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Ecdysteroid Titer, Responsiveness of Prothoracic Gland to Prothoracicotropic Hormone (PTTH), and PTTH Release of the Recessive Trimolter Strain of *Bombyx mori* in Extra-ecdysed Larvae by JHA and 20E Application

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ABSTRACT—Application of 20-hydroxyecdysone (20E) or juvenile hormone analogue (JHA) to the last (fourth) larval instar of a recessive trimolter *Bombyx* strain induced extra larval ecdysis. The ecdysteroid titer, the PTTH-secreting activity of the brain, and the ecdysteroid-secreting activity from the PG were compared among the untreated and 20E- and JHA-treated recessive trimolter. The ecdysteroid titer in the hemolymph and ecdysteroid secretory activity of the PG decreased in the mid-fourth larval instar in untreated control larvae, while those in 20E- and JHA-treated larvae did not decrease during the same stage. An application of JHA enhanced the PTTH-secreting activity of the brain. Results indicate that the recessive trimolter strain of *Bombyx mori* lost the responsiveness of the PG to PTTH in the early stage of the fourth larval instar, resulting in precocious metamorphosis. Results also indicate that the application of 20E induced extra larval ecdysis by maintaining substantial levels of ecdysteroid in the hemolymph. However, JHA is suggested to induce larval ecdysis through activation of the brain and maintenance of the responsiveness of the PG to PTTH.

INTRODUCTION

Changes in hemolymph ecdysteroid titer in the last (fifth) larval instar of *Bombyx mori* differ from those of the penultimate larval instar (Calvez *et al*., 1976). Hemolymph ecdysteroid titer in the fifth larval instar decreases to an undetectable level in the mid-stage, but a substantial hormone titer can be detected even at the minimal level in the penultimate larval instar. Gu *et al*. (1996) reported that the undetectable ecdysteroid titer was the result of deficiency in the signal transduction pathway of the prothoracic gland (PG) cells to the prothoracicotropic hormone (PTTH) in the final larval instar.

The juvenile hormone (JH)-secreting activity of the *corpora allata* (CA), which controls larval-pupal transformation, was reported to be maintained by ecdysteroid *in vitro* in *Manduca sexta* (Granger *et al*., 1987; Whisenton *et al*., 1985; Watson *et al*., 1986) and *B. mori* (Gu and Chow, 1996). Gu and Chow (1996) reported that in the last larval instar of *B. mori*, a low hemolymph ecdysteroid titer inactivated CA activity, resulting in larval-pupal transformation. From these results, Gu *et al*. (1996) speculated that the final larval instar was brought about by the deficiency in the signal transduction pathway of the PG cells to PTTH, resulting in the decrease of ecdysteroid titer and the inactivation of CA. To confirm whether or not this hypothesis was generalized, this study was conducted using a recessive trimolter strain of *B.mori*.

The recessive trimolter strain of *B. mori* exhibits an interesting feature, namely, that precocious metamorphosis occurs at the end of the fourth larval instar. The trimolter can be changed to the tetramolter by application of either juvenile hormone analogue (JHA, methoprene; Komori, 1981) or 20-hydroxyecdysone (20E) (Gu *et al*., 1995). Thus, the recessive trimolter offers the endocrinological organs in the shifting process from ultimate to penultimate. A comparative study of endocrinological evidence in the recessive trimolter after treatment with two types of hormones has not been carried out. The present experiment was conducted to clarify what happened in a recessive trimolter in which extra larval ecdysis was induced by 20E and JHA. In the present study, we compared untreated and hormone-treated larvae of a *Bombyx* race, European No. 7 trimolter. We observed ecdysteroid titer in the hemolymph, PTTH-secreting activity of the brain, and ecdysteroid-secreting activity of the PG in both kinds of larvae.

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MATERIALS AND METHODS

Experimental animals

The tetramolter strain (N124 x C124) and the recessive trimolter (European No. 7) of *Bombyx mori* were used. All of the larvae were reared on fresh mulberry leaves at 25°C under a 12 hr light-12 hr dark photoperiod. Larvae were collected within 4 hr after each ecdysis, and this time was designated as the 0 hr of the fourth or fifth larval instar.

