Eur. J. Entomol. **91**: 189–196, 1994
ISSN 1210–5759

Eclosion hormone-like immunoreactivity in the nervous system of *Bombyx mori* (Lepidoptera: Bombycidae) and *Antheraea yamamai* (Lepidoptera: Saturniidae) before and after hatching

SHIN-ICHI NAYA[1], KOICHI SUZUKI[1], HAJIME FUGO[2] and FRANTIŠEK SEHNAL[3]

[1]Faculty of Agriculture, Iwate University, Morioka 020, Japan

[2]Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183, Japan

[3]Institute of Entomology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic

Embryonic diapause, eclosion hormone, neurohormones, KK-42, silkworm, silkmoth, midgut

Abstract. A monoclonal antibody to *Bombyx mori* eclosion hormone (EH) reacted in whole mounts of the nervous system from pharate and freshly hatched larvae of this species with 4 ventromedial brain neurons and a pair of nerve fibers runing down the ventral nerve cord. Weak immunoreactivity was occasionally detected in similar whole preparations of the nervous system from *Antheraea yamamai*. In paraffin sections of the nervous system of *A. yamamai*, an EH-like antigen was found in 4 ventromedial brain neurons, in 2 or 3 pairs of neurons in each of the ventral ganglia, and in a pair of nerve fibers extending through the nerve cord. The intensity of immunostaining in diapausing and post-diapausing pre-hatched larvae, and in the newly hatched first-instar larvae, was similar. Midgut endocrine cells were also examined but no reaction with the anti-EH antibody was detected.

INTRODUCTION

A fully apolyzed insect often rests in the old exuvium as a pharate stage for several hours or even days or months, until a specific environmental cue triggers the release of a peptidic neurohormone, called the eclosion hormone (EH), which stimulates the ecdysial behaviour. In Lepidoptera, EH appears to be produced by 2 pairs of brain neurons, from where it is transported via the nerve cord to release sites in the proctodeal nerve (Hewes & Truman, 1991). The chemical structure of EH has been elucidated in *Manduca sexta* (Marti et al., 1987; Kataoka et al., 1987) and *Bombyx mori* (Kono et al., 1987). The EHs of these two species comprise 62 amino acids, and their sequences are 80% identical.

Eclosion hormone activity, as revealed in assays using pharate adults, is detectable already in the embryo (Truman et al., 1981b). In *Hyalophora cecropia*, it rises gradually from about the middle of embryonic development, drops at the time of embryonic ecdysis, and then remains at a low level through hatching (Truman et al., 1981a). A similar profile of EH activity was found in *Samia cynthia*, except that in this species the rise continues throughout hatching (Saito et al., 1990). On the other hand, Fugo et al. (1985) reported for *Bombyx mori* that the EH activity increases until one day before hatching, and then drops to a half during hatching. It was suggested that EH may actually induce hatching behaviour. Such a role of EH would be particularly important in those insects, whose development is interrupted by diapause in the stage of a morphologically fully formed pharate

first-instar larva. This pharate larva moves little until certain environmental conditions terminate the diapause (Umeya, 1950). Mobility then increases and becomes hatching behaviour, leading to escape from the egg.

Suzuki et al. (1990) examined how diapause just before hatching is controlled in *Antheraea yamamai*. They suggested that a repressive factor, which is produced somewhere in the mesothorax, inhibits production of a maturation factor, originating in the second to fifth abdominal segments and terminating diapause. It is worth noting that, as with ecdysial behaviour in postembryonic stages, hatching behaviour, once initiated, can proceed in the absence of the brain but requires stimulation from within the abdomen. These similarities, and the reports on EH activity in embryos, led us to explore whether or not the maturation factor could be EH. In the present paper we examine the spatial pattern of EH production and its possible changes from diapausing to newly hatched larvae. Because we used an antibody to *Bombyx* EH, we first verified the immunoreactivity in *B. mori*, a species which does not have diapause in the pharate first-instar larva. In *A. yamamai* we studied natural termination of diapause as well as its artificial break by the imidazole derivative KK-42 (Suzuki et al., 1989; Kuwano et al., 1991; Fujisawa et al., 1992).

