Diapause is an alternative developmental pathway with its own metabolic demands. In the insect life cycle it is accomplished by dynamic changes in the developmental, behavioral and physiological events. Generally, diapause is influenced by environmental factors like temperature, humidity, light and nutrition. However, it is controlled by various genes which are expressed at different levels depending upon external stimuli. On initiation of diapause, metabolic activities are suppressed which facilitates the insect to extend its food reserves so as to bridge the hostile conditions. During diapause, survival is also enhanced by the synthesis of polyols, other cryoprotectants and several longevity classes of heat shock proteins. On termination of diapause, the metabolic rate rapidly increases initiating development and hence, predictably genes involved in the metabolic suppression would be down regulated and those involved in the initiation would be up regulated. There is sufficient database available that describes physiological features of diapause, its environmental regulation and the hormonal control mechanism. However, the molecular aspects involved in the diapause mechanism still remains obscure. In this study, an attempt is made to decipher various molecular events during embryonic diapause in multivoltine silkworm, B. mori.

Firstly, the differential expression of metabolic enzyme and heat-shock protein genes (Hsp) during early embryogenesis in diapause and non-diapause eggs of the silkworm Bombyx mori was quantified and confirmed through semi-quantitative RT-PCR. Data analysis revealed that, the phosphofructokinase (PFK) expression started at a higher level in the early stage (6h after oviposition) in non-diapause eggs, but at lower level in diapause induced eggs. However, the PFK gene expression in diapause
eggs was comparatively higher than in non-diapause eggs. PFK is known to facilitate the use of carbohydrate reserves. Hence, lower level of PFK gene expression in the early stage of diapause induced eggs but a comparatively higher level of expression than in non-diapause eggs indicates enzyme inactivation via protein phosphorylation during early embryogenesis followed by de-phosphorylation at a later stage. The Sorbitol Dehydrogenase-2 (SDH-2) gene was down regulated in diapause induced eggs up to 24h and its overall expression level in diapause induced eggs coincided with that of PFK gene at 48h in non-diapause eggs. Sorbitol is a protectant and is found to reveal an initial temporary accumulation during carbohydrate metabolism. The down regulation of SDH-2 gene observed during the first 24 hours in diapause induced eggs demonstrates the requirement of sorbitol as a protectant. However, since the diapause process culminates by 48h, the SDH-2 gene expression increased and coincided with that of PFK gene expression. The trehalase (Tre) gene activity regulates the uptake and use of sugar by the tissues. The lowered expression in diapause induced eggs compared to non-diapausing eggs of this gene indicates the non requirement of sugar by tissues since the metabolic activities are at minimum level. The non-diapause eggs revealed maximum expression of GPase gene with major fluctuations as well as an overall higher expression compared to diapause induced eggs. Since, the diapause process requires less energy source, the same is reflected by the lower activity of the gene. Heat shock protein genes (Hsp20.4, 40, 70, and 90) revealed differential levels of expression in both the eggs at all stages of embryonic development indicating absence of any specific role in the diapause process. The present study thus provides an
overview of the differential expression levels of metabolic enzyme and Hsp genes in non diapause and diapause induced eggs of multivoltine silkworm B. mori within 48h after oviposition, confirms the major role of some of the metabolic enzyme genes in early embryogenesis.

Investigations were also carried out on the differential expression of diapause related genes (five metabolic, five heat shock protein and one translational regulatory) in HCl treated (non-diapause) and untreated (diapause) B. mori bivoltine eggs during early embryogenesis (upto 48h following oviposition). Data analysis revealed the up regulation of Sorbitol dehydrogenase upon HCl treatment, indicating increased glycogen synthesis for further embryonic development, and, down regulation of Phosphofructokinase 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 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, the crucial role of Sorbitol dehydrogenase and Phosphofructokinase genes in diapause termination was proved as evidenced by HCl treatment, while the other genes did not have any major roles.

