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Changes in Diapause Related Gene Expression Pattern during Early Embryonic Development in HCl-treated Eggs of Bivoltine Silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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ABSTRACT

Investigation of differential expression of diapause related genes (five metabolic, five heat shock protein and one translational regulatory) in HCl-treated (non-diapause) and untreated (diapause) eggs of B. mori *during early embryogenesis (up to 48h following oviposition) revealed the up-regulation of sorbitol dehydrogenase upon HCl treatment, indicating increased glycogen synthesis for further embryonic development but, down-regulation of phosphofructo kinase gene expression after 18h of oviposition indicating an arrest of glycerol and sorbitol conversion. The expression of poly A binding protein gene expression was higher upon HCl treatment, revealing the initiation of translation. The expression levels of other genes analyzed did not vary significantly, except for Hsp90 and Hsp40, which were up-regulated on acid treatment until 18h. Thus, Sorbitol dehydrogenase and phosphofructo kinase genes have a crucial role in diapause termination as evidenced by HCl treatment, while the other genes did not have major roles.*

Key words: hibernation; acid treatment; metabolic genes; heat shock protein genes

INTRODUCTION

Diapause is an alternative developmental pathway with its own metabolic demands. In the insect lifecycle, it is accomplished by the dynamic change of developmental, behavioral and physiological events. Generally, diapause is influenced by the environmental factors such as temperature, humidity, light and nutrition. However, it is controlled by various genes which are expressed at different levels depending upon external stimuli (Saravanakumar and Ponnuvel 2007).

On initiation of diapause, metabolic activities are suppressed and the suppression facilitates the insect to extend its food reserves to bridge the hostile conditions. During diapause, survival is also enhanced by the synthesis of polyols and other cryoprotectants and several longivity classes of heat shock proteins. On termination of diapause metabolic rate rapidly increases, initiating the development hence, predictably genes involved in the metabolic suppression would be down-regulated and those involved in the initiation would be up-regulated (Hahn and Denlinger 2007). The accommodation of the neurons in the brain during the embryonic development causes various morphological and morphometric changes and these changes can regulate the expression of different developmental genes. The different brain specific gene expression was studied during the post-embryonic development in ant *Acroyrmex*

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octospinosus, which showed similar features to those described for other insects (Soares et al. 2004).

In the silkworm *Bombyx mori* (L.) (Lepidoptera: Bombycidae), diapause is triggered by a neuropeptide diapause hormone (DH), released from the neurosecretory cells of the subesophageal ganglion (SG), in the mother via corpora cardia – corpora allata into the hemolymph, which then targets the developing ovaries (Yamashita 1996; Mitsumasu et al. 2008). Only eggs originating from the oocytes receiving the DH signal stage enter diapause at gastrula (Homma et al. 2006). Multiple genes are expressed during the induction, initiation and termination stages of diapause. There is a sufficient database available that describes the physiological features of diapause, its environmental regulation and the hormonal control mechanism (Hahn and Denlinger 2010). Robich et al. (2007) studied the molecular mechanism of diapause in *Culex pipiens* and identified 32 genes that were either up-regulated or down-regulated during the early, late or throughout dormancy. The embryonic development in *B. mori* is interesting because in certain races, embryonic development precedes uninterrupted (non-diapause), while in some, it is characterized by the diapause. The diapause eggs can be artificially treated with HCl to continue embryonic development without interruption. Therefore, silkworm eggs provide an ideal material for examining the adaptability of glycogen metabolism and its role in diapause (Chandrashekar and Bali 1987). Glycogen is stored in the eggs of the silkworm and is utilized as and when energy is required during the course of diapause as well as embryonic development (Chino 1958). The activation of glycogen phosphorylase (GPase) and glucose-6-phosphate dehydrogenase gene (G6PD) activity was assayed in the direction of glycogen breakdown. The absence of phosphofructokinase (PFK) and high activity of glucose-6-phosphate dehydrogenase gene (G6PD) gene suggests predominant pathway in carbohydrate metabolism in the early stages of embryogenesis (Kageyama and Ohnishi 1973). With the onset of embryonal diapause in silkworm, *B. mori* is accompanied by the conversion of glycogen into glycerol and sorbitol. The phosphofructokinase (PFK) acts as a primary enzyme playing a key role in the conversion. Diapause termination is followed by glycogen synthesis from these sugar alcohols, i.e., glycerol and sorbitol. The sorbitol is then converted to glycogen, which is a source of energy for embryogenesis. The conversion is controlled by the key enzyme NAD dependent sorbitol dehydrogenase-2(SDH-2) (Chino 1958). Trehalose is the sugar used for inter tissue transport of carbohydrate fuel in the insects and is typically made from glycogen reserves in the fat body and exported into the hemolymph for uptake and use by other tissues (Wyatt 1967). The gene expression of ovary trehalase (Tre) in the silkworm is induced by the diapause hormone (*DH*) (Ikeda et al. 1993; Su et al. 1994). The pivotal role in the insects is achieved by the action of key enzyme trehalase (Tre). The poly A binding protein (PABP) plays an important role in mRNA stabilization and translation. The storage of mRNAs in stress granules during hypo-metabolism is observed to be a useful mechanism to preserve the valuable pool of un-translated mRNAs until normal conditions are re-established. This provides a very rapid re-initiation of the translation of key transcripts to provide protein products that are needed immediately during arousal from the hypo metabolic state. The poly A binding protein (PABP) are prominent protein constituent of stress granules. Hahn and Denlinger (2010) observed that the expressions of PABP gene remained unchanged throughout the diapause period of *Cx. pipiens* but was down-regulated in non-diapause mosquitoes. The orthologous sequence of this gene was retrieved from *B. mori* EST database to analyse the expression levels in diapausing and non-diapausing eggs of the silkworm *B. mori.*

