CHAPTER 3

Results

3.1 Molecular characterization of *OfDH-PBAN* cDNA

3.1.1 Cloning of *OfDH-PBAN* cDNA

Using the degenerated primers OfDPF and OfDPR (Fig. 3.1), 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. 3.1), 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 open reading frame (ORF) represents 597 nucleotides encoding a 199-amino acid long polypeptide. The ORF is terminated by a TAA stop codon that is 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. 5). Consequently, the cDNA is believed to encode the precursor polyproteins for DH at D^{24}-L^{46}, α-SGNP at V^{97}-L^{103}, β-SNGP at S^{106}-L^{125}, PBAN at L^{129}-L^{165} and γ-SGNP at T^{168}-L^{175}, all of which share the same C-terminal motif FXPR/KL (Fig. 3.2A).

By homology search, the *OfDH-PBAN* amino acid sequence deduced from the other known *DH-PBANs* shows the following 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 3.1). At the amino acid level, *OfDH* is 35-92%

identical to other known *DHs*, and *OfPBAN* is 35-60% identical to other known *PBANs*. *Of-α-SGNP* is 86-100% identical to those from other species, whereas *Of-β-SGNP* and *Of-γ-SGNP* show 38-75% and 50-88% similarity with those from other species (Fig. 3.2B).

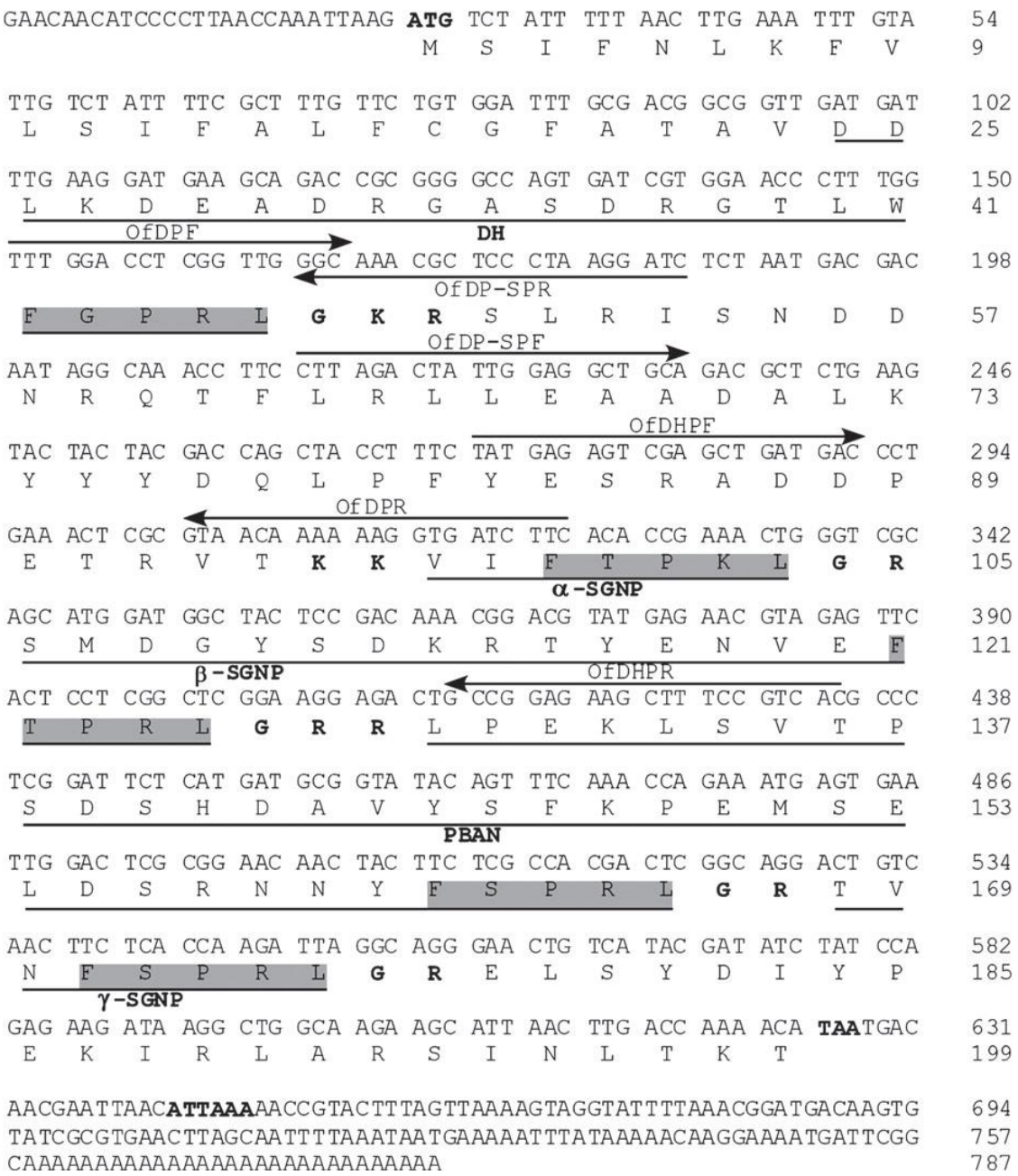
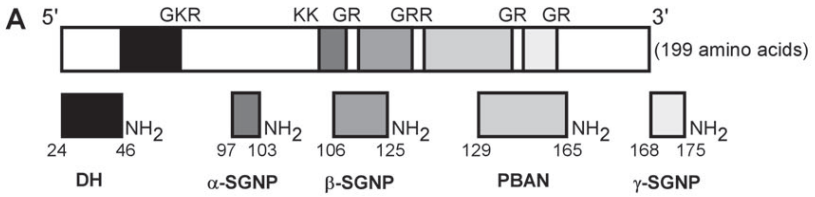


Figure 3.1 Nucleotide sequence and deduced amino acid sequence of the *OfDH-PBAN* cDNA. The suggested start (ATG) and stop (TAA) codons, and polyadenylation signal (ATTAAA) are shown in bold letters. The five presumptive peptides are underlined, and endoproteolytic cleavage sites are printed in bold. Arrows over the nucleotide sequences

represent the position of the different synthetic primers used in 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 RACE 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').



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	RLKDSGLAPP------DE--YRTPELLDAR-AQYFSPRL	41	GGSMTFSPRL	63
Adoxophyes sp.	----NDLKE-DGEREANSDRQG-LWFGPRL	35	VIFTPKL	100	----NADE--DQQQSVDFTPRL	42	---QSEAVTSSD--EQVY--RQDMSPVDGR-LKYFSPRL	35	--TVKLTPRL	63

Figure 3.2 Schematic drawing of the DH-PBAN precursor polyprotein in *O. 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 3 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 to *O. fuscidentalis*, which are calculated following 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.).

Table 3.1 Homology of the deduced amino acid sequence of the OfDH-PBAN from the bamboo borer, *O. 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

3.1.2 Phylogenetic analysis

A phylogenetic tree was constructed using the neighbour-joining method (Fig. 3.3). *Omphisa fuscidentalis* clusters together with *Helicoverpa assulta, H. armigera, Helicoverpa zea, Heliothis virescens, Spodoptera exigua, Spodoptera littoralis* (Noctuidae), *Antheraea pernyi, Samia cynthia ricini* (Saturniidae) and *M. vitrata* (Crambidae) with 97-100% bootstrap support, 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.

