ECDYSONE CHANGES IN THE HAEMOLYMPH OF TWO SILKWORMS (*BOMBYX MORI* AND *PHILOSAMIA CYNTHIA*) DURING LARVAL AND PUPAL DEVELOPMENT

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1. Introduction

The earliest attempts to determine ecdysone titers in insect tissue were made with bioassays on *Calliphora* [1], *Bombyx mori* [2] or *Sarcophaga* [3]. Recently developed physiochemical methods have however given a new scope to this essential aspect of insect physiology. In Lepidoptera some measurements have been made on *Pieris brassicae* late larvae and pupae, using a mass fragmentographic method [4] and on *Manduca sexta* late larvae, using a radioimmunoassay (RIA) [5]. Except for the work of Shaaya and Karlson [2] (who worked on total extracts of *Bombyx mori*), the above-mentioned researchers deal only with either larvo-nymphal or imaginal ecdysis. Therefore it seemed important to determine in the same insect the variations of circulating ecdysone at the following three points of its development: larval, larvo-pupal and imaginal ecdyses.

Ecdysone levels were determined by RIA in *Bombyx mori* and in *Philosamia cynthia*, another silkworm which undergoes a pupal diapause. A single hormonal peak was found at each developmental stage. The maximum values of these peaks in *Bombyx* were respectively 550, 900 and 6000 ng/ml; their duration varied at each stage, depending upon the extent of morphogenetic processes. Moreover, thin layer chromatography (TLC) was combined with RIA in order to determine the ratio of the two hormonal forms, α- and β-ecdysone, at certain critical periods of development.

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2. Materials and methods

2.1. *Experimental organisms*

Bombyx mori silkworms of European breed 200 X 300 were reared on fresh *Morus alba* leaves at 22 ± 0.5°C under natural light conditions. *Philosamia cynthia* larvae were reared on fresh *Ailanthus glandulosa* leaves under the same conditions. The larvae were exposed to light for eight hours every day to induce a pupal diapause in all insects. The development of the silkworms was carefully synchronized by selecting the larvae after each ecdysis.

2.2. *Haemolymph samples*

A sample of haemolymph (20–200 μl) was taken from one insect of each species every day by removing a proleg (larvae) or an antenna (pupae). This operation was repeated several times for several assays. The samples were plunged into liquid nitrogen and lyophilised. Male and female *Bombyx* pupae were separated.

2.3. *Radioimmunoassays*

Using the method developed by de Reggi et al. [6], each RIA was performed on the haemolymph of one larva or pupa. In this report the word 'ecdysone' designates all RIA active material. The anti-ecdysone serum used shows a reduced affinity for dehydroecdysones and inactive derivatives.

2.4. *Combined TLC/RIA analysis*

Biological extracts were taken from hemolymph samples using methanol/water (70 : 30, v/v) solvent (300 μl). 20 μl of this solution were chromatographed on pre-coated plates of silica gel (Merck F-254) in a solvent system of chloroform/methanol (80 : 20, v/v) (2 X1 h). Samples of α- and β-ecdysone were run as standards (α-ecdysone was kindly supplied by J. P. Delbecque, and β- was purchased from Schwarz-Mann). The gel was then divided according to the position of the standards, scraped off and suspended in methanol/water. After removing the gel, the solvent was evaporated, and the RIA activity of each fraction was determined.

This method provided two kinds of information. First the respective quantities of α- and β-ecdysone, and second that part of the total RIA activity is due to other ecdysteroids.

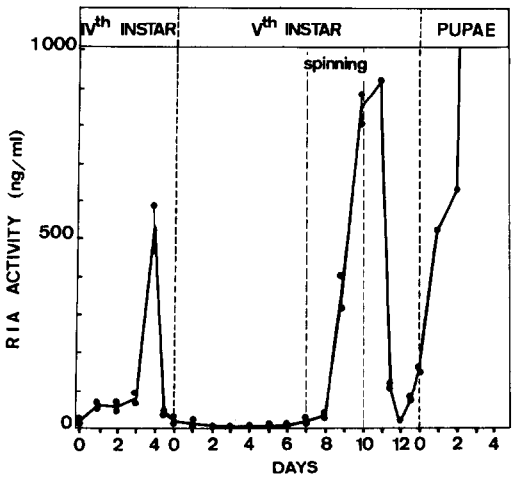

Fig.1. Ecdysone changes in the haemolymph of *Bombyx mori* larvae during the 4th and 5th larval instars as determined by radioimmunoassay. The days 'zero' correspond to each ecdysis.

