Ecdysteroid and juvenile hormone changes in *Bombyx mori* eggs, related to the initiation of diapause

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We describe a method for the routine determination of changes in juvenile hormone levels in insect eggs. The hormones are first converted into their diol derivatives, then they are purified from other lipids and separated by high-performance liquid chromatography (HPLC). The radioimmunoassay of the fractions was then determined. The method permits the simultaneous assay of ecdysteroids, and it was used for determining the hormonal changes in *Bombyx* eggs during the pre-diapause development. Our major finding is that the hormonal content of eggs dramatically increased prior to the initiation of diapause. This hormonal rise included ecdysone, 20-OH-ecdysone and 3 juvenile hormones. The HPLC retention time of the latter corresponded to JH_1, JH_2 and JH_3. Subsequently, the embryos entered diapause and the hormonal content of eggs was reduced to traces of ecdysteroids. These dramatic changes in juvenile hormone levels during early embryogenesis raise a number of issues which are developed in the discussion.

Ecdysteroid *Juvenile hormone* (Bombyx mori *egg)* *Diapause initiation* *Hormone level changes* *Embryogenesis*

1. INTRODUCTION

Ecdysteroids and juvenile hormones (JH) are involved in the control of the life cycle of insects, including oogenesis and embryonic development. This has been carefully established for ecdysteroids. However, studies on juvenile hormones have been hampered by the lack of reliable data on the in vivo concentration and nature of the hormones. Several techniques have been developed for this purpose, namely biological tests [1,2], gas chromatography coupled to mass spectrometry (GC-MS) [3,4] and radioimmunoassay (RIA) [5]. Owing to these techniques, active investigations are in progress in this field. Some difficulties persist, however. Because of the lipophilic properties of the hormones, JH quantification is especially difficult in material which contains high concentrations of lipids, as whole insect body and eggs. For each of the above methods, these compounds present specific difficulties in the analysis of relevant hormones. In the RIA, lipids would create micelles and micro-fat globules in the antibody solution and, because of their poor solubility in water, JHs and the labelled hormone would be trapped in these particles. The RIA method developed in [6] counters this problem by chemically converting JHs into their diol derivatives. This conversion increases their solubility in water and makes their separation from other lipids much easier. Here, we describe the procedure designed for routine RIA assays in developing eggs, which contain a wide spectrum of lipids. The technique was used to determine the changes in JH levels during the early embryogenesis of *Bombyx mori*, from oviposition until diapause entrance. The protocol allows us to simultaneously determine the variations of both

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JHs and ecdysteroids, the two hormonal families being assayed from the same egg sample.

2. MATERIALS AND METHODS

We used silkworms of the European line 200 × 300 from Lyon. Eggs of this univoltine strain begin to develop immediately after laying but developing stops 4–5 days later as diapause starts [7]; at this time the embryo appears as a germ band. Diapause is broken by allowing eggs to hibernate at 7°C for 3 months and then incubating them at 22°C. Larvae hatch 15–16 days later. Diapause can be also terminated by a 20-OH-ecdysone treatment [8].

Newly-laid eggs undergo color changes, due to the progressive coloration of serosal cuticle. They are first yellow and then turn pink, brown and lastly gray. Yellow, pink and brown stages last about 24 h. Such changes are specific to diapausing eggs and were used as criterias for monitoring prediapausal development.

2.1. *Hormonal assay*

Samples pooling 200 eggs of each stage were collected (gray eggs were sampled immediately after the brown–gray color transition). They were homogenized in 1500 μl hexane plus 500 μl methanol and 250 μl water. The homogenate was gently centrifuged to separate the hexane phase, which contains the juvenile hormones, from the methanol/water phase, that contains the ecdysteroids. The latter phase was further rinsed with 1 ml hexane, then the 2 hexane fractions were pooled.

RIAs were performed on the 2 extracts.

2.2. *Radioimmunoassay of JHs*

We used a four-step procedure: (1) hormones were converted into their diol derivatives; (2) purified from other lipids; and (3) separated by HPLC; then (4) the RIA activity of HPLC fractions was determined.

(1) *Conversion into diols:* The hexane extract was dry-evaporated and the residue was dissolved in 1.5 ml dioxane plus 130 μl 0.5 N H_2SO_4. The solution was incubated at 40°C for 15 h.

(2) *Purification:* This step was achieved by: (i) solvent partitioning; (ii) mini-silicagel column; and (iii) thin-layer chromatography (TLC).

At the end of the incubation mentioned above, 1.5 ml hexane plus 2 ml 0.01 N NaOH were added and the solution was vigorously shaken for 1 min and then centrifuged. JH diols are in the upper hexane phase, while many of the fatty acids and other polar lipids remain in the dioxane/water phase. The hexane was loaded onto a silicagel column (7 × 30 mm) equilibrated in hexane. The effluent was discarded and the column was rinsed with 2 ml hexane. JH diols were then eluted with 2 ml acetone. Most apolar lipids were eliminated at this step. The latter acetone solution was loaded onto a TLC silicagel plate which was developed twice with heptane/dioxane (70/30, v/v) (the migration area was 10 cm long). The diol containing area was cut out and eluted with 1 ml acetone.