Application of 20E

20-hydroxyecdysone (Sigma Chemical Company, St. Louis, MO) was dissolved in distilled water and applied on the mulberry leaves that were fed to the larvae. A concentration of 20 ppm was used because it was the lowest concentration capable of inducing extra larval molting in all of the larvae (data not shown).

Application of JHA

JHA (methoprene; Zoecon Corporation, Palo Alto, CA) was diluted with acetone to a concentration of 1 mg/ml and topically applied to the larvae. Two μg/larva was applied along the dorsal surface, since this dose was the most effective for inducing extra larval molting: 0.1 μg/larva was not effective, 1 μg/larva induced an extra larval ecdysis in 50% of the treated larvae, and 10 μg/larva induced larval-pupal intermediates.

Radioimmunoassay (RIA)

Thirty microliters of hemolymph or 30 μl of a PG incubation medium was extracted with 200 μl methanol, and then the methanol extract was prepared for RIA according to Takeda *et al*. (1986). The amount of ecdysteroids was determined by RIA according to the methods of Takeda *et al*. (1986). RIA was carried out using 20E as a standard compound. Tritiated ecdysone (70 Ci/mmol) was purchased from Du Pont/New England Nuclear Research Products (Boston, MA).

PTTH preparation

To examine the responsiveness of the PG to PTTH, PTTH solution was prepared according to Gu *et al*. (1996). Brain-*corpora cardiacum-corpora allata* (BR-CC-CA) complexes were dissected from day-0 last-instar tetramolter larvae and incubated in 100 μl Grace's medium (Gibco BRL, Life Technologies, Grand Island, NY) for 4hr. The complexes were removed, and the obtained medium was used as a PTTH source.

The responsiveness of the PG to PTTH

PG responsiveness to PTTH was estimated according to Gu *et al*. (1996). From a pair of glands, one gland was incubated in a medium containing PTTH, and the other was incubated in a medium without PTTH as a control. After incubation for 6 hr (within the linear increase of production), the amount of ecdysteroid in the media was determined by RIA. Though RIA was carried out using 20E as a standard compound, the released ecdysteroid is known to be ecdysone. Therefore, the levels of ecdyseroid released from the PGs were underestimated because the binding capacity of 20E to the antiserum is three-fold higher than that of ecdysone in the used range of concentration (Takeda *et al*., 1986).

PTTH release from BR-CC-CA complexes

Levels of PTTH released from BR-CC-CA complexes were determined according to the method described by Shirai *et al*. (1993). BR-CC-CA complexes were dissected and incubated in a 100 μl Grace's medium for 12 hr, and then the amount of PTTH secreted into the medium was indirectly estimated by the amount of ecdysteroid released from the PG (day-6 tetramolter last-instar larvae) incubated with a BR-CC-CA incubation medium or a control medium. The amount of ecdysteroid in the medium was determined by RIA.

RESULTS

Hemolymph ecdysteroid titer in tri- and tetramolter larvae

Levels of hemolymph ecdysteroid were determined from the third larval ecdysis to the wandering stage in the last (fourth) larval instar of trimolter larvae and from the third larval ecdysis to the wandering stage in the last (fifth) larval instar of tetramolter larvae. The fluctuation of hemolymph ecdysteroid titer showed a small peak at 24–36 hr, followed by a decrease at 48 hr after the third larval ecdysis of trimolter larvae (Fig. 1). After remaining at a low level until 120 hr, the hemolymph