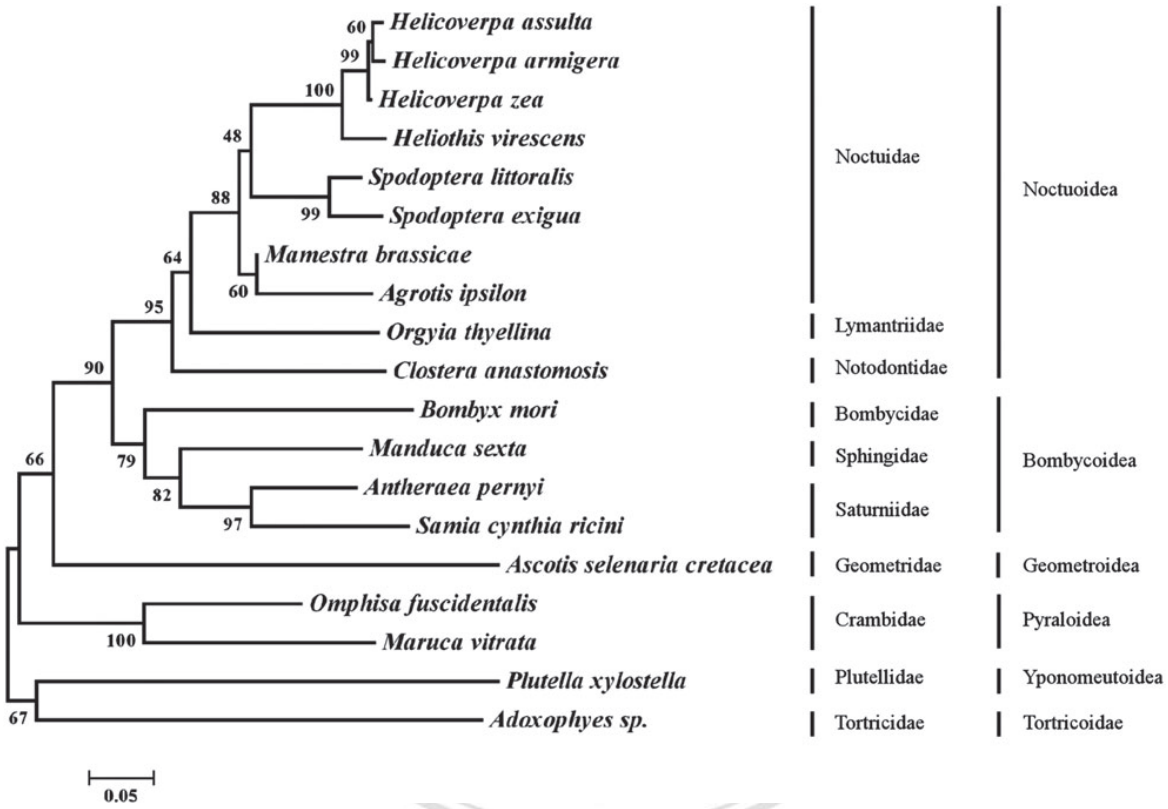


Figure 3.3 Phylogenetic tree inferred from the known lepidopteran DH-PBAN amino acid sequences by the neighbour joining method. The neighbour joining tree was constructed using MEGA5 software (www.megasoftware.net) based on Dayhoff matrix (PAM) with 1000 bootstrap replicates. The number above branches 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 on the right.

3.2 *OfDH-PBAN* mRNA expression

3.2.1 Tissue distribution and developmental expression of *OfDH-PBAN*

The expression of the *OfDH-PBAN* mRNA was examined by Quantitative real-time PCR. Total RNA was isolated from SG, 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 *OfDH-PBAN* mRNA was expressed in the SG, detectable at much lower levels in other neural tissues, and not detected in the non-neural tissues (Fig. 3.4). Furthermore, the expression of the *OfDH-PBAN* transcript was detected during larval and pupal development. The expression level of *OfDH-PBAN* mRNA was consistently high during the 5th instar of larval development, then moderately high from October to December (larval stage) and significantly increased in January (larval stage), reaching the maximum level in March (larval stage). After pupation, the expression sharply decreased to a low level (Fig. 3.5).

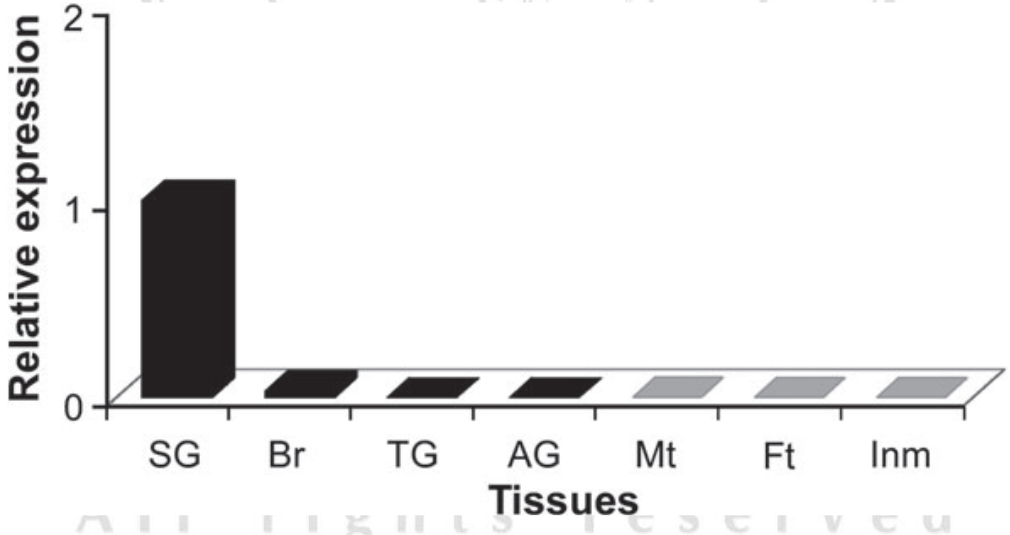


Figure 3.4 Tissue expression of *OfDH-PBAN* mRNA from young 5th instar larvae of *O. fuscidentalis*. Total RNA (1.0 µg) was isolated from various tissues and mRNA expression was determined by Q-RT-PCR. Suboesophageal ganglion (SG), brain (Br), thoracic ganglia (TG), abdominal ganglia (AG), Malpighian tubules (Mt), fat body (Ft) and integument (Inm). 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.

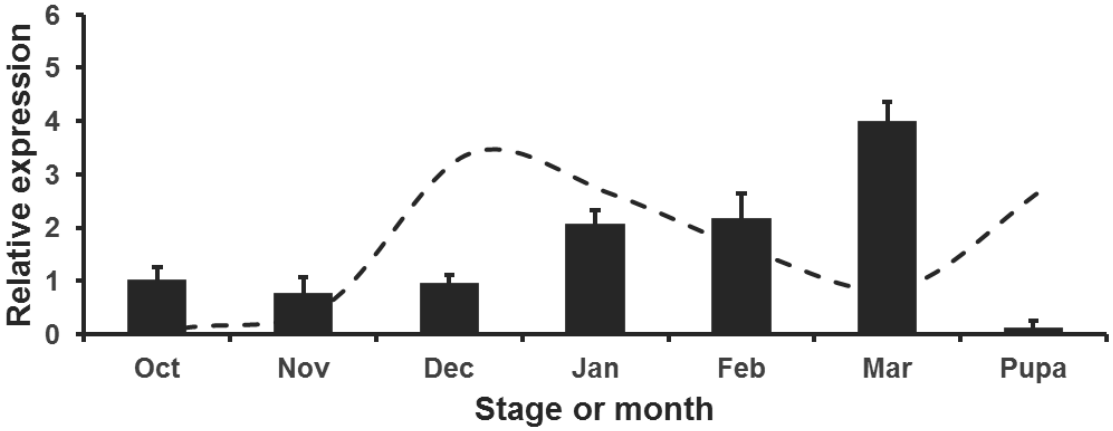


Figure 3.5 Developmental changes in the expression levels of *OfDH-PBAN* mRNA in the subesophageal ganglion of *O. fuscidentalis* during diapause (October to March) and post-diapause (pupation) as measured by Q-RT-PCR. The results are expressed as the relative expression after normalization against endogenous 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 are significantly difference (ANOVA, $n = 3$, $P < 0.05$). The dotted line represents the hemolymph ecdysteroid titer (Singtripop *et al*., 1999).

3.2.2 Effect of photoperiod on *OfDH-PBAN* mRNA expression

Because there was an evidence that photoperiod induced pupation in diapausing *O. fuscidentalis* larvae, the effect of photoperiod on the expression of *OfDH-PBAN* mRNA by quantitative real-time PCR was also examined in the SG of larvae reared at 25 ºC combined with various photoperiods (LD 0:24 h, LD 2:22 h, LD 14:10 h and LD 18:6 h). Results showed that the photoperiod had an effect on the expression level of *OfDH-PBAN* mRNA. Under the photocycles of LD 2:22 h, LD 14:10 h and LD 18:6 h during larval diapause the expression level *OfDH-PBAN* mRNA was low during the first twenty days of the larval stage. At day-25, the expression was significantly higher, and it dropped abruptly to a minimum in the pupal stage (Fig. 3.6).

