APPENDIX A

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Molecular cloning, developmental expression and tissue distribution of diapause hormone and pheromone biosynthesis activating neuropeptide in the bamboo borer *Omphisa fuscidentalis*

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Abstract. Diapause, an arrested period of post-embryonic development in insects, is under the control of hormonal interactions. In the bamboo borer *Omphisa fuscidentalis* Hampson (Lepidoptera: Crambidae), larvae remain in diapause for as long as 9 months during the dry season, from September to the following June, although the factors that regulate larval diapause are poorly understood. The present study describes the cloning and expression analysis of the diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) precursor of *O. fuscidentalis* (*Ompfu-DH-PBAN* cDNA), aiming to reveal how it may be involved regulating larval diapause in this species in combination with environmental factors. The open reading frame (ORF) of the cDNA encodes a 199-amino acid precursor protein that contains DH, PBAN and three other neuropeptides, all of which share a conservative C-terminal pentapeptide motif FXPR/KL (X = G, T or S). The *Ompfu-DH-PBAN* is highly similar (74%) to the DH-PBAN of the legume pod borer (*Maruca vitrata*). A quantitative real-time polymerase chain reaction reveals that *Ompfu-DH-PBAN* mRNA is expressed only in neural tissues and that expression is highest in the suboesophageal ganglion. In addition, the expression level of *Ompfu-DH-PBAN* mRNA in the suboesophageal ganglion is consistently high during the fifth larval instar, increasing moderately in early diapause before reaching a peak during late diapause. After pupation, expression of the Ompfu-DH-PBAN precursor decreases to a low level. In addition to endocrine factors, the results demonstrate that photoperiod increases the expression level of *Ompfu-DH-PBAN* mRNA in larval diapause. These results also suggest that the expression of the *Ompfu-DH-PBAN* gene correlates with larval diapause development and may be activated by photoperiod in *O. fuscidentalis*.

Key words. Diapause hormone, *Omphisa fuscidentalis*, pheromone biosynthesis activating neuropeptide.

Introduction

Diapause is a state of developmental arrest that is common among insects and is under the control of hormonal interactions (Denlinger, 2002). In most multivoltine species, diapause occurs in response to environmental cues, which is referred to

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'facultative diapause', although, in univoltine species, diapause is genetically determined to occur at a certain stage in each gen- eration regardless of the environmental factor; this is referred to as 'obligatory diapause' (Denlinger, 1985). In insects that undergo a complete metamorphosis, diapause can occur in any stage of their growth and is regulated by the neuroendocrine system (Singtripop *et al.*, 1999).

In general, diapause is controlled by two types of factors:endocrine and environmental. The suboesophageal ganglion, which is located under the brain, is where the diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) is synthesized. DH-PBAN is an important insect neuropeptide that is reportedly involved in diapause regulation. DH-PBAN induces embryonic diapause by inhibiting ovarian development in *Bombyx mori* and terminates pupal diapause by activating the prothoracic gland (PG) to synthesize ecdysone in *Helicoverpa* spp. Moreover, DH-PBAN stimulates sex pheromone biosynthesis in female moths for male attraction (Denlinger, 1985; Zhang *et al.*, 2004b). In *B. mori*, DH stimulates the transcription of the trehalase gene in ovaries, thereby increasing trehalase activity, which facilitates the higher accumulation of glycogen in eggs, a prerequisite for diapause initiation during which sufficient reserves are sequestered for survival in the diapause period and for post-diapause develop- ment (Shiomi *et al.*, 1994; Su *et al.*, 1994; Hahn & Denlinger, 2011). Although DH-PBAN genes are widely studied in more than 30 insect species spanning four orders (Choi *et al.*, 2010), there is no previous knowledge of the genomic structure of DH-PBAN or gene expression profile in the bamboo borer *O. fuscidentalis* Hampson, a member of the lepidopteran family Crambidae.

In addition to endocrine factors, environmental factors are crucial for the control of diapause in many temperate insect species (Tauber *et al.*, 1986; Danks, 1987; Hodek & Hodková, 1988; Hodek, 2002). Diapause termination by photoperiod and high temperature is reported in the flies *Calliphora vicina* (Vinogradova, 1974) and *Lucilia sericata* (Tachibana & Numata, 2004). In the zygaenid moth *Pseudopidorus fasciata*, the larvae are highly sensitive to photoperiod, and both diapause induction and termination are dependent on whether the night-lengths exceed the critical night length or not (i.e. 10.5 h for diapause induction and 10 h for diapause termination) (Wei *et al.*, 2001; Li *et al.*, 2003). Furthermore, the expression of the DH gene has been shown to be temperature dependent in *B. mori* and *Helicoverpa* spp. In addition, the environmental signal of low temperature acts on the brain to cause release a factor that regulates the suboesophageal ganglion to synthesize and secrete FXPRLamide peptides into the haemolymph and act on the PGs to synthesize ecdysone, and then ecdysone directly causes the pupae to break diapause in *Helicoverpa* spp. (Zhao *et al.*, 2004).