Heat shock proteins (HSPs) represent a ubiquitous component of stress response in diverse organisms (Lindquist and Craig 1988), suggesting their role played in the rapid increase in stress tolerance during early diapause. The up-regulation of HSPs appears to be common in diapausing insects at different developmental stages (Storey and Storey 2004). The HCl treatment has been shown to be useful to prevent the entry into diapause or to break diapause and has made it possible to provide newly hatched *B. mori* larvae whenever farmers required to rear them (Andrewartha 1952). The treatment of newly laid bivoltine silkworm eggs with HCl breaks the diapause, forming non-hibernating eggs. The HCl treated eggs undergo developmental changes and hatch after nine days of incubation, while the non-treated eggs enter hibernation and hatch after four months

preservation under 5ºC. As the onset of diapause occurs predominantly in the early stage, i.e., within 30h of oviposition, it is worth analyzing the differential gene expression at this crucial period to find out the significant up- as well as down-regulation of diapause related genes involved in non-diapause process. Earlier findings indicated that few metabolic and heat shock protein genes were differentially expressed during the onset of diapause (Saravanakumar et al. 2008; Ponnuvel et al. 2010). Based on the available information, an attempt has been made to investigate the molecular aspects of these major genes involved in diapause phenomenon in HCl treated (non-diapause) and untreated (diapause) eggs during early embryogenesis.

MATERIALS AND METHODS

Strain selection and sample preparation

The bivoltine silkworm strain CSR 2 (evolved by CSRandTI, Mysore) was selected for the study. The larvae of this strain were reared as per standard rearing methods (Krishnaswami 1978) and newly deposited disease-free eggs were collected for the study. To break the diapause and to obtain non-diapause bivoltine silkworm eggs, fifty disease-free bivoltine eggs were treated with HCl (specific gravity of 1.110) at 25º C for 90 min. After acid treatment, the eggs were thoroughly washed in running water to remove the traces of acid and air-dried.

RNA isolation

The diapause and HCl treated (non-diapause) egg samples were collected at 0 h and then after every 6 h up to 48 h. Total RNA was extracted from the eggs using TRIzol reagent (Invitrogen) and quantified by measuring the UV absorbance at 260 or 280 nm. The total RNA samples were denatured in formaldehyde, formamide and electrophoresed in 2.0% agarose gels.

cDNA preparation

The first strand cDNA was synthesized utilizing RNA (2µg) treated with 0.5μl of DNase buffer and 0.5μl of DNase (Invitrogen) for 15 minutes. Then, the reaction was terminated by heating at 75ºC for 10 minutes and 1μl 10 mM dNTP, oligo $(dT)_{18}$ (Eurofin India Pvt Ltd, Bangalore) was added, followed by incubation at 65ºC for 5 min. Finally, 1X reverse transcriptase buffer (4µl), 5mM DTT (1μl) and M-MLV Superscript III reverse transcriptase (Invitrogen) (1μl) was added to obtain a final volume of 20μl. The reaction was terminated by heating at 75ºC for 10 min according to the manufacturer's protocol.