3. Results

Because of good agreement in the results, two determinations per day were judged sufficient. The two corresponding values are shown separately on the graphs. RIA activity is expressed as ng of ecdysterone-equivalent.

3.1. *Bombyx mori*

Figure 1 reveals large changes in haemolymph ecdysone content of larvae. At each instar a high peak precedes molting. During the 4th larval instar a low but significant level of ecdysone is observed during the feeding period. The peak (550 ng/ml) occurs on the 4th day, when the larvae stop feeding, and then falls before ecdysis. The ecdysone titer continues to decrease during the first day on the 5th larval instar, after which no ecdysone is detected in the haemolymph. Silkworms start spinning their cocoons before the ecdysone level rises. Ecdysone reaches 900 ng/ml on the 11th day, falls a few hours before the larvo–pupal ecdysis and is 150 ng/ml at ecdysis. During pupal development, a single large peak appears (fig.2). In males and females the highest ecdysone concentration found was on days 4/5 and was about 6000 ng/ml, i.e. 1.3×10^{-6} M. The concentration then decreases sharply in both sexes, being only 10–20 ng/ml a few hours before emergence. During most of the pupal development (9 days) the ecdysone titer is at or above 500 ng/ml, the highest value found during the fourth larval instar.

3.2. *Philosamia cynthia*

The ecdysone variations in *Philosamaia* are similar to those observed previously (fig.3); a peak appears just before molting starts. Nevertheless, the

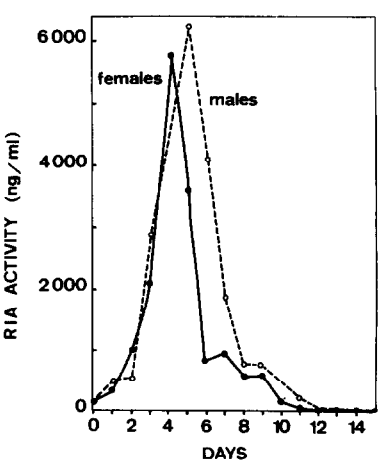

Fig.2. Ecdysone changes in the haemolymphs of *Bombyx mori* pupae, as determined by radioimmunoassay. The emergence occurs at days 15–16.

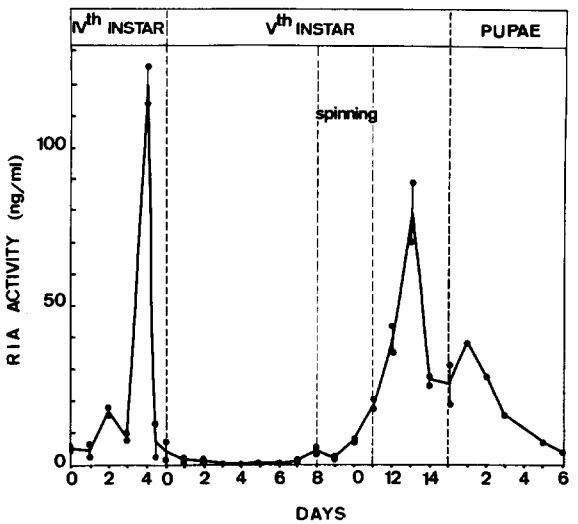

Fig.3. Ecdysone changes in the haemolymph of *Philosamia cynthia* larvae during the 4th and 5th larval instars, as determined by radioimmunoassay. The days 'zero' correspond to each ecdysis.

hormonal titers are five times lower than in *Bombyx*. Moreover, the peak of the 5th stage occurs later with respect to the spinning period in *Philosamia* than in *Bombyx*; it is also smaller (90 ng/ml) than in the 4th stage (110 ng/ml). In diapausing pupae, the ecdysone titer falls rapidly and becomes negligible five days after ecdysis.

3.3. *Determination of the α:β ratio*

A combination of TLC and RIA analysis of the haemolymph samples was performed on *Bombyx* larvae and pupae (only male pupae were used for this test). The results are summarized in table 1. In larvae, the α- and β-ecdysone fractions accounted for 88% of the total RIA activity. The other ecdysteroids detected were either non-migrating conjugates or more apolar materials, corresponding to 3-dehydro-α- (or β-) ecdysone fractions. The sum of α- and β-ecdysone is lower in pupae, particularly in 3 day-old pupae because of the higher proportion of more apolar compounds. If we consider the α- and β-fractions exclusively, it appears that 95% of the hormonal peak in fourth instar larvae is made up of the β- form. At the peak of hormonal activity, in fifth instar larvae, this proportion falls to 65% on day 10 and then to 12% on day 11. In three to five day-old male pupae, no or very little RIA activity is found in the β-ecdysone fraction.

