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Intensity of Larval Diapause in the Bamboo Borer, *Omphisa fuscidentalis*

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ABSTRACT—Larvae of the bamboo borer, *Omphisa fuscidentalis*, enter larval diapause in September and pupate in the following June (Singtripop *et al*., 1999). We examined the changes in the responses of larvae to exogenous 20-hydroxyecdysone (20E) in order to estimate the progress of diapause development. In this respect, we adopted two terms, responsiveness and sensitivity of larvae to 20E. Responsiveness was estimated by the percentage of larvae that pupated, and sensitivity was evaluated by the duration from the day of 20E injection to pupation. The responsiveness of larvae declined gradually from September to November when larvae were least responsive to 20E, and then increased markedly from January to February. This indicates that the intensity of diapause increases from September to November and terminated gradually thereafter. Thus the sequence of events as the larval responses to 20E is characterized by a V-shaped curve. Sensitivity of larvae to 20E was at the same level from September to December, and increased remarkably from December to January. The abrupt increase in the sensitivity occurred one month earlier than the bottom of the V-shaped curve of larval responsiveness, suggesting that the increases in the responsiveness and sensitivity in the latter half of diapause may be brought about by respective mechanisms.

Key words: 20-hydroxyecdysone, responsiveness, sensitivity, development, tropical insect

INTRODUCTION

Insect diapause has been studied mostly on species in the temperate zones where insects must survive winter, a season with low temperature and food deficiency. By contrast, insects in the tropics may not suffer the environmental extremes as the temperate insects do, though a dry season comes around every year in some regions. There are several tropical insects with a long period of diapause in their life cycles. Larvae of the anthophorid bee, *Epicharis zonata*, are in diapause for nine month during rainy season in French Guyana (Roubix and Michener, 1980), and adult of the endomychid beetle, *Stenotarus rotundus*, for almost 10 months in Panama (Wolda and Denlinger, 1984). The bamboo borer, *Omphisa fuscidentalis*, is a tropical moth found in bamboo forests of northern Thailand. Bamboo borer larvae enter a diapause after maturation in the last stadium in September, and the diapause lasts for 9 months to the following June (Singtripop *et al*., 1999). In the tropical species with a long diapause period, synchronized progress and termination of diapause must be important as same as in temperate species in order to synchronize the post-embryonic development for breeding, but little is known about the regulation of diapause in those insects.

Larval diapause is a developmental arrest. Since growth and differentiation is controlled strictly by the biologically active ecdysteroid, 20-hydroxyecdysone (20E), the diapause is regarded mostly as an ecdysone-deficiency syndrome, which is caused primarily by a shutdown of ecdysone production essential for further growth and differentiation. Although diapause is merely an ecdysone-deficiency syndrome, responses of diapause larvae and pupae to exogenous 20E change with the progress of diapause development (Denlinger, 1985). For the maintenance and termination of diapause, there are various physiological events including decreased activity of brain to produce and/or release prothoracicotropic hormone (PTTH), competency of prothoracic gland to respond to PTTH, and responsiveness of tissues to 20E. The effective dose of 20E required for diapause termination changes with the progress of diapause in pupae of *Sarcophaga argyrostoma* (Gibbs, 1976),

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S. crassipalpis (Denlinger, 1976), *Heliothis punctiger* (Browning, 1981), and *Mamestra configurata* (Bodnaryk, 1977). As chilling progresses in *Hyalophora cecropia*, pupae increase their responsiveness to ecdysteroids (Waldbauer *et al.*, 1978). If tissue responsiveness to 20E reflects the intensity of diapause, the intensity may be estimated by 20E injections.

In insect species used for examining the change in the hormone sensitivity through larval diapause period, 20E injection causes various responses. Exogenous 20E induces a stationary molt to larvae in some species (*Chilo suppressalis*, Yagi and Fukaya, 1974; *Diatraea grandiosella*, Yin and Chippendale, 1973), while the treatment results in pupation in other species (*Nomia melanderi* and *Megachile rotudata* , Hsiao and Hsiao, 1969; *Laspeyresia pomonella*, Sieber and Benz, 1980). Injection of 20E, however, mostly yields malformed individuals with both larval and pupal characteristics (Denlinger, 1985). In *D. grandiosella* (Yin and Chippendale, 1973), the type of molt induced by 20E changes along with the progress of diapause development. Those various responses to 20E make it difficult to examine the change in 20E sensitivity of diapause larvae.

