CHAPTER 4

Discussion and Conclusions

4.1 Discussion

4.1.1 Molecular characterization and gene expression of *OfDH-PBAN* in the subesophageal ganglion

A full-length *OfDH-PBAN* cDNA was obtained from the subesophageal ganglion of *O. fuscidentalis* larvae using the strategy of RT-PCR and RACE. The nucleotide sequence of the *OfDH-PBAN* cDNA encodes a 199 amino acids precursor peptide containing sequences for five FXPRL peptides (DH, PBAN, and α-, β- and γ-SGNP) that are produced by a postendoproteolytic process at six cleavage sites identified in other DH-PBAN cDNAs (Iglesias *et al*., 2002). All of the expected endoproteolytic cleavage sites (G-K-R or G-R-R or G-R) are present in the sequence isolated. The structural organization of *OfDH-PBAN* mRNA is similar to that reported in other lepidopteran species (Sato *et al*., 1993; Choi *et al*., 1998, 2004; Jacquin-Joly *et al*., 1998; Ma *et al*., 1998; Duportets *et al*., 1999; Xu and Denlinger, 2003, 2004; Wei *et al*., 2004; Zhang *et al*., 2004a; Lee and Boo 2005; Xu *et al*., 2007; Jing *et al*., 2007; Uehara *et al*., 2011; Chang and Ramasamy, 2014). From the alignment, the deduced sequence shows similarity with the other known DH-PBANs: 74% with *M. vitrata*, 64% with *A. pernyi*, 63% with *H. armigera*, 62% with *S. exigua*, 61% with *B. mori* and 54% with *P. xylostella*.

Among the peptides encoded by the *OfDH-PBAN* cDNA, it is well known that DH induces embryonic diapause in *B. mori* (Yamashita, 1996) and breaks pupal diapause and stimulates pupal development in moths from the family Noctuidae (Xu and Denlinger, 2003; Zhang *et al*., 2004b). However, there is no further evidence to suggest a role for DH in regulating larval diapause in other species of lepidopteran insects. Thus, it cannot be confirmed how the OfDH is involved in regulating larval diapause in *O. fuscidentalis*. However, it is perhaps remarkable that OfDH lacks a

histidine between residues A^{33}-S^{34}, which is exclusively conserved in closely related species from Bombycoidea and Noctuoidea. A future study featuring a comparison of OfDH peptides with DH peptides from other species of lepidopteran insects may advance our understanding of the structural and functional variations of the DH neuropeptide.

The OfPBAN exhibits low similarity (35-60%) to other known PBAN peptides, whereas although the C-terminal YFSPRL motif is highly conserved within lepidopteran species (Fig. 3.2B). Amidation of the Leu residue at the C-terminus of OfPBAN should occur as in the other PBAN peptides, because this amidation is necessary for pheromonotropic activity of PBAN molecules (Kitamura *et al*., 1989; Raina *et al*., 1989; Lee and Boo, 2005). Moreover, the amino acid sequence alignment of OfPBAN possesses a single methionine residue and is similar to the report for *B. mori* and *H. zea* (Kitamura *et al*., 1989). Therefore, it is conceivable that the oxidized form of PBAN might be necessary to induce full biological activity in *O. fuscidentalis*, although as yet there is no experimental evidence that this is the case.

The α-SGNP, whose biological function has been demonstrated in *H. zea*, in which it exhibited pheromonotropic activity (Ma *et al*., 1996) and showed binding to the PBAN receptor, suggests that α-SGNP acts physiologically like PBAN (Choi *et al*., 2003). The predicted peptide sequence of Of-α-SGNP is highly conserved among other examined α-SGNPs, but it is not clear whether Of-α-SGNP performs a function similar to that in other lepidopteran species. The Of-β-SGNP shows the highest divergence among the five FXPRL peptides compared (Fig. 3.2B), exhibiting only 38-75% homology with β-SGNP from other species. Recently, Helze-β-SGNP has been shown to play a role in epidermal melanization in the larval stage (Raina *et al*., 2003), suggesting that Of-β-SGNP might have a similar role. However, the specific physiological functions of the Of-β-SGNP and Of-α-SGNP still need to be investigated.

