Prevention of Pupal Diapause by the Application of Juvenile Hormone Analogue to the Last Instar Larvae of *Mamestra brassicae*

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When JHA (ZR-515) was applied to late last instar larvae of *Mamestra brassicae* destined for pupal diapause, some of the resulting pupae failed to enter diapause. The roles of the brain and the prothoracic glands in these pupae continuing adult development were examined. Extirpation of the brain within 10 hr after pupal ecdysis did not prevent adult development, but ablation of the prothoracic glands at the same stage disrupted metamorphosis. These results suggest that the brain does not play an important role in the prevention of pupal diapause by JHA application. Brains from JHA-treated pupae which failed to enter the diapause had enough prothoracicotropic hormone (PTTH) activity to cause pupation of dauer larvae. Brains with no PTTH activity could not be activated by *in vivo* culture in the haemocoel of isolated pupal abdomens to which JHA was then applied. However, the pupal inactive brains did acquire PTTH activity when β -ecdysone was injected into the pupae. It is proposed, based on these results, that an increase in the PTTH activity of the brain is elicited by ecdysone from prothoracic glands activated by JHA. It is concluded from these results that JHA applied to the last instar larvae are maintained even at the pupal stage and activates the pupal prothoracic glands to secrete ecdysone, which the ecdysone plays an important role to prevent to enter the pupal diapause. Furthermore, the ecdysone also activates the diapause pupal brain to secrete PTTH which in turn activates the prothoracic glands; probably this hormone secreted in this way takes part in the induction of diapause prevention.

INTRODUCTION

In some lepidopterous insects, it has been reported that the pupal prothoracic glands of both diapausing pupae and brainless pupae can be activated by the application of juvenile hormone (JH) (GILBERT and SCHNEIDERMAN, 1959; KRISHNAKUMARAN and SCHNEIDERMAN, 1965; RÖLLER and DAHM, 1968; RÖLLER et al., 1969). Furthermore, RIDDIFORD (1972) found that juvenile hormone analogue application to late last instar larvae of the Cecropia silkworm prevented the resulting pupae from entering pupal diapause. In *Mamestra brassicae*, it appeared that the prothoracic glands are activated by prothoracicotropic hormone (PTTH) during the last larval instar and the pupal stages, but that activation can also be obtained by JHA (ZR-515) application to late last instar larvae and to pupae (HIRUMA et al., 1978a).

A manifestation of prothoracic gland activation by JHA treatment of late last instar

larvae was the prevention of pupal diapause in many of the resulting pupae. The present paper will discuss the mechanism of prevention of pupal diapause by JHA application in *M. brassicae*.

MATERIALS AND METHODS

Rearing of insect. Larvae of the cabbage armyworm, *M. brassicae*, were reared on an artificial diet (AGUI et al., 1975) under aseptic conditions until the penultimate instar, and then maintained semi-aseptically during the last instar. Diapausing pupae were obtained from larvae reared under a short day photoperiod (12L : 12D) at 23°C. Under these conditions, the larvae purged their guts and entered the wandering stage 6 days after the last larval ecdysis. Non-diapausing pupae were obtained from larvae reared under a long day photoperiod (16L : 8D) at 25 °C. In this case, gut purge and wandering began 5 days after the last larval ecdysis.

Hormone treatment. The juvenile hormone analogue (JHA) ZR-515 (Zoecon) (provided by Otsuka Pharmaceutical Co., Ltd., Tokushima) was dissolved in acetone to give final concentrations of 1, 5 and 10 µg/µl. JHA was topically applied to the dorsal region of *M. brassicae* larval abdomen. Control animals were treated with 1 µl of acetone applied in the same manner.

Crystals of β -ecdysone (purchased from Rhoto Pharmaceutical Co., Ltd., Ohsaka) were dissolved into a 0.9% NaCl solution, and the final concentration was adjusted to 1 µg/µl. Four µl of this solution were injected into pupal abdomens by microsyringe. Four µl of 0.9% NaCl solution were injected into control abdomens.