The larval diapause in the bamboo borer lasts as long as 9 months, and the developmental stage to enter diapause is the time of onset of wandering, that is after ceasing of feeding but before the onset of gut purge (Singtripop *et al.*, 1999). In addition, topically applied juvenile hormone analogue (JHA) breaks the diapause through an activation of prothoracic gland (Singtripop *et al.*, 2000). These indicated that 20E injection might yield a single type of larval response, pupation, and therefore this species appeared suitable to examine the progress of diapause using the sensitivity to 20E. In the present study, we show that both responsiveness and sensitivity of larvae to 20E changes along with the progress of diapause, and that the diapause intensity increases in the first one-third of the diapause period.

MATERIALS AND METHODS

Animals

Larvae of *Omphisa fuscidentalis* were collected monthly from the bamboo, *Dendrocalamus membranaceus* in a forest in Amphur Maewang, Chiang Mai Province, Thailand, in 1998–1999. They were kept in plastic containers (12×14×8 cm) with wet paper towel at 25°C in continuous darkness (Singtripop *et al.*, 1999).

Hormones

20-Hydroxyecdysone (20E, Sigma, St. Louis, MO) was dissolved in distilled water at 1 mg/ml and stored at –35°C until the use. The stock solution was diluted to various concentrations with water and a 5 μl aliquot was injected into each larva. The treatment was performed from September 1998 to April 1999: September 16, October 17, November 11, December 16, January 16, February 1, March 26, April 26 and May 19. Fifteen larvae were used in each month and were observed daily for 6 weeks after the injections unless mentioned otherwise. We recorded the percentage of pupated animals that was referred to as responsiveness of larvae to 20E, and the duration from the day of 20E treatment to the day of the formation of pupal cuticle of Grade 2 (Singtripop et al., 2000), that was defined as sensitivity of larvae to 20E.

RESULTS

Break of larval diapause by 20-hydroxyecdysone

To examine the effective dose of 20E to induce pupation, larvae were injected with various doses of 20E in January 1998. Larvae treated with more than 1 μg actively moved on the day of injection and the following day if touched, but became motionless 2 days after the injection. Between one and two days after the injection, they produced one or two pieces of frosty feces, and occasionally their hindgut was partially ruptured outside. Three to four days after becoming motionless, the larvae produced a tanned pupal cuticle beneath the larval cuticle but did not shed the old larval cuticle. Fig. 1A shows the dose response of pupal cuticle formation to 20E. At doses more than 1 μg, all the larvae deposited pupal cuticle except one of fifteen larvae received 4 μg 20E. A dose of 0.25 μg was effective but 0.1 μg was not effective at all. These results showed that diapause larvae were competent to respond to 20E and the response was not stationary molt but only pupation.

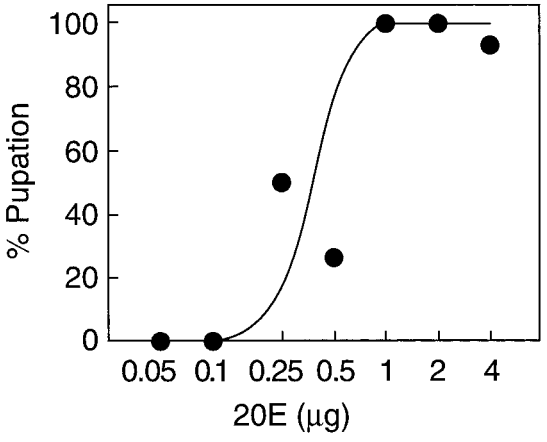

Fig. 1. Induction of pupation by 20E in the diapause larvae of *Omphisa fuscidentalis* in January 1998. Percent pupation (responsiveness), which is the percentage of larvae producing pupal cuticle (Grade 2; Singtripop *et al.*, 2000), is shown as a function of 20E doses. Fifteen larvae were used for each dose.

