MINI-REVIEW

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Circadian clock and ecdysteroid titers in the wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) larvae

BRONISŁAW CYMBOROWSKI

Department of Invertebrate Physiology, Warsaw University, Żwirki i Wigury 93, 02-089 Warszawa, Poland

Physiology, wax moth, *Galleria mellonella*, ecdysteroids, circadian clock, prothoracic glands

Abstract. In this mini-review, data on the circadian rhythmicity in ecdysteroid synthesis by the prothoracic glands of final instar larvae of the wax moth (*Galleria mellonella*), as well as ecdysteroid titers in their hemolymph are summarized. It was found that at the low temperature of 18°C and in continuous darkness (DD), the last instar of the wax moth larvae did not pupate. When these larvae were transferred to 30°C and kept in DD, they immediately initiated development and pupated in a circadian manner. They exhibited the circadian rhythmicity of ecdysteroid titers in their hemolymph, with a period of approximately 24 hours.

The mechanism underlying circadian rhythmicity of ecdysteroid titers in the hemolymph of *Galleria mellonella* larvae was investigated. It was found that brain removal or head-ligating of larvae in which developmental processes had been synchronized by transfer from 18°C to 30°C, did not abolish daily rhythms of ecdysteroid titers in the hemolymph. The observed rhythms persisted in continuous darkness for 5 days after treatment, which suggests that the circadian clock driving these rhythms is not located within the brain.

In further experiments, it was shown that the prothoracic glands, taken from temperature-synchronized last instar larvae of *Galleria,* exhibited a daily rhythm of ecdysteroid synthesis, with a maximum coinciding with the peak of ecdysteroid titers found in controls. Therefore, it is suggested that the circadian rhythm of ecdysteroid titers in the hemolymph of these larvae is regulated by a clock, located probably within the prothoracic glands.

The processes of ecdysis and eclosion of many insects are subject to circadian timing, which has been analyzed at the neuroendocrine level, in several cases involving adult eclosion, larval moulting and also pupation. It has been shown that the release of two neurosecretory hormones, eclosion hormone and prothoracicotropic hormone (PTTH), which are involved in triggering these events, is under strict temporal control by circadian oscillator or oscillators. Several experimental techniques, particularly ablation and transplantation, have been used to localize the oscillators or the, so called, biological clocks, which time PTTH and eclosion hormone release from the insect brain (Truman, 1980; Takeda, 1984). For example, the gating of larval ecdysis results from circadian control of PTTH release into the hemolymph of the saturniid moth *Samia cynthia ricini* (Fujishita & Ischizaki, 1981). However, the final timing of these and many other events may be controlled by a circadian clock(-s) located within the prothoracic glands (Mizoguchi & Ischizaki, 1982, 1984; Cymborowski, et al., 1989). So far, it is evident that ecdysteroids, produced by prothoracic glands (PG), synchronize the cellular and molecular events which occur during the insect development. They appear to be able to act directly on the circadian system timing ecdysis (Ampleford & Steel, 1982, 1985). However, the activity of ecdysteroids,