The bamboo borer *O. fuscidentalis* is a univoltine lepidopteran insect that experiences an annual severe dry season in Northern Thailand, Laos and Myanmar. The fifth-instar larvae enter diapause and remain inside the internode of bamboo culm for 9 months, from September until the next June. Preliminary observations show that the inside of a bamboo shoot is com- pletely dark. There is practically no fluctuation of humidity with a lowest relative humidity of 95% and a highest of 100% throughout the year. The lowest monthly average temperature is >20 ℃, with the daily temperature change inside a bamboo shoot adhering to that of the outside with a delay of 1 h or less. Thus, pre-diapause preparation and diapause development of the bamboo borer larvae occur in a relatively stable environment. Pupation of individual larvae of a single colony appears to occur synchronously because development of adults from pupae in an individual internode is well synchronized. This indicates that the break of diapause must be environmentally regulated. Photoperiod is a common environmental cue for breaking diapause. The bamboo culm 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 approximately $1 \times 10{-}21\%cm^{-1}$ (Singtripop *et al.*, 1999).

In several lepidopteran insects, such as the Southwestern corn borer *Diatraea grandiosella* (Chippendale & Yin, 1979), the European corn borer *Ostrinia nubilalis* (Bean & Beck, 1980, 1983) and the rice stem borer *Chilo suppressalis* (Agui & Hiruma, 1977), larval diapause is induced by a high juvenile hormone titre in the haemolymph that is maintained throughout diapause. During the long larval diapause in *O. fuscidentalis*, the ecdysteroid titre in the haemolymph is very low and prothoracic glands exhibit low secretory activity (Singtripop *et al.*, 1999). An application of a juvenile hormone analogue (JHA) to diapausing larvae induces an increase in the ecdysteroid titre leading to diapause termination. However, the brain is not involved directly in the activation of the PG by JHA. After JHA treatment, the PG activity increases over several days and the haemolymph ecdysteroid titre increases after 2 weeks, indicating that JHA may not simulate ecdysteroid biosynthesis in PG directly (Singtripop *et al.*, 2000), and also suggesting that hormonal maintenance of larval diapause is different between *O. fuscidentalis* and the other borer species noted above. In addition, photoperiod probably plays an important role in the termination of larval diapause in *O. fuscidentalis* because adult female moths lay an egg cluster on newly grown bamboo shoots in early August. The entrance hole for the first-instar larvae is left on the bamboo shoot. The size of the hole increases continuously until pupation occurs inside the bamboo shoot in the middle of June. This suggests that the light intensity may increase as the hole size increases (T. Singtripop, unpublished data). However, the mechanism for breaking larval diapause by photoperiod is still unknown. Thus, studying the roles of both endocrine and environmental factors may help our understanding of the mechanisms of larval diapause regulation in *O. fuscidentalis*.

The present study describes the cloning and sequencing of the *O. fuscidentalis* DH-PBAN precursor cDNA (*Ompfu-DH-PBAN* cDNA) using the reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) strategies. The expression profile of *DH-PBAN* mRNA in the suboesophageal ganglion and other tissues during the developmental stages is examined using the combined method of quantitative real-time PCR. Furthermore, the effect of photoperiod on *Ompfu-DH-PBAN* gene expression is exam- ined, which is a key factor for the termination of larval diapause.

Materials and methods

Insects

Omphisa fuscidentalis larvae were obtained from bamboo forests in the Maewang District, Chiang Mai Province, Thailand. The diapausing fifth-instar larvae were collected from October to March, and pupae were collected in April. Larvae were reared in containers (12 × 14 × 8 cm3) lined with sheets of wet paper towel and were maintained continuously in the dark at 25 ∘C and 95% relative humidity.

Total RNA extraction, cDNA synthesis and PCR amplification

Total RNA was isolated from suboesophageal ganglion, other neural tissues (e.g. brain, thoracic ganglia and abdominal ganglia) and non-neural tissues (e.g. fat body, integument and Malpighian tubules) with the single-step method of acid guanidinium thiocyanate phenol chloroform (AGPC) extraction in accordance with the manufacturer's instructions (Chomczynski & Sacchi, 1987). Prior to cDNA synthesis, the RNA was treated with RNase-free DNaseI (Fermentas, Lithuania) to eliminate contaminating DNA. A quantity of 1 µg of total RNA was used to generate first-strand cDNA with an oligo-dT primer and M-MuLV-Reverse Transcriptase (Fermentas). Degenerate primers were designed based on conserved regions of Ompfu-DH-PBAN found in other lepidopteran species (*B. mori DH-PBAN*, GenBank accession number S50045; *Helicoverpa armigera DH-PBAN* AF492474; *Manduca sexta DH-PBAN* AY172672; *Agrotis ipsilon DH-PBAN* AJ009674; *Spodoptera exigua DH-PBAN* AY628764). Conservation was estimated from sequence alignments using clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). PCR amplification was performed using degenerate primers: OfDPF (5'-TGG TTC GGH CCY AGR HTN GGS-3') and OfDPR (5'-GAA GAT BAC YTT YTT BGT HAC-3') (Fig. 1) based on known DH-PBAN cDNA conserved sequences with the reaction conditions: one cycle for 3min at 94 ∘C, followed by 30s at 94∘C, 45 s at 56 ∘C and 30 s at 72 ∘C for 40 cycles, and then a final extension step for 2min at 72 ∘C.