Monthly changes in responsiveness to 20E

Effects of various doses of 20E on the diapause larvae were examined monthly, and the results were expressed as the percentage of pupated animals (percent pupation) (Fig. 2). In September when the bamboo borer larvae matured and entered larval diapause, 60% of the larvae pupated after the treatment with 0.5 μg 20E, while no pupation was observed at and less than 0.25 μg. Responses of larvae in October were similar to those in September. In November, only 18% of larvae pupated at a dose of 0.5 μg, showing that November larvae were less responsive to 20E than September and October ones. In December, the dose-

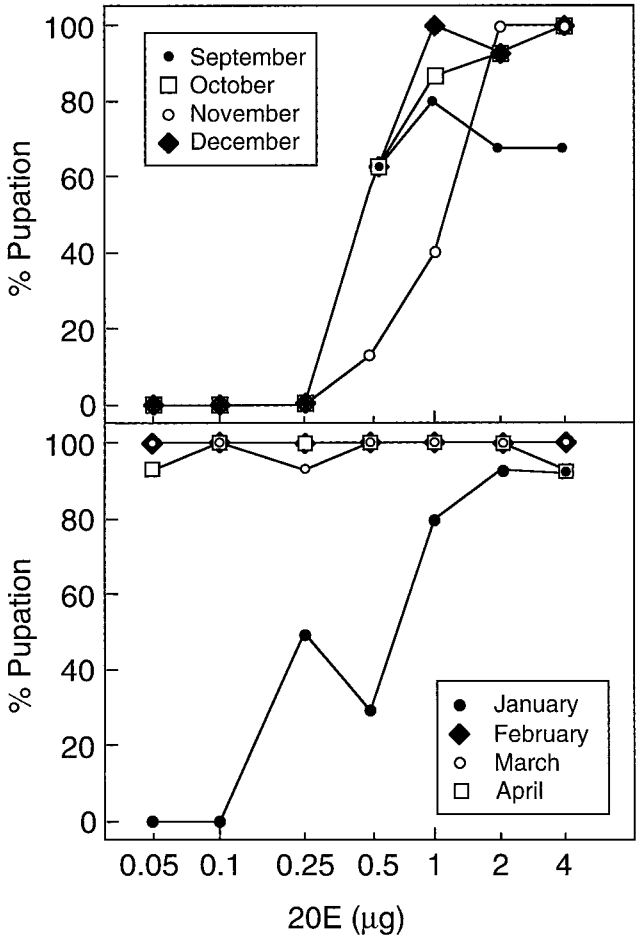

Fig. 2. Monthly changes in responsiveness (percent pupation) of *Omphisa* diapause larvae to various doses of 20E. Larvae received a single injection of 7 different doses of 20E in each month from September 1998 to April 1999. Fifteen larvae were used for each dose in each month.

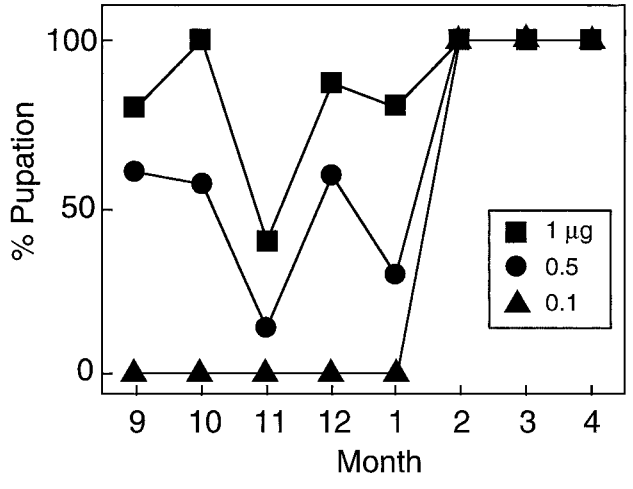

Fig. 3. Intensity of diapause in the bamboo borer larvae. Percent pupation at three selected doses of 20E in Fig. 2 is shown as a function of months examined.

response was similar to those in September and October. In January, a dose of 0.25 μg caused pupation in 56% of larvae. January larvae were thus more responsive to 20E than before. In and after February, all the larvae pupated at a dose of as less as 0.05 μg.

Fig. 3 shows monthly changes in percent pupation of the bamboo borer larvae to three selected doses of 20E as depicted from the data in Fig. 2. The number of larvae that pupated at a dose of 1 μg in October, December and January was almost the same as in September, but the number in November was much lower. Similar tendency was observed at a dose of 0.5 μg 20E.