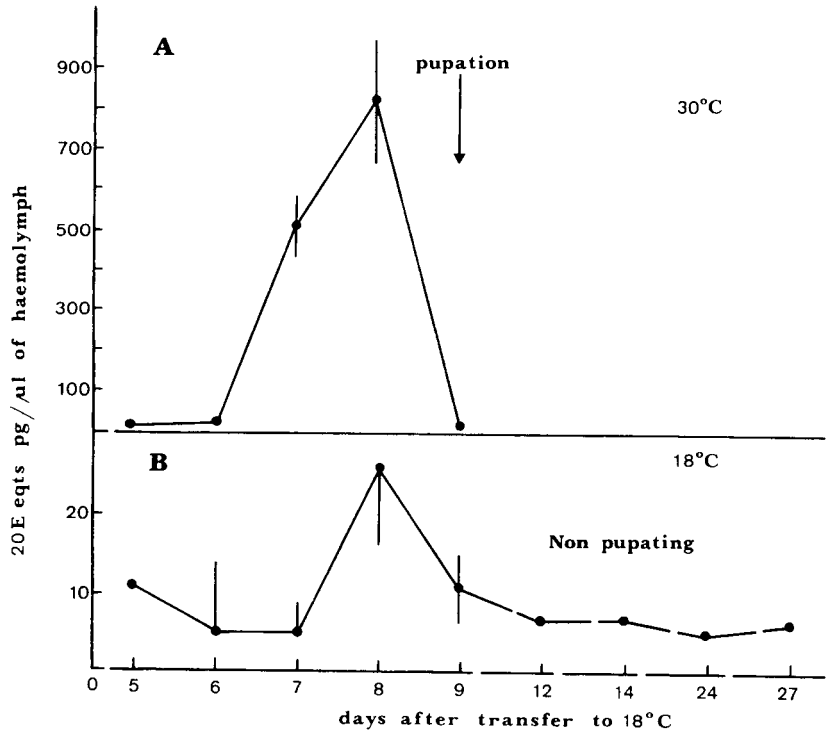

Fig. 1. Effects of lower temperature on ecdysteroid titers in the haemolymph of larvae of *Galleria mellonella* transferred from 30 to 18°C on the 1st day of the last instar. A – control, last instar larvae reared at 30°C; B – 1-day-old last instar larvae transferred to 18°C. Samples of haemolymph were assayed by EIA. Each point represents mean (± SD) of 3–6 separate determinations. Data are presented as pg of 20-hydroxyecdysone (20E) equivalents (eqts) per µl of haemolymph. (Modified from Mikołajczyk & Cymborowski, 1993).

during intermoult and the mechanism underlying circadian rhythmicity of ecdysteroid titer in insect hemolymph remains unknown.

In this mini-review the laboratory data accumulated on the mechanism of circadian rhythmicity of ecdysteroid titers in the hemolymph of final instar larvae of the wax moth (*Galleria mellonella*) are summarised. All experiments were performed on larvae reared in constant darkness (DD), as under natural environment in beehives, and at a constant temperature of either 30°C or 18°C. It was found that at the low temperature of 18°C the last instar wax moth larvae did not pupate and remained as larvae for a long period of time (up to about one year). When such larvae were transferred to the optimal temperature of 30°C they immediately initiated further development and pupated in a circadian manner, with period close to 24 hours (Śmietanko et al., 1989).

Using the enzyme immunoassay (EIA) method of Porcheron et al. (1989) for measuring the ecdysteroids titer, it was found that larvae of *Galleria mellonella* reared continuously at 30°C and under DD conditions had, in the second half of the last instar, a large increase