(3) *HPLC:* The acetone was evaporated and the dry residue was redissolved in 300 μl methanol. The latter was injected onto a Bondapak C-18 column (3.4 mm i.d. × 300 mm) which was eluted with an acetonitrile/water gradient (40/60 to 60/40 (v/v); duration 30 min; flow 1 ml/min). Fractions of 1 ml were collected. JH_3 diol was eluted in fractions 15–16, JH_2 diol in fractions 20–21 and JH_1 diol in fractions 24–25.

(4) *RIA:* We determined the RIA activity of each HPLC fraction. For this purpose, 250 μl 0.1 M citrate buffer were added to a suitable aliquot of the fraction, which was lyophilized and then redissolved in 250 μl water. Lastly, this solution was assayed for JHs, as in [6]. Only fractions corresponding to JH_1, JH_2 and JH_3 diols showed any RIA activity.

The protocol was primarily designed for routine JH assays rather than the recovery of the highest yield of hormone. The latter should be almost constant from one experiment to another, since the high sensitivity of the technique does not allow the use of any labelled JH as internal standard. Thus, the percentage of JH recovered at each step of the procedure was repeatedly checked by adding ^3H-labelled JH_1 (Sigma) to control samples of eggs. Table 1 shows that the final yield of hormone recovery was close to 37%, thus the actual hormone level was accurately assumed to be 2.7-times the result given by the RIA.

Table 1

Percentage of ^{3}H-labelled JH_1 recovery at each step of the procedure (mean value from 10 replicates and standard error of the mean)

Step	% Hormone recovery
1. Initial extract	93% ± 1.5
2. Solvent partitioning	81% ± 2.0
3. Silicagel column	63% ± 2.2
4. TLC	45% ± 2.6
5. HPLC	37% ± 3.1

2.3. *Radioimmunoassay of ecdysteroids*

Methanol (500 μl) was added to the methanol/water extract and the solution was centrifuged to discard the precipitated protein and the insoluble material. Then, an aliquot of the supernatant was injected onto a Merk Lichrosorb RP-18 HPLC column (3.4 × 130 mm) which was eluted by the following gradient: acetonitrile/0.1% trifluoroacetic acid (TFA) (0/100–30/70, v/v; duration 20 min; flow 1 ml/min). Fractions of 1 ml were collected; 20-OH-ecdysone was eluted in fraction 15 and ecdysone in fraction 17. Our attention was focused on these two compounds. Thus, fractions 12–19 were lyophilized. The residue was dissolved in 250 μl of citrate buffer and the solution was assayed as in [9]. Only fractions corresponding to ecdysone and 20-OH-ecdysone showed any RIA activity.

All the results are expressed as μmol hormone × mg eggs; this corresponds to a molar concentration if we consider that the specific density of eggs is equal to 1.

The experiment was performed two times. Repeated experiments yielded reproducible consistent results.

3. RESULTS

Fig.1 shows the mean values of the 2 experiments performed. It shows that the hormonal content of *Bombyx mori* eggs underwent dramatic changes during the early embryonic development.

Yellow eggs: Newly-laid eggs contained high levels of both ecdysone and 20-OH-ecdysone, the latter being at the highest concentration (~4 × 10^{-7} μmol/mg); however, no JHs were detectable during this stage.

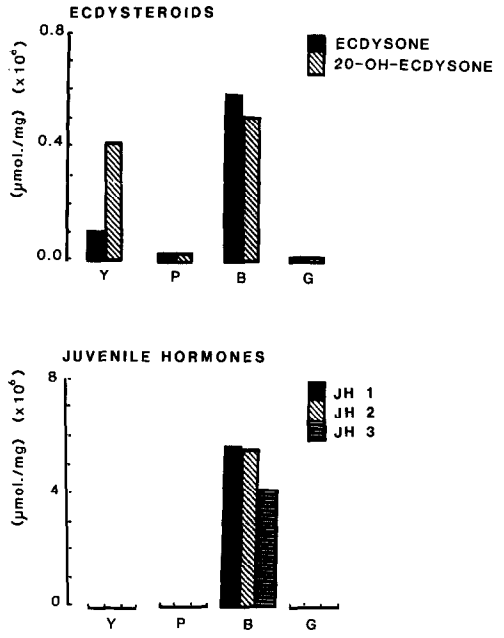

Fig.1. Changes in ecdysteroid (ecdysone and 20-OH-ecdysone) and in juvenile hormone levels in *Bombyx mori* eggs, during the early embryonic development. Pre-diapausal development is correlated to changes in the egg shell color: yellow (Y), pink (P), brown (B) and gray (G). The brown–gray color transition coincides with the onset of diapause.

Pink eggs: Ecdysteroids were drastically reduced during the second day of embryonic development and no JH appeared so that the hormonal content of these eggs was very low.