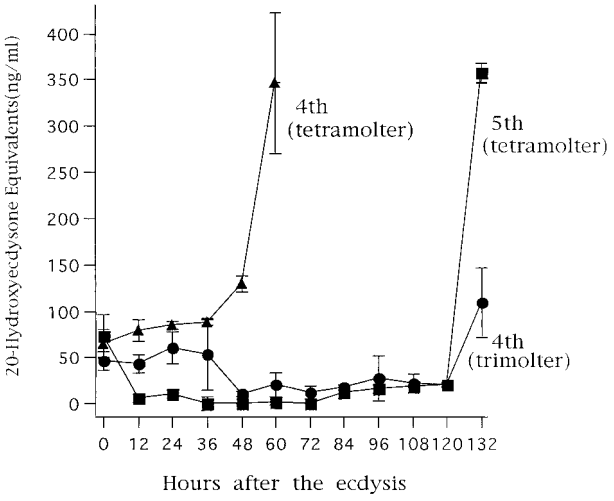


Fig. 1. Hemolymph ecdysteroid titer (ng/ml) after the third larval ecdysis of the trimolter E7 strain (●–●) and after the third (▲–▲) and the fourth (■–■) larval ecdysis of the tetramolter of *Bombyx mori*. RIA was carried out using 20E as a standard compound. Each datum point represents the mean and SEM of 3–4 insects.

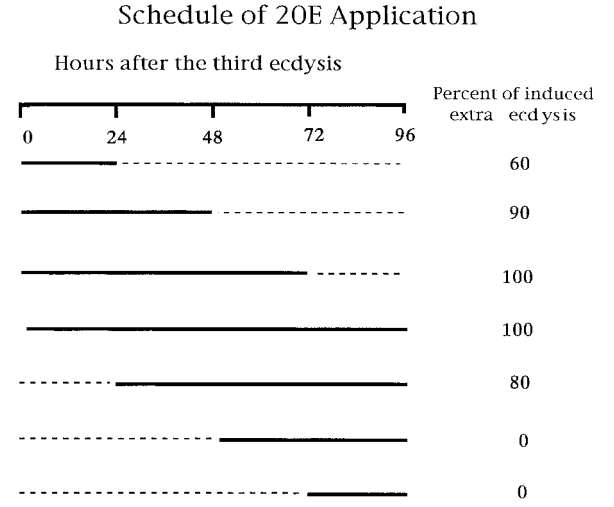


Fig. 2. Effects of 20E feeding time on the induction of the extra larval ecdysis of the trimolter. Solid lines indicate the period of feeding with mulberry leaves treated with 20E (20ppm). Dotted lines indicate the period of feeding with mulberry leaves without 20E. Numbers on the right of each schedule indicate the percent of induced extra larval ecdysis (n=10).

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ecdysteroid titer increased to about 100 ng/ml at 132 hr, whereas that of the fourth larvae of the tetramolter did not decrease during the fourth larval stage. In the tetramolter larvae, the hemolymph ecdysteroid titer of the fifth larval instar decreased to a low level 12 hr after the fourth larval ecdysis, and this amount did not clearly increase before the wandering stage.

Effects of 20E application

As shown in Fig. 2, extra larval ecdysis was induced in all of the trimolter larvae when they were fed mulberry leaves applied with 20E from 0 to 72 hr or to 96 hr after the third ecdysis. By contrast, other feeding schedules did not bring about 100% extra larval ecdysis. Treatment starting at 48 hr induced no extra larval ecdysis. These results indicated that the most effective time-point for 20E application in order to induce extra larval ecdysis was 24–48 hr after the third larval ecdysis.

Effect of JHA application

The effective timing of JHA application for inducing an extra larval ecdysis of trimolter larvae was from 0 to 48 hr after the third larval ecdysis, and application at 24 hr proved to be the most effective (Table 1). None of the present trials yielded a 100% rate of extra ecdysis. Based on these results, we applied JHA 24 hr after the third larval ecdysis in the following experiments.