These results indicated that the intensity of larval diapause was highest in November, after which it became gradually weak. The larvae did not respond to 0.1 μg 20E at all from September to January, but all of the larvae responded to the same dose in February. This indicates that responsiveness of larvae to 20E dramatically increased from January to February.

Changes in sensitivity to 20E

We recorded the mean duration from the day of 20E treatment to the day of the formation of pupal cuticle and expressed the results with the term, sensitivity, which represents how fast larvae respond to 20E by pupation (Fig. 4). In September through December, larvae pupated within 10 days after 20E injections of more than 0.25 μg 20E except for the data at a dose of 1.0 μg in November when 40% of larvae pupated in 23±2 days after the injection. No pupation was observed at a dose of 0.25 μg or less in those months. In January, 0.5 μg 20E induced pupation 16±8 days after the injection, which was 5–7 days longer than those in September through December. At 0.25 μg, pupation occurred but the time of pupation was 25±6 days. From February to April, larvae pupated within 6 weeks of the treatment with the dose as low as 0.05 μg. In May, 6 of 15 larvae pupated between day 46 and 56 of the treatment with 0.05 μg, and we combined these animals with those pupated within 6 weeks for calculating the mean day of pupation. Surprisingly, larvae in May were less sensitive than those in the prior 3 months.

The changes in sensitivity are clearly shown in Fig. 5 where the days required for pupation after injection are plotted against the months examined. At 2 μg, the times of pupation in all months were similar. If larvae responded to 20E, they pupated within 10 days after injection with no exception. At a dose of 1 μg 20E, pupation occurred within 23±2 days in November larvae, indicating that November larvae were extraordinarily less sensitive to 20E than those in other months. At 0.5 μg, pupation occurred within 10 days after injection from September through December, while the mean days to pupation in January larvae were 16, significantly longer than that in the early months. April larvae pupated 21 days after 0.5 μg injection, which was the longest among the monthly-examined larvae.