The phylogenetic tree based on the ORF sequences of known DH-PBANs was constructed, demonstrating that Of-DH-PBAN is relatively closer to DH-PBANs from Crambidae than from other lepidopteran families. However, the function of DH-PBAN remains unreported in this family.

In the present study, all the predicted Of-peptides share an identical sequence in a homologous domain at the C-terminal region: FXPRL-NH_2. The FXPRL-NH_2 peptides are involved in many physiological processes in insects, including stimulation of hindgut contractions in the cockroach, *Leucophaea maderae* (Holman *et al*., 1986), regulation of cuticular melanization and reddish colouration in larvae, *Spodoptera litura* and stimulation of sex pheromone production in the adult stage (Matsumoto *et al*., 1990), induction of embryonic diapause in *B. mori* (Yamashita, 1996) and stimulation of oviduct contractions in *Locusta migratoria* (Fonagy *et al*., 1992). Thus, these five peptides appear to be multifunctional, and may play conserved roles in the coordination of physiological processes in *O. fuscidentalis*.

DH-PBAN neuropeptides have been widely shown to be primarily synthesized and secreted from the SG in moth species. In the present study, the expression of *OfDH-PBAN* mRNA was highest in the SG. Low expression was also detected in the brain, thoracic and abdominal ganglia, but no expression was detected in non-neural tissues. This tissue distribution pattern of *DH-PBAN* mRNA was similar to that reported in *M. sexta* (Xu and Denlinger, 2004), *H. virescens* (Xu and Denlinger, 2003) and *H. zea* (Ma *et al*., 1996). In the larval stages of *O. fuscidentalis*, the expression levels of the *OfDH-PBAN* mRNA in the SG were higher than at the pupal stage, similar to the reports for diapausing *M. sexta* (Xu and Denlinger, 2003) and *S. exigua* (Xu *et al*., 2007). These findings suggest that the *OfDH-PBAN* transcript might not be necessary to pupal development but may be involved in larval development and may be related to many other biological processes. However, the potential functions of OfDH-PBAN during development remain to be further investigated.

At present, it is known that an endocrine factor (OfDH-PBAN) has an effect on larval diapause regulation in *O. fuscidentalis*. Moreover, another factor that might have an effect on regulation of larval diapause in *O. fuscidentalis* is a photoperiod. For diapause regulation, photoperiod is less important than temperature in *B. mori*, but photoperiod is the most important factor in many other species of insects. Previous studies have shown that larval diapause is terminated by photoperiod in *O. fuscidentalis* (Singtripop, T., unpublished data), as it is in the flies, *C. vicina* (Vinogradova, 1974) and *L. sericata* (Tachibana and Numata, 2004). In *B. mori* and a *Helicoverpa spp*.,

expression of the DH gene is temperature dependent (Xu *et al*., 1995; Zhao *et al*., 2004). Hence, it may be assumed that photoperiod might affect the expression level of *OfDH-PBAN* mRNA in *O. fuscidentalis*. In the current study, photoperiod is shown to have an effect on the expression level of *OfDH-PBAN* mRNA during larval diapause. Moreover, the *OfDH-PBAN* mRNA level increases rapidly before the pupal stage, and falls abruptly to a minimum in the pupal stage under photoperiods of LD 2:22 h, LD 14:10 h and LD 18:6 h (Fig. 3.6). This suggests the possibility that photoperiod might stimulate the secretion of DH-PBAN inform the SG, which then acts on the prothoracic glands to stimulate release ecdysone, which then has an effect on larval diapause termination. Accordingly, the increased expression of *OfDH-PBAN* mRNA before entering the pupal stage may be associated with an increment of ecdysteroid titer in the hemolymph. In *O. fuscidentalis*, the hemolymph ecdysteroid concentration is low during larval diapause and increases prior to the pupal stage, thereby presumably stimulating pupal metamorphosis (Singtripop *et al*., 1999). Based on the previous results, it is suggested that photoperiod might be able to break larval diapause in *O. fuscidentalis*, depending on the stage of development. However, this hypothesis needs to be confirmed through further experiments.