RACE

Two specific primers, OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3'), were synthesized for RACE based on the cDNA sequences obtained from internal amplification. 3' and 5' RACE were conducted using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, California) in accordance with the manufacturer's instructions. The 5'-ready-cDNA had a nested universal primer (5'-AAG CAG TGG TAT CAA CGC AGA GT-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3'). The 3'-ready-cDNA had a nested universal primer (Clontech) and OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3').The reaction mixture was subjected to one cycle for 5 min at 94 ∘C, 45 cycles that consisted of 94 ∘C for 30 s, 68 °C for 30 s and 72 °C for 30 s, and then a final extension step for 2 min at 72 °C.

Cloning and sequencing

The PCR products were separated on 1.5% agarose gel and purified using a GEL/PCR Purification Mini Kit (Favorgen Biotech Corp., Taiwan). Purified DNA fragments were subcloned into pTZ57R/T vectors using an InsTAclone PCR cloning Kit (Fermentas) and amplified in *Escherichia coli* DH5α competent cells (One Shot; Invitrogen, Carlsbad, California) after transformation. Recombinant plasmids were isolated using a GeneJET Plasmid Miniprep Kit (Fermentas), and DNA sequencing was performed by an ABI PRISM Bigdye Terminator (version 3.1) cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems). The cDNA sequences of DH-PBAN were searched against the GenBank database using FASTA (European Molecular Biology Laboratory, European Bioinformatics Institute).

Quantitative real-time PCR (Q-RT-PCR)

Total RNA was isolated from suboesophageal ganglion by the AGPC method (Chomczynski & Sacchi, 1987) and then reverse transcribed (1.0 µg) using oligo-dT primer and reverse transcriptase (Fermentas, Hanover, Maryland). The primers used for amplifying the *Ompfu-DH-PBAN* were: forward primer (5'-TAT GAG AGT CGA GCT GAT GAC-3') and reverse primer (5'-GTG ACG GAA AGC TTC TCC GG-3'). The primers for *OfRpL3* (*O. fuscidentalis* ribosomal protein L3, GenBank accession number EF453378) were forward primer (5'-TCT ACC CCA AGA AGA GGT CTC G-3') and reverse primer (5'-ACG ACA GTC CTC AGA CAT GTG C-3'). *OfRpL3* was used as an endogenous control. Quantitative real-time PCR was conducted using an EXPRESS SYBR Green ER qPCR Supermix Universal (Invitrogen) and an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, California). One reaction contained 1 µL of template cDNA sample and 0.2 µm primers in a final reaction volume of 20 µL. The thermal cycling parameters were 94 ∘C for 5min, followed by 40 cycles at 94 ∘C for 20 s, 60 ∘C for 30 s and 72 ∘C for 40 s. After Q-RT-PCR, the absence of undesired by-products was confirmed by an automated melting curve analysis and agarose gel electrophoresis of the PCR product. The amplification efficiency for both the reference and target genes was analyzed. The relative expression level of *Ompfu-DH-PBAN* from samples with different treatments was estimated by a comparative CT method (ΔΔCT) for relative quantitation of gene expression. The *C*t (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the background level. The dynamic range of both the target (*Ompfu-DH-PBAN*) and normalizer (*OfRpL3*) were determined. After normalization with *OfRpL3* [i.e. Δ*C*t =*C*t (Ompfu-DH-PBAN) −*C*t(OfRpL3)], the Δ*C*t value of the treatment group was further compared with that of the control group, known as the calibrator (i.e. ΔΔ*C*t). The relative expression