4. Discussion

Our results on *Bombyx* and *Philosamia* confirm the main conclusions drawn by Shaaya and Karlson [2] but our graphs show sharper peaks of ecdysone activity as a result of more precise measurements and/or because we were considering only the haemolymph- and not the whole body-ecdysone. We conclude that (1) the ecdysone level rises just before the third and fourth larval moults; (2) hormone activity is low when ecdysis takes place: (3) in *Bombyx* hormone activity rises again just prior to

Table 1
Partitioning of RIA activity in *Bombyx mori* between α- and β-ecdysone and other ecdysteroids, as observed by combined thin-layer chromatography and radioimmunoassay analysis

	Larvae			Nymphs	
	Fourth instar	Fifth instar			
	Day 4	Day 10	Day 11	Day 3	Day 5
(α + β) / Total (per cent)	88	n.a.	87	53	78
α / (α + β) (per cent)	5	35	88	99	98

n.a. = non assayed

larvo–pupal ecdysis and there is a high ecdysone peak during the first part of pupal development; (4) the duration of high ecdysone content in the haemolymph varies with the instar.

Moreover in both silkworms the haemolymph is completely devoid of ecdysone for several days or hours preceding an intense period of hormonal activity (feeding period of the 5th larval instar, pharate pupae of *Bombyx*). Lack of synthesis or intense functioning of the metabolic pathways described by Hoffmann [7] may account for this total absence of ecdysone.

However, we stress three problems: (1) the differences observed between the two Lepidopterae; (2) the duration of high ecdysone titer; (3) the different results on total extracts and haemolymph samples of *Bombyx mori* pupae.

4.1. *Analyses of interspecific differences*

The hormonal profiles observed show marked interspecific variations, in particular since the absolute values of ecdysone titers are much lower in *Philosamia* than in *Bombyx*. It is remarkable that the hormonal behaviour of two so closely related species can be so different. Can this fact be interpreted to mean that *Philosamia* tissues have a higher affinity for ecdysone? On the other hand, one might expect that the diapausal interruption of pupal development would modify the ecdysone profile in the latter species. It does undoubtedly lead to the fall of ecdysone titer in pupae. The pupal diapause probably also causes the lowering and delaying of the peak in late larvae. However, Calvez [8] assumed that in *Pieris brassicae* the ecdysone peak values observed in the 5th larval instar are unmodified in insects reared under light conditions inducing pupal diapause.

4.2. *Significance of ecdysone peak duration*

In silkworms the ecdysone peak lasted from 4–5 days in prepupae; during the larval–pupal development, the ecdysone titer stayed for 9 days as high or higher than the maximum value observed during the 4th larval instar. These high ecdysone concentrations must be linked to the particular nature of larvo–nymphal ecdysis and to metamorphosis. Many target tissues, especially imaginal discs can at the last larval stage and in pupae be the site of cell differentiation and morphogenesis [9,10]. So we think that the exposure time of the cells to the hormone is an important differentiation factor, as suggested by in vitro experiments [10–12].

4.3. *Total ecdysone and circulating ecdysone in pupae*

The ecdysone titers in total tissue extracts from *Bombyx* pupae [13] undergo sex-dependent variations. A first peak, comparable to the circulating ecdysone peak that we described above, occurs in both males and females. A second peak appears only in females and is due to ovarian contribution. In good agreement with this observation, it has been recently shown that oocytes of *Bombyx* contain a high titer of ecdysone which rises during egg maturation [14]. This ovarian ecdysone, which appears in many insects [15–17] is certainly synthetized in situ, seeing that Hoffmann et al. [18] demonstrate that ecdysone is produced by follicular cells in the ovary of *Locusta migratoria*. It is noteworthy that ovary-generated ecdysone is not detectable in the haemolymph of late pupae of Bombyx.

4.4. *α:β ratio*

One final point worth mentioning is the characterization of the hormonal forms which are found in *Bombyx*. The quasi-totality of RIA activity in larvae is provided by α- and β-ecdysone. In contrast, for pupae, the proportion of conjugates or more apolar forms (probably inactivated products) is much higher. The still other more exciting results are the variations observed in the α : β ratio. In fourth instar larvae, the hormonal form is β-ecdysone, but the situation changes during the fifth stage. The proportion of α-ecdysone rises to 35% at the beginning of the hormonal peak and to 88% the next day. Lastly, in pupae no or very little β-ecdysone is found. Thus, when Butenandt and Karlson [19] extracted from *Bombyx* pupae the hormonal factor responsible for ecdysis, they logically found α-ecdysone.

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