MATERIALS AND METHODS

Insects and egg treatments

The wild silkmoth, *Antheraea yamamai* Guérin-Méneville (Lepidoptera: Saturniidae) was reared outdoors on the Japanese oak, *Quercus acutissima* Carruth, or indoors on the artificial diet produced by the Nippon Chlorella Company (Japan). Adults were transferred to small outdoor cages in which they mated and deposited eggs. Egg masses were collected daily, and dipped for 10 min in 0.5% chlorinated lime to separate individual eggs. All eggs were kept at 25°C for 10 days, when fully formed but virtually immobile first-instar larvae had developed. These were maintained in perpetual diapause when the eggs were further kept at 25°C. Other eggs were chilled for 10 months at about 4°C, and then used as post-diapause eggs. Upon their transfer to 25°C, the larvae inside began to wiggle within 24 hr, and in 48 to 60 hr they hatched. Diapause in some of the eggs that had never been chilled, was terminated artificially: the chorion was broken and the larva treated with 0.1 µg KK-42 (1-benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl]imidazole) in 0.5 µl acetone (Suzuki et al., 1989). The compound was synthetized and kindly provided by Kuwano et al. (1985). Immunohistochemistry was undertaken using diapausing larvae (one month in 25°C), pharate larvae (48 to 24 hr before hatching), and newly hatched larvae (within 2 hr of eclosion).

Daizo and N4 races of the domestic silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) were reared on mulberry leaves at 25°C and a 12 hr photoperiod. First-instar larvae about one day before hatching and within 2 hr of hatching were used for immunohistochemistry.

Antibody and immunohistochemistry

A monoclonal antibody (IgG), which was raised against a synthetic peptide corresponding to amino acids 49–61 of *B. mori* EH (Kono et al., 1990), was made available to us by Prof. A. Suzuki of the University of Tokyo. Our immunohistochemical procedures were similar to those used by Kono et al. (1990).

For whole mount immunostaining, the brains of pharate adults, and the entire central nervous system of the pharate or hatched first-instar larvae of *A. yamamai* and *B. mori*, were fixed in Bouin's fluid for 4 hr. After washing in TTBS (50 mM Tris-buffered 0.8% NaCl saline, pH 7.4 (=TBS) containing 0.05% Tween-20), and removal of the surface sheath from the brains of pharate adults, the tissues were immersed in anti-EH(49-61)IgG solution (1 : 500) containing 2% Triton X-100. Overnight incubation at 4°C was followed by washing in TTBS, and the application of horseradish peroxidase-linked anti-mouse IgG 1 : 500; (AMS, Jackson Immuno-Research Laboratories) for 2 hr at room temperature. Tissues washed in TBS were afterwards incubated for 10 min in 1.3 mM diaminobenzidine and 0.02% H_2O_2 in TBS. The reaction was stopped by transferring the tissues to clean TBS. Processed tissues were dehydrated in an ethanol series, cleared overnight in methylsalicylate, and mounted.

The whole mount staining gave satisfactory results in *B. mori* but mostly failed in *A. yamamai*. Therefore, immunohistochemistry in this species was performed on sections. The brain or the whole nervous system was fixed in Bouin-Hollande-Sublimate fluid (Eckert & Ude, 1983) for 4 hr, dehydrated in ethanol series and embedded in paraffin. Serial sections 2 μm thick were mounted on slides, deparaffinized and hydrated, treated for 3–5 min with Lugol solution (10 mg KI and 5 mg iodine/1 ml water) and, after brief washing, for 3 min with 5% $Na_2S_2O_3$, and eventually transferred to phosphate buffered saline (PBS, 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.115% Na_2HPO_4, pH 7.4) containing 1–2% Triton X-100 and 0.05% sodium azide. Non-specific IgG binding was minimized by treating the sections with 5% goat serum (5 min at room temperature). Subsequent incubations in primary and secondary antibodies and the immunostaining were carried out as described above for the whole mounts. The sections were then lightly counterstained with Meyer's haematoxylin and mounted.