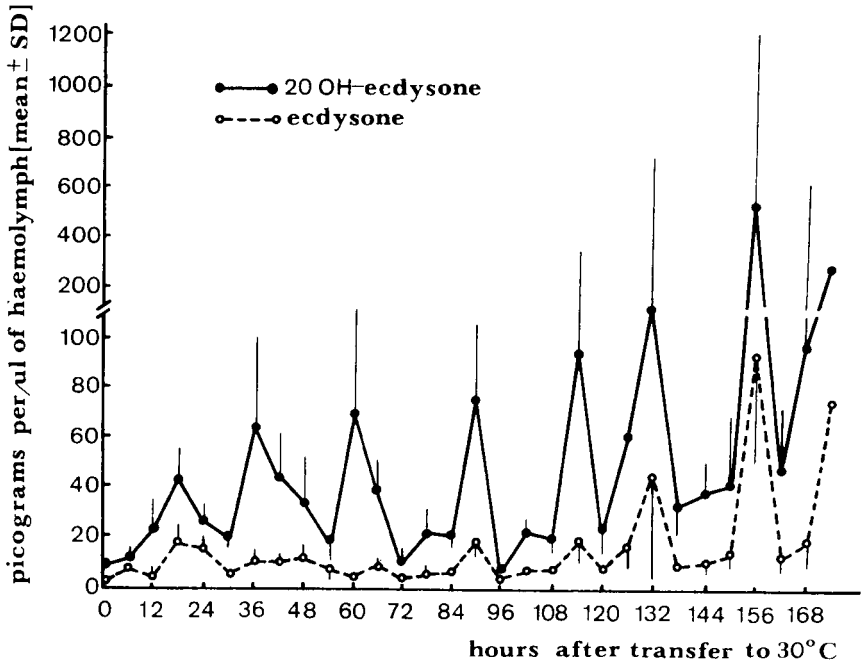

Fig. 2 (upper). Ecdysteroid titers in the haemolymph of the last instar larvae of *Galleria mellonella* reared for 90 days at 18°C and then transferred to 30°C at which the measurements were made every 6 hours until pupation. Ecdysteroids were determined by RIA method. Each point consists of three to six replications. Bars indicate standard deviations. (After Cymborowski et al., 1989).

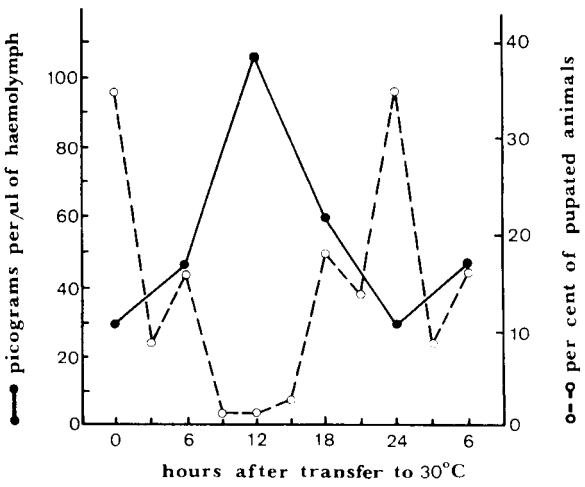

Fig. 3 (right). Circadian rhythms in ecdysteroid titers (mean values for 7 days) and pupation rhythms (means for 10 days) of the last instar larvae of *Galleria mellonella* kept for 90 days at 18°C and then transferred (day 0) to 30°C. (After Cymborowski et al., 1989).