Table 1. The effect of JHA application timing on the induction of extra-ecdysis of trimolter *Bombyx mori**

Time of JHA application (after the third ecdysis)	Number of insects	Percentage of insects entering extra-molting
0	10	60±10
24	10	87± 6
48	10	47± 6
72	10	3± 6
96	10	0

* Data indicate means and SEM of three different experiments

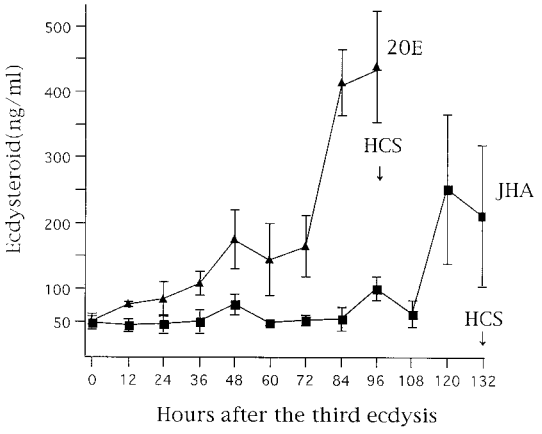


Fig. 3. Hemolymph ecdysteroid titer (ng/ml) of the hormone-treated trimolter E7 strain of *Bombyx mori* after the third larval ecdysis: ▲–▲, larvae fed mulberry leaves treated with 20E (20ppm) from the third ecdysis to 72 hr after the third larval ecdysis; ■–■, larvae treated with JHA by topical application. JHA (2 μg/larva) was applied on the back of the insects at 24 hr after the third ecdysis. RIA was carried out using 20E as a standard compound. Each datum represents the mean and SEM of 3–4 insects. HCS indicates the timing of head-capsule slippage.

Hemolymph ecdysteroid titer after 20E application and JHA application

Hemolymph ecdysteroid levels were determined for the larvae that were fed with 20E from 0 to 72 hr and those applied with JHA at 24 hr of the fourth instar of the trimolter (Fig. 3).

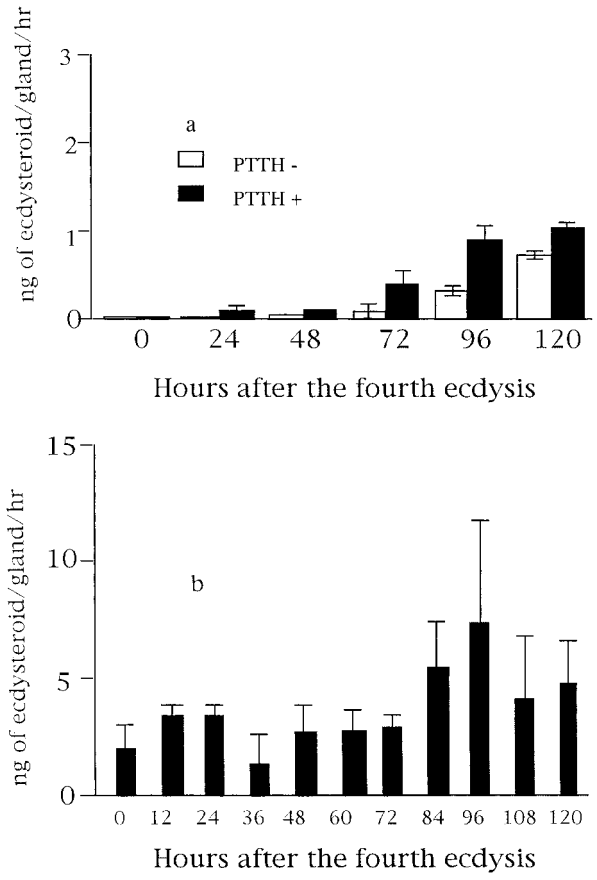


Fig. 4. (a) PG responsiveness to PTTH of the tetramolter last larval instar. A pair of PG was dissected; then, one gland from the dissected pair was incubated in a medium containing PTTH, and the other gland was incubated in a medium without PTTH. PTTH sources were prepared as described in Materials and Methods. After incubation for 6 hr, the released ecdysteroid in the medium was determined by RIA. RIA was carried out using 20E as a standard compound. Open and closed columns represent secreted ecdysteroid from the PG without PTTH and from the PG with PTTH, respectively. Each datum represents the mean and SEM of 3–4 glands from different insects.
(b) PTTH-secreting activity of BR-CC-CA complexes at indicated stages of the tetramolter last larval instar. BR-CC-CA complexes were dissected and incubated in a 100 ml Grace medium for 12 hr. The amount of PTTH secreted into the medium was indirectly estimated by determination of the amount of ecdysteroid released from the PG (day-6 tetramolter last-instar larvae) incubated with BR-CC-CA incubation medium or control medium. Secreted ecdysteroid levels were determined by RIA. Each datum represents the mean amount in BR-CC-CA incubation medium minus the amount in the control medium and the SEM of 3–4 glands.