Brown eggs: in contrast to pink eggs, very high hormone concentrations were observed in brown eggs. Ecdysone and 20-OH-ecdysone levels reached about 5 × 10^{-7} μmol/mg. Simultaneously three juvenile hormones appeared. They reached levels as high as 5 × 10^{-6} μmol/mg for JH_1 and JH_2, and 4 × 10^{-6} μmol/mg for JH_3. It should be emphasized that this pattern of JH RIA activity was consistent with the A_{225} of the fractions. Such high hormonal concentrations were specific to the brown egg stage.

Gray eggs: nearly all the hormone disappeared and only a few residual ecdysteroids were still observed. This situation remained unchanged during the diapause period.

4. DISCUSSION

We found that JH levels can reach very high values in very young embryos of *Bombyx* and that they can undergo rapid and dramatic changes, varying from undetectable level to $>5 \times 10^{-6}$ μmol/mg in about 24 h. This phenomenon is a common observation for ecdysteroids but not for JH. In [4], the MS-CG technique was used for determining the JH content of eggs from various species and they found rather low values (0.09–5.4 ng/g; i.e., about 0.3×10^{-9}–18×10^{-9} μmol/mg). On the other hand, high JH levels have been described in eggs and embryos of the cockroach *Nauphoeta* [11], using the *Galleria* bioassay, but all the JH material appeared after the dorsal closure, during the later stages of embryonic development; JHs were not detected during early embryogenesis.

The presence of high JH concentration in very young embryos raises a number of questions. At this early stage, apparently no differentiated structure is present which can be responsible for hormone synthesis. As for ecdysteroids, it is a well-known fact that a specific metabolic pathway leads to the release of ecdysone and 20-OH-ecdysone in developing eggs. During their maturation, oocytes are filled with conjugated ecdysteroids produced by the ovary. These conjugates are subsequently hydrolyzed during the embryonic development and the free hormones are released [11]. This raises the possibility that a comparable metabolic pathway could be involved in the production of JHs in developing eggs, as for the production of ecdysones.

4.1. *Nature of the JH RIA-active material*

At least 5 different juvenile hormones have been already found in insect embryos [12a] and perhaps others are as yet undescribed, forcing prudence regarding interpretations as to the exact nature of the compounds discovered in *Bombyx mori* eggs. We can say that these products are active in our test and thus belong to the juvenile hormone family. Moreover, according to their HPLC elution pattern, we assume that they are JH_1, JH_2 and JH_3. However, this interpretation requires further confirmation.

4.2. *Physiological significance of the hormonal patterns*

It is generally assumed that 20-OH-ecdysone stimulates the synthesis of embryo's cuticles, as it does in larvae. Thus, the ecdysteroids observed in *Bombyx* eggs immediately after egg-laying are probably involved in the triggering of the deposition of the serosal cuticle, a phenomenon which occurs on the first day of embryogenesis [12b].

However, the second period of hormonal activity, which appeared during the brown-egg stage and which included both ecdysteroids and JH is undoubtedly related to the initiation of diapause. This assumption is supported by the fact that this brown-egg stage is specific to diapausing embryos; it does appear in univoltine strains. At the same time (i.e., 3 days after oviposition) non-diapausing eggs would have reached the metamerisation stage, and thus they would have a very different hormonal pattern [13]. Generally speaking, diapause initiation in insect larvae and pupae is associated with a decrease in ecdysteroid titers and, conversely, diapause can be terminated by injecting 20-OH-ecdysone [14]. This has also been demonstrated for embryogenesis [8,15]. Thus the presence of ecdysone and 20-OH-ecdysone during the brown stage of *Bombyx mori* eggs is unexpected. However, it should be underlined that this ecdysteroid increase is accompanied by a simultaneous rise in JH titers. The effects of the former can be altered by the action of the latter. Indeed larval–pupal diapause is assumed to be regulated by JH. However, whether diapause maintenance needs high JH concentrations or not is unclear. In some cases diapause can be induced by application of JH analogue and diapausing animals have relatively high JH titers (*Chilo*, [16]) whereas in other cases diapause is induced by removal of corpora allata and diapausing individuals have low JH titers (*Leptinotarsa*, [17]). Such conflicting results can translate differences in the physiology of diapause from one insect species to another. In any case, our findings show that the levels of both ecdysteroids and juvenile hormones must be taken into consideration. The most probable conclusion is that the diapausal breakdown of *Bombyx* embryonic development is triggered by the combined action of the ecdysones and the 3 juvenile hormones.

We stress that after the diapause has been initiated only traces of ecdysones remain in the eggs, the juvenile hormones being undetectable. This situation remained unchanged until embryogenesis resumed. After termination of diapause, drastic and fast changes in ecdysteroid and in juvenile hormone levels are observed again. As seen before diapause, each day of embryonic development has its own specific hormonal pattern [13]. The effects of these hormones and the mechanisms by which they control the embryogenesis of insects is still unknown.

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