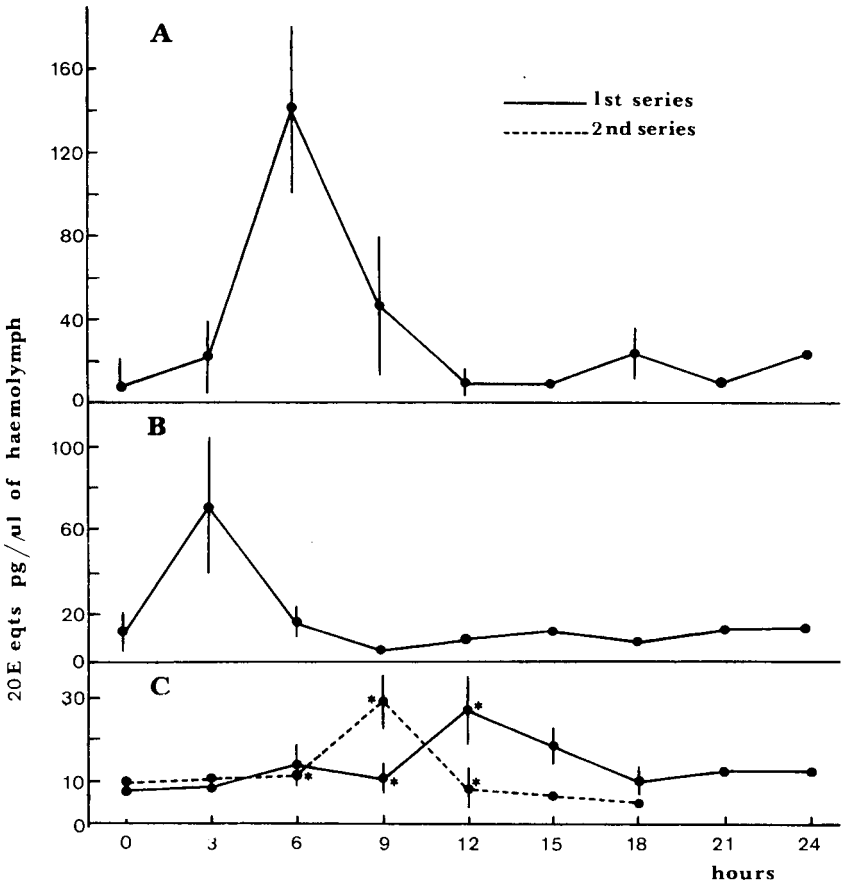

Fig. 4. Daily changes in ecdysteroid titers in haemolymph of the last instar larvae of *Galleria mellonella* on day 5 after treatment and transfer from 18 to 30°C. A – control larvae, B – brainless larvae, C – larvae ligated behind the head. Samples of haemolymph were assayed by EIA method. Each point represents mean (± SD) of 4–7 separate determinations. Data are presented as pg of 20-hydroxyecdysone (20E) equivalents (eqts) per µl of haemolymph. Asterisks indicate statistical significance at $p < 0.05$. Modified from Cymborowski et al., 1991.

in haemolymph ecdysteroid levels, reaching a peak of about 800 pg of 20-hydroxyecdysone equivalents/µl of haemolymph on day 8, which was followed by a rapid decrease to an undetectable level, by the time of pupation on day 9 (Fig. 1A). In larvae transferred to 18°C, on the first day of the last instar, and then reared continuously at this temperature without pupating, haemolymph ecdysteroid levels remained very low (less than 10 pg of 20-hydroxyecdysoneequivalents per microlitre of haemolymph), except for one small peak of about 27 pg of 20-hydroxyecdysone equivalents/µl of haemolymph, which occurred on day 8, after transfer to 18°C (Fig. 1B). This small peak was not sufficient to elicit further development leading to pupation and the larvae went into diapause (Mikołajczyk & Cymborowski, 1993).

