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Role of low ecdysteroid levels in the early last larval instar of *Bombyx mori*

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Abstract. The relationship between juvenile hormone and ecdysteroid titers during the early stage of the last larval instar of the silkworm, *Bombyx mori*, was examined in this study. When larvae were fed 20-hydroxyecdysone-supplemented mulberry leaves throughout the last larval instar, 100% underwent supernumerary larval molting instead of metamorphosis. The application of juvenile hormone mimic during the early last instar did not induce supernumerary larval molting, but did delay metamorphosis. Temporal and quantitative ecdysteroid titer measurements revealed that in normal larvae the titers maintained very low levels (3–12 ng/ml) during the early stage of the last instar; however, in 20-hydroxyecdysone-treated larvae, levels ranging from 24 to 45 ng/ml were detected, and a major peak (246 ng/ml) was observed on day 6. These results show that very low ecdysteroid titer levels during the early stage of the last larval instar may play an important role in initiating decreases in juvenile hormone titers as well as in directing metamorphosis.
Key words. Low ecdysteroid titers levels; supernumerary larvae; metamorphosis; *Bombyx mori.*

In holometabolous insects, juvenile hormone titers decline to undetectable levels by the midpoint of the last larval instar. It has previously been demonstrated that this decline is due to the combined effects of the inactivation of the corpora allata[1] and the elevation of juvenile hormone esterase levels[2]. In contrast, ecdysteroid titers maintain very low levels during the early stage of the last larval instar[3]. It is suspected that in some insect species larval-pupal metamorphosis does not depend solely on the inactivation of the corpora allata, but also on the simultaneous inactivation of the prothoracic glands[4,5]. However, the actual role of the inactive prothoracic glands, and the relationship between juvenile hormone and ecdysteroid titers during the early stage of the last instar, are not very clear.

In *Bombyx mori*, the endocrine system underlying larval-larval molting is completely different from that underlying larval-pupal metamorphosis[6,7]. One difference is observable in the changes of hemolymph ecdysteroid titers; the titers maintain levels from 30 to 50 ng/ml during the early penultimate instar[8], but become undetectable during the early last instar[9]. Observed differences in the biosynthetic activity of the prothoracic glands between the penultimate and last instars appear to account for these different ecdysteroid titer levels[10]. However, the reason for such a difference is unclear, and no attempt has yet been made to clarify the role of the observed low ecdysteroid levels in the early last larval instar.

Our previous study[11] of *B. mori* recessive trimolters showed that ecdysteroid titer levels exhibited characteristic changes during the last (fourth) larval instar. We found that the decrease which occurs after the small increase in ecdysteroid titers during the early last instar may play a role in directing precocious metamorphosis. In the early penultimate (fourth) instar of tetramolter silkworms, precocious metamorphosis can be induced when a decrease in ecdysteroid titers is artificially produced by treatment with KK-42[12] or by exposure to high temperature and moisture[13]. These results led us to consider the possibility that those very low ecdysteroid titer levels observed in the early stage of the last larval instar of tetramolter silkworms may also be an important signal for larvae to undergo metamorphosis.

Here we report that when low ecdysteroid levels in the early last instar of tetramolter silkworms are artificially elevated by treating the food source with 20-hydroxyecdysone, a decline in juvenile hormone titers is prevented, and therefore supernumerary larval molting can be induced. Evidence that low ecdysteroid levels in the early last instar may be a prerequisite for larvae to undergo metamorphosis is presented.

Materials and methods

Experimental animals. Larvae of the silkworm, *B. mori* (Hounan), were reared on fresh mulberry leaves at 24 ± 1 °C under a 12 L:12 D photoperiod. Newly-ecdysed last instar larvae were collected and used for experiments.

Application of hormones. 20-hydroxyecdysone (Sigma Chemical Co., St. Louis, MD) was dissolved in distilled water to a concentration of 20 ppm and applied topically to mulberry leaves. Last instar larvae were fed these treated mulberry leaves and the development of larvae was carefully observed. Initial feeding of the 20-hydroxyecdysone-treated leaves took place on different days during the last instar.