Implantation of ganglia. Brains and suboesophageal ganglia were extirpated from donor pupae, rinsed in a 0.9% NaCl solution, and were implanted into ethyl ether anaesthetized neck-ligated 5-day-old last instar larvae for the examination of their PTTH activity. The recipient larvae had been reared under long day conditions, as the larva at this stage has been known not to pupate for at least 30 days after ligation. Implantation was performed through the proleg, and the proleg was tied off with silk thread after implantation in order to prevent leakage of the blood. Implantations into pupal isolated abdomens were made through the segmacoria, and the wound site was sealed with paraffin wax (m.p. = 56–58°C). The abdomens were isolated from freshly ecdysed pupae reared under short day conditions. The isolated abdomens did not undergo adult development for at least 60 days.

Extirpation of the brains and the prothoracic glands. The pupal brain was extirpated with forceps under CO₂-anaesthesia and the wound site was sealed with paraffin wax. For the extirpation of prothoracic glands, pupae were anaesthetized with CO₂, and their prothoracic glands were cauterized by radio frequency microcautery through the cuticle. These wound sites were also sealed with paraffin wax immediately after the operation.

RESULTS AND DISCUSSION

The effects of JHA on the disturbance of entering diapause were observed following daily topical applications of 10, 5 and 1 µg of JHA or 1 µl of acetone to short-day last instar larvae from the 6th day after the last larval ecdysis to one day before pupal ecdysis. Table 1 shows that 88.9, 61.5 and 65.0% of the resulting living pupae failed to enter diapause when larvae were treated with 10, 5 and 1 µg JHA, respectively. All larvae non-

treated or treated with acetone pupated normally, entered diapause and did not undergo eye pigmentation, which is a criterion for adult development, for at least 60 days after pupation. However, many of the pupae resulting from larvae treated with 10 μ g of JHA died within a few days after pupation as a result of incomplete pupal ecdysis. Among the living pupae, an abnormality of the wing cover was frequently observed. Although many of the larvae topically treated with JHA failed to enter the diapause, some entered diapause (Table 1). It is speculated that in the latter case, JHA treatment could not activate the prothoracic glands enough to cause adult development, as larvae had been strongly destined for pupal diapause.

It has been proposed that the absence of the PTTH is responsible for pupal diapause (WILLIAMS, 1946; 1947). Thus, JHA application might interfere with entering pupal diapause by altering the prothoracicotrophic activity of the brain or the synthetic activity of the prothoracic glands. To distinguish between these two possibilities, the following experiments were performed. Five μ g of JHA or 1 μ l of acetone was topically applied to last instar larvae in the same manner as previous experiments, and their brains

Table 1. DISTURBANCE OF PUPAL DIAPAUSE FOLLOWING TOPICAL APPLICATION OF JHA TO LARVAE

Treatment ^a	No. of larvae used	No. of pupae obtained	No. of living pupae	% of adult development	Average days required for E. P. ^b (mean $\pm$ SD)
10 μ g JHA	35	35	18	88.9	16.1 $\pm$ 6.5
5 μ g JHA	15	15	13	61.5	16.4 $\pm$ 4.5
1 μ g JHA	23	23	20	65.0	17.5 $\pm$ 6.7
Acetone	12	12	10	0	—
No treatment	12	12	11	0	—

^a JHA was topically applied once a day to the short-day larvae from the 6th day of the last instar to the day before the pupal ecdysis. One μ l of acetone was applied to control larvae.

^b E. P. = eye pigmentation, used as the criterion for adult development.

Table 2. DISTURBANCE OF DIAPAUSE IN DEBRAINED OR PROTHORACIC GLAND-ABLATED PUPAE FOLLOWING JHA TREATMENT OF LAST INSTAR LARVAE

Treatment ^a	No. of larvae used	% failing to diapause	Average days required for E. P. ^b (mean $\pm$ SD)
<i>Brain extirpation^c</i>			
5 μ g JHA	12	41.7	16.8 $\pm$ 2.8
Acetone	11	0	—
No treatment	11	0	—
<i>Prothoracic gland ablation^d</i>			
5 μ g JHA	10	0	—
Acetone	9	0	—
No treatment	10	0	—

^a and ^b See the caption of Table 1.

^c Brains were extirpated within 10 hr after pupation.