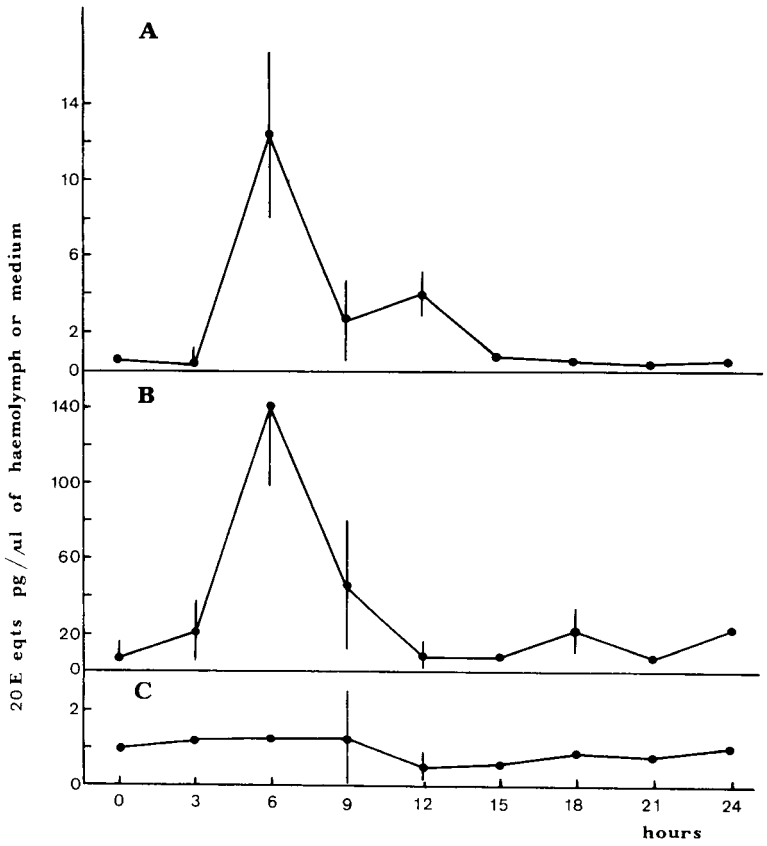

Fig. 5. Kinetic of synthesis of ecdysteroids by prothoracic glands of *Galleria mellonella* in vitro at successive hours during a 24-hour period on day 5 after transfer of the larvae from 18 to 30°C (A). Ecdysteroid titers in the haemolymph of the control larvae kept for three weeks at 18°C and then transferred to 30°C (B). Samples were taken every 3 hours on day 5 after transfer. Ecdysteroid titers in the haemolymph of 5-day-old larvae reared at 30°C (C). Aliquots of culture or samples of haemolymph were assayed by EIA. Each point represents the mean (± SD) of 6–8 separate determinations. In (A) data are presented as pg of 20-hydroxyecdysone (20E) equivalents (eqts) synthesized and released in culture medium per µl of medium. (After Cymborowski et al., 1991).

When the last instar larvae of *Galleria*, after spending about three months at 18°C and in constant darkness, were transferred to 30°C and further kept in DD, they initiated development immediately and pupated within 9–10 days. In the haemolymph of these larvae, determined by the RIA method of De Reggi et al. (1975), a circadian rhythm of ecdysteroid titers, with a period close to 24 hours, could be detected (Cymborowski et al., 1989). It is worth mentioning that, during 7 consecutive days, each subsequent ecdysteroid peak was higher than the previous one (Fig. 2). Pupation of these larvae was rhythmic. The majority of them pupated within a single gate of about 3 hours between 18 and 24 hr (Fig. 3).

In further experiments, the mechanism underlying the circadian rhythmicity of ecdysteroid titers in this insect was investigated. It was found that brain removal or head-ligating

of larvae, in which developmental processes had been synchronized by transfer from 18°C to 30°C, did not abolish circadian rhythms of ecdysteroid titers in the hemolymph (Cymborowski et al., 1991). Observed rhythms persisted even for 5 days after these treatments (Fig. 4), which suggested that the circadian clock driving these rhythms is not located within the brain.

This suggestion was confirmed by in vitro experiments, in which the prothoracic glands were taken from larvae every 3 hours during the 24 hr period, 4 days after the larvae had been transferred from 18°C to 30°C. The purpose of this experiment was to establish whether the peak of ecdysteroid titers found in the haemolymph of control larvae (see Fig. 1A) coincided with the maximum of synthetic activity of the prothoracic gland. As can be seen in Fig. 5, there is indeed such correlation. The in vitro maximum occurred at the same time as the peak of ecdysteroid titer of the haemolymph of the control larvae, transferred from 18°C to 30°C four days earlier (Fig. 5B). In another control group of larvae, kept at 30°C throughout the whole development (5-day-old last instar larvae), such a peak in ecdysteroid titer could not be detected (Fig. 5C).

These results suggest that ecdysteroid synthesis by prothoracic glands exhibit significant daily increases and decreases, being most active at the time when the peak of ecdysteroids is observed in the haemolymph of the control temperature-synchronized larvae. Therefore, it appears that the clock driving these rhythms is located within the prothoracic glands of the larvae of *Galleria*. Whether these glands respond promptly to phase shifts of light and/or temperature pulses has, yet, to be shown. It seems that ecdysteroids may be considered as a primary pacemaker for imposing rhythmicity over a wide variety of developmental processes, and act to maintain temporal order during the development. In this sense, ecdysteroids may act as time-keeping compounds in insect body. Daily releases of these could act as an internal Zeitgeber (timegiver), preparing the morphological, as well as physiological backgrounds for future developmental events, such as those of the last larval instar of *Galleria*, culminating in pupal ecdysis (Cymborowski et al., 1989).

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