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Contributed Paper

Effects of Photoperiod on Larval Diapause Termination in the Bamboo Borer, *Omphisa fuscidentalis* Hampson

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ABSTRACT

The larval diapause termination in bamboo borer, *Omphisa fuscidentalis* is under the control of ecdysteroid hormone which can be induced by photoperiod. In this study, the effects of photoperiod on levels of ecdysteroid titer and the termination of larval diapause of *O. fuscidentalis* were investigated. The larval diapause was induced by two photoperiodic conditions (LD 2:22 and LD 18:6) following which pupation occurred. Percentage of pupation within 60 days of observation in LD 2:22 and LD 18:6 were 67% and 78%, respectively. The day of pupation was first observed during 19-36 days of observation while it was 43-65 days for larvae kept in the dark (control). The ecdysteroid titer of larvae under various photoperiodic conditions was measured by enzyme-linked immunosorbent assay (ELISA). The level of ecdysteroid titer of the larvae significantly increased on day 20 with LD 2:22 and day 15 with LD 18:6. The expression of ecdysone receptors, *OfEcR-A* mRNA and *OfEcR-B1* mRNA, were performed. The expression of *OfEcR-A* mRNA for LD 2:22 or LD 18:6 were significantly higher than that in the control larvae after day 10 and *OfEcR-B1* mRNA were significantly higher than that in the control larvae after day 5. This level correlated with the increment of ecdysteroid titer, suggesting that photoperiod induces larval diapause termination by increasing the ecdysteroid titer in hemolymph and *OfEcR* mRNA expression in the epidermis.

Keywords: photoperiod, larval diapause, bamboo borer, ecdysteroid, ecdysone receptor

1. INTRODUCTION

The photoperiod is the most important cue initiating diapause in insects [1-2] because photoperiod is used to predict the oncoming of adverse seasons through the use of a photoperiodic clock system [3]. Therefore, Photoperiodic control of diapause induction and termination has been study in a large number of insect species [4-6]. More experiment study the photoperiodic control diapause and diapause termination by exposed under the series of photoperiod (24-h light-dark cycles). The photoperiodic responds

was measured by determined the duration of diapause and rate of diapause under various of photoperiod [7]. There are two major types of photoperiodic response [4, 8] that are a short day response, diapause is induced by long photoperiod such as *Drosophila auraria* and *D. triauraria* [9-10] and a long day response, diapause is induced by short photoperiod such as *Pseudopidorus fasciata, Riptortus clavatus* and *Ostrinia furnacalis* [7, 11-12]. In the fall webworm, *Hyphantria cunea,* the pupa are highly sensitive to photoperiod and diapause termination are dependent on whether the day length exceed the critical day length (CDL) (i.e. 14 h 30 min, 14 h 25 min and 13 h 30 min at 22, 25 and 28 °C, respectively) [6].

The bamboo borer, *Omphisa fuscidentalis* is a moth in the order Lepidoptera and is found in Northern Thailand, Laos and Myanmar [13]. The larvae have five instars and enter obligatory diapause in the fifth instar for a period from September to May and larval diapause termination occurs in June. During the long larval diapause, the larvae remain inside the internode of bamboo culm [13]. Preliminary observations show that changes of humidity and temperature had no effect on the termination of larval diapause [13]. Moreover, pupation of individual larvae of a single colony appears to occur synchronously because development of adult from pupae in the individual internode is well synchronized in *O. fuscidentalis*. This suggests that the break of larval diapause must be environmentally regulated.

In addition to photoperiod, the endocrine factor effects on the larval diapause regulation in *O. fuscidentalis* [14]. During the long larval diapause in *O. fuscidentalis*, the ecdysteroid titer in the hemolymph is very low and prothoracic glands exhibit low secretory activity [13]. Moreover, 20E is tightly involved in the termination of the larval diapause by increasing the ecdysteroid titer and *EcR* mRNA expression in the prothoracic gland (PG) of *O. fuscidentalis* [15], same as the report in *Manduca sexta* [16]. Thus, studying the roles of endocrine factors may help our understanding of the mechanisms of photoperiod on the termination of larval diapause in *O. fuscidentalis.* However, the mechanism for breaking larval diapause by photoperiod is still unknown.