A juvenile hormone mimic (hydroprene, Zoecon Corp., CA) was dissolved in acetone (HPLC grade) to a concentration of 1 mg/ml; 10 µl of solution was applied

topically near the dorsal midline of larval abdomens. Control specimens were treated with 10 µl of acetone only.

Radioimmunoassay of hemolymph ecdysteroid levels. Individual hemolymph samples (100–200 µl from each animal) were collected and stored at −70 °C until assays were conducted. Using methanol, ecdysteroid was extracted from the hemolymph, after which titers were determined by radioimmunosassay (RIA) according to procedures described in previous studies[14,15].

In vitro assay of prothoracic gland activity. Prothoracic glands were dissected, rinsed in insect Ringer's[10], and individually incubated in 100 µl of Grace's tissue culture medium (Gibco, Grand Island, NY). Incubations were continued for 8 h at 25 °C in high humidity, after which each medium was assayed for ecdysteroid content using an ecdysteroid RIA. Ecdysone was used as the unlabeled ligand for the RIA; synthesis is expressed in ng ecdysone. Ecdysone and tritiated ecdysone (70 Ci/mmol) were purchased from Sigma and New England Nuclear Co., respectively.

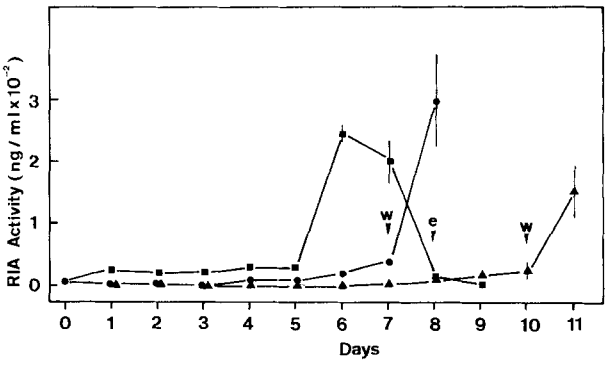

Figure 2. Effects of 20-hydroxyecdysone feeding or hydroprene application on hemolymph ecdysteroid titers during the last larval instar. Squares represent larvae that were fed 20-hydroxyecdysone-supplemented mulberry leaves throughout the instar. Triangles represent larvae treated with hydroprene during the first three days of the last instar. Circles represent control larvae which were treated with acetone and reared on normal mulberry leaves. e, supernumerary larval molting; w, wandering, Ecdysteroid quantities were determined by RIA and calibrated using 20-hydroxyecdysone as a standard. Each point represents a mean value from four separate assays. Bars indicate S.D.

Results

Effects of 20-hydroxyecdysone feeding. Changes in hemolymph ecdysteroid levels during the last larval instar are shown in figures 1 and 2. In newly-ecdysed last instar larvae, the ecdysteroid level was 12 ± 5 ng/ml. In control larvae, the titers remained at very low levels (3–12 ng/ml) during the first three days; on day 4, a slight increase in titer level was observed, and a major increase was detected after day 7 (when larvae began wandering). In treated larvae, ecdysteroid titers increased within one hour following 20-hydroxyecdysone feeding (fig. 1), then remained at a constant level between 24 and 45 ng/ml throughout the first five days (fig. 2). These levels then increased dramatically to a major peak (246 ng/ml) on day 6.

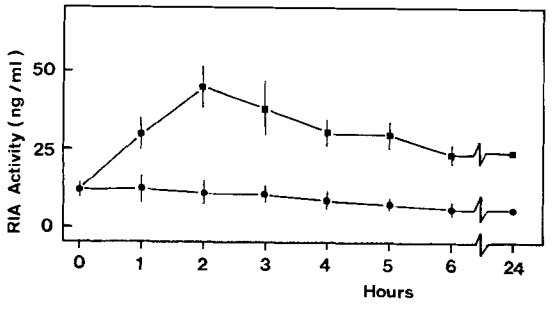

Figure 1. Effects of 20-hydroxyecdysone feeding on hemolymph ecdysteroid titers during the first 24 h of the last larval instar. Newly-ecdysed last instar larvae were fed 20-hydroxyecdysone-supplemented mulberry levels throughout the instar (squares); control larvae (circles) were reared on normal mulberry levels. Ecdysteroid quantities were determined by RIA and calibrated using 20-hydroxyecdysone as a standard. Hour 0 corresponds to the first feeding of last instar larvae. Each point represents a mean value from four separate assays. Bars indicate S.D.

