefly to the lack of appropriate bioassays. To overcome this barrier,

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