Doses of less than 0.5 μg induced pupation in January

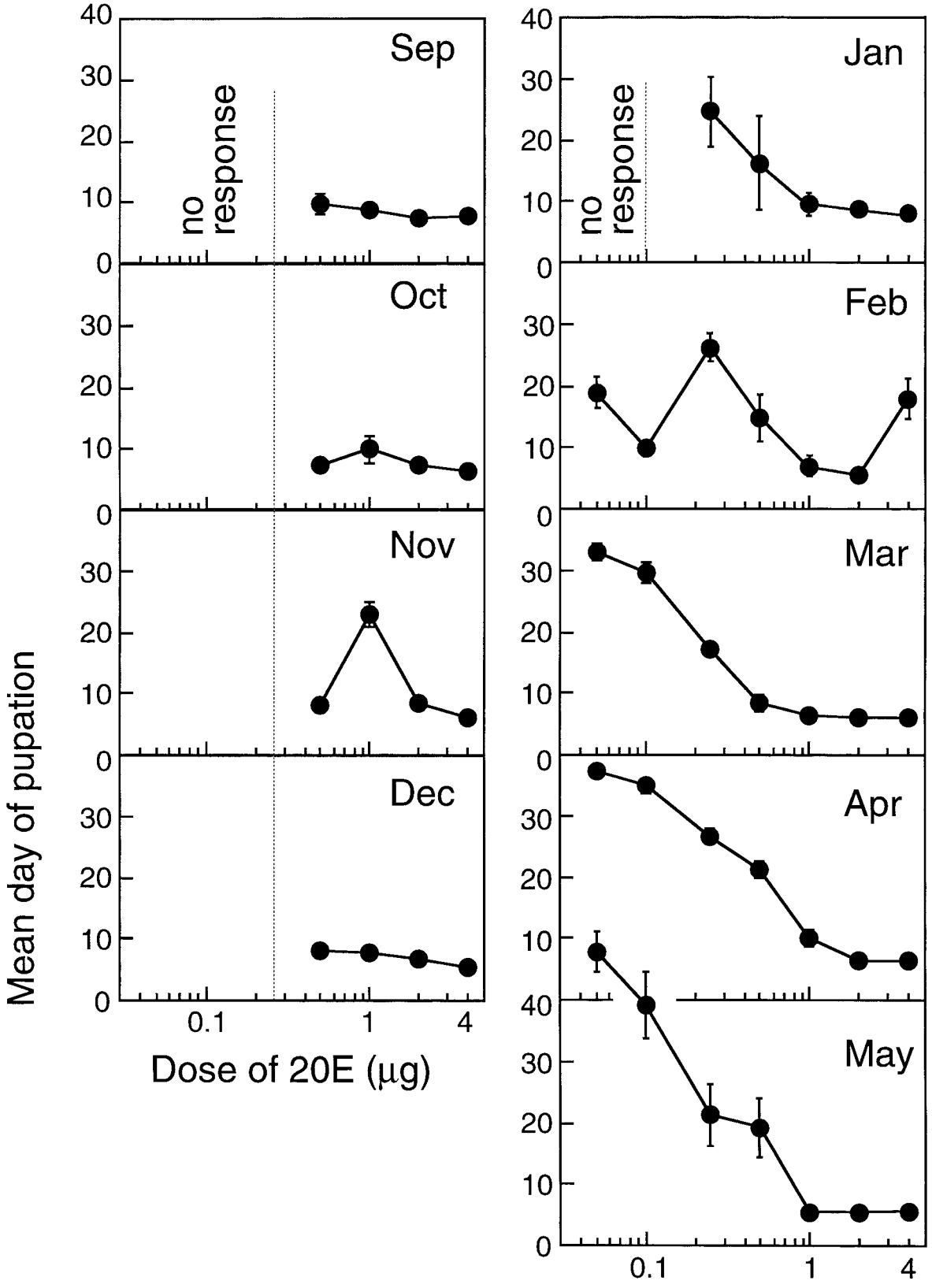

Fig. 4. Monthly changes in sensitivity to various doses of 20E of *Omphisa* diapause larvae. Larvae were injected with various doses of 20E and observed 6 weeks thereafter except for 0.05 μg in May when larvae were observed until day 56 when the last larva pupated. See text for details. No datum point is shown for doses of 0.25 μg or less in September through December and of 0.1 μg or less in January since none of larvae pupated at those doses. Each datum point is a mean±SEM of 15 larvae.

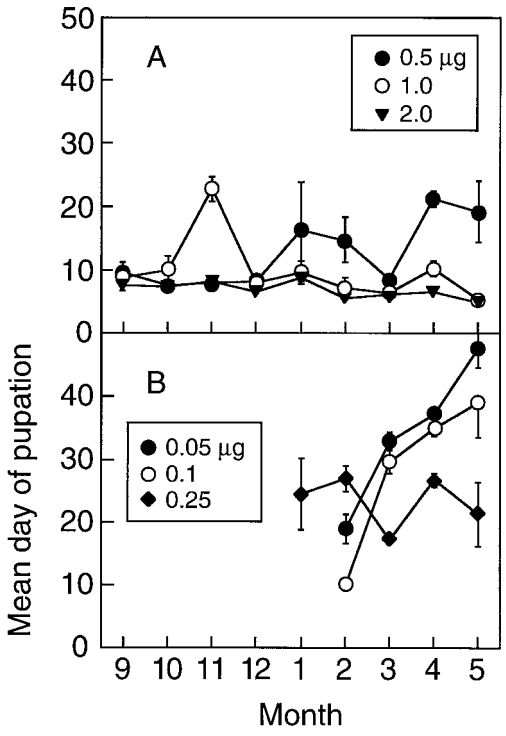

Fig. 5. Comparison of sensitivity of *Omphisa* diapause larvae to various doses of 20E. Day of pupation in Fig. 4 is plotted against the months examined. Details are the same as in Fig. 4.

and later months (Fig. 5B). At 0.25 μg, day of pupation was similar in all 5 months with small fluctuations. By contrast, at the doses of 0.1 and 0.05 μg, pupation took longer period in later months. At 0.1 μg, pupation occurred about 10 days after the injection in February while it took about 4-times longer in May. Similar result was also obtained at 0.05 μg. These results showed that larvae became less sensitive successively after February.