Identification of metabolic genes and primer design

The *B. mori Tre* (Acc No. D13763) sequence was retrieved from the database and the forward (5'-cgctgcttcattacgttcaa-3') and reverse (5'-tgttcgggtttttcaaggac-3') primer were designed for cDNA sequence. Similarly, the *SDH-2* gene cDNA sequence (Acc No. DQ443393) was retrieved and primers were designed for the cDNA sequence as forward (5'-gatggtagcgagtgggaaaa-3') and reverse (5'–gatagcccaagcaaggttca-3'); and *PABP* gene (Acc No. NM_001098353) cDNA sequence was retrieved and primer designed as forward (5'- tggacgtgcacaaaagaaag- 3') and reverse (5'- gagggagcgatggtacaaaa – 3'). However, as the sequences for the other genes like *GPase, PFK* and *G6P-DH* were not reported in *B. mori*, the heterologus gene sequences available in *Drosophila melanogaster* and *Antheraea paukstadtorum* were retrieved and used for identification of homologous corresponding gene sequences in the *Bombyx* silk database. The *D. melanogaster GPase* gene was homology searched with the silk database (http://morus.ab.a.u-tokyo.ac.jp) and the sequence (Acc No.NRPG1576), which showed the maximum homology with the *Bombyx GPase* gene was selected. Based on the gene sequence, the forward (5'-gatggtgatcgagaacatcg-3') and reverse (5'-caagctgcgagtttaaagtacaa-3') primers were designed. Similarly, *PFK* gene cDNA sequence (Acc No. ovS302A08f) retrieved from the silk database formed the basis for designing the *PFK* forward (5'-atgaactccatgcacctagc-3') and reverse (5'-caatattttgattaccacgatgga-3') primers. The *A. paukstadtorum G6P-DH* gene was also searched in silk database and the gene sequence (Acc No. fcaL-P16_F_O12) which showed homology with *Bombyx G6P-DH* was retrieved. Subsequently, the forward (5'-aacctttgccgcataacac-3') and reverse (5'- gctgacatcatcacgtccaa-3') primers were designed for the *G6P-DH* gene sequence.

Identification of heat shock protein genes and primer design

The heat shock protein genes were analyzed for diapause and HCl treated eggs. The *B. mori Hsp*

20.4 gene sequence was obtained from the database (Acc No. AF315318) and forward (5'-ttttggccttgccttaacac-3') and reverse (5'-ttcgctctggtccttgatct-3') primers were designed. *Hsp* 20.8 gene sequence (Acc No. AF315317) was also searched from the database and forward (5'-ctaaccccgaacgacatgct-3') and reverse (5'-gatgtacccatcggcagtct-3') primers were designed. Similarly, *Hsp* 40 gene sequence (Acc No. AB206400) and *hsp 70* (Acc No. DQ311189.1) gene sequences were obtained and forward (5'-tcggacgatgacatcaagaa-3') and reverse (5'-cccgggcgatatcttctaat-3') primers for *Hsp* 40 and forward (5'- gaacacactcgctgcacatc-3') and reverse (5'-gaggagtgcccaagatcgac-3') primers for *hsp 70* were designed. Based on *Hsp* 90 cDNA (Acc.No.AB060275) sequence, the requisite forward (5'-ttcccagttcattggctacc-3') and reverse (5'-tcttgcgcttcttgttttca-3') primers were designed.

Reverse transcription polymerase chain reaction (RT pcr) analysis

The PCR amplification was performed in a 25μl reaction mixture containing 2.0μl 10X reaction buffer (100mM Tris-HCL, pH 8.3, 500mM KCl), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 picomoles of forward and reverse primers, 0.3 U of *Taq* DNA polymerase (MBI Fermentas) with 1μl first strand cDNA as template. β actin (FP 5'cactgaggctcccctgaac 3' and RP 5' ggagtgcgtatccctcgtag 3') (Bangalore Genei) was used as an internal standard. The PCR amplification was carried out under the following conditions: 94°C for 3min followed by 27 cycles of 94°C for 30s, 50°C for 30s, 72°C for 2 min and a final extension of 7 min at 72°C.