^d Prothoracic glands were ablated within 10 hr after pupation.

were extirpated or prothoracic glands were ablated within 10 hr after the ensuing pupation. Table 2 shows that 42% of the debrained pupae which had been treated with JHA showed eye pigmentation within 17 days after pupation. The control group of debrained pupae treated with acetone did not undergo eye pigmentation for at least 60 days after pupation. By contrast, pupae with ablated prothoracic glands did not undergo eye pigmentation within 60 days after pupation, regardless of the treatment. The results suggests that the prevention of pupal diapause by JHA treatment is not due to an increase in the PTTH activity of the pupal brain with a subsequent stimulation of ecdysone synthesis by the prothoracic glands but rather to the direct stimulation by JHA of the biosynthetic activity of the prothoracic glands until the pupal stage, because the prothoracic glands of the last instar larvae and of the pupae in *M. brassicae* have been already shown to be activated by JHA (HIRUMA et al., 1978a). This idea receives some support from the finding that JH maintains the activity of the prothoracic glands over long period in *Antheraea polyphemus* pupae (GILBERT, 1962). If this hypothesis is true, it is unclear why the ecdysone produced by the prothoracic glands of JHA-treated larvae up to the time of prothoracic glands ablation is insufficient to prevent pupal diapause. BODNARYK (1977) reported that a low sensitivity to β -ecdysone exists in diapause pupae of *Mamestra configurata* during 0 to 12 hr after pupation. Thus, adult development might not be initiated by the β -ecdysone (converted from α -ecdysone produced by the prothoracic glands) within 10 hr after pupation, even if JHA treatment had maintained high prothoracic glands activity after pupation.

Cecropia silkmoth pupae also failed to enter the diapause when a single injection of JH analogue is applied to post-spinning stage larvae or prepupae which were destined for entering diapause (RIDDIFORD, 1972). Since RIDDIFORD has postulated that in Cecropia silkmoth, the JH analogue prevents diapause by activating the pupal brain, experiments were designed to examine the possible activation of the brain of *M. brassicae* by JHA application. Five μ g of JHA or 1 μ l of acetone were topically applied to larvae from day 6 of the last instar to one day before pupal ecdysis. Brains were dissected from the resulting pupae within 10 hr after pupation, and the brain was implanted into neck-ligated larva. Table 3 shows that implantation of brains taken from pupae which had been treated with JHA as larvae induced pupation in 33.3% of the ligated larvae. By contrast, the larvae implanted with brains from control pupae did not pupate within 25 days after implantation. The results indicate that JHA application to last instar *M. brassicae* larvae destined for pupal diapause can activate the pupal brain to secrete

Table 3. PUPATION OF NECK-LIGATED DAUER LARVAE SUPPLIED WITH BRAINS FROM PUPAE RESULTING FROM JHA-TREATED LAST INSTAR LARVAE

Treatment of brain donor ^a	No. of larvae used	% pupated	Average days required for pupation (mean $\pm$ SD)
5 μ g JHA	9	33.3	13.3 $\pm$ 2.5
Acetone	11	0	—
No treatment	15	0	—

^a See the caption of Table 1.

Brains taken from the resulting pupae were removed within 10 hr after pupation and one brain was implanted into each neck-ligated dauer larva.

Experimental animals were observed for 25 days after treatment.

Table 4. PUPATION OF NECK-LIGATED DAUER LARVAE SUPPLIED WITH BRAINS
PREVIOUSLY CULTURED *in vivo* IN ISOLATED PUPAL ABDOMENS
TOPICALLY TREATED WITH JHA

Implant	No. of larvae used	% pupated	Average days required for pupation (mean $\pm$ SD)
JHA-treated brain ^a	15	6.7	13.0
Acetone-treated brain ^a	13	7.7	12.0
Intact brain ^b	17	0	—

^a Brains taken from 7-day-old last instar larvae destined for diapause were cultured for 2 days in isolated pupal abdomens, which were topically treated with 5 μ g of JHA or 1 μ l of acetone each day. Following recovery, these brains were implanted into neck-ligated dauer larvae, each host receiving one brain. Experimental animals were observed for 25 days after treatment.

^b Brains from 7-day-old last instar larvae destined for diapause were implanted.

PTTH, although activation of the pupal brain by JHA does not play an important role for the prevention of diapause as shown in Table 2.