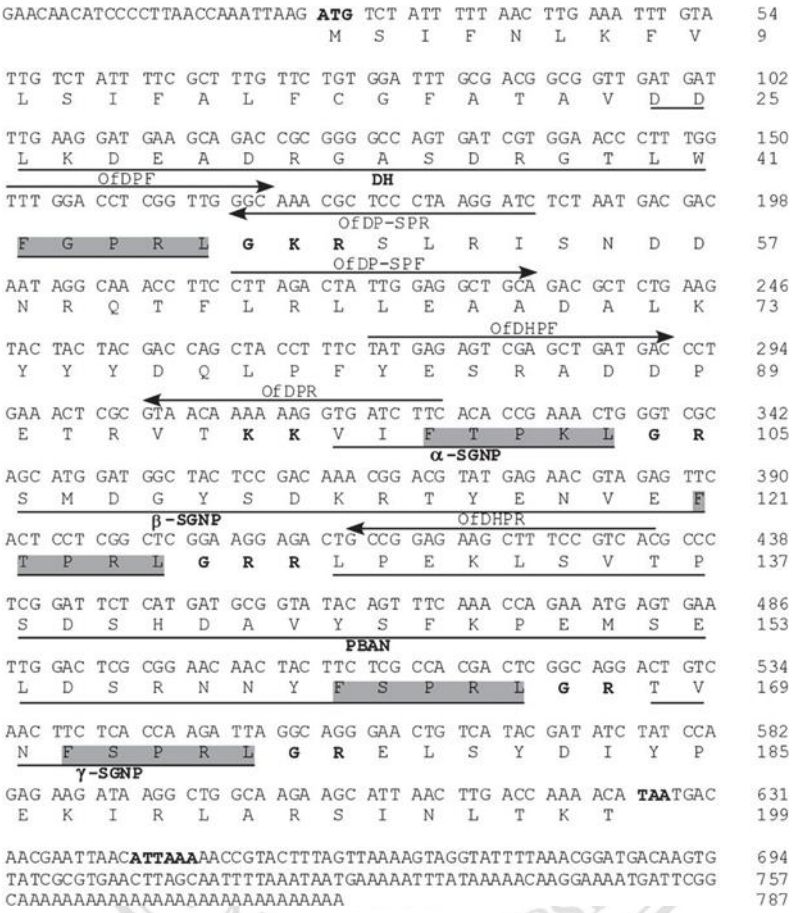


Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the *Ompfu-DH-PBAN* cDNA. The suggested start (ATG) and stop (TAA) codons, and polyadenylation signal (ATTAAA), are shown in bold. The five presumptive peptides are underlined, and endoproteolytic cleavage sites are shown in bold. Arrows over the nucleotide sequences represent the position of the different synthetic primers used in the polymerase chain reaction (PCR). Degenerate primers are OfDPF (5'-TGG TTC GGH CCY AGR HTN GGS-3') and OfDPR (5'-GAA GAT BAC YTT YTT BGT HAC-3'). Specific primers for rapid amplification of cDNA ends and PCR are OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3').

level was determined using the formula: $2^{-\Delta\Delta C}{}_{t}$ (Pfaffl, 2001; Dorak, 2006). The relative expression was calculated using three samples from independent experiments. The data were subjected to one-way analysis of variance (anova) followed by Tukey's test for multiple comparisons. $P < 0.01$ was considered statistically significant. Data are presented as the mean ± SEM.

Effect of photoperiod on the gene expression of DH-PBAN

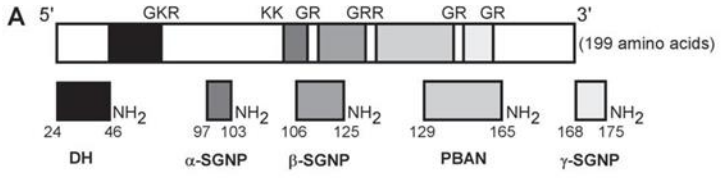
Larvae were reared at 25 °C combined with various photocycles (LD 0 : 24, 2 : 22, 14 : 10 and 18 : 6 h). The number of larvae used in each tested photocycle was at least 300. The *Ompfu-DH-PBAN* mRNA expression level was examined every 5 days until the larvae entered pupation.

Results

Cloning of Ompfu-DH-PBAN cDNA

Using the degenerate primers OfDPF and OfDPR (Fig. 1), an amplified DNA fragment of the expected 177 bp size was obtained. After cloning and sequencing, the deduced amino acid sequence shows 57–63% identity to other known DH-PBAN cDNAs. Two specific primers, OfDP-SPF for the 3'-RACE and OfDP-SPR for the 5'-RACE (Fig. 1), were synthesized based on the sequence of the 402-bp fragment. After amplification and cloning, a 493-bp fragment from 5'-RACE and a 533-bp fragment from 3'-RACE were obtained.

The full-length cDNA (787 bp) contains a 5' untranslated region of 27 nucleotides, and the ORF represents 597