Semi-quantitative measurement

The semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method was adopted according to Goto and Kimura (2004) for the quantification of mRNA. In this experiment, PCR reactions with 30 cycles were fixed for metabolic genes, *Hsp* genes and β actin. After PCR, gel electrophoresis was carried out wherein equal volumes of the PCR product of each sample were run in triplicate on a 1.5% agarose gel. The PCR products were detected under UV after staining with ethidium bromide; the intensity of stained products quantified by a Densito quantifying image analyzer (Syngene Gel documentation system, Madison, USA) and mean values were calculated. The RT-PCR was performed in *Pectoralis major* muscle cDNA samples isolated from the meats of two phenotypically distinct chicken lines to investigate the α-ryr and β-ryr gene expression by real-time RT-PCR approach using β actin gene as internal standard (Oda et al. 2009). The β-actin gene of *B. mori* was used as an internal control to normalize the gene expression in diapause and non-diapause eggs.

RESULTS

Metabolic enzyme and translational regulatory gene expression

The expression of metabolic enzyme genes, *viz.*, phosphofructo kinase (PFK), sorbitol dehydrogenase (SDH-2), trehalase (Tre), glycogen phosphorylase (GPase), glucose-6-phosphate dehydrogenase (G6PD) and poly A binding protein (PABP) involved during early embryogenesis (6 to 48 h after oviposition) were analyzed in diapause and non-diapause (HCl treated) eggs of the bivoltine silkworm *B. mori* (Fig. 1).

***Phosphofructo kinase* gene (PFK)**

The present study revealed that phosphofructo kinase (PFK) expression levels were similar up to 18 h in both type of eggs. However, the diapause eggs revealed a higher trend compared to the HCl treated (non-diapause) eggs that continued until 48 h. In the HCl treated (non-diapause) eggs PFK expression sharply declined from 18 to 24 h with no further expression until 48 h.

***Sorbitol dehydrogenase* gene (SDH)**

The SDH gene did not reveal much expression until 48 h in diapause eggs. In the HCl treated (non-diapause) eggs, the gene expression level was similar to diapause eggs until 6 h and then increased sharply at 12 h to reach a maximum at 18 h. This was followed by a sharp decline until 30 h with a sharp increase at 36 h. No expression was observed at 42 and 48 h.

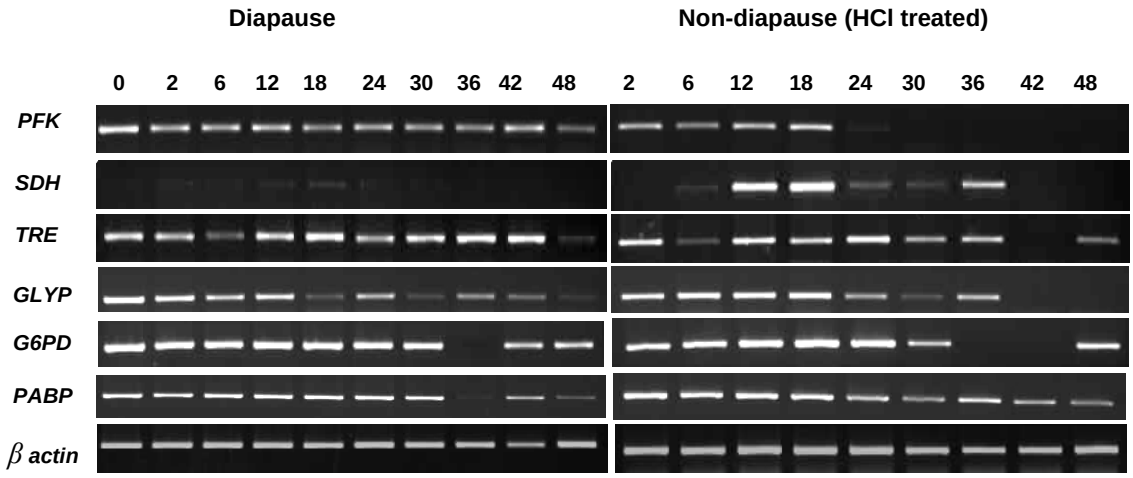

Figure1 - Metabolic gene and translational regulatory gene expression patterns in diapause and HCl treated (non-diapause) eggs of silkworm *Bombyx mori.* The RNA from diapause and HCl treated (non-diapause) induced eggs was isolated at 6 hourly intervals from 0 to 48h after oviposition and the gene products were run on 1.5% agarose gel. *β actin* was used as internal standard to confirm equal loading. Each sample was run in triplicate.