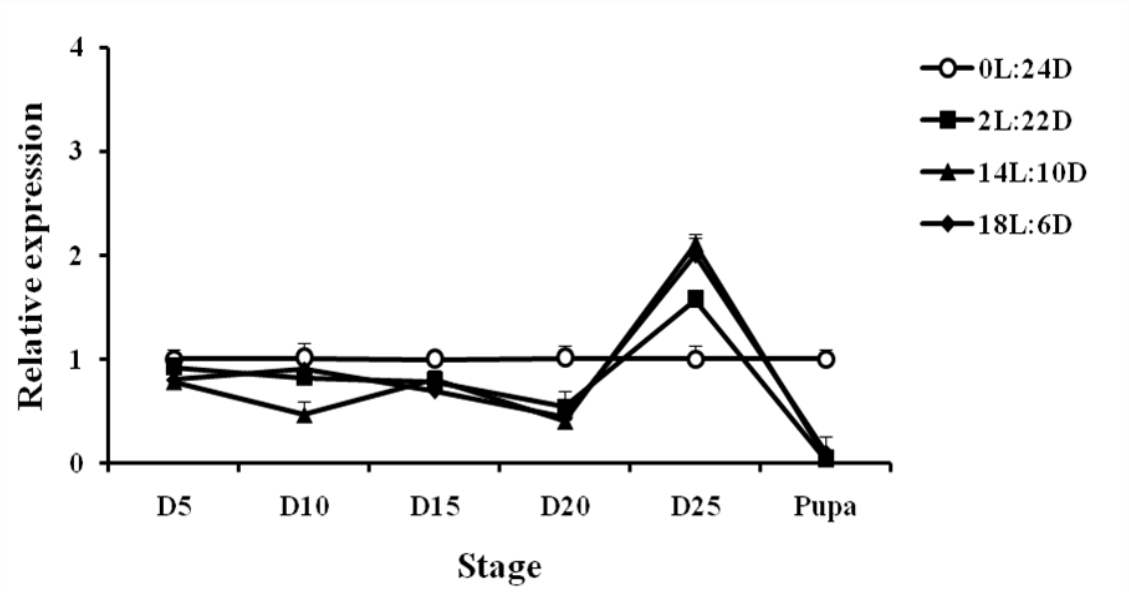


Figure 3.6 Effect of diapause-inducing photoperiod on the expression of *OfDH-PBAN* gene in the suboesophageal ganglion of diapausing larvae of *O. fuscidentalis*. Larvae were reared at 25 ºC under various photoperiods (LD 0:24 h, LD 2:22 h, LD 14:10 h, and LD 18:6 h). The *OfDH-PBAN* mRNA levels were 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 Q-RT-PCR. 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. See also the legend to Fig. 3.5.

3.3 Molecular characterization of *OfMet, OfE75C* and *OfHR3* cDNA

3.3.1 Cloning of *OfMet* cDNA

The partial sequence of *OfMet* contains 748 nucleotides, encoding a protein of 248 amino acids. By homology search (Table 3.2), the deduced protein sequence of OfMet revealed the highest homology with *B. mori* (63%), followed by *D. plexippus* (62%), *O. brumata* (38%) and *H. armigera* (36%). We have confirmed the molecular structure similarity of known Met genes among species in the order Lepidoptera. The OfMet amino acid sequences were aligned with those of two other insect species, the protein sequence of OfMet is most homologous to Met from *B. mori* of the family Bombycidae and least similar to that of *O. brumata* in the Geometridae (Fig. 3.7).

Table 3.2 Homology of the deduced amino acid sequences of the OfMet from the bamboo borer, *O. fuscidentalis* with Methoprene tolerant (Met) of other lepidopteran species.

Species	**Identity (%)**	**Accession Number**
Bombyx mori	63.4	D4Q9H9
Danaus plexippus	62.4	G6CT04
Operophtera brumata	37.7	A0A0L7LC43
Helicoverpa armigera	35.7	A0A023PPA4

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OfMet      DKTGALRLTAHYLRAHQYVFGDSIGQGNPQFSAMSARALLSLLKGFLLTTTYRGLIVVVS    60
BmMet      DKTGVLRLTAHYLRAHQYVFCNKMVHTNPDFNPEFTDAVLKLFNGFLITTTYRGIIVVVS    60
ObMet      DKTSVLRLAANKLRNE-HVFGNTIKCSHIETWSPAFLKFFDLIGGIMFTVTCRGRICIIS    59
           ***..***:*. ** . :** :.:   . :        .:.*: *:::*.* ** * ::*

OfMet      QNVQQYLGYTELDLLGQNVFNIIHEDDRQLMRDQLMPKKNMLGPNGELLVPEEPEGNRMV    120
BmMet      KNVHQYLGFPELDLLGQNLVNLTHPRDRQMLLEKLKPRSQVLGPNGELLIPNEPDGVYKV    120
ObMet      PNIQEKLGYCYVDLLGLDLYNYVHPDDKEILHQHIYPHELQTGSDSRL------------    107
            *::: **:  :**** :: *  *  *:::: ::: *:.   * :..*

OfMet      AQILAGEKRRFIIRFKKFCQRSEPCQYVTCHVEGTLRKSDRACRGYNRCCQMVRRARARG    180
BmMet      VEGLRREKRSFTIRLKKQGPRSEPAQYVMCHIEGSFRKADGANHTLSRCCQVVRRSRTRG    180
ObMet      ----YEQHHNFNIRIKRAGARSDPVRYERCRIDGMLRKSDKAIANAVQDERVIRRQRVRQ    163
                 ::: * **:*:   **:* :*  *:::* :**:* *     :  :::** *.*

OfMet      D-NPCSSGNDIVFVGVVRVATETFITESNMESYRMEYRTRHSIDGQIIQSEQRISLVTGY    239
BmMet      E-APECSGNDIVFIGVVRPSVETFHSESRMESFCMEYRTRHSVDGQIVQCEQRISLVTGY    239
ObMet      NRTFSSSGNDFVFIGMIHVLSSNLPPRILPPTAYSEYWTRHMIDGRIVQCDQSISLAVGY    223
           :   .****:**:*:::   ..:  .   :  ** *** :**:*:*.:* ***..**

OfMet      MTHERARLV      248
BmMet      MTHEVKGVN      248
ObMet      MTDEVTGTS      232
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Figure 3.7 Clustal W2 alignment of deduced amino acid sequence of OfMet with sequences from two other insect species (BmMet, *B. mori* and ObMet, *O. brumata*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.

3.3.2 Cloning of *OfE75C* cDNA

The partial sequence of *OfE75C* contains 449 nucleotides, encoding a protein of 149 amino acids. By homology search (Table 3.3), the deduced protein sequence of OfE75C revealed the highest homology with *B. mori* and *M. sexta* (96%), followed by *A. aegypti* (78%). We have confirmed the molecular structure similarity of known E75C genes among species in the order Lepidoptera. The OfE75C amino acid sequences were aligned with those of two other insect species, the protein sequence of OfE75C is most homologous to E75C from *B. mori* and *M. sexta* of the family Bombycidae and Sphingidae, respectively (Fig. 3.8).

Table 3.3 Homology of the deduced amino acid sequence of the OfE75C from the bamboo borer, *O. fuscidentalis* with E75C of other lepidopteran species.

Species	**Identity (%)**	**Accession Number**
Bombyx mori	96.1	Q8WSA1
Manduca sexta	96.1	Q5YB71
Aedes aegypti	77.5	Q0JRL0

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OfE75C      SVIQCMRPPPPPPP---PRLLKPSSFEEPSSSIPDLEFDGTTVLCRVCGDKASGFHYGVH   57
MsE75C      SVIQCMRPPPPPPPPPPAPRLHKPPSFEEPSSSIPDLEFDGTTVLCRVCGDKASGFHYGVH   60
BmE75C      SVIQCMRPPPPPPPPPPPPRLLKPPSFEEPSSSIPDLEFDGTTVLCRVCGDKASGFHYGVH   60
            **************   *** ** ***********************************

OfE75C      SCEGCKGFFRRSIQQKIQYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGMSRDAVRFGR   117
MsE75C      SCEGCKGFFRRSIQQKIQYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGMSRDAVRFGR   120
BmE75C      SCEGCKGFFRRSIQQKIQYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGMSRDAVRFGR   120
            ************************************************************

OfE75C      VPKREKARILAAMQQSSTSRAHEQAATAELDD   149
MsE75C      VPKREKARILAAMQQSSTSRAHEQAAAAELD-   151
BmE75C      VPKREKARILAAMQQSSSSRAHEQAAAAELD-   151
            *****************:********:****
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Figure 3.8 Clustal W2 alignment of deduced amino acid sequence of OfE75C with sequences from two other insect species (MsE75C, *M. sexta* and BmE75C, *B. mori*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.