B

Species	DH		α-SGNP		β-SGNP		PBAN		γ-SGNP	
O. fuscidentalis	---VDDLKD-EADRGA-SDRGT-LWFGPRL	(%)	VIFTPKL	(%)	-SMDGYSD-KRTYENVEFTPRL	(%)	-LPEKLSVTPSDSHDAVYSFKPEMSELDSR-NNYFSPRL	(%)	--TVNFSPRL	(%)
M. vitrata	---LDDSKD-EADRGA-SDRGT-LWFGPRL	92	VVFTPKL	86	SIGGVFQD--KKYDNVEFIPRL	47	-IPDALPVTPS--DDDVYSFKPDSGEVDRR-TSYFNPRL	60	--KVSFSPRL	75
S. c. ricini	---TNDVKD-EGDRGAHSDRGS-LWFGPRL	79	VIFTPKL	100	RASNAYQE-KRTYENVEFTPRL	70	-LTEDMPATPTDQE--MF--DQDPEQIDTR-TRYFSPRL	42	--TMTFSPRL	75
M. sexta	---SNDIKD-EGDRGAHSDRGA-LWFGPRL	79	VIFTPEL	86	SIGDIYQE-KRTYENVEFTPRL	55	-ISEDMPATPSDQEYPMY--HPDPEQIDTR-TRYFSPRL	40	---THFSPRL	75
A. pernyi	---SNDIKD-EGDKGAHSDRGS-LWFGPRL	75	VIFTPKL	100	SLDDSTQEKRVFYENFEFTPRL	75	-LSDDMPATPKDQE--MY--HQDPEQVDTR-TRYFSPRL	36	--TITFSPRL	75
B. mori	----TDMKD-ESDRGAHSERGA-LWFGPRL	78	IIFTPKL	86	-----SVAKPQTHESLEFIPRL	50	-LSEDMPATPADQE--MY--QPDPEEMESR-TRYFSPRL	45	--TMSFSPRL	75
B. mandarina	----TDMKD-ESDRGAHSERGA-LWFGPRL	78	IIFTPKL	86	-----SVAKPQTHDSLEFIPRL	44	-LSEDMPATPADQE--IY--QPDPEVMESR-TRYFSPRL	42	--TMSFSPRL	75
C. anastomosis	TNNDNTMKDGGADRGAHSDRGG-LWFGPRL	75	VVFTPKL	86	--SMAYDD--KSYENVEFTPRL	67	-LADDMPATPSDQE--YY--RQDPEQIDSR-SNYFSPRL	45	--TMTLTPRL	50
A. ipsilon	--SSNDVKDGGADRGAHSDRGG-MWFGPRI	71	VIFTPKL	100	--SLSYED--KMFDNVEFTPRL	56	-LADDTPATPADQE--MY--RPDPEQIDSR-TKYFSPRL	39	--TMNFSPRL	88
O. thyellina	--TGNDVKDDGQDRVAHSDRGGQLWFGPRL	71	VIFTPKL	100	-SLSTYEE--KLYDNVEFTPRL	58	-LSDDMPATPPDQE--YY--RPDPEQIDSR-TKYFSPRL	42	--TMTFSPRL	75
H. armigera	--NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	--SLAYDD--KSFENVEFTPRL	61	-LSDDMPATPADQE--MY--RQDPEQIDSR-TKYFSPRL	39	--TMNFSPRL	88
H. zea	--NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	--SLSYDD--KSFENVEFTPRL	61	-LSDDMPATPADQE--MY--RQDPEQIDSR-TKYFSPRL	41	--TMNFSPRL	88
H. assulta	--NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	--SLAYDD--KSFENVEFTPRL	61	-LSDDMPATPADQE--MY--RQDPEQIDSR-TKYFSPRL	41	--TMNFSPRL	88
H. virescens	--NNNDDKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	--SLAYDD--KSFENVEFTPRL	61	-LADDMPATPADQE--MY--RQDPEQIDSRRTKYFSPRL	41	--TMNFSPRL	88
S. exigua	--NNNEIKDGGSDRGAHSDRAG-LWFGPRL	67	VIFTPKL	100	--SLAYDD--KVFENVEFTPRL	61	-LSDDMPATPADQE--LY--RPDPDQIDSR-TKYFSPRL	42	--TMNFSPRL	88
S. littoralis	--NNNEIKDGGSDRGAHSDRAG-LWFGPRL	67	VIFTPKL	100	--SLAYDD--KVFENVEFTPRL	61	-LADDMPATPADQE--LY--RPDPDQIDSR-TKYFSPRL	42	--TMNFSPRL	88
A. S. cretacea	----ENFKEENFDRNIRSGRAN-VVFKPIL	65	VIFTPKL	100	----------SVDFTPRL	75	QLVDDVPQ--------RQ--QIEEDRLGSR-TRFFSPRL	36	-TTMNFSPRL	88
P. xylostella	----DDLKD-EDIQRDARDRAS-MWFGPRL	61	VIFTPKL	100	SMEDPYEE--KRSYDVDFTPRL	38	RLNDSGLAPP------DE--YRTPELLDAR-AQYFSPRL	41	GGSMTFSPRL	63
Adoxophyes sp.	----NDLKE-DGEREANSDRQG-LWFGPRL	35	VIFTPKL	100	----NADE--DQQQSVDFTPRL	42	---QSEAVTSSD--EQVY--RQDMSPVDGR-LKYFSPRL	35	--TVKLTPRL	63

Fig. 2. Schematic drawing of the diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) precursor polyprotein in *Omphisa fuscidentalis*. (A) *DH-PBAN* cDNA encoding pre-prohormone consisting of 199 amino acids. It is presumed that the pre-prohormone undergoes post-translational processing via a series of enzymatic steps that cleave the GKR, KK, GRR and three GR sequences, and further modification by amidation at the C-terminal amino acid of the intermediate peptide substrates to yield the signal sequence (SS) and peptide hormones DH, α-, β- and γ-SGNP, and PBAN, similar to other Lepidopteran DH-PBAN precursor polyproteins. (B) Sequence alignment of DH, PBAN and three additional neuropeptides from 18 lepidopteran species, in addition to *O. fuscidentalis*. The percentages represent the amino acid similarities compared with *O. fuscidentalis*, which are calculated after clustalw2 alignment. The GenBank accession numbers of these sequences are: AFX71575 (*Maruca vitrata*), AAP41132 (*Samia cynthia ricini*), AAO18192 (*Manduca sexta*), AAR17699 (*Antheraea pernyi*), AAB24327 (*Bombyx mori*), AAM88285 (*Bombyx mandarina*), ABR04093 (*Clostera anastomosis*), CAA08774 (*Agrotis ipsilon*), BAE94185 (*Orygia thyellina*), AAL05596 (*Helicoverpa armigera*), AAA20661 (*Helicoverpa zea*), AAC64293 (*Helicoverpa assulta*), AAO20095 (*Heliothis virescens*), AAT64424 (*Spodoptera exigua*), AAK84160 (*Spodoptera littoralis*), BAF64458 (*Ascotis selenaria cretacea*), AAX99220 (*Plutella xylostella*) and AAK72980 (*Adoxophyes* sp.).

