J. Ecophysiol. Occup. Hlth. 11 (2011) 53-68

Diapause specific expressed sequence tags of *Antheraea mylitta* Drury

P. K. Mishra, A. Kumar, Lily Jaiswal, S. K. Sharan, D. Kumar, J. P. Pandey, A. K Sinha, and B. C. Prasad

Silkworm Physiology Laboratory, Central Tasar Research and Training Institute, Piska Nagri, Ranchi-835303, Jharkhand, India

Abstract: *Daba bivoltine ecorace of tropical tasar silkworm Antheraea mylitta Drury undergoes facultative pupal diapause and shows different type of voltinism. During course of its long pupal diapause, erratic, unseasonal and unsynchronised emergence of adults is noticed and losses of seed stock range in between 10 - 30%. In order to avoid all these problems, proper understanding of induction, maintenance and termination state of diapause of this economically important insect is felt essential. The presence of diapause specific expressed sequence tags (ESTs) through PCR clones of Hsp70, Hsp23, hexamerins and PCNA genes have been reported in the present study. The ESTs obtained form the primers of Hexamerins were only seen when pupae were 65 and 165 days old. ESTSs obtained form the primers designed form Hsps 