***Trehalase gene* (Tre)**

Tre expression levels in the diapause and non-diapause eggs were similar until 12 h. However, in the diapause eggs, the expression levels slightly increased at 18 h but dropped at 24 h with a gradual increase until 42 h and decrease at 48 h. In the HCl treated eggs, the gene expression fluctuated from 12 h to 48 h.

***Glycogen phosphorylase* (GPase)**

GPase expression in the diapause eggs declined until 18 h and fluctuated till 36 h, followed by the gradual decrease till 48 h. The expression levels of the HCl treated (non-diapause) eggs did not change from 6 to 18 h but were at a level higher than that of the diapause eggs. The GPase expression gradually decreased till 30h to sharply increase by 36 h, followed by sharp decrease at 42 h. No expression was observed at 42 and 48 h.

***Glucose- 6-phosphate dehydrogenase gene* (G6PD)**

The G6PD gene expression in the diapause eggs revealed a gradual decline till 30h with no expression at 36 h. However, the expression levels increased from 36 to 48 h. In the HCl treated (non-diapause) eggs, the expression pattern increased until 18 h and later declined until 36 h. In general, similar expression pattern was observed in both the eggs.

***Poly A binding protein gene* (PABP)**

The PABP gene expression also was stable in the diapause eggs until 30 h, but declined to undetectable levels at 36 h, rose sharply at 42 h and further declined by 48. In contrast, the expression levels in the HCl treated (non-diapause) eggs gradually declined till 30 h. Later, it increased sharply up to 36h followed by gradual decline till 48 h.

***Heat shock protein* (Hsp) expression**

Like the metabolic enzyme genes, the heat shock protein genes were also analyzed after every 6 h following the oviposition up to 48 h in the diapause and HCl treated (non-diapause) eggs (Fig. 2).

***Hsp* 20.4**

The Hsp 20.4 gene expression levels in the diapause eggs declined till 6 h and slightly increased till 18 h. Later, it steadily decreased up to 24h, followed by no expression from 30 h till 48 h. The HCl treated (non-diapause) eggs revealed a decreasing pattern till 30 h, except for a slight increase at 6 and 24 h respectively. However, expression was observed from 30 h till 42 h with nil expression by 48h.

***Hsp* 20.8**
The expression levels of Hsp 20.8 revealed a similar pattern in both the type of eggs till 12 h. Later, both the eggs revealed a fluctuating expression up to 48h.

***Hsp* 40**
The Hsp 40 expression levels in the diapause eggs revealed a decreasing pattern till 30 h, with a sharp dip at 6 h and sharp rise at 12h. Later, the expression levels increased to some extent by 36 h to decline to nil by 48 h. The HCl treated (non-diapause) eggs revealed an increasing pattern and recorded a peak level expression at 18 h, which declined sharply till 30 h, followed by sharp rise at 36 h and fall by 42 - 48 h.

***Hsp* 70**
The Hsp 70 gene expression varied greatly in the diapause eggs and the expression levels decreased sharply up to 6 h, followed by sharp increase up to 24 h, which recorded a peak expression. This was followed by sharp decline by 30 h and a slight rise and fall till 48 h. The overall expression of HCl treated (non-diapause) eggs revealed a decreasing pattern till 48h.

***Hsp* 90**
The Hsp 90 gene expression levels in general increased up to 18 h with no expression recorded at 30 h in both the cases but at a higher level in HCl trated (non-diapause) eggs. However, in the diapause eggs, there was a slight decrease from 0 to 6 h, which later increased up to 18 h, followed by a dip till 30 h. A low level of expression was observed at 36 h, which gradually decreased till 48 h. In the HCl (non-diapause) treated eggs, a gradual rise was observed from 0 to 18 h, followed by a sharp dip at 24 – 30 h. This was followed by a sharp rise up to 36h, a sharp decline up to 42 h and then a slight increase till 48 h.