nucleotides encoding a 199-amino acid long polypeptide. The ORF is terminated by a TAA stop codon followed by a 160-nucleotide long 3' untranslated region. A consensus polyadenylation signal (ATTAAA) was found 9 bp upstream of the polyA tail. A hydrophobic sequence from M^{1} to V^{23} serves as a signal peptide (Von Heijne, 1985). There are six potential endoproteolytic cleavage sites at G^{47}-K^{48}-R^{49}, K^{95}-K^{96}, G^{104}-R^{105}, G^{126}-R^{127}-R^{128}, G^{166}-R^{167} and G^{176}-R^{177} (Fig. 1). Consequently, the cDNA is assumed to encode the precursor polyproteins for DH at D^{24}-L^{46}, α-SGNP (α-suboesophageal neuropeptide) at V^{97}-L^{103}, β-SGNP (β-suboesophageal neuropeptide) at S^{106}-L^{125}, PBAN at L^{129}-L^{165} and γ-SGNP (γ-suboesophageal neuropeptide) at T^{168}-L^{175}, all of which share the same C-terminal motif FXPR/KL (Fig. 2A).

By a homology search, the Ompfu-DH-PBAN amino acid sequence deduced from the other known DH-PBANs shows the homology: 74% with *M. vitrata*, 64% with *Antheraea pernyi*, 63% with *H. armigera*, 62% with *S. exigua*, 61% with *B. mori* and 54% with *Plutella xylostella* (Table 1). At the amino acid level, Ompfu-DH is 35–92% identical to other known DHs, and Ompfu-PBAN is 35–60% identical to other known PBANs. Ompfu-α-SGNP is 86–100% identical to those from other species, whereas Ompfu-β-SGNP and Ompfu-γ-SGNP show 38–75% and 50–88% similarity to those from other species (Fig. 2B).

Phylogenetic analysis

A phylogenetic tree was constructed using the neighbour-joining method (Fig. 3). *Omphisa fuscidentalis* clusters together with *Helicoverpa assulta*, *H. armigera*, *Helicoverpa zea*, *Heliothis virescens*, *S. exigua*, *Spodoptera littoralis* (Noctuidae), *A. pernyi*, *Samia cynthia ricini* (Saturniidae) and *M. vitrata* (Crambidae) with 97–100% bootstrap support,

Table 1. Homology of the deduced amino acid sequence of the Ompfu-DH-PBAN from the bamboo borer *Omphisa fuscidentalis* with diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) of other lepidopteran species

Species	Identity (%)	Similarity (%)	Accession Number
Maruca vitrata	73.7	88.4	M9P2L6
Antheraea pernyi	63.6	86.4	Q6SYA3
Helicoverpa armigera	63.0	83.5	Q95UR4
Spodoptera exigua	61.9	84.3	Q6RKA1
Bombyx mori	61.3	82.9	H9IWL9
Plutella xylostella	53.9	77.5	Q2M4G0

Forming a monophyly of the superfamilies Noctuoidea, Bombycoidea and Pyraloidea. The families Noctuidae, Lymantriidae and Notodontidae form a monophyletic clade comprising the superfamily Noctuoidea. The sister group to the Noctuoidea is the Bombycoidea, to which the families Saturniidae, Sphingidae and Bombycidae belong. The Geometroidea (represented by *Ascotis selenaria cretacea*) and the Pyraloidea form a trichotomy with the Noctuoidea plus Bombycoidea clade. The sister taxon to the above clade is the Yponomeutoidea plus Tortricoidea. On the whole, the DH-PBAN protein sequence similarity is correlated with the basic taxonomic relationships among the species and infers the feasibility and sensitivity of the DH-PBAN gene sequences as a phylogenetic marker in the class Insecta.

Tissue distribution and developmental expression of Ompfu-DH-PBAN

The expression of the *Ompfu-DH-PBAN* mRNA was examined by Q-RT-PCR. Total RNA was isolated from suboesophageal ganglion, other neural tissues (e.g. brain, thoracic ganglia and abdominal ganglia) and non-neural tissues (e.g. fat body, integument and Malpighian tubule). The results showed that *Ompfu-DH-PBAN* mRNA was expressed in the suboesophageal ganglion, detectable at much lower levels in other neural tissues, and not detected in the non-neural tissues (Fig. 4). Furthermore, the expression of the *Ompfu-DH-PBAN* transcript was detected during larval and pupal development. The expression level of *Ompfu-DH-PBAN* mRNA was consistently high during the fifth instar of larval development, then moderately high from October to December (larval stage), and significantly increased in January (larval stage), reaching a maximum level in March (larval stage). After pupation, the expression sharply decreased to a low level (Fig. 5).