3.3.3 Cloning of *OfHR3* cDNA

The partial sequence of *OfHR3* contains 984 nucleotides, encoding a protein of 327 amino acids. By homology search (Table 3.4), the deduced protein sequence of OfHR3 revealed the highest homology with *P. xuthus* (89%), followed by *H. armigera* (83%), *B. mori* (81%), *P. interpunctella* (73%) and *C. fumiferana* (68%). We have confirmed the molecular structure similarity of known HR3 genes among species in the order Lepidoptera. The OfHR3 amino acid sequences were aligned with those of three other insect species, the protein sequence of OfHR3 is most homologous to HR3 from *P. xuthus*, *H. armigera* and *B. mori* of the family Papilionidae, Noctuidae and Bombycidae, respectively (Fig. 3.9).

Table 3.4 Homology of the deduced amino acid sequence of the OfHR3 from the bamboo borer, *O. fuscidentalis* with HR3 of other lepidopteran species.

Species	**Identity (%)**	**Accession Number**
Papilio xuthus	88.9	I4DLS9
Helicoverpa armigera	82.8	Q9BMC6
Bombyx mori	81.0	Q9U5G6
Plodia interpunctella	72.6	Q6PWP7
Choristoneura fumiferana	68.0	Q27547

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OfHR3       TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF   60
PxHR3       TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF   60
HaHR3       TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF   60
BmHR3       TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRY   60
            ************************************************************:

OfHR3       HRAQMRAQTDAAPDSVYDAQQQTPSSSDQFHGHYNGYPAYGSPLSSYGYNNAGPALPSNM   120
PxHR3       HRAQMRAQTDAAPDSVYDAQQQTPSSSDQFHGHYNGYPGYSSPLSSYGYSGAGPALTSNM   120
HaHR3       HRAQMRAQTDTAPDSVYDAQQQTPSSSDQFHGHYNGYPGYGSPLSSYGYNNAGPALQSNM   120
BmHR3       HKAQMRVQADAAPDSVYDAQQQTPSSSDQFHGHYNSYPGYGSPLSSYGYNNAGPALPSNM   120
            *:****.*:*:************************.**.*.********. ***** ***

OfHR3       SGMQAAPPQQQSYDVSADYVDST-TYEPKQ-TGFLDTDFIG   159
PxHR3       N-IPPQQQQPQPYDVSADYVDSTTTYEPKQTGGFLDPDFIG   160
HaHR3       GGIQPQAPQQQPYDVSADYVDSTTAYEPKQTEGFLDPDFIS   161
BmHR3       SGMQPQPPAQPPYEVSGDYVDSTTTYEPKQ-TGFLDADFIS   160
              :         *:**.****** :*****  **** ***.
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Figure 3.9 Clustal W2 alignment of deduced amino acid sequence of OfHR3 with sequences from three other insect species (PxHR3; *P. xuthus*, HaHR3; *H. armigera* and BmHR3; *B. mori*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.

3.4 *In vivo* effects of JHA on *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA expression in both SG and PG

We induced pupation by treating diapausing larvae with JHA and examined the effect of JHA on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in both SG and PG (Fig. 3.10). After the application of 0.5 µg JHA, *OfMet* mRNA levels in the SG were low on day 0, while levels of *OfMet* mRNA in the PG were low until day 1. Thereafter, it then increased and remained until reaching a maximum level on day 8, decreased on day 10 and abruptly dropped to a minimum level until G3 stage (Fig. 3.10A). In addition, *OfDH-PBAN* mRNA levels in the SG were low from days 0 to 4, and then gradually increased until reaching a maximum level on day 12, while *OfDH-PBAN* mRNA levels in the PG were low from days 0 to 8, and then increased dramatically until reaching a peak on day 12. Expression of both SG and PG then abruptly dropped to a low level at G0-G3 stage (Fig. 3.10B). Interestingly, *OfMet* and *OfDH-PBAN* in the PG was expressed much later than *OfMet* and *OfDH-PBAN* in the SG. In the SG, the expression level of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA was expressed at a very low level between day 0 to G3 stage (Fig. 3.10C-I). By contrast, *OfEcR-A* and *OfEcR-B1* mRNA levels in PG were low from days 0 to 12, and then increased dramatically to a high level at G0-G3 stage. The expression level of *OfEcR-A* mRNA reached a maximum level at G1 and G2 stage, but the *OfEcR-B1* mRNA level peaked at G1 stage (Fig. 3.10C and D). In addition, the expression level of *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in the PG was low between day 0 and G0 stage, then rapidly increased to the maximal value at the G1 stage and decreased at G2 and G3 stage (Fig. 3.10E-I).

These results indicated that JHA had an effect on expression of *OfMet* and *OfDH-PBAN* mRNA in SG and PG. Interestingly, *OfMet* and *OfDH-PBAN* in the SG was expressed much faster than *OfMet* and *OfDH-PBAN* in the PG. Moreover, JHA affected on expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in PG during pupal stage.

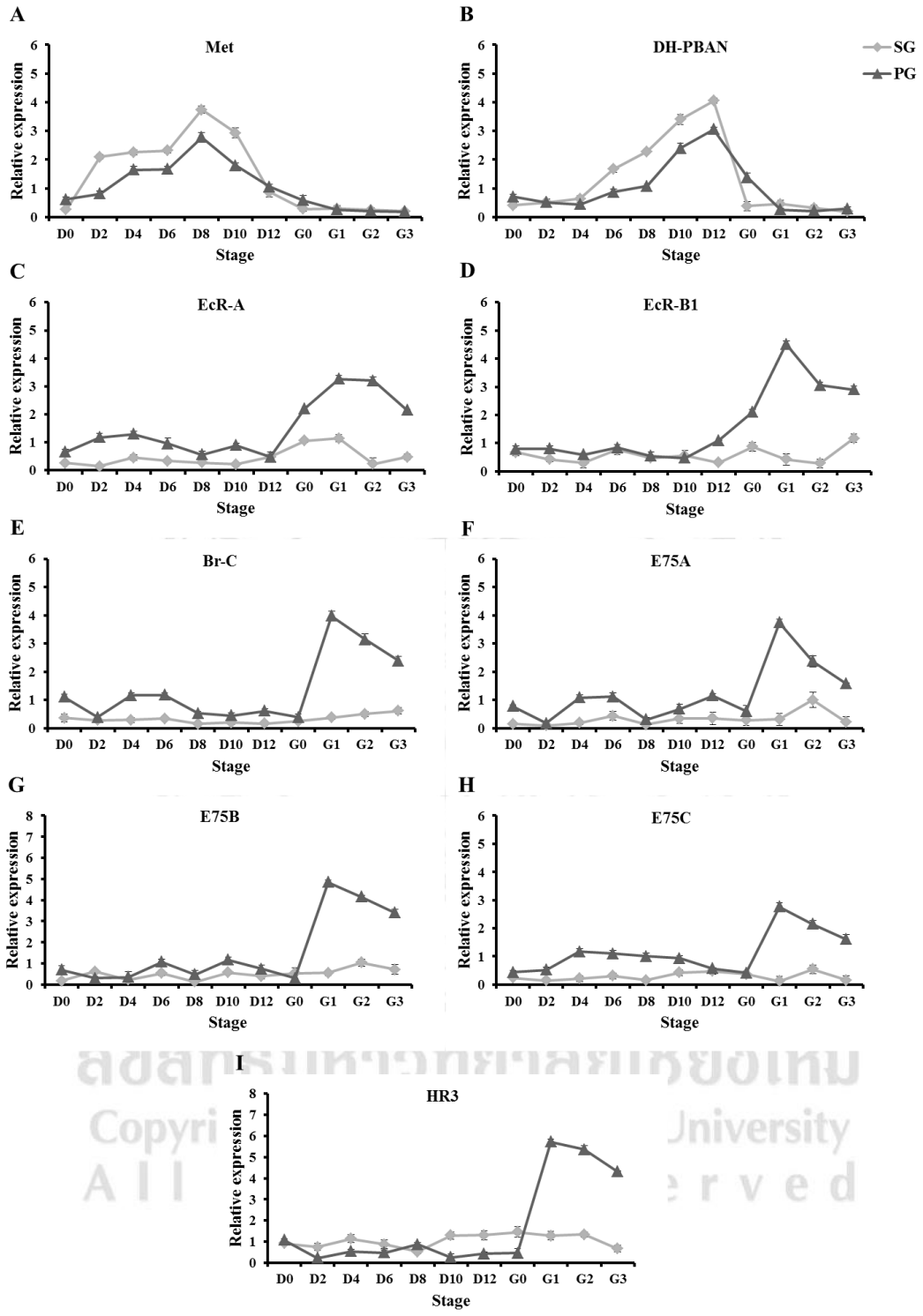

Figure 3.10 Induction of JHA on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in SG and PG of diapausing larvae. Larvae were applied by 0.5 µg JHA, and *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) mRNA levels were determined from the total of pool RNA after treatment; D0-D12, 0-