In this study, the effect of photoperiod on the hemolymph ecdysteroid titer and the expression of *OfEcR* genes in the epidermis are examined.

2. MATERIALS AND METHODS

2.1 Animals

O. fuscidentalis diapausing larvae were obtained from a bamboo forest in Maewang District, Chiang Mai Province, Thailand on January 2014.

2.2 Induction by Photoperiod

The larvae were kept in transparent plastic boxes (12×14×8 cm) lined with wet paper towels. The boxes were placed at 25°C under LD 0:24 (continuous dark as control), LD 2:22 and LD 18:6 until the larvae pupated. Stages of pupation are described according to Singtripop *et al.* [13].

2.3 Hemolymph Collection

Hemolymph of *O. fuscidentalis* under various photoperiods was collected by incision of the 2nd prolegs. The hemolymph (30 µl) was mixed with 270 µl methanol and centrifuged at 10,000×g for 5 mins. The supernatant was transferred to a small test tube and dried *in vacuo* at room temperature. The residue was stored at -20°C [13] for measuring 20E concentration by enzyme-linked immunosorbent assay (ELISA).

2.4 Ecdysteroid Analysis

Ecdysteroid was measured with 20-hydroxyecdysone EIA kit (Cayman Co, Germany). The competitive immunoassay system utilizes immobilized 20-hydroxyecdysone-specific antibodies and an ecdysone-acetylcholinesterase enzyme (AChE) provided by this kit is 100% binding to ecdysone. The sample was diluted with EIA buffer. This assay was performed in a 96-well microplate precoated with mouse antirabbit IgG. Samples were analyzed at least in triplicates. A 50 µl of sample was incubated in 96 well microplates. The amount of ecdysteroid in each sample was compared with the 20-hydroxyecdysone standard curve and expressed as ng/ml 20-hydroxyecdysone equivalents. The colorimetric reaction was measured at 405 nm.

2.5 Standard Curve Preparation

A serial 2 fold dilution of standard 20E was prepared from 10 ng/ml solution. An eight point standard curve was then produced.

2.6 Total RNA Extraction, cDNA Synthesis, and PCR Amplification for *EcR-A* and *EcR-B1* Expression

Total RNA was isolated from the epidermis of *O. fuscidentalis* with the single-step method of acid guanidinium triocyanate phenol chlorofrom (AGPG) extraction [17]. RNA was determined by UV absorbance at OD 260. Total RNA (0.5 µg) isolated from dorsal abdominal epidermis of fifth instar larvae to pupae was primed with a reverse transcriptase (Fermentus, Hanover, Maryland) and 1 µl oligo-dT primer (0.1 µg/µl) in a total volume of 1 ml according to the manufacturer's protocol. The transcription process included 3 steps: annealing at room temperature for 10 min; cDNA synthesis at 42°C for 60 min and enzyme inactivation at 70°C for 10 min. The cDNA was mixed with 80 µl TE then stored at -30°C until further use. PCR amplification was performed using specific primers of *EcR-A* and *EcR-B1* (*OfEcRF* and *OfEcR-R*). After electrophoresis, the *Ec*R mRNA expression profile in the epidermis was examined using the combined method of semi-quantitative RT-PCR.

2.7 Semi-quantitative RT-PCR

The amplified product was resolved in 1.5 % agarose gel and the DNA was visualized by eithidium bromide using an UV transilluminator and photographed. Band intensities were estimated using ImageJ. Relative expression for each gene was calculated relatively to the control gene (*RpL3*) [18].

2.8 Data Analysis

Data were subjected to One-way Analysis of variance (ANOVA) followed by the Turkey's test for multiple comparisons. Treatment differences was considered significant at $P < 0.05$.

3. RESULTS

3.1 Effects of Various Photoperiodic Induction on the Percentage of Larval Diapause Termination

Diapausing larvae were reared under two photoperiod conditions (LD 2:22 and LD 18:6) in comparison with the control (dark condition, without light exposure) at 25°C. Percentage of pupation was observed after 60 days (Figure 1). Percentage of pupation under the control was 22% while the pupation under LD 2:22 was 67% and under LD 18:6 was to 78%.