Effect of photoperiod on Ompfu-DH-PBAN expression

Because there was evidence that photoperiod induced pupation in diapausing *O. fuscidentalis* larvae, the effect of photoperiod on the expression of *Of-DH-PBAN* mRNA by Q-RT-PCR was also examined in the suboesophageal ganglion of larvae reared at 25 ∘C combined with various photocycles

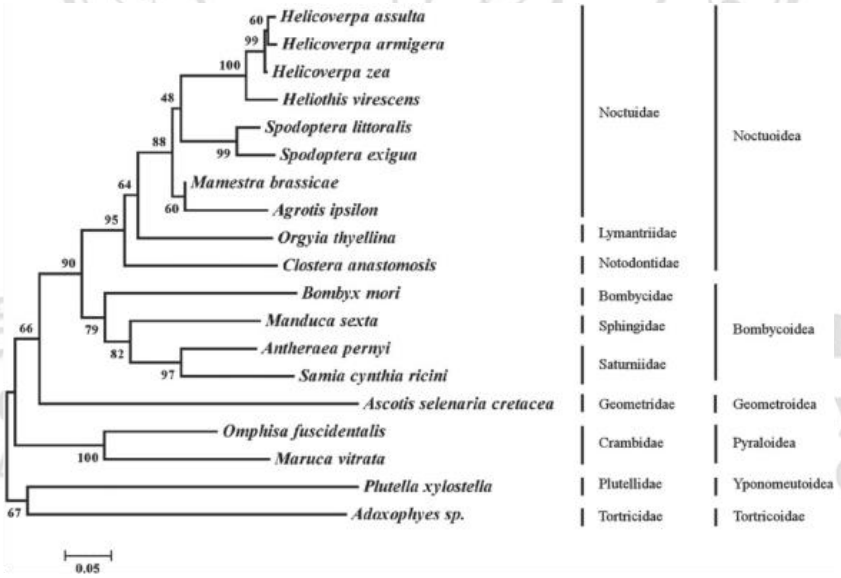


Fig. 3. Phylogenetic tree inferred from the known lepidopteran diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) amino acid sequences by the neighbour-joining method. The neighbour-joining tree was constructed using mega5 (http://www.megasoftware.net) based on the Dayhoff matrix (PAM) with 1000 bootstrap replicates. The number above branches shows the percentage bootstrap support above 50%. The scale on the bottom indicates the number of substitutions per amino acid site. The corresponding taxonomic families and superfamilies of the taxa and clades are shown to the right.

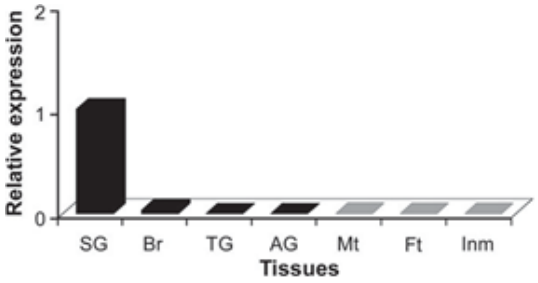


Fig. 4. Tissue expression of *Ompfu-DH-PBAN* mRNA from young fifth-instar larvae of *Omphisa fuscidentalis*. Total RNA (1.0 μg) was isolated from various tissues and mRNA expression was determined by a quantitative real-time polymerase chain reaction. SG, suboesophageal ganglion; Br, brain; TG, thoracic ganglia; AG, abdominal ganglia; Mt, Malpighian tubules; FB, fat body; Inm, integument. The data represent mean values of three independent samples, normalized relative to ribosomal protein RpL3 transcript levels. The integument was taken as the calibrator sample.

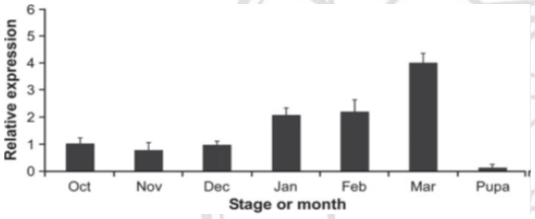


Fig. 5. Developmental changes in the expression levels of *Ompfu-DH-PBAN* mRNA in the subesophageal ganglion of *Omphisa fuscidentalis* during diapause (October to March) and post-diapause (pupation) as measured by a quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous ribosomal protein mRNA *OfRpL3*. Expression is relative to the gene expression in diapausing larvae collected from October (assigned a value of 1). Each value is the mean±SEM of three independent experiments. Means with different letters indicate a significantly difference (anova: n=3, P<0.05)

(LD 0 : 24, 2 : 22, 14 : 10 and 18 : 6 h). The results showed that the photoperiod had an effect on the expression level of *Ompfu-DH-PBAN* mRNA. Under the photocycles of LD 2 : 22, 14 : 10 and 18 : 6 h during larval diapause, the expression level *Ompfu-DH-PBAN* mRNA was low during the first 20 days of the larval stage. At day 25, expression was significantly higher, dropping abruptly to a minimum in the pupal stage (Fig. 6).