12 days after treatment, G0-G3, stages in pupal development. The larvae became motionless before the formation of a brown cuticle (G0). The body colour then changes from creamy to light yellow (G1). The next day, the dorsal epidermis turns light brown (G2). Approximately 1 day later, the entire body becomes brown (G3) (Singtripop *et al*., 2000). For the controls, diapausing larvae were applied by acetone. Total RNA was isolated from SG or PG of staged *O. fuscidentalis* larvae (as above) and mRNA levels were analyzed using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against the expression of endogenous *OfRpL3*. Values are relative to the level of expression in the different controls collected at the same time points as the treated samples. Each value is the mean ± SEM of three independent experiments. Means with different letters are significantly different (ANOVA, $n = 3$, $P < 0.01$).

3.5 *In vivo* effects of 20E on *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA expression in both SG and PG

We also examined the effects of 20E on the expression of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in both SG and PG (Fig. 3.11). In larvae injected with 0.5 µg 20E, the expression level of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG was low from day 0 to G3 stage and was not significant when compared with control (Fig. 3.11A and B). Moreover, *OfEcR-A* and *OfEcR-B1* mRNA levels in the SG were low from days 0 to 10, and then increased gradually until reaching a maximum level at G3 stage, while *OfEcR-A* and *OfEcR-B1* mRNA levels in the PG were low on days 0 to 10, then increased to a maximum level in the G1 stage and decreased again between G2 and G3 stage (Fig. 3.11C and D). Furthermore, the expression level of *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA was low between days 0 and G0 stage, then increased dramatically and peaked at G1 stage, followed by a decline at G2 and G3 stage (Fig. 3.11E-I).

These results indicated that 20E had no effect on expression of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG but had effect on expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in both SG and PG during pupal stage.

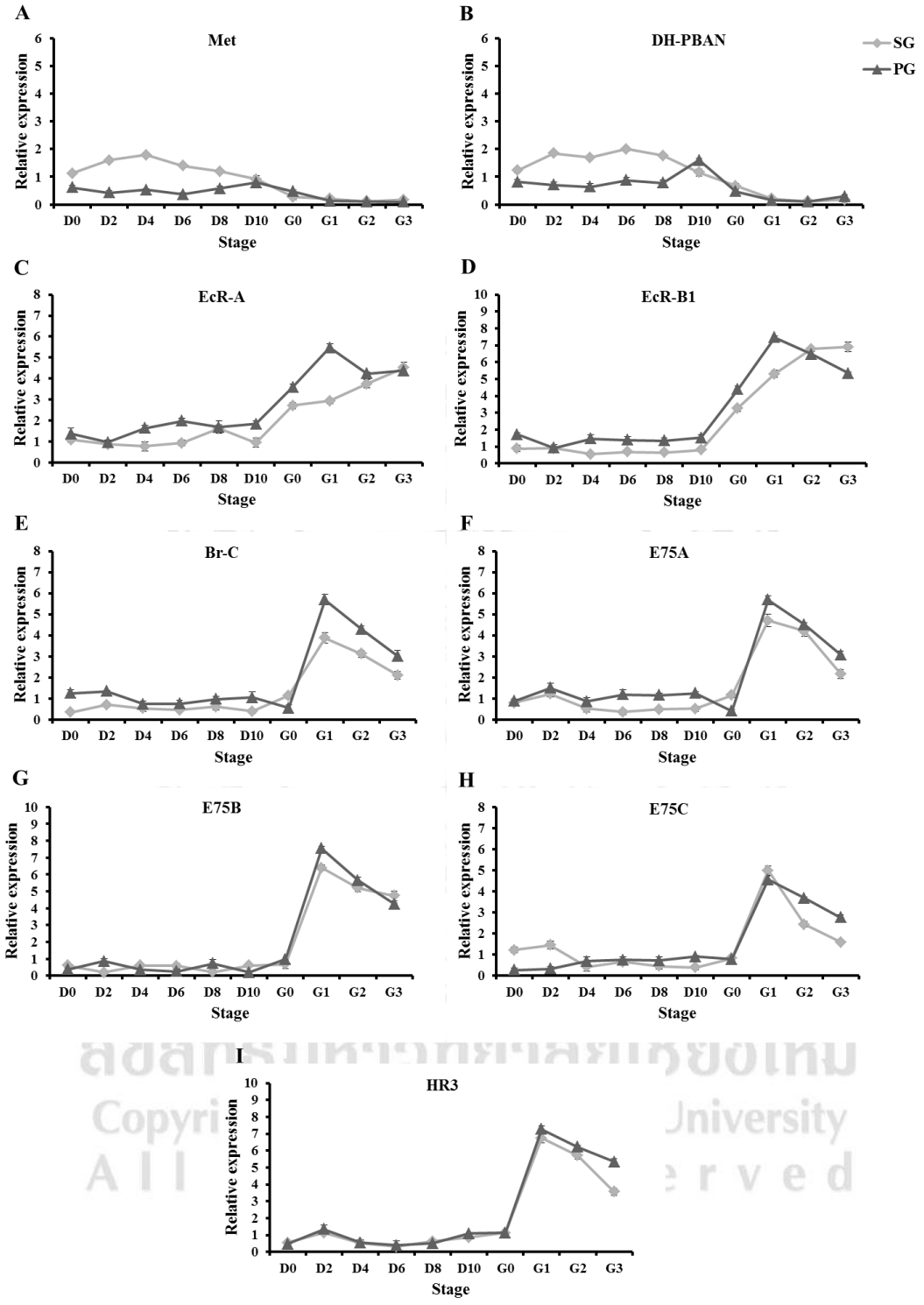

Figure 3.11 Induction of 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in SG and PG of diapausing larvae. Larvae were injected with 0.5 µg of 20E, and *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) mRNA levels were determined from the total of pool RNA after treatment;

D0-D10, 0-10 days after treatment, G0-G3, stages in pupal development. The larvae became motionless before the formation of a brown cuticle (G0). The body colour then changes from creamy to light yellow (G1). The next day, the dorsal epidermis turns light brown (G2). Approximately 1 day later, the entire body becomes brown (G3) (Singtripop *et al*., 2000). For the controls, diapausing larvae were injected by distilled water. Total RNA was isolated from SG or PG of staged *O. fuscidentalis* larvae (as above) and mRNA levels were analyzed using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against the expression of endogenous *OfRpL3*. Values are relative to the level of expression in the different controls collected at the same time points as the treated samples. Each value is the mean ± SEM of three independent experiments. See also the legend to Fig. 3.10.

3.6 *In vitro* effects of JHA and 20E on *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA expression in the SG

To understand how hormonal mechanism on termination of larval diapause is regulated by JHA, the SG was cultured in Grace's medium containing both JHA at 0.1 µg/50 µl and 20E at 0.1 µg/50 µl for different continuous time exposures. The induction patterns of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression was examined under these experimental conditions.