RESULTS

In preliminary experimens, we applied anti-EH antibody to the whole mounts of brains from *B. mori* 1–2 days before imaginal emergence. The results (Fig. 1A) confirmed that the antibody reacts in pharate adult brain with two pairs of ventromedial neurosecretory cells, and with axons emanating from these cells (Truman & Coppenhaver, 1989; Kono et al., 1990). No other immunoreactive neurons were identified. Immunostaining of the whole central nervous system from pharate first-instar larvae (about one day before hatching) of *B. mori* also revealed the two pairs of EH-positive ventromedial neurons (Fig. 1B), which could clearly be distinguished when the brain was viewed from different angles. Their axons could not be traced within the brain but immunoreactive fibers, possibly originating in these cells, were found along the ventral nerve cord. They seem to be located on the dorsal surface rather than inside the ganglia (Figs 1C, D). No immunopositive perikarya were found in the ventral nerve cord.

Identical procedures of whole mount immunostaining was applied to the pharate and freshly ecdysed first instar larvae of *A. yamamai* within 2 hr of hatching, but only in one of ten nervous systems examined did we find a lightly stained axon running through the ventral ganglia. By contrast, application of EH-antibody to the sections of *A. yamamai* nervous system consistenly yielded a good response. Two pairs of immunoreactive neurons were clearly identified in the ventromedial region of the brain (Fig. 2A). They seemed to be further apart from one another (Fig. 2B) than the immunoreactive cells seen in the whole mounts of *B. mori* brains (Figs 1A, B).

In *A. yamamai* we also found EH-positive neurons in the ventral ganglia (Table 1). No immunostained neurons were detected in the suboesophageal and the terminal abdominal ganglion but in all other ganglia we could distinguish 3 to 6 neurons per ganglion. Hence, we assume that all thoracic and all but the last abdominal ganglia contain 2-3 pairs of neurons with an EH-like antigen. From the examples in Figs 2C, E, and F, one can see that the neurons are located dorsally and somewhat posteriory to the neuropile. In one section (Fig. 2D) we were fortunate enough to be able to trace an immunopositive fiber running through the ganglia and their connectives.

No obvious differences in the immunoreactivity of either axons or neurons were detected when the diapausing, post-diapausing, and newly hatched larvae were compared (Table 1). Treatment of diapausing larvae with KK-42 also had no effect on the immunostaining, which was consistently localized in the neurons and nerve fibres described above, and nowhere else. Special attention was paid to the midgut, which contains endocrine cells reacting with antibodies to various neurohormones (Žitňan et al., 1993). No reaction with