The Suppressive Subtractive Hybridization (SSH) technique was utilized to study the differential regulation of diapause specific genes using SSH, 186 cDNA clones were isolated from both diapause and nondiapause eggs sequenced. Of the sequenced clones, 29 which matched with the silkbase entries could be classified into six functional groups viz., regulatory, food utilization, stress response, metabolic, ribosomal and transposable
elements. Under the regulatory group, 12 genes were identified, while, under food utilization, one gene i.e., taste receptor type 2 member 117 was identified. Under stress response, 4 genes viz., one Heat shock cognate 70kDa protein and 3 of the ubiquitin family, and under the metabolic group also, 4 genes viz., 3 belonging to chitin family and one as propanediol utilization protein were identified. Under the ribosomal group, 7 proteins mostly 60s ribosomal protein subunits as well as transposable elements and one gene with negative regulation of transcription were identified. The qPCR analysis confirmed the expression of 11 of these genes, wherein, 3 genes were upregulated during diapause and another 7 during non-diapause, while, one gene remained unchanged.

In *Bombyx mori* the embryonic diapause is normally induced by the diapause hormone in *Bombyx mori*. Studies indicate that, *Antheraea yamamai* paralytic peptide (Antya-ParP), when injected into the pupae; produced diapausing eggs even if the subesophagial ganglion is deprived. Hence, a comparison of this gene expression in diapause induced and non-diapause eggs at different time intervals after oviposition was studied to investigate the role of paralytic peptide (PP) as well as paralytic peptide binding protein (PP-BP) in diapause induction in polyvoltine silkworms. Additionally, the multigene organization of PP-BP in the *B. mori* genome was also investigated. Results revealed an upregulation of PP at 18h as well as PP-BP at 12 and 18h after oviposition. The tissue specific expression analysis revealed that, PP-BP is highly expressed in fat body followed by egg and brain while no expression was observed in midgut. The expression levels of PP and PP-BP in diapause and non-diapause eggs from 0h to 48h were also validated.
through realtime PCR which revealed that PP is highly expressed at 18 and 24h while PP-BP expression is higher at 12 and 18h time intervals suggesting their possible role in diapause induction. The whole genome survey of the ENF-BP paralogous sequences revealed a total of 46 *B. mori* PP-BP homologs that are classified into 3 categories viz., ENF-BP, Typical 30KPs and serine/threonine rich 30KPs. These paralogous sequences are distributed on chromosomes 7, 20, 22 and 24, all 30KP and S/T rich 30KP proteins are present in the same locus of chromosome 20.

The molecular mechanism involved in diapause was also analyzed using a genome wide microarray in diapause induced and non-diapause eggs to identify additional embryonic diapause related genes. In diapause eggs, 638 genes were upregulated and 1136 genes down regulated at 18 h after oviposition, whereas, 675 genes were up regulated and 595 genes down regulated at 30 h after oviposition. The genes identified were classified based on their function, into three categories, viz, molecular function, biological process and cellular component. Real-time PCR analysis confirmed the expression of 20 genes, the relative transcript levels of *aquaporin* gene being higher among the 20 genes, followed by *SDH-2* and *cytochrome b5* in diapause eggs, while, *Kruppel homolog*, *period* and *Relish* were higher in non-diapause eggs. The upregulation of *SDH-2* and *cytochrome b5* indicates a rapid increase in the metabolic rate in diapause-destined embryos prior to the onset of diapause within 36 hours as a preparatory phase for diapause.

This work represents the first attempt in investigating the molecular aspects involved in induction of embryonic diapause in multivoltine silkworm eggs through semi quantitative PCR, SSH and
microarray techniques. Semi quantitative PCR demonstrated a major role of metabolic enzymes and minor role of Hsp genes during early embryogenesis in diapause induced and non-diapause eggs of silkworm *B. mori*. The differential regulation of diapause related genes specifically involved in early embryonic diapause has been demonstrated through suppressive subtractive hybridization and microarray techniques. The expression levels of genes identified through SSH and microarray were also validated through qPCR. The up and down regulated genes could be classified into several distinct functional groups and the functional aspects of individual genes could also be probed. This dataset will serve as a material for formulating detailed experimental investigation in the future by other investigators to characterize genes of interest involved in diapause. In addition, germplasm/ breeders stock/ commercially authorized multivoltine silkworm races can be identified for induction of diapause and long term preservation based on specific gene expression that are upregulated during the process of diapause.