We remarked that in the presence of JHA alone at 0.1 µg/50 µl, the *OfMet* mRNA levels were low at 0 h, and then rapidly increased to the maximal value at 0.5 h, followed by a decline from 1 to 24 h (Fig. 3.12A), while the expression level of *OfDH-PBAN* was low at 0 h, reached a maximum at 1 h and then decreased at 4-24 h (Fig. 3.12B). By contrast, JHA did not significantly up-regulate *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression (Fig. 3.12C-I). In the presence of 20E alone, *OfMet* and *OfDH-PBAN* mRNA levels were constitutively expressed at a low level over time (Fig. 3.12A and B) whereas the level of *OfEcR-A* and *OfEcR-B1* mRNA was low at 0 h, then dramatically increased at 0.5 h, reached the highest level at 1 h, followed by a large decrease at 4 h and remained steady at a low level until 24 h (Fig. 3.12C and D). The relative expression of *OfBr-C*, *OfE75A* and

OfHR3 was low between 0 and 0.5 h, then increased dramatically to the maximal value at 4 h and decreased at 8-24 h (Fig. 3.12E, F and I), while the *OfE75B* and *OfE75C* mRNA levels were low at 0-1 h, rapidly increased to a maximum level at 4 h, followed by a decline at 8 h and remained at a low level until 24 hour after 20E application (Fig. 3.12G and H). Moreover, we performed a time course experiment to determine how the presence of the two hormones together affected gene expression as a function of time. In the presence with 0.1 µg/50 µl of JHA and 20E together, *OfMet* and *OfDH-PBAN* transcripts showed temporal patterns similar to those obtained in response to JHA alone (Fig. 3.12A and B). The *OfEcR-A* and *OfEcR-B1* mRNA levels were low at 0 and 1 h, and then increased until reaching a maximum level at 1 h, followed by a decline from 4 to 24 h (Fig. 3.12C and D). In addition, the expression level of *OfBr-C*, *OfE75A* and *OfE75C* mRNA was low between 0 and 0.5 h, while *OfE75B* and *OfHR3* mRNA levels were low from 0 to 1 h. Thereafter, expression then increased until reaching a maximum level at 4 hour and declined at 8-24 h after application of the two hormones (Fig. 3.12E-I). These results indicated that JHA alone had effect on expression of *OfMet* and *OfDH-PBAN* mRNA (Fig. 3.12A-B), while 20E alone had effect on expression ecdysone receptor genes and ecdysone inducible genes in SG (Fig. 3.12C-I).

We next examined the dose-dependence on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression by adding various concentrations of JHA and 20E (0.005-1 µg/50 µl) to the medium. The total RNA was collected and isolated from the SG. On the basis of results of the time course experiment, the SG was incubated with various concentrations of JHA for 0.5 h (Fig. 3.13). The results showed that *OfMet* and *OfDH-PBAN* mRNA levels were low at 0.005 µg/50 µl JHA, then moderately high from 0.01-0.05 µg/50 µl JHA and reached a maximum at 0.1-1 µg/50 µl JHA (Fig. 3.13A and B), while *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were constitutively expressed at a low level over dose of JHA (Fig 3.13C-I). By contrast, the SG was incubated with different concentrations of 20E for 4 h (based on the results of the time course experiment). 20E did not significantly up-regulate *OfMet* and *OfDH-PBAN* mRNA expression (Fig. 3.13A and B) whereas *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005 µg/50 µl 20E and gradually increased until reaching a maximum level at 0.1-1 µg/50 µl 20E (Fig. 3.13C-I).

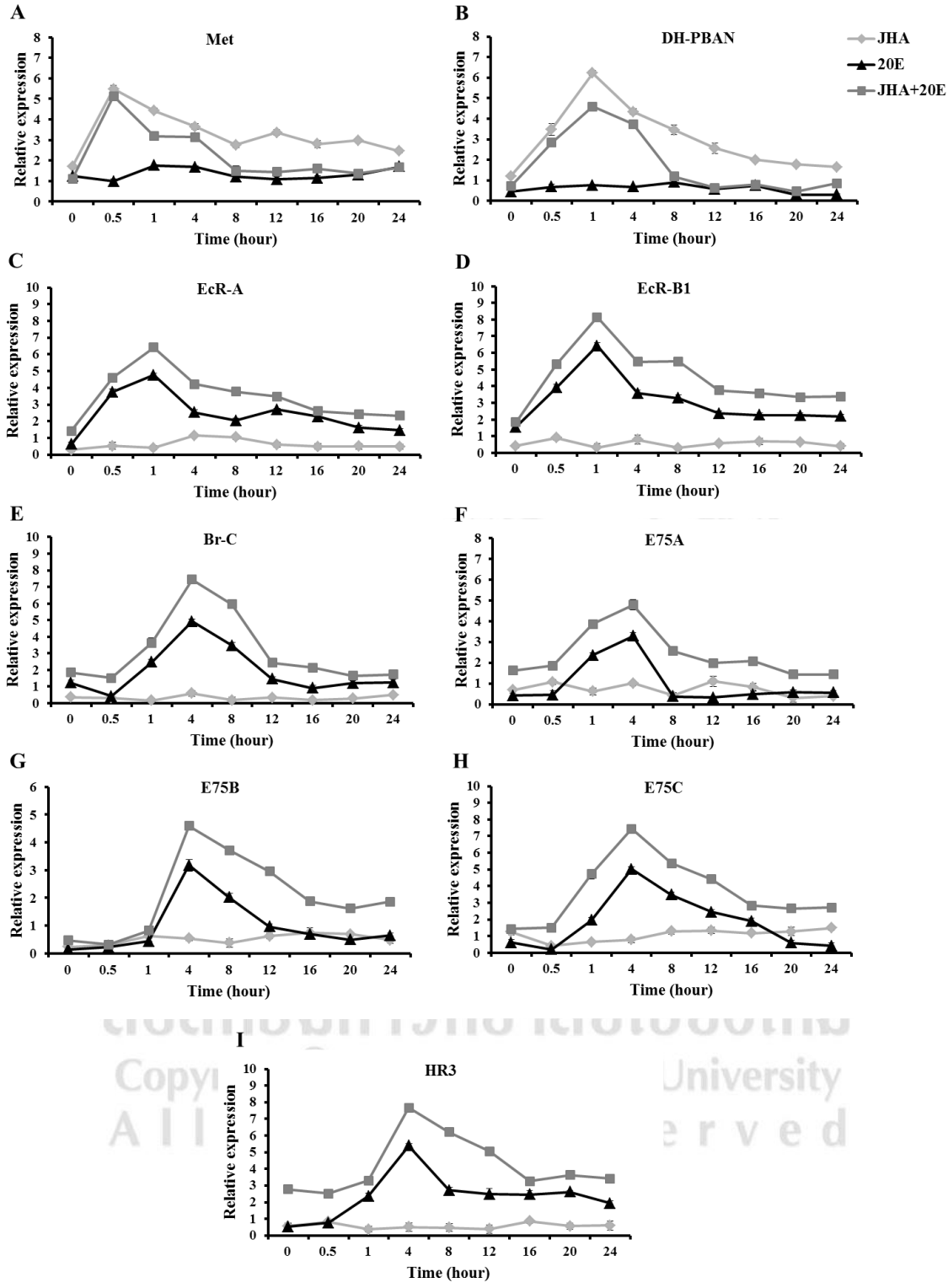

Figure 3.12 Induction of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the SG of diapausing larvae *in vitro*. Time course analysis of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and

OfHR3 (I) mRNA after treatment with 0.1 µg/50 µl of JHA alone, 0.1 µg/50 µl of 20E alone or 0.1 µg/50 µl of JHA and 20E together determined at 0-24 h. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Expression is relative to the gene expression in the different controls (SG incubated in medium without hormonal additions were collected at the same time points as treated samples). Each value is the mean ± SEM of three independent experiments. Means with different letters are significantly different (ANOVA, $n = 3$, $P < 0.01$).