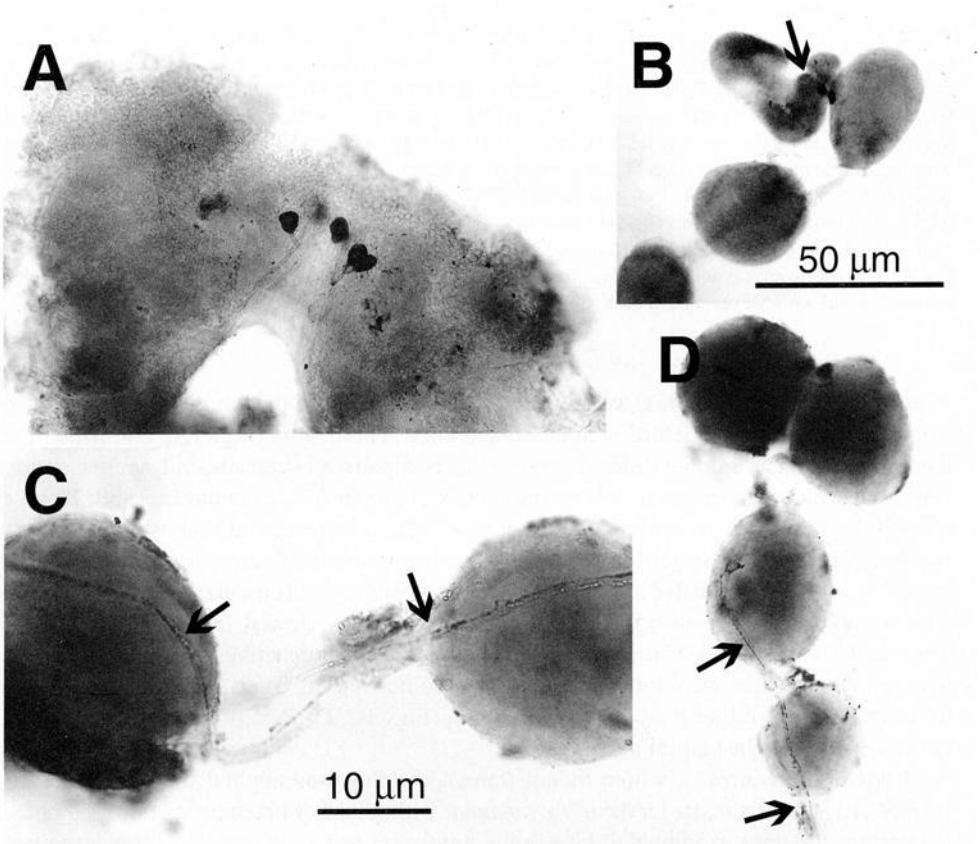

Fig. 1. Immunohistochemical detection (arrows) of eclosion hormone (EH)-like material in *Bombyx mori* (whole mounts). A. Brain of a pharate adult with immunoreactive material confined to two pairs of ipsilateral medial neurons and their axons. B. Presumably identical cells in the brain of pharate first-instar larva (about one day before hatching). C, D. Occurrence of immunoreactive material in a nerve running along the nerve cord. Bar in B indicates magnification also of A and D.

TABLE 1. EH-immunoreactivity in silk moths around the time of hatching.

Species & stage	Insect number	Brain (4 neurons)	Nerve cord (axons)	Neurons in ventral ganglia			
				SG	T_{1-3}	A_{1-7}	A_8
Bombyx:							
pharate, –24hrs[s,t]	9	++	++	–	–	–	–
hatched, +2 hr[t]	4	++	++	–	–	–	–
Antheraea:							
diapause[s]	6	++	+	–	+	+	–
KK-42 (–48 to –24 hr)[s]	6	++	+	–	+	+	–
hatched, +2 hr[s]	6	++	+	–	+	+	–
hatched, +2 hr[t]	4	–	(+)	–	–	–	–

[t] Whole mounts, [s] Sections. The time relates to the moment of eclosion (0 hr). Diapausing larvae were from eggs incubated at 25°C for 1 month. Treatment with KK-42 was applied to such larvae.

DISCUSSION

Our immunohistochemical data show that *A. yamamai* contains antigens recognized by the antibody to the EH of *B. mori*. Immunoreactive neurons in the pars intercerebralis of *A. yamamai* are apparently homologous with the EH-containing ventromedial neurosecretory cells (type-V) of *B. mori* (Kono et al., 1990) and *M. sexta* (Truman & Copenhaver, 1989). Occurrence of the antigen in longitudinal nerve fibres in the nerve cord of *A. yamamai* suggests that EH is transported from these ventromedial brain cells to release sites in the proctodeal nerves, as demonstrated in *M. sexta* by Hewes & Truman (1991). In our study we did not attempt to identify the proctodeal nerves but the presence of EH-like material in axons runing from the brain to the end of the nerve cord was obvious in the whole mounts of *B. mori* nervous system. Our data suggest that various Lepidoptera produce structurally similar EHs in type-V brain neurons, and transport the EHs down the nerve cord, as soon as a larva is formed in the egg.

Copenhaver & Truman (1986) demonstrated that their polyclonal antibody to *M. sexta* EH reacted with 5 pairs of lateral neurosecretory cells (type-Ia_2) in the brain of this species. Immunoreaction with both type-V and type-Ia_2 cells was confirmed by Homberg et al. (1991) who used the same antibody. By contrast, Beckage et al. (1994), who employed another polyclonal antibody to conspecific EH, were able to detect in *M. sexta* only the type-V cells. Using a monoclonal antibody to EH of *B. mori*, Kono et al. (1990) also stained only type V but not type-Ia_2 cells in the brain of this species. A plausible explanation for these observations and our results is that type-Ia_2 cells contain an antigen that is recognized by the polyclonal antibody of Copenhaver & Truman (1986) but not by two other antibodies to EHs. This antigen may be different from the EH because EH mRNA could not be detected in the Ia_2 neurons (Horodyski et al., 1989).