The following experiments examined whether isolated brains from larvae destined for diapause could be directly activated by JHA treatment. Brains from 7-day-old last instar larvae destined for diapause were implanted into isolated pupal abdomens in order to make closed milieu in that of the resulting pupae applied JHA to the late last instar larvae. Five μ g of JHA was applied topically to the isolated abdomens once a day for 2 days, and then the implanted brains were dissected out and one of them was reimplanted into the neck-ligated larva. As controls, either brains taken out from isolated pupal abdomens which had been treated with 1 μ l of acetone or intact brains from 7-day-old last instar larvae were implanted singly into neck-ligated larvae. Table 4 shows that there is no detectable difference between the PTTH activity of JHA-treated brains and that of the control. Since topically applied JHA easily penetrates the pupal integument of *M. brassicae* (HIRUMA et al., 1978a), it appears that JHA does not directly activate the pupal brain.

It has been shown that pupal brain of *M. brassicae* can be activated by ecdysone *in vitro* (AGUI and HIRUMA, 1977) and that PTTH release from cerebral neurosecretory cells can be inhibited by JHA treatment (HIRUMA et al., 1978b). It is possible, therefore, that the brain is activated by the ecdysone produced by the prothoracic glands in response to JHA application in the last larval instar. To determine whether the brain of diapausing pupae could be activated by β -ecdysone, 4 μ l of a 1 μ g solution of β -ecdysone or an equal volume of 0.9% NaCl solution were injected into 1-day-old and 30-day-old diapausing pupae. The brains and suboesophageal ganglia of these pupae were removed 24 hr after hormone injection and implanted into neck-ligated larvae. Table 5 shows that the implantation of brains from 1-day-old β -ecdysone-treated pupae caused pupation in 33.3% of the neck-ligated larval hosts. Brains from 30-day-old β -ecdysone-treated pupae caused pupation of the host larvae to a lesser extent. Suboesophageal ganglia from β -ecdysone-treated pupae and brains from 1-day-old pupae injected with 0.9% NaCl solution did not elicit pupation of host animals. These results indicate that injected β -ecdysone can activate the brains of the diapausing pupae, although the degree of activation appears to depend on the age of the pupae at the time of injection. On

Table 5. PUPATION OF NECK-LIGATED DAUER LARVAE IMPLANTED WITH BRAINS FROM β -ECDYSONE INJECTED DIAPAUSING PUPAE^a

Treatment of donor pupae ^b	Implant ^c	Age of donor (Days after pupation)	No. of larvae used	% pupated	Average days required for pupation (mean $\pm$ SD)
4 μ g β -Ecdysone	Br	1	12	33.3	11.8 $\pm$ 2.2
4 μ g β -Ecdysone	SG	1	14	0	—
0.9% NaCl Solution	Br	1	16	0	—
4 μ g β -Ecdysone	Br	30	18	16.7	13.3 $\pm$ 1.2
4 μ g β -Ecdysone	SG	30	15	0	—
0.9% NaCl Solution	Br	30	25	0	—

^a Brains were dissected from diapausing pupae 24 hr after β -ecdysone injection, and one brain was implanted into each neck-ligated larva. Experimental animals were observed for 25 days.

^b β -Ecdysone was injected in 4 μ l of 0.9% NaCl solution; 4 μ l of 0.9% NaCl solution was injected into each control donor.

^c Br = Brain, SG = Subesophageal ganglion.

Fig. 1. Diagrammatic representation of pupal diapause prevention by JHA application to last instar larvae of *M. brassicae*.

PG; Prothoracic gland, Br: Brain, PTTH; Prothoracicotropic hormone.

Detail explanations, see text. Arrows show stimulation.

the basis of these results and on the finding that diapausing pupal brains can be activated directly by β -ecdysone *in vitro* (AGUI and HIRUMA, 1977), there is distinct possibility that the ecdysone produced by the prothoracic glands activate the diapause brains.

The results of the experiments described in this communication strongly suggests that JHA treatment of late last instar larvae can stimulate the pupal prothoracic glands to produce ecdysone, and that the ecdysone plays a role to prevent to enter the pupal diapause. While, the ecdysone thus produced can also activate the brain by acting as a positive feedback regulator for the synthesis or release of PTTH, which the tropic neurohormone stimulate ecdysone biosynthesis by the prothoracic glands as well known fact. Ecdysone itself can also apparently activate the prothoracic glands of *M. brassicae* (AGUI and FUKAYA, 1973) and those of some other lepidopterous insects (WILLIAMS, 1952; SIEW and GILBERT, 1971; KIMURA and KOBAYASHI, 1975). Thus any or all of these three mechanisms would affect a high titer of the ecdysone following JHA treatment of last instar larvae and prevent the resulting pupae from entering diapause (Fig. 1).

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