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Hemolymph ecdysteroid titer after 20E treatment increased at 48 hr (Fig. 3), instead of decreasing levels observed in untreated trimolter larvae (see Fig. 1). Hemolymph ecdysteroid remained at a relatively high level until 72 hr and then increased again at 84 hr. Most of the treated larvae showed head-capsule slippage (HCS) at 96 hr.

After JHA application, the level of hemolymph ecdysteroid remained at about 50 ng/ml until 108 hr and then increased to about 200 ng/ml at 120 hr. Most treated larvae showed HCS at 132 hr. We could collect larvae which would experience extra ecdysis by their behavior after 120 hr; however, within 108 hr after the third larval ecdysis, we could not discriminate between larvae that would molt to larvae and molt to pupae. Fig. 3 shows combined results from about 90% of larvae that entered an extra larval molt (or not). Even in this case, no decrease in ecdysteroid level was found (Fig. 3). In JHA-treated larvae, the rise in ecdysteroid level and subsequent ecdysis occurred later than in 20E-treated larvae.

PTTH release and PTTH-stimulated ecdysteroid secretion of the PG in the 5th larval instar of the tetramolter

Ecdysteroid-secreting activity of the PG in the fifth larval instar of the tetramolter was extremely low until 72 hr, increased to about 0.3 at 96 hr, and then rose to about 0.75 ng/gland/hr at 120 hr after the fourth ecdysis (Fig. 4a, -PTTH). PG responsiveness to PTTH was very low until 48 hr and increased at 72 hr. The rate of ecdysteroid secretion from the PG was about 0.4 at 72 hr and then rose to about 0.9 ng/gland/hr at 96 hr (Fig. 4a, +PTTH).

Significant activities of PTTH secretion of the brain were found throughout the fifth larval instar (Fig. 4b). Almost constant activities were observed from 0 to 72 hr except 0 and 36 hr, and elevated activities were seen at 84 and 96 hr.

PTTH release and PTTH-stimulated ecdysteroid secretion of the PG in the 4th larval instar of the trimolter

The ecdysteroid-secreting activities of the PG were low until 96 hr, with the lowest level at 72 hr (Fig. 5a, -PTTH). The responsiveness of the PG to PTTH became relatively higher 96 hr after the third larval ecdysis (Fig. 5a, +PTTH). PTTH secretory activities of the brain were found at 24 and after 48 hr (Fig. 5b). Especially, extremely elevated activities were observed at 84 and after 144 hr (Fig. 5b).

PTTH release and PTTH-stimulated ecdysteroid secretion of the PG in the 20E-treated fourth larval instar of the trimolter

The ecdysteroid-secreting activities of the PG in the 20E-treated trimolter larvae (PTTH-) were low until 48 hr, increas-