In addition, the partial sequence of *OfMet*, *OfE75C* and *OfHR3* cDNA encodes 248, 149 and 327 amino acids, respectively. A comparison of amino acid sequence with other Mets and E75Cs showed high identity with *B. mori* of the family Bombycidae, while the protein sequence of OfHR3 showed high identity with *P. xuthus* of the family Papilionidae.

4.1.2 Hormonal mechanisms on the termination of larval diapause in the bamboo borer, *Omphisa fuscidentalis*

Though there is no previous evidence demonstrated the effect of JHA on the expression of *DH-PBAN* genes in other insect's tissues, our previous experiment showed that the expression of *OfDH-PBAN* mRNA in SG was much higher than PG (data not shown), indicating that *DH-PBAN* gene is synthesized primarily in SG (Ma *et al*., 1994). Hence, we assumed that JHA might activate the DH-PBAN biosynthesis in SG directly. Here, we show that both *in vivo* and *in vitro* experiments results displayed a similar pattern of responsiveness to *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*,

OfE75A, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression. Interestingly, JHA induces the expression of *OfMet* and *OfDH-PBAN* mRNA in SG and PG. This result of *OfMet* expression coincides with the effect of JH on the induction of *BgMet* mRNA in whole body of *Blattella germanica* (Lozano and Belles, 2014). Recently, Met has been proclaimed as the JH receptor (Jindra *et al*., 2013, 2015). This correlates with our results in which the induction of Met was shown as a rapid response to JHA, indicating that Met may play the role mediating JH-20E crosstalk in termination process of larval diapause. These results provide evidence that JH itself plays a crucial role in regulating the expression of its receptor (Orth *et al*., 1999). However, *OfMet* and *OfDH-PBAN* was up-regulated by JHA alone and *OfMet* and *OfDH-PBAN* in the SG was expressed much faster than *OfMet* and *OfDH-PBAN* in the PG, suggesting that one of the functions of JH in the termination of larval diapause may be to stimulate *OfMet* and *OfDH-PBAN* expression directly in the SG. In addition, our preliminary experiment showed that exogenous DH could also breaks larval diapause of *O. fuscidentalis* by increasing levels of ecdysteroid in the haemolymph (Subta, P., unpublished data). This result was correlated with the previous study obtained from *Helicoverpa spp*. (Zhao *et al*., 2004). Nevertheless, it is still unknown how DH activates prothoracic glands to release ecdysone, and then stimulate larvae to break diapause. We suggest that *OfDH-PBAN* genes might be a haemolymph factor to terminate larval diapause, after it was synthesized from the SG and released into the haemolymph in *O. fuscidentalis*.

JHA had no effect on increase in *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in SG. This result of *OfEcR-A* and *OfEcR-B1* was agreement with that obtained on *EcR* homologous gene in the *M. sexta* epidermis which JH prevented the 20E-induced metamorphic switching by regulating the induction of *EcR* by 20E (Hiruma *et al*., 1999). The effect of JHA on *OfE75* expression was similar to the reports for *D. melanogaster* (Beckstead *et al*., 2007) and *M. sexta* (Keshan *et al*., 2006; Zhou *et al*., 1998a). On the other hand, our study demonstrated that JHA increased the level of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in PG, while 20E increased the level of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in both SG and PG. These results correspond to the reports that JHA and 20E is tightly involved in the termination of the larval diapause by increasing the ecdysteroid titre and *EcR* mRNA expression in the PG