3.2 Effects of Various Photoperiodic Conditions on the Duration Time of Larval Diapause Termination

In the same experiment, we observed duration time of pupation after photoperiodic induction (Figure 2). In the control condition, we found that the first day of pupation was observed on day 43 while it was 19 days and 20 days in LD 2:22 and LD 18:6 respectively. The continuance of pupation was observed. 3, 5 and 10 pupae were found in control condition at mean 50±6.08 days, 55.2±9.04 days and 65.4±13.52 days. The duration time of pupation in control conditions was significantly longer than that in the LD 2:22 (mean 23.7±4.16 days, 30.2±9.42 days and 36.1±9.07 days) and LD 18:6 (mean 24±6.08 days, 28±7.11 days and 36.8±10.71 days). However, the duration of pupation was not significant different between the LD 2:22 and LD 18:6.

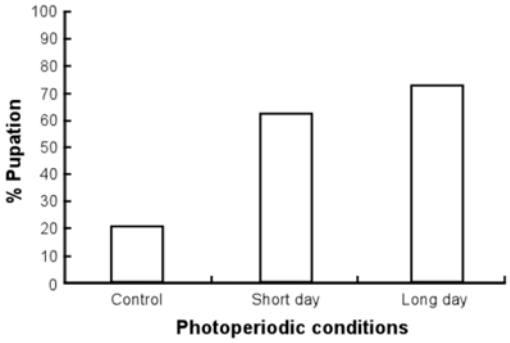

Figure 1. Percentage of pupation in *O. fuscidentalis* after photoperiodic induction. Diapausing larvae were reared at 25°C for 60 days under various photoperiodic condition; control condition (LD 0:24), short day condition (LD 2:22) and long day condition (LD 18:6). (n=18).

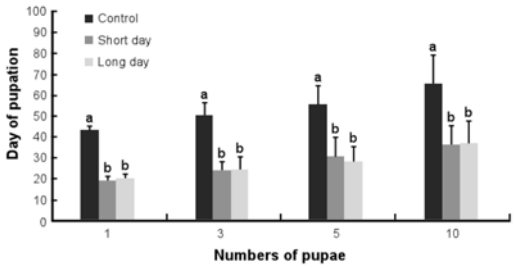

Figure 2. Duration time of pupation in *O. fuscidentalis* after photoperiodic induction. Diapausing larvae were reared at 25°C under various photoperiodic condition; control condition (LD 0:24), short day condition (LD 2:22) and long day condition (LD 18:6). Each value is the mean±SD. Means with different letters indicate significantly difference (ANOVA, n=1, 3, 5, 10, $P<0.05$).

3.3 Effects of Photoperiod on Hemolymph Ecdysteroid Titer in Diapause Larvae

Because the photoperiod affected the duration time of pupation in *O. fuscidentalis*, it suggests that the ecdysteroid level might be changed due to the effect of photoperiod on pupation. We therefore studied the relationship of photoperiod and hemolymph ecdysteroid titer during pupation in diapausing larvae (Figure 3). The hemolymph ecdysteroid levels of diapausing larvae on day 5 and day 10 after photoperiodic induction were not significantly different between control, LD 2:22 and LD 18:6, while the hemolymph ecdysteroid concentration of diapausing larvae kept under two different of photoperiod (LD 2:22 and LD 18:6) increased on day 15 and 20 after photoperiodic induction. The hemolymph ecdysteroid levels of diapause larvae reared under LD 18:6 condition were significantly higher than that of larvae reared under LD 2:22 and the control. The prepupae were

observed after day 20 in both LD 2:22 and LD 18:6, while in control was observed after day 43. In addition, the hemolymph ecdysteroid titer of prepupae reared under control, LD 2:22 and LD 18:6 were high (255±51, 166±51 and 253±31 ng/ml) but they were not significantly different between groups. The results suggest that the photoperiod had an effect on the hemolymph ecdysteroid levels of diapausing *O. fuscidentalis* larvae.