In both pharate and newly hatched first-instar larvae of *A. yamamai* we found EH-positive material also in 2–3 pairs of cells in the thoracic and in all but the last abdominal ganglia. We could not test the specificity of this reaction by using antiserum saturated with the antigen, because the antigen was not available to us. Our data, however, is consistent with other observations on the production of EH in abdominal ganglia.

First, the production of EH in ventral ganglia around the time of eclosion was indicated by the results of bioassays for the EH activity. It was found that pharate first-instar larvae of *H. cecropia* (Truman et al., 1981b) and *S. cynthia* (Saito et al., 1990) contained EH activity both in the head and in the remainder of the body. The presence of EH in the posterior body part could not be attributed solely to its transport from the brain, because ligation just behind the head did not prevent an increase of EH activity in the abdomen (Saito et al. 1990).

Further support for our work comes from recent immunohistochemical investigations on the last instar larvae of *M. sexta*. Beckage et al. (1994) found that parasitism of *M. sexta* by a braconid wasp enhances immunostaining for several neurohormones, including occurrence of immunoreactive neurons that cannot be detected in the control larvae. The most striking effect of parasitism is the presence of EH-immunoreactivity in 2 pairs of neurons in the ventral ganglia. It must be emphasized that an autologous antiserum was used and that the immunoreaction failed to occur when this antiserum was saturated with EH (D. Žitňan, pers. commun.). Hence, it is reasonably certain that ventral ganglia of the parasitized larvae contain neurons producing the EH.