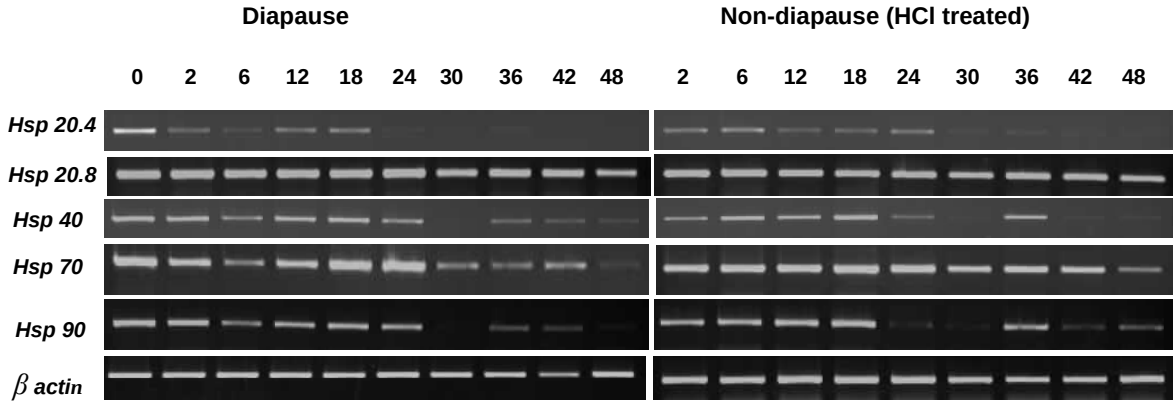

Figure 2 - Heat shock protein gene expression patterns in diapause and non HCl treated (non-diapause) eggs of silkworm *Bombyx mori.* The RNA from diapause and HCl treated (non-diapause) induced eggs was isolated at 6 hourly intervals from 0 to 48h after oviposition and the gene products were run on 1.5% agarose gel. *β actin* was used as internal standard to confirm equal loading. Each sample was run in triplicate.

DISCUSSION

The silkworm *B. mori* follows a system of hibernation adopted by insects during their life cycle (Andrewartha 1952). Only eggs originating from the oocytes that receive the DH signal enter the diapause at the gastrula stage (Homma et al. 2006). The bivoltine silkworm eggs enter diapause and hatch after four months preservation under 5º C. Alternatively, the diapause can be broken by treating the eggs with HCl immediately after oviposition. Reports have indicated that Tre, GPase, G6PD and SDH genes expressed at different levels in various target tissues of *B. mori* are actively involved in the diapause process and in the eggs, diapausing is completed by 24-36 h (Sakano et al. 2004; Saravanakumar et al. 2008; Ponnuvel et al. 2010). The present study is an

analysis of the expression of selected metabolic enzymes, translational regulatory gene and HSP genes within 48 h of oviposition in both the diapause and HCl treated (non-diapause) bivoltine silkworm eggs.

Metabolic enzyme genes and translational regulatory gene expression

The onset of embryonic diapause in the silkworm, *B. mori*, is accompanied by the conversion of glycogen into glycerol and sorbitol with PFK playing a key role in the conversion. The higher trend of PFK gene expression in the diapause eggs compared to HCl treated (non-diapause) eggs in the present study confirmed the PFK role in conversion. In freeze tolerant goldenrod gall fly (*Eurosta solidaginis*), glycerol is utilized in the pentose phosphate pathway. However, when the temperature drops below 5ºC, PFK expression is strongly inhibited, diverting the metabolism from glycerol to sorbitol synthesis (Storey and Storey 1983; Tsumuki et al. 1987). Less expression of the PFK gene in the HCl treated (non-diapause) eggs from 24 to 48 h indicated the probable enzyme inactivation via protein phosphorylation during the early embryogenesis.

Diapause termination is followed by glycogen synthesis from glycerol and sorbitol, which is controlled by the NAD – sorbitol dehydrogenase (SDH) *(*Yaginuma and Yamashita 1979*).* The present study also revealed nil expression of the gene from 0 to 48 h in the diapause eggs. In the eggs treated with HCl, the SDH activity increased rapidly (Yaginuma et al. 1990). Similar type of SDH gene expression pattern, i.e., higher level of expression from 6 to 18 h, was observed in the HCl treated (non-diapause) eggs suggesting that SDH was a key enzyme for sorbitol degradation at the termination of diapause in *B. mori* eggs.

Glycogen phosphorylase (GPase) is activated by anaerobic condition in diapause and non-diapause eggs (Yamashita et al. 1975). In the present study also, both the diapause and HCl treated (non-diapause) eggs revealed equivalent *GPase* gene expression at a high level, indicating similar glycogen requirement in both the types of eggs.

In an earlier study, it was observed that *G6PD* activity was maintained at a high level throughout the diapause and embryonic development that dropped suddenly before the hatching (Suzuki and Miya 1975; Horie et al. 2000). The present results revealed the same high expression levels of G6PD up to 30h, except for a sharp decline at 36 h and the expression levels increased by 48 h.