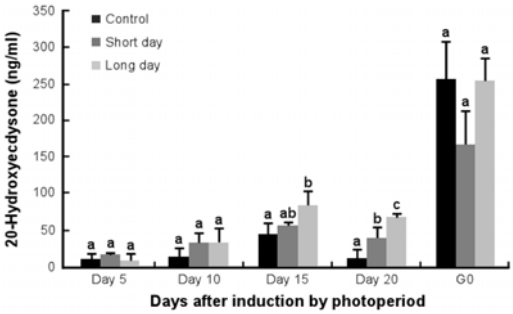

Figure 3. Ecdysteroid titer in hemolymph of diapause larvae in *O. fuscidentalis* (day 5-20 and prepupae, G0) after photoperiodic induction. Diapausing larvae were reared at 25°C under various photoperiodic condition; control condition (LD 0:24), short day condition (LD 2:22) and long day condition (LD 18:6). Each value is the mean±SD. Means with different letters indicate a significantly difference (ANOVA: n=3, P<0.05).

3.4 Ecdysone Receptor mRNA Expression in Epidermis were Induced by Photoperiod

Because, photoperiod increased hemolymph ecdysteroid concentration and induced pupation in *O. fuscidentalis*, the expression of *EcR-A* and *EcR-B1* mRNAs might correlate with hemolymph ecdysteroid concentration. In this study we examined the pattern of *OfEcR-A* and *OfEcR-B1* mRNAs expression in epidermis of *O. fuscidentalis* when reared under different photoperiod conditions. The effect of photoperiod on *OfEcR-A* mRNA expression in epidermis is shown in Figure 4. In day 5, *OfEcR-A* mRNA level were low in all treatment groups. In LD 2:22, *OfEcR-A* mRNA increased significantly on day 10 to day 20 when compared with control but was not significant in prepupal period. In LD 18:6, *OfEcR-A* mRNA significantly increased from day 10 until prepupal period when compared to control. The effect of photoperiod on *OfEcR-B1* mRNA expression is shown in Figure 5. The expression in LD 2:22 and LD 18:6 increased significantly compared to the control on day 5 until prepupal period. In LD 2:22, the expression was high on day 10 and prepupal period. In LD 18:6, the *OfEcR-B1* mRNA was high on day 20 and prepupal period. The results showed that the photoperiod increased the expression levels of *OfEcR* mRNA in epidermis, which was high in late diapause larvae until the diapause larvae termination.

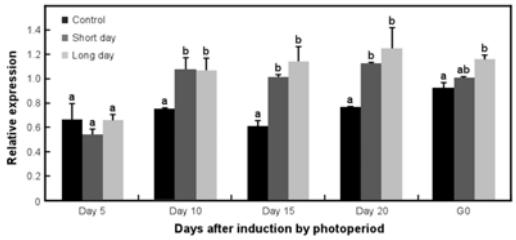

Figure 4. The photoperiod induction of *OfEcR-A* mRNA expression in the epidermis in diapausing larvae of *O. fuscidentalis*. Diapausing larvae were reared at 25°C under various photoperiodic condition; control condition (LD 0:24), short day condition (LD 2:22) and long day condition (LD 18:6). The level of *OfEcR-A* mRNA were determined from total RNA after photoperiodic induction; day 5-20 and the diapause larvae enter prepupae (G0) after induction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Each value is the mean±SD. Means with different letters indicate significantly difference (ANOVA, P<0.05).

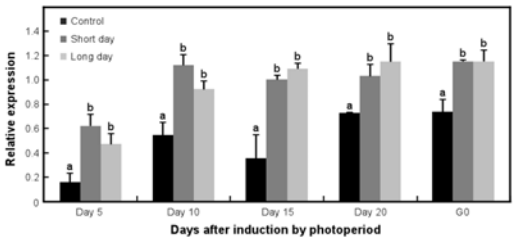

Figure 5. The photoperiod induction of *OfEcR-B1* mRNA expression in the epidermis in diapausing larvae of *O. fuscidentalis*. Diapausing larvae were reared at 25°C under various photoperiodic condition; control condition (LD 0:24), short day condition (LD 2:22) and long day condition (LD 18:6). The level of *OfEcR-B1* mRNA were determined from total RNA after photoperiodic induction; day 5-20 and the diapausing larvae enter prepupae (G0) after induction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Each value is the mean ±SD. Means with different letters indicate significantly difference (ANOVA, $P<0.05$).