Discussion

A full-length *Ompfu-DH-PBAN* cDNA has been obtained from the suboesophageal ganglion of *O. fuscidentalis* larvae using the strategy of RT-PCR and RACE. The nucleotide sequence of the *Ompfu-DH-PBAN* cDNA encodes a 199 amino acid precursor peptide containing sequences for five FXPRL peptides (DH, PBAN, and α-, β- and γ-SGNP) that are produced by

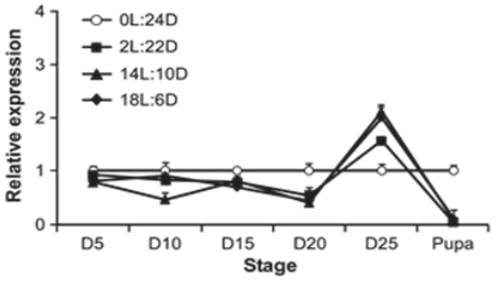


Fig. 6. Effect of diapause-inducing photoperiod on the expression of *Ompfu-DH-PBAN* gene in the suboesophageal ganglion of diapausing larvae of *Omphisa fuscidentalis*. Larvae were reared at 25 ∘C under various photocycles (LD 0 : 24, 2 : 22, 14 : 10, and 18 : 6 h). The *Ompfu-DH-PBAN* mRNA levels were determined from total RNA at various during diapause (D5–D25, day after feeding) and post-diapause (pupation) under each photocycle. Total RNA was isolated from the suboesophageal ganglion of staged *O. fuscidentalis* larvae, and mRNA levels were analyzed using a quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. The different controls that were collected at the same time points as treated samples were used as the calibrator sample. The data represent mean values of three independent experiments.

a post-endoproteolytic 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 isolated sequence. The structural organization of *Ompfu-DH-PBAN* mRNA is similar to that reported for 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 & Denlinger, 2003, 2004; Wei *et al.*, 2004; Zhang *et al.*, 2004a; Lee & Boo, 2005; Jing *et al.*, 2007; Xu *et al.*, 2007; Uehara *et al.*, 2011; Chang & Ramasamy, 2014). From the alignment, the deduced sequence shows similarity to 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 *Ompfu-DH-PBAN* cDNA, it is well known that DH induces embryonic diapause in *B. mori* (Yamashita, 1996) and also breaks pupal diapause and stimulates pupal development in moths from the family Noctuidae (Xu & Denlinger, 2003; Zhang *et al.*, 2004b). However, there is no evidence to suggest a role for DH in regulating larval diapause in other species of lepidopteran insects. Thus, at the present, it cannot be confirmed how the Ompfu-DH may be involved in regulating larval diapause in *O. fuscidentalis*. However, it is remarkable that Ompfu-DH lacks a histidine between residues A33-S34, which is exclusively conserved in closely-related species from Bombycoidea and Noctuoidea. Future studies featuring a comparison of Ompfu-DH 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 Ompfu-PBAN exhibits low similarity (35–60%) to other known PBAN peptides, whereas the C-terminal YFSPRL motif is highly conserved within lepidopteran species (Fig. 2B). Amidation of the Leu residue at the C-terminus of Ompfu-PBAN 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 & Boo, 2005). Moreover, the amino acid sequence alignment of Ompfu-PBAN possesses a single methionine residue, similar to *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 available to indicate that this is the case.

The biological function of α-SGNP is demonstrated in *H. zea*, in which it exhibits pheromonotropic activity (Ma *et al.*, 1996) and shows binding to the PBAN receptor, suggesting that α-SGNP acts physiologically similar to PBAN (Choi *et al.*, 2003). The predicted peptide sequence of Ompfu-α-SGNP is highly conserved among other examined α-SGNPs, although it is not clear whether Ompfu-α-SGNP performs a function similar to that in other lepidopteran species. The Ompfu-β-SGNP shows the highest divergence among the five FXPRL peptides compared (Fig. 2B), exhibiting only 38–75% homology with β-SGNP from other species. Helze-β-SGNP is reported to play a role in epidermal melanization in the larval stage (Raina *et al.*, 2003), suggesting that Ompfu-β-SGNP might have a similar role. However, the specific physiological functions of the Ompfu-β-SGNP and Ompfu-α-SGNP still need to be investigated.

A phylogenetic tree based on the ORF sequences of known DH-PBANs demonstrates that Ompfu-DH-PBAN is relatively closer to DH-PBANs from Crambidae than from other lepidopteran families. In any case, the function of DH-PBAN remains unreported in this family.

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

At present, it is known that an endocrine factor (Ompfu-DH-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 environmental (photoperiod). For diapause regulation, photoperiod is less important than temperature in *B. mori*, although photoperiod is the most important factor in many other species of insects. Previous studies show that larval diapause is terminated by photoperiod in *O. fuscidentalis* (T. Singtripop, unpublished data), as is also the case in the flies *C. vicina* (Vinogradova, 1974) and *L. sericata* (Tachibana & 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 *Ompfu-DH-PBAN* mRNA in *O. fuscidentalis*. In the present study, photoperiod is shown to have an effect on the expression level of *Ompfu-DH-PBAN* mRNA during larval diapause. Moreover, the *Ompfu-DH-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, 14 : 10 and 18 : 6 h (Fig. 6). This suggests that photoperiod might stimulate the secretion of DH-PBAN to inform the suboesophageal ganglion, which then acts on the prothoracic glands to stimulate release ecdysone, with a consequent effect on larval diapause termination. Accordingly, the increased expression of *Ompfu-DH-PBAN* mRNA before entering the pupal stage may be associated with an increment of ecdysteroid titre in the haemolymph. In *O. fuscidentalis*, the haemolymph ecdysteroid concentration is low during larval diapause and increases prior to the pupal stage, thereby presumably stimulating pupal metamorphosis (Singtripop *et al.*, 1999). Accordingly, 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 in additional experiments.

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