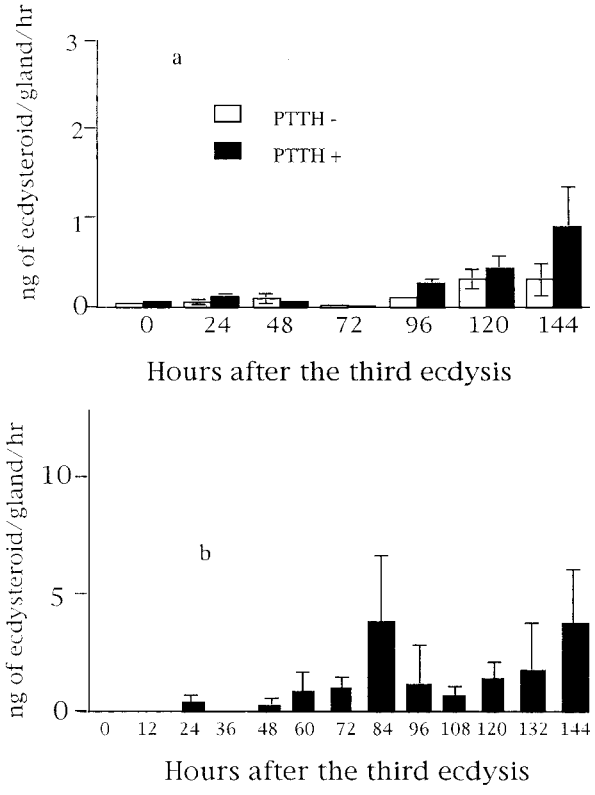


Fig. 5. (a) PG responsiveness to PTTH of the recessive trimolter E7 strain in the fourth larval instar. The experimental methods and representation of data are described in Fig. 4 (a).
(b) PTTH-secreting activity of BR-CC-CA complexes at indicated stages of the recessive trimolter E7 strain in the fourth larval instar. The experimental methods and representation of data are described in Fig. 4 (b).

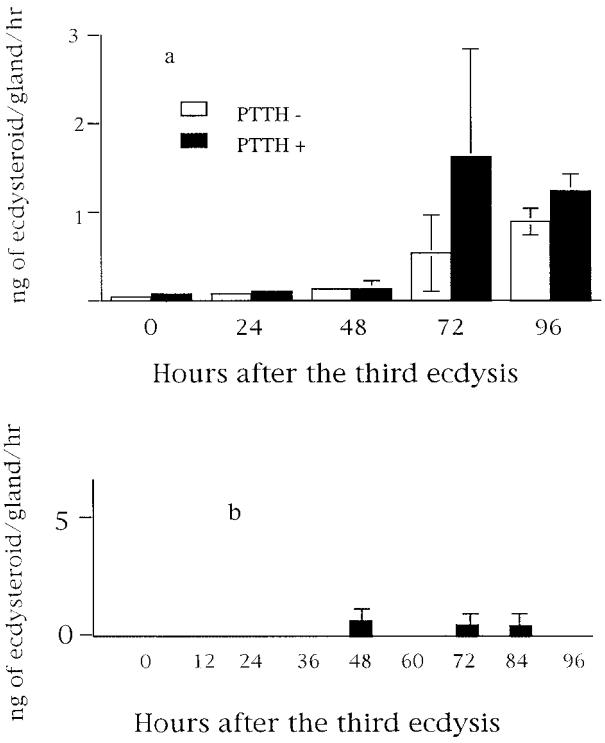


Fig. 6. (a) PG responsiveness to PTTH of the recessive trimolter E7 strain fed mulberry leaves treated with 20E (20ppm) from 0 to 72 hr after the third ecdysis. The experimental methods and representation of data are described in Fig. 4 (a).
(b) PTTH-secreting activity of BR-CC-CA complexes at indicated stages of the recessive trimolter E7 strain treated with 20E during the fourth larval instar. The experimental methods and representation of data are described in Fig. 4 (b).

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ing at 72 hr (Fig. 6a). The glands did not respond to PTTH until 48 hr but were stimulated at 72 hr. The PTTH secretory activities of the brain were extremely low except for 48, 72, and 84 hr (Fig. 6b).

PTTH release and PTTH-stimulated ecdysteroid secretion of the PG in the JHA-treated fourth larval instar of the trimolter

The ecdysteroid-secreting activities of the PG in the JHA-treated trimolter (-PTTH) were low until 72 hr and then rose from 96 hr (Fig. 7a, -PTTH). Responsiveness of the PG to PTTH was found at all stages (Fig. 7a, +PTTH). BR-CC-CA of the JHA-treated larvae had substantial activities of PTTH secretion during the third larval ecdysis (Fig. 7b). PTTH secretory activities were observed from earlier stages in the case of JHA treatment, as compared to 20E-treated larvae.