Changes in prothoracic gland biosynthetic activity during the last instar of treated larvae were also measured; results are presented in figure 3. Prothoracic glands from control larvae did not produce ecdysteroid during the first three days, after which they showed an increase in activity; a major increase was observed after day 7. Like the control larvae, the prothoracic glands of larvae treated with 20-hydroxyecdysone did not produce ecdysteroid during the first three days of the last instar. They then increased their activity on day 5, and a major

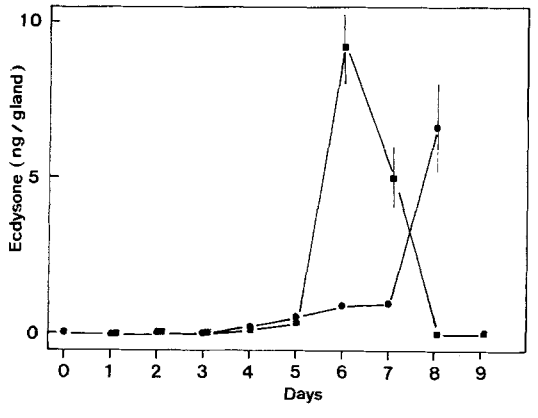

Figure 3. Effects of 20-hydroxyecdysone feeding on prothoracic gland biosynthetic ability during the last larval instar. Newly-ecdysed last instar larvae were fed 20-hydroxyecdysone-supplemented mulberry leaves throughout the instar (squares); control larvae (circles) were reared on normal mulberry leaves. Prothoracic glands from treated and control larvae were dissected and incubated in Grace's tissue culture medium for 8 h; ecdysteroid synthesis was quantified by RIA and calibrated using ecdysone as a standard. Each point represents a mean value from four separate assays. Bars indicate S.D.

Effects of 20-hydroxyecdysone feeding on the development of last instar larvae

Day*	Superlarvae	Pupae	Characteristics
0	10	0	Small amount of pupal cuticle on head
1	10	0	Some pupal cuticle on head and legs
2	6	4	Superlarvae with more pronounced pupal cuticle and pupae showing accelerated metamorphosis
3	0	10	Pupae showing accelerated metamorphosis

20-hydroxyecdysone was dissolved in distilled water to a concentration of 20 ppm, then applied topically to mulberry leaves. Last instar larvae were fed these treated mulberry leaves and the development of larvae was carefully observed. The initial feeding of 20-hydroxyecdysone began on different days of the last instar. *'Day' refers to the day on which feeding with 20-hydroxyecdysone started.

peak was observed on day 6 (when larvae began slipping their head capsules to the supernumerary larval instar). These results clearly show that, following 20-hydroxyecdysone feeding, prothoracic gland activity during the first three days was not significantly increased compared to that observed in control larvae.

After 20-hydroxyecdysone feeding, the development of larvae was carefully examined during the subsequent stage; results are shown in the table. We found that when treatment with 20-hydroxyecdysone began on day 0 of the last instar, supernumerary larval molting occurred on day 8. These supernumerary larvae always showed a few pupal patches on their heads; some fed sporadically during the supernumerary larval instar, but all larvae ultimately died. Hemolymph ecdysteroid titer levels remained very low (5–11 ng/ml) during the early stage of the supernumerary larval instar – similar to those levels observed in the early last instar of control larvae.

When larvae received 20-hydroxyecdysone starting on day 1 of the instar, they underwent supernumerary larval molting between day 8 and day 10. However, these superlarvae exhibited more pupal characteristics than those which first received 20-hydroxyecdysone on day 0. When larvae received 20-hydroxyecdysone starting on day 2, 60% underwent supernumerary larval molting; the remainder began wandering on day 6 – one day earlier than control larvae. Accelerated pupal development was more pronounced when 20-hydroxyecdysone treatment began on a later day; when larvae first received 20-hydroxyecdysone on day 3, all larvae began wandering on day 6 and no supernumerary larvae were induced.