(Singtripop *et al*., 2008). However, the expression level of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA induced by JHA much later than observed for 20E *in vitro*. These results indicate that JHA did not up-regulate *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression, but these genes were directly up-regulated by 20E in both tissues. It has been reported *in vitro* that the *EcR* genes were directly stimulated by 20E in the larvae of Lepidopterans such as *M. sexta* (Jindra *et al*., 1996), *P. interpunctella* (Siaussat *et al*., 2004), and *B. mori* (Sekimoto *et al*., 2006). Moreover, exogenous 20E induces the *EcR* genes (Henrich *et al*., 2003; Riddiford *et al*., 2003; Hossain *et al*., 2006). In addition, the expression of ecdysone inducible genes were directly induced by 20E such as *Br-C* (Zhou *et al*., 1998b, 2001; Sekimoto *et al*., 2006), *E75* (Matsuoka and Fujiwara, 2000; Swevers *et al*., 2002; Siaussat *et al*., 2004; Bigot *et al*., 2012; Li *et al*., 2015) and *HR3* (Palli *et al*., 1996; Eystathioy *et al*., 2001). Furthermore, JH affects the expression of ecdysone inducible genes such as *Br-C* (Zhou and Riddiford, 2002; Minakuchi *et al*., 2011), *E75* (Dubrovskaya *et al*., 2004; Dubrovsky *et al*., 2004) and *HR3* (Siaussat *et al*., 2004), but how JH modifies the expression of these genes has been unknown.

In addition for *in vivo* experiment, the expression of *OfMet* and *OfDH-PBAN* mRNA before entering the pupal stage and the expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in pupal stage may be associated with an increment of JH and 20E titer in the hemolymph. In *O. fuscidentalis*, the hemolymph ecdysteroid concentration is low during larval diapause and increases prior to the pupal stage, thereby presumably stimulating pupal metamorphosis (Singtripop *et al*., 1999). However, JH titer during development of *O. fuscidentalis* has not been determined, but it has been one report to approximate the JH titer indirectly is though the study of juvenile hormone binding protein (JHBP) titre in fat body found that the *OfJHBP* gene expression was high in the diapause period and low from the late-diapause until pupation (Ritdachyeng *et al*., 2012). Accordingly, JH may play a role in the termination of larval diapause by although changes in the JH titer in the hemolymph toward diapause termination remain to be determined to confirm those results above.

Based on the previous results obtained in the JHA-treated diapausing larvae, JHA can breaks larval diapause of *O. fuscidentalis* by increasing the ecdysteroid titer in

hemolymph after brain removal and treatment with 1 µg JHA (Singtripop *et al*., 2000) (Fig. 4.1A) and JHA did not activate the ecdysteroid biosynthesis in PG directly (Singtripop *et al*., 2008) (Fig. 4.1B), suggesting that JHA may induce the expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* via *OfMet* and *OfDH-PBAN* in SG and then act on PG to release ecdysone. Thus, the ecdysteroids produced by the PG may have up-regulated the *OfEcR* genes in PG cells, which in turn causes induction of ecdysone inducible genes and termination of larval diapause in *O. fuscidentalis* (Fig. 4.1B). Moreover, we demonstrated that simultaneous treatment with JHA and 20E increases *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression relative to the application of either JHA or 20E alone. This suggests that the two hormones regulate the expression of these genes and JH may be involved in the up-regulation of these genes expression in both SG and PG.

The finding in this study may help us to understand the crosstalk between JH and 20E on termination of larval diapause in *O. fuscidentalis* which JH might play a crucial role in regulating ecdysteroid signaling pathway in this mechanism. However, this hypothesis needs to be confirmed through further experiments.