4. DISCUSSION

The larval diapause of *O. fuscidentalis* maintained under photoperiod LD 2:22 and LD 18:6, the larval diapause termination within 60 days of observation was 67% and 78%, respectively, while the larval diapause termination was 22% under the control (Figure 1). The duration time of diapause termination was not significant different between LD 2:22 and LD 18:6, which was terminated during 19-36 days. The duration time of diapause termination under two photoperiod was shorter than control which during 45-65 days (Figure 2). This result suggests that the larval diapause of *O. fuscidentalis* was terminated by photoperiod in agreement with the study of Singtripop. Moreover, it has been reported that photoperiod can break larval diapause in *Calliphora vicina* and *Lucicia sericata* [19-20]. In many insect species, the diapause induction respond to short-day photoperiod (day length < CDL) and diapause termination was induced by long-day photoperiod in constant temperature [21-22]. There are different in *O. fuscidentalis*, which the larval diapause maintained under the control and terminated under LD 2:22 (long-day) and LD 18:6 (short-day).

In natural condition, the larval diapause of *O. fuscidentalis* is inside of the bamboo clum. The bamboo clum wall is more than 1 cm thick and it may be impossible for light to pass through this because the light permeability of the wall is approximaltely 1×10^{-21}% cm^{-1} [13], indicating that the photoperiod might not had effect on larval diapause regulation. However, the photoperiod probably plays an important role in termination of larval diapause in *O. fuscidentalis* in June (natural condition) because adult female moth lay cluster of egg on newly grown bamboo shoots in early August. The entrance hole for the first-instar larvae is left on the bamboo shoot, which in turn causes the size of the hole increases continuously until pupation occurs inside the bamboo shoot in the middle of June. Thus, the light intensity may increase as the hole size increases (T. Singtripop, unpublished data) correlate with our results obtained which photoperiod can break larval diapause of *O. fuscidentalis* in laboratory, which is dependent on developmental profile.

In addition, the ecdysteroid is the important hormone for control insect development and diapause regulation [23]. During the long larval diapause in *O. fuscidentalis*, the hemolymph ecdysteroid titer was low [13] but it was increased within 20 days when induced by photoperiod (Figure 3), suggesting that photoperiod stimulate secretion of ecdysteroid in the hemolymph. However, the photoperiod had

no effect on termination in early larval diapause of *O. fuscidentalis* because in this stage the hemolymph ecdysteroid concentration less than 3 ng/ml [13]. This indicates that the induction of larval diapause termination by photoperiod, depending on the hemolymph ecdysteroid concentration. Thus, increasing of hemolymph ecdysteroid concentration was induced by photoperiod when the level above the threshold.

Hence, it may be assumed that the photoperiod might affect the expression level of *OfEcR* mRNA in epidermis of *O. fuscidentalis*. In the present study, the expression of *OfEcR-A* and *OfEcR-B1* increased in the larval diapause under LD 2:22 and LD 18:6 which correlated with the hemolymph ecdysteroid titer (Figure 4 and Figure 5) because increasing in ecdysteroid level stimulated *Ec*R mRNA expression in the larvae of Lepidopterans [16, 24-25]. Therefore, increasing of *OfEcR* mRNA expression in epidermis of diapausing *O. fuscidentalis* larvae may be involved with photoperiod and the hemolymph ecdysteroid titer. Interestingly, *OfEcR-B1* mRNA is more sensitive than *OfEcR-A* mRNA because *EcR-B1* may control proliferative and remodeling events of cell [26] whereas *EcR-A* may be associated with final differentiation events [27].

Based on the previous results obtained in the effects of various photoperiodic on diapausing larvae, the photoperiod able to break larval diapause in *O. fuscidentalis*, indicating that photoperiod might stimulate PG to release ecdysteroid into the hemolymph. Thus, the ecdysteroid produced by the PG may be induced the *OfEcR* genes expression in epidermis, which in turn causes termination of larval diapause in *O. fuscidentalis*. However, this hypothesis needs to be confirmed through further experiments.

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