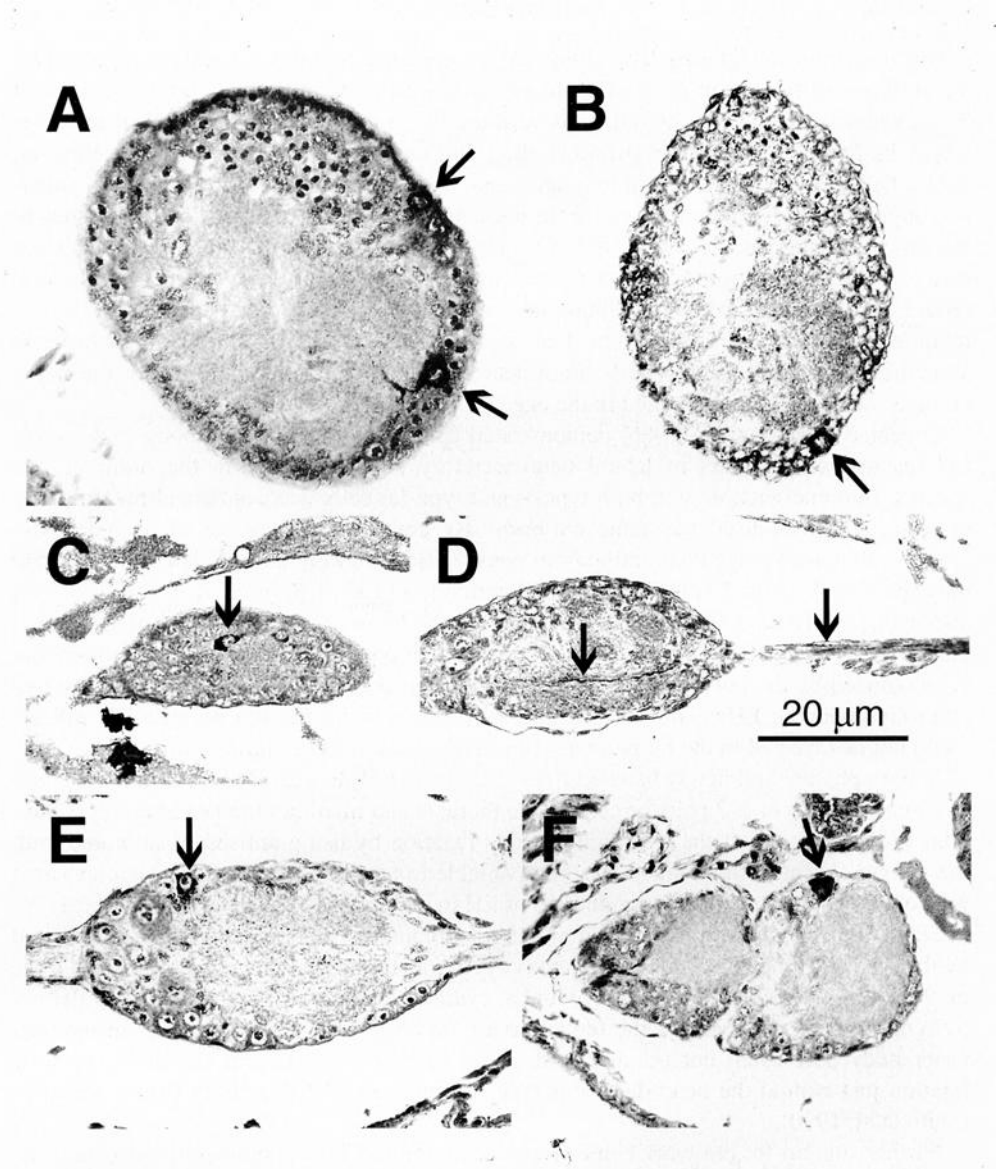

Fig. 2. EH-like antigen (arrows) in the nervous system of first-instar larvae in *Antheraea yamamai* (sagittal sections). A. Two immunoreactive neurons near pars intercerebralis in a newly hatched larva. B. One of those neurons in a diapausing larva. C. Immunoreactive neuron in the metathoracic ganglion of a diapausing larva. D. Immunoreactive material in a nerve fiber running through the metathoracic ganglion and adjacent connective of a pharate larva. E. Third abdominal ganglion with one reacting cell in a diapausing larva. F. Last two abdominal ganglia, of which the penultimate contains a reacting cell, in a newly hatched larva.

Summarizing all these data we tentatively suggest that most if not all ventral ganglia of caterpillars contain 2–3 pairs of neurons capable of producing EH. It seems that normally they secrete EH prior to and/or at hatching but, under stress conditions such as parasitism, the secretion is also resumed in later developmental stages. In *A. yamamai*, neither natural nor artificial termination of the first-instar diapause, nor hatching of the larvae, are associated with a dramatic change in EH immunoreactivity.

ACKNOWLEDGEMENTS. The study was performed during a stay of F. Sehnal at the Iwate University. A scholarship from the Japan Society for the Promotion of Science, which made this stay possible, is gratefully acknowledged. We also thank Dr E. Kuwano for the gift of KK-42, and Dr A. Suzuki for the gift of anti-EH antibody. For helpful criticism of the manuscript we are obliged to Dr. G. Goldsworthy, Dr. D. Žitňan and two anonymous reviewers.

REFERENCES

BECKAGE N., ŽITŇAN D. & SEHNAL F. 1994: Effects of parasitism by Cotesia congregata on the neurosecretory system of the tobacco hornworm, Manduca sexta. In Loeb M.A. & Borkovec A.B. (eds): *Insect Neurochemistry and Neurophysiology*. CRC Press, New York, in press.

COPENHAVER P.F. & TRUMAN J.W. 1986: Identification of cerebral neurosecretory cells that contain eclosion hormone in the moth Manduca sexta. *J. Neurosci.* **6**: 1738–1747.

ECKERT M. & UDE J. 1983: Immunocytochemical techniques for the indentification of peptidergic neurons. In Strausfeld N.J. (ed.): *Functional Neuroanatomy*. Springer, Berlin, pp. 267–301.

FUGO H., SAITO H., NAGASAWA H. & SUZUKI A. 1985: Eclosion hormone activity in developing embryos of the silkworm, Bombyx mori. *J. Insect Physiol.* **31**: 293–298.

FUJISAWA T., KUMAGI T., KUWANO E. & SUZUKI K. 1992: Improved method of artificial hatching using imidazole derivatives and effect of the injection of 20-hydroxyecdysone on diapause breakdown in pharate first-instar larvae in the wild silkmoth, Antheraea yamamai. *J. Seric. Sci. Jpn.* **61**: 207–214 (in Japanese, English summary).

HEWES R.S. & TRUMAN J.W. 1991: The roles of central and peripheral eclosion hormone release in the control of ecdysis behavior in Manduca sexta. *J. Comp. Physiol. (A)* **168**: 697–707.