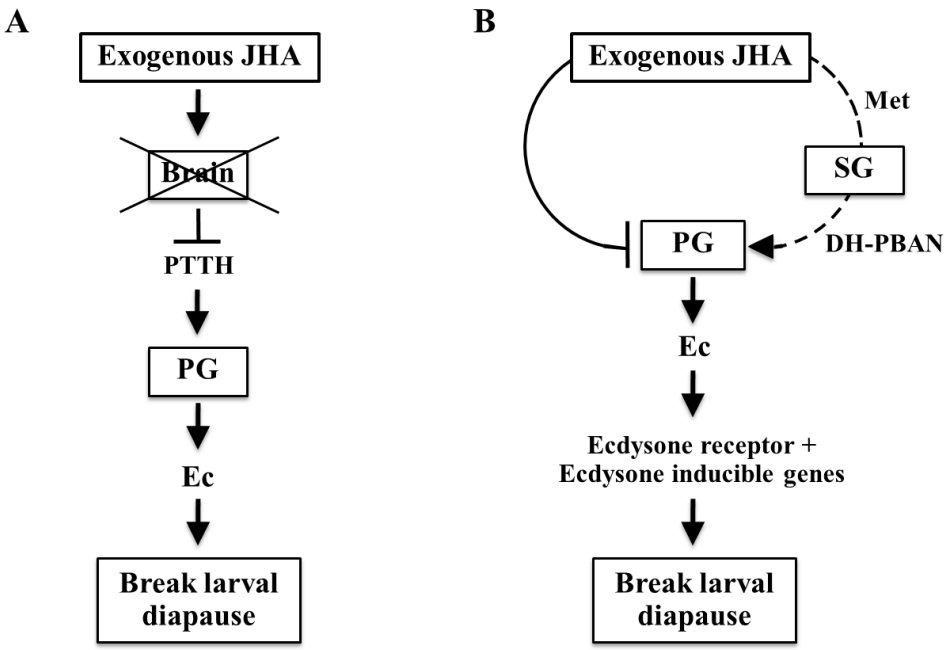

Figure 4.1 Schematic diagrams of JH signaling pathway underlying termination of larval diapause by JHA in *O. fuscidentalis*. (A) JHA treated larvae after brain removal (Singtripop *et al*., 2000). (B) Effects of JHA on PG and SG. See text for details.

4.2 Conclusions

In the bamboo borer, *O. fuscidentalis*, the *DH-PBAN* gene was cloned and sequenced. The full-length *OfDH-PBAN* cDNA consists of 787 bp, including a 597 bp open reading frame encoding 199 amino acids. The cDNA is believed to encode the precursor polyproteins for DH, PBAN, and α-, β- and γ-SGNP, all of which share the same C-terminal motif FXPR/KL. The SG is the primary site of *OfDH-PBAN* mRNA expression.

The effect of endocrine factor on larval diapause regulation in *O. fuscidentalis* was determined indirectly by the examination of relative expression of *OfDH-PBAN* mRNA level in the SG. *OfDH-PBAN* mRNA levels were high during the 5th instar of larval development, reached maximal levels when they enter late-diapause of larval stage and decreased in the pupal stage. Moreover, the environmental factor (photoperiod) had an effect on the expression level of *OfDH-PBAN* mRNA during larval diapause which the *OfDH-PBAN* mRNA level increases rapidly before the pupal stage, and falls abruptly to a minimum in the pupal stage under photoperiods of LD 2:22 h, LD 14:10 h and LD 18:6 h. These results suggest that the increased expression of *OfDH-PBAN* mRNA during larval diapause plays an important role in regulation larval diapause development and may be activated by photoperiod in *O. fuscidentalis*.

In vivo and *in vitro* gene expression experiments demonstrated how hormonal mechanism on termination of larval diapause in the bamboo borer is regulated by JHA and 20E. Both *in vivo* and *in vitro* experiments show a similar pattern of responsiveness to *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression which JHA had effect on expression of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG whereas 20E had no effect on expression of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG, indicating that *OfMet* and *OfDH-PBAN* mRNA was up-regulated by JHA alone. Moreover, 20E had effect on expression *OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA in both SG and PG, while JHA had effect on expression *OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA in PG. In addition, we observed that increase in *OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* gene expression in the presence with 0.1 µg/50 µl of JHA and 20E together was higher than SG and PG

incubated in medium applied with either JHA or 20E alone, suggesting that the presence of both hormones induced an increase in induction level of these genes in both tissues.

Finally, JHA stimulate *OfMet* and *OfDH-PBAN* expression directly in the SG and both JHA and 20E are involved in the regulation *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* expression that results in termination of larval diapause of the bamboo borer.