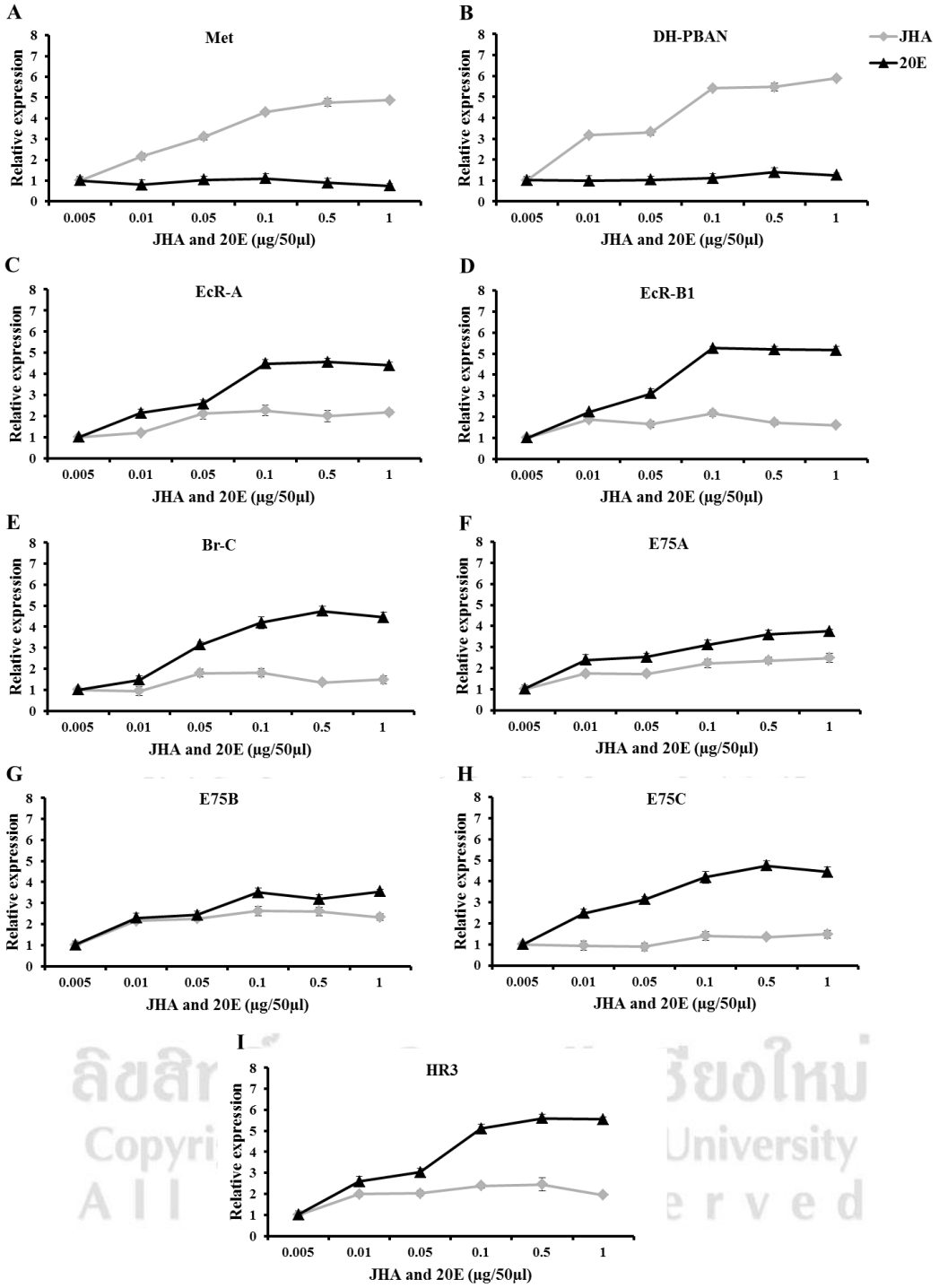

Figure 3.13 Concentration responses of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA to JHA and 20E (from 0.005 to 1 µg/50 µl) in the SG of diapausing larvae *in vitro*. Dose response of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) at 0.5 h after treatment with different concentrations of JHA and at 4 h after treatment with different concentrations of 20E. Total RNA was isolated and

mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The data represent mean values of three independent samples, normalized relative to *OfRpL3* transcript levels. 0.005 µg/50 µl of JHA alone or 0.005 µg/50 µl of 20E alone was taken as the calibrator sample.

3.7 *In vitro* effects of JHA and 20E on *OfDH-PBAN, OfMet, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA expression in the PG

We also examined the effect of JHA and 20E on the change of *OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA expression in PG, the PG was incubated in Grace's medium containing both 0.1 µg/50 µl of JHA and 0.1 µg/50 µl of 20E as a function of time. In the presence of JHA alone, the results showed that the relative expression of *OfMet* mRNA was low at 0 h, rapidly increased at 0.5 h, peaked at 1 h and then decreased at 4-24 h, while *OfDH-PBAN* was low between 0 and 1 h, then increased suddenly to a maximum level at 4 h, followed by a decrease at 8 h and remained at low levels from 12-24 h (Fig 3.14A and B). Moreover, *OfEcR-A* and *OfEcR-B1* mRNA levels were low from 0-4 h, and then increased dramatically to the maximal value at 8 h, followed by a decline at 12-24 h (Fig. 3.14C and D). In addition, the expression level of *OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA was low from 0 to 8 h, and then increased rapidly to a maximum level at 12 h. Expression of *OfBr-C, OfE75A, OfE75B, OfE75C* and *OfHR3* mRNA then declined and remained steady at a low level until 24 h after JHA addition (Fig. 3.14E-I). In the presence of 20E alone, *OfMet* and *OfDH-PBAN* mRNA levels remained at a low level at 0-24 h and were not significant when compared with control (Fig. 3.14A and B). By contrast, the relative expression of *OfEcR-A* and *OfEcR-B1* mRNA was low at 0 h, then rapidly increased to a maximum level at 0.5 h and decreased at 1 h. Expression then declined abruptly to low levels until 24 h (Fig. 3.14C and D). The relative level of *OfBr-C, OfE75A* and *OfE75B* mRNA expression was low at 0 h, and then increased dramatically until reaching a maximum level at 1 h. Thereafter, it decreased at 4 h and then remained at low levels from 8-24 h (Fig. 3.14E, F and G). *OfE75C* and *OfHR3* mRNA levels gradually increased and peaked at 4 h, followed by a decline at 8-24 h after the application of 20E (Fig. 3.14H and I). In combination of both JHA at 0.1 µg/50 µl and 20E at 0.1 µg/50 µl for the time course experiment, *OfMet* and *OfDH-PBAN*

mRNA expression showed a similar pattern on those obtained in response to JHA alone (Fig 3.14A and B), while the relative level of *OfEcR-A* and *OfEcR-B1* mRNA was low at 0 h, then increased dramatically until reaching a maximum level at 0.5 h and gradually decreased to a low level (Fig. 3.14C and D).The expression level of *OfBr-C*, *OfE75A*, *OfE75B* and *OfE75C* mRNA increased gradually and peaked at 1 h whereas *OfHR3* mRNA levels increased gradually until reaching a maximum level at 8 h. Thereafter, expression of these five genes then gradually decreased to a low level after application of the two hormones (Fig. 3.14E-I).

These results indicated that JHA alone showed effect on expression of *OfMet* and *OfDH-PBAN* mRNA (Fig. 3.14A-B) whereas JHA and 20E had effect on expression of ecdysone receptor genes and ecdysone inducible genes (Fig. 3.14C-I). In addition, the increase in *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in PG cultured with both JHA and 20E was higher than PG incubated in medium contained either JHA or 20E. This observation suggested that the presence of both hormones induced an increase in expression level of these genes in PG (Fig. 3.14C-I).

We next examined the dose-dependence on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression by adding various concentrations of JHA and 20E (0.005-1 µg/50 µl) to the medium. The total RNA was collected and isolated from the PG. Based on the previous results of the time course experiment, the PG was incubated with various concentrations of JHA for 4 h (*OfMet* and *OfDH-PBAN*), for 8 h (*OfEcR-A* and *OfEcR-B1*) or for 12 h (*OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3*) (Fig. 3.15). The results showed that *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005 µg/50 µl JHA, then gradually increased to a high level at 0.1-1 µg/50 µl JHA (Fig. 3.15A-I). Moreover, the PG was incubated with different concentrations of 20E for 1 h (based on the results of the time course experiment). 20E did not significantly up-regulate *OfMet* and *OfDH-PBAN* mRNA expression (Fig. 3.15A and B), while *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005 µg/50 µl 20E, then gradually increased at 0.01 and 0.05 µg/50 µl 20E and peaked at 0.1-1 µg/50 µl 20E (Fig. 3.15C-I).