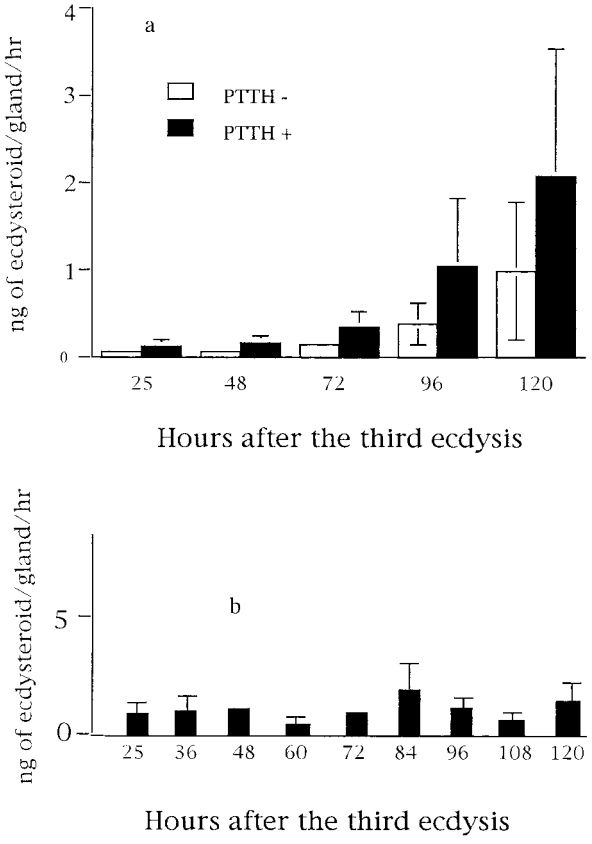


Fig. 7. (a) PG responsiveness to PTTH of the recessive trimolter E7 strain treated with JHA during the fourth larval instar. The experimental methods and representation of data are described in Fig. 4 (a). JHA (2 μg/larva) was applied on the back of the insects at 24 hr after the third ecdysis.
(b) PTTH-secreting activity of BR-CC-CA complexes at indicated stages of the recessive trimolter E7 strain treated with JHA during the fourth larval instar. The experimental methods and representation of data are described in Fig. 4 (b).

DISCUSSION

The hemolymph ecdysteroid titer remained at the levels of 50 ng/ml until 36 hr and then decreased in the mid-stage of the fourth (last) larval instar, 48 hr after the third larval ecdysis of the recessive trimolter used in this experiment (Fig. 1). Gu *et al*. (1992) observed a similar phenomenon in another trimolter *Bombyx* strain, rt. In spite of the substantial existence of hemolymph ecdysteroid, the ecdysteroid-secreting activity of the PG at 0 and 24 hr was not high in the trimolter strain (Fig. 5a). The reason remains unclear at present. By contrast, in the tetramolter larvae, hemolymph ecdysteroid titer decreased 12 hr after the fourth larval ecdysis but was maintained at substantial levels even at minimal levels during the fourth instar (Fig. 1). These results of the present study suggest the possibility that a low titer of hemolymph ecdysteroid brought about precocious pupation in the recessive trimolter, as Gu *et al*. (1996) stated referring to the induction of the final larval stage.

Unresponsiveness of the PG to PTTH was observed in the early fifth larval instar of the tetramolter (Fig. 4a). However, PTTH secretion from the brain was observed in the tetramolter throughout the fifth larval instar (Fig. 4b); this result corresponds well with previous reports (Shirai *et al*., 1993; Dai *et al*., 1995), in which PTTH in the hemolymph was found during the early stage of the last larval instar of *B. mori*. This implies that the decrease of hemolymph ecdysteroid was brought about by unresponsiveness of the PG to PTTH in the last larval instar of *B. mori*. The present results agree with the report by Gu *et al*. (1996).

Okuda *et al*. (1985) reported that the responsiveness of the PG to PTTH in the penultimate larval instar was higher than that of the last larval instar of *Bombyx mori*. Gu *et al*. (1996) supported this finding by following the change of the signal transduction pathway in the PG to PTTH. They observed lower levels of cAMP production in the PG in response to PTTH in the early stage of the fifth larval instar of *B. mori*. Our present findings (Fig. 1, Fig. 5a) suggest that the PG in the fourth larval instar of the recessive trimolter lost its responsiveness to PTTH during the feeding stage in the fourth larval instar. In other words, the PG of the recessive trimolter changed to that of the last larval type; it lost responsiveness to PTTH, resulting in a decrease of hemolymph ecdysteroid in the mid-stage of the fourth larval instar of the recessive trimolter.