DISCUSSION

Although larval diapause is a period of developmental arrest, physiological conditions do not remain in a static state through the diapause period (Denlinger, 1985). Intensity of diapause and/or progress of diapause are estimated by the animal responses to exogenous hormones. In several lepidopterans, an injection of 20E into larvae at early or mid-diapause results in a stationary (larval-larval) molt, while in late diapause 20E induces larval molt instead. This indicates that a high JH concentration is involved in maintaining the larval diapause by suppressing the function of brain-prothoracic gland axis (Denlinger, 1985; Rountree and Bollenbacher, 1986; Sakurai, 1990), and a decrease in JH concentration is one of the indications for diapause termination (Yin and Chippendale, 1973; Yagi and Fukaya, 1974; Sieber and Benz, 1980). In the bamboo borer, diapause larvae responded to moderate doses of 20E by producing tanned pupal cuticle even in the earliest stage of diapause. This implies that the JH titer in hemolymph of the diapause larvae is low or negligible. Thus the lack of ecdysteroid may be the main cause of diapause but JH is not involved in such a lack. In the bamboo borer, the intensity of diapause must change with the progress of diapause since the responses of the diapause larvae to different doses of exogenous 20E were different month by month.

The present study led us to take account of two different factors in understanding the 20E effects. One is the percentage of larvae that respond to 20E, that reflects assumably the intensity of diapause, and the other is the duration from the day of 20E injection to pupation, reflecting the sensitivity of larvae to 20E. Bamboo borer larvae become mature and enter diapause in September (Singtripop *et al*., 1999). They did not respond to 20E doses of 0.25 μg or less from September to December. Thus the responsiveness of larvae to 20E was low at early to mid-diapause and was lowest in November. In February and later months, larvae respond to a single injection of 20E as low as 0.05 μg, showing that responsiveness of larvae to 20E dramatically increased from January to February. Consequently the pattern of the change in the responsiveness exhibits a V-shaped curve, once intensified and then terminated towards the end of diapause. This pattern is in good accordance with those in pupal diapause in *Heliothis punctiger* (Browning, 1981) and *M. configurata* (Bodnaryk, 1977), in which pupal responsiveness to 20E is high in early diapause, decreases by mid-diapause and again increases in late diapause. Accordingly such changes in the responsiveness may be common in diapause at larval and pupal stages.

Comparison of the mean days of pupation after the 20E injection in each month shows that there are two types of responses, immediate and delayed responses. In pupal diapause, the immediate and delayed responses are frequently observed. In *Sarcophaga*, moderate doses of 20E terminate diapause immediately, and smaller doses fail to break diapause immediately but shorten diapause period (Zdarek and Denlinger, 1975). Similarly in larval diapause of *Omphisa*, the immediate response within 10 days was observed when a dose of 0.5 μg or more was injected in early to mid-diapause except for the 1 μg in November. This result imply that the exogenous 20E at moderate doses stimulated directly the target epidermal cells to produce the pupal cuticle because in lepidopteran final instar larvae it takes several days from 20E injection to pupal cuticle formation. From January to May, however, larvae did not respond immediately to 0.5 μg 20E. The pupation occurred 17 days after the treatment in January and 20 days in May. This indicates that the larvae, probably epidermal cells in late diapause are less sensitive than those in early to mid-diapause though the reason is obscure.

Lower doses of 20E less than 0.5 μg failed to induce the pupal cuticle deposition during September to January. From February to May, the low doses effectively induced the pupation but the deposition of pupal cuticle occurred more than 20 days after 20E injection, which appears to be a delayed response. In the delayed response, the epidermis

must not be the direct target of exogenous 20E from the point of view of a long latent period. The delayed response is assumed to be a stimulative activity of exogenous 20E for ecdysone production by prothoracic gland (Denlinger, 1985). In *Manduca* last instars, prothoracic glands with low secretory activity are activated by a low concentration of 20E through a positive feedback mechanism (Sakurai and William, 1989). If this is the case in the bamboo borer, exogenous 20E may stimulate the prothoracic gland, gradually lead to the activation of the gland, and result in an induction of pupal cuticle deposition. It is possible that the physiological state of endocrine system, especially the prothoracic gland, may change after January so that the positive feedback response is switched on.

Prothoracic glands of diapause larvae produce minute but significant amount of ecdysone (Singtripop *et al*., 2000), and hemolymph ecdysteroid concentration is at a 10^{-8} M level (0.01–0.03 μg/ml) during mid- to late diapause (Singtripop *et al*., 2002). Nevertheless the diapause is not terminated in January, indicating that the concentration is not enough for the positive feedback activation of prothoracic gland. Thus responsiveness of the gland to 20E as well as ecdysteroid threshold for the positive feedback may be controlled by different mechanisms from those for maintenance and termination of larval diapause.

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