Effects of juvenile hormone mimic application. To clarify the effect of juvenile hormone on changes in hemolymph ecdysteroid titers and on the development of larvae, hydroprene was applied topically to last instar larvae each day during the first three days. Changes in hemolymph ecdysteroid titers and the development of larvae were compared with control larvae. Ecdysteroid titers in treated larvae remained at very low levels during the early stage (similar to those levels in early last instar control larvae; see fig. 2). However, the major increase eliciting larval-pupal metamorphosis occurred on day 11 – three days later than in control larvae.

Discussion

Our results clearly show that when ecdysteroid titer levels, which are very low during the early stage of the last larval instar of *B. mori*, are artificially elevated to levels ranging from 24 to 45 ng/ml (similar to those levels observed during the early penultimate larval instar) via food source treatment with 20-hydroxyecdysone, larvae undergo supernumerary larval molting instead of metamorphosis. The responsiveness of larvae to treatment is dependent on the time at which larvae first receive 20-hydroxyecdysone. Pupal characteristics in the subsequent instar become more pronounced as larvae receive their initial doses of 20-hydroxyecdysone later during the first three days, after which accelerated pupal development is observed rather than supernumerary larval stages.

Since juvenile hormone titers decline dramatically during the first three days of the last instar[7,16], juvenile hormone levels that are present when larvae first received 20-hydroxyecdysone may account for differences in the responsiveness of larvae to this treatment. In addition, our results also show that during the first three days of the last instar, as soon as the very low ecdysteroid levels are artificially elevated by 20-hydroxyecdysone treatment, subsequent declines in juvenile hormone levels are also prevented. The application of a juvenile hormone mimic does not affect ecdysteroid levels during the early last instar, and no supernumerary larval molting can be induced; however, larval-pupal metamorphosis is delayed. Therefore, our results show that very low ecdy-steroid levels play an important role in initiating the decline in juvenile hormone, as well as in directing larval-pupal metamorphosis.

In several Lepidopteran species (such as *B. mori* and *Manduca sexta*), perfect supernumerary larvae cannot be induced; the endocrine system is already committed to undergoing metamorphosis after the last larval ecdysis[17–20]. In this study, our induced supernumerary larvae always showed some pupal cuticle on their heads and legs. This shows that the change of pupal commitment in these epidermal regions may occur before the last larval ecdysis, and may appear earlier than in other epidermal regions. It has previously been demonstrated in several insect species that different epidermal regions change their commitment at different juvenile hormone levels[21–23]. Thus, our results imply that the endocrine system can be completely reversed from metamorphosis to larval molting by 20-hydroxyecdysone treatment, but

that the effect of juvenile hormone decline on some epidermal regions may be irreversible. Similar results have been reported for *M. sexta*[24, 25] and other insect species[1].

It may be possible that when larvae receive 20-hydroxyecdysone from food, the elevated hemolymph ecdysteroid levels observed during the early stage of the last instar is due to increased secretory activity of the prothoracic glands, since a positive feedback in prothoracic gland biosynthetic activity has previously been reported[26]. However, our present results do not support this hypothesis. We found that when newly-ecdysed last instar larvae received 20-hydroxyecdysone throughout the last instar, prothoracic gland biosynthetic ability did not significantly increase as compared with that observed in control larvae during the early last instar; the prothoracic glands of treated larvae produced no ecdysteroid during the first three days, just like those of control larvae. Therefore, those elevated hemolymph ecdysteroid levels observed during the early stage of the last instar in treated larvae may be due in part to the 20-hydroxyecdysone (which directly entered the hemolymph) and in part to the unknown ecdysteroid metabolites that were detectable in our RIA system. Whatever the mechanism by which hemolymph ecdysteroid titers are elevated following 20-hydroxyecdysone treatment, the maintenance of juvenile hormone levels during the last larval instar may be mediated through brain activity. The decline in juvenile hormone levels of normal last instar larvae is due both to the cessation of allatotropic hormone secretion and to the synthesis and secretion of allatostatic hormone[1]. Further study is needed to clarify this regulatory mechanism.

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