Trehalase (Tre) is distributed in almost all the tissues and organs of insects in different forms at various levels throughout the life cycle (Chen et al. 2010). Predominance of Tre gene was reported in the midgut throughout larval, pupal as well as adult development and far less in other tissues (Su et al. 1993; 1994). In the present study, higher expression level of Tre gene was observed after 12 h in the diapausing eggs, while, in the HCl treated (non-diapausing) eggs, the level increased up to 48 h after a sharp decline at 42 h. This indicated that the Tre gene was selectively up-regulated during the early diapause (within 48h of oviposition) as considerable energy was required during the early onset of diapause, for which trehalose was used as an energy source.

Hahn and Denlinger (2010) extensively reviewed the molecular events involved in the egg, pupal and adult diapause and observed that many genes were shutdown during the diapause while a small group of genes were up-regulated. Several classes of genes up-regulated as well as down-regulated in the diapause have been identified using suppresive substractive hybridisation (SSH) and microarray (Xia et al. 2007). Robich et al. (2007) identified 32 diapause related genes, which were specifically expressed during dormancy in *Culex pipiens.* Among these, the expressions of PABP gene remained unchanged throughout the diapause period of *C. pipiens*. However, PABP gene was down-regulated in non-diapause mosquitoes. It is also reported that PABP gene is involved in stage specific regulation in eukaryotic system (Storey and Storey 2004). In the present study the comparatively higher expression of *PABP* gene observed in non-diapause eggs indicated that these eggs might tend to produce more transcripts for its embryonic development and at higher concentration, PABP might regulate the early embryonic development. Since limited mRNA transcripts were present in the diapause eggs, the PABP requirement was also lower in the diapause eggs.

Heat shock protein (Hsp) expression

Although the molecular regulation of diapause remains largely unknown, there are many research findings suggesting the involvement of heat-shock proteins in the expression of diapause or dormancy (Rinehart 2007). Hsps are normally expressed in response to stress, acting as the molecular

chaperones to prevent the abnormal folding of proteins. Generally, the Hsps are ubiquitously expressed in all the stages of embryo development (Sonodo et al. 2006). The present study revealed no significant variation in Hsp expression, except for Hsp 90 and Hsp 40, which showed maximum expressions up to 18h after the oviposition in both the diapause and HCl treated (non-diapause) eggs. However, Hsp 20.4 expression was low in both the diapause and HCl treated (non-diapause) eggs, while the Hsp 20.8 expression did not vary much even though it fluctuated. In *B. mori*, Hsp 70 is synthesized in the diapausing and non-diapausing egg development at the early germ - anlage stage (Coulon and Dorel 1991; Rinehart et al. 2000) and also after the heat shock or acid treatment during the embryogenesis, except for two unaffected phases, namely the pre-blastodermic and deep diapause stages. The synthesis of Hsp70 could be induced again immediately by the end of the reactivation phase at 5ºC for 35 days (Coulon and Mathelin, 1991). The present study revealed that the Hsp 70 gene expression was lower in the diapause eggs compared to non-diapause eggs during the rest of the hours, except for an increase during 18 to 24 h.

Studies in *Drosophila triauraria* and the blow fly *Lucilia silicate* indicated no evidence of Hsp90 involvement in the diapause (Goto and Kimura, 2004). In contrast to this, in *S. crassipalpis* Hsp90 transcripts were down-regulated during the diapause (Rinehart and Denlinger, 2000). In flesh flies, the Hsp90 was up-regulated in response to cold shock (Joplin, 1990) but not involved in the diapause (Rinehart and Denlinger 2000). The present results revealed that the Hsp 90 gene expression was up regulated in HCl treated (non-diapause) eggs compared to the diapause eggs, indicating a significant role for Hsp 90 in early embryonic development of HCl treated (non-diapause) *B. mori* eggs.

The results of this study, thus, gave an insight into the molecular underpinnings involved in the process of diapause, which offered new tools that might prove valuable for developing the biomarkers to monitor the developmental status of various pests. Further studies on stage-specific protein profiles or mRNA expression patterns could prove useful for predicting the timing of diapause and its termination in most insect species in the field. This may also provide a unique opportunity to investigate the conserved regulatory mechanisms that sequentially halt and reinitiate the development as well as to understand the molecular basis of insect seasonality and other related topics.

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