Application of 20E to the recessive trimolter around 48 hr in the fourth larval instar induced an extra larval ecdysis (Fig. 2). Instead of decreasing, as observed in untreated larvae (Fig. 1), hemolymph ecdysteroid titer after 20E application remained at relatively high levels until 72 hr (Fig. 3). These results support the notion that the recessive trimolter becomes a precocious pupa as a result of the decrease in ecdysteroid level.

The function of ecdysteroid to maintain the JH-producing activity of the CA has been reported by Whisenton *et al*. (1985), Granger *et al*. (1987), and Watson *et al*. (1986) in *Manduca*. These authors observed that 20E effectively stimulated the CA through BR-CC. Gu and Chow (1996) also reported that the CA activity of *B. mori* was maintained by 20E *in vitro*. These reports support the assumption that the decrease of hemolymph ecdysteroid around 48 hr in the fourth-instar larvae of the trimolter brings about precocious metamorphosis by inac-

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tivating the CA; this suggests that maintenance of hemolymph ecdysteroid, by 20E application, inhibited the inactivation of CA, resulting in extra larval ecdysis in the recessive trimolter. Moreover, PTTH release from the BR-CC-CA of the 20E treated larvae was lower than that of untreated larvae (Fig. 5b, Fig. 6b). This suggests that 20E inhibited PTTH release from the BR, although the reason remains unclear at present.

It is reported that the ecdysteroid-secreting activity of the PG was inhibited by JHA in the early stage but activated by JHA in the late stage of the last larval instar in *Mamestra brassicae* (Hiruma and Agui, 1982) and *B. mori* (Dedos and Fugo, 1996). However, ecdysteroid-secreting activity was maintained by JH in the penultimate larval instar in *M. brassicae* (Hiruma, 1986), *Rhodnius prolixus* (Garcia *et al*., 1987), and *M. sexta* (Lonard *et al*., 1993). Thus, JH is known to activate the PG in the penultimate larval instar but not in the early stage of the last larval instar. Unlike what was observed in untreated larvae (Fig. 5b), application of JHA in the early stage of the fourth larval instar of the recessive trimolter increased PTTH-secreting activity of the brain (Fig. 7b) and ecdysteroid secretion of the PG (Fig. 7a). On the other hand, 20E-treatment did not stimulate larval PG to secrete ecdysteroid (Fig. 6a), and it inhibited the PTTH-secreting activity of the brain (Fig. 6b). Application of JHA in the early stage in the fourth instar of the trimolter probably activated the PG via activation of the brain (Fig. 7a, 7b). This implies that JHA activated the PG, as several authors observed in the penultimate larval instar, because the recessive trimolter larvae are not determined to be the last larval instar and have the PG of the penultimate type until about 24 hr after the third larval ecdysis. In the present result, the most effective timing inducing extra larval ecdysis by JHA-application (24 hr, Table 1) was earlier than that of 20E (48 hr, Fig. 2), which indicates that CA inactivation brought about the unresponsiveness of the PG to PTTH in the recessive trimolter of *B. mori*. From these findings, it is suggested that the final larval instar is started by the inactivation of CA, resulting in the unresponsiveness of the PG to PTTH, and that a following low level of ecdysteroid maintains the inactivation of CA in *B. mori* .

In the recessive trimolter strain of *B. mori*, it is suggested that the PG unresponsiveness to PTTH and the subsequent decrease in hemolymph ecdysteroid titer resulted in precocious metamorphosis in the fourth larval instar and that 20E application induced extra larval ecdysis by maintaining hemolymph ecdysteroid, while JHA induced extra larval ecdysis through activation of the brain and maintenance of the responsiveness of the PG to PTTH.

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