HOMBERG U., DAVIS N.T. & HILDEBRAND J.G. 1991: Peptide-immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth Manduca sexta. *J. Comp. Neurol.* **303**: 35–52.

HORODYSKI F.M., RIDDIFORD L.M. & TRUMAN J.W. 1989: Isolation and expression of the eclosion hormone gene from the tobacco hornworm, Manduca sexta. *Proc. Natl. Acad. Sci. USA* **86**: 8123–8127.

KATAOKA H., TROETSCHLER R.G., KRAMER S.J., CESARIAN B.J. & SCHOOLEY D.A. 1987: Isolation and primary structure of the eclosion hormone of the tobacco hornworm, Manduca sexta. *Biochem. Biophys. Res. Commun.* **146**: 746–750.

KONO T., NAGASAWA H., ISOGAI A., FUGO H. & SUZUKI A. 1987: Amino acid sequence of eclosion hormone of the silkworm, Bombyx mori. *Agric. Biol. Chem.* **51**: 2307–2308.

KONO T., NAGASAWA H., ISOGAI A., FUGO H. & SUZUKI A. 1990: A monoclonal antibody against a synthetic carboxy-terminal fragment of the eclosion hormone of the silkworm, Bombyx mori: characterisation and application to immunohistochemistry and affinity chromatography. *Zool. Sci.* **7**: 47–54.

KUWANO E., TAKEYA R. & ETO M. 1985: Synthesis and anti-juvenile hormone activity of 1-substituted-5-[(E)-2,6-dimethyl-1,5-heptadienyl]imidazoles. *Agric. Biol. Chem.* **49**: 483–486.

KUWANO E., FUJISAWA T., SUZUKI K. & ETO M. 1991: Termination of egg diapause by imidazoles in the silkmoth, Antheraea yamamai. *Agric. Biol. Chem.* **55**: 1185–1186.

MARTI T., TAKIO K., WALSH K.A., TERZI G. & TRUMAN J.W. 1987: Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm Manduca sexta. *FEBS Lett.* **219**: 415–418.

SAITO H., FUGO H., NAKAJIMA M. & MUKAIYAMA F. 1990: Eclosion hormone activity during the embryonic development of the saturniid silkmoth, Samia cynthia ricini Donovan (Lepidoptera: Saturniidae). *Appl. Entomol. Zool.* **25**: 85–93.

SUZUKI K., FUJISAWA T., KURIHARA M., ABE S. & KUWANO E. 1989: Artificial hatching in the silkworm, Antheraea yamamai: application of KK-42 and its analogs. In Akai H. & Wu Z.S. (eds): *Wild Silkmoths '88.* International Society for Wild Silkmoths, Tokyo, pp. 79–84.

SUZUKI K., MINAGAWA T., KUMAGAI T., NAYA S., ENDO Y., OSANAI M. & KUWANO E. 1990: Control mechanism of diapause of the pharate first-instar larvae of the silkmoth Antheraea yamamai. *J. Insect Physiol.* **36**: 855–860.

TRUMAN J.W., TAGHERT P.H., COPENHAVER P.F. & TUBLITZ N.J. 1981a: Hormonal control of ecdysis through embryonic and post-embryonic development. In Sehnal F., Zabźa A., Menn J.J. & Cymborowski B. (eds): *Regulation of Insect Development and Behaviour.* Wrocław Technical Univeristy Press, Wrocław, pp. 1011–1020.

TRUMAN J.W., TAGHERT P.H., COPENHAVER P.F., TAUBLITZ N.J. & SCHWARTZ L.M. 1981b: Eclosion hormone may control all ecdysis in insects. *Nature* **291**: 70–71.

TRUMAN J.W. & COPENHAVER P.F. 1989: The larval eclosion hormone neurones in Manduca sexta: identification of the brain-proctodeal neurosectory system. *J. Exp. Biol.* **147**: 457–470.

UMEYA Y. 1950: Studies on embryonic hibernation and diapause in insects. *Proc. Japan Acad.* **26**: 1–9.

ŽITŇAN D., ŠAUMAN I. & SEHNAL F. 1993: Peptidergic innervation and endocrine cells of insect midgut. *Arch. Insect Biochem. Physiol.* **22**: 113–132.

Received June 11, 1993; accepted February 21, 1994