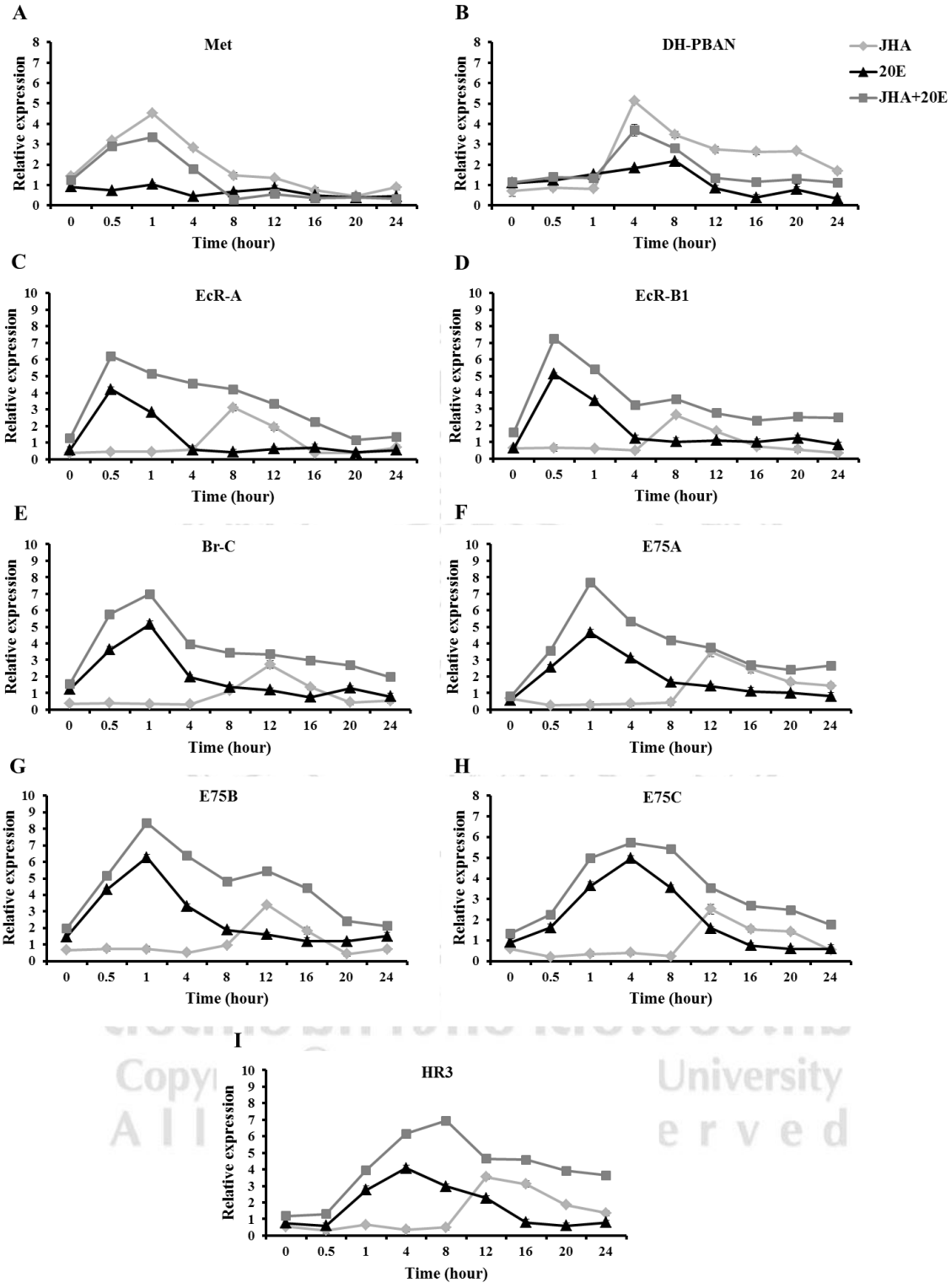

Figure 3.14 Induction of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the PG of diapausing larvae *in vitro*. Time course analysis of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and

OfHR3 (I) mRNA after treatment with 0.1 µg/50 µl of JHA alone, 0.1 µg/50 µl of 20E alone or 0.1 µg/50 µl of JHA and 20E together determined at 0-24 h. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Expression is relative to the gene expression in the different controls (PG incubated in medium without hormonal additions were collected at the same time points as treated samples). Each value is the mean ± SEM of three independent experiments. See also the legend to Fig. 3.12.

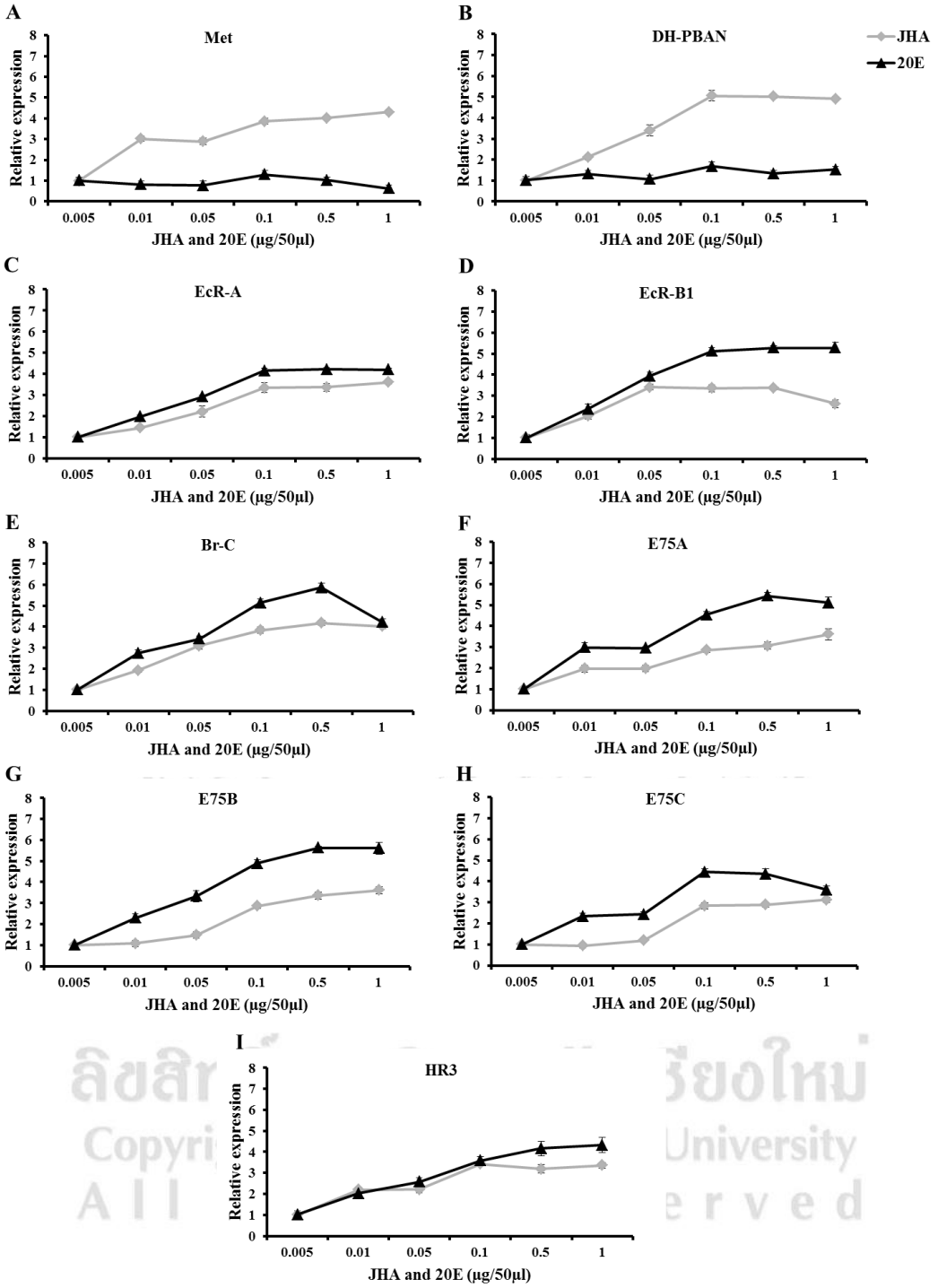

Figure 3.15 Concentration responses of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA to JHA and 20E (from 0.005 to 1 µg/50 µl) in the PG of diapausing larvae *in vitro*. The PG was incubated with different concentrations of JHA for 4 h to measure mRNA levels of *OfMet* (A) and *OfDH-PBAN* (B), for 8 h to measure *OfEcR-A* (C) and *OfEcR-B1* (D) or for 12 h to measure *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I). Effect of 20E doses on

OfMet (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) at 1 h after treatment with different concentrations of 20E. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The data represent mean values of three independent samples, normalized relative to *OfRpL3* transcript levels. 0.005 µg/50 µl of JHA alone or 0.005 µg/50 µl of 20E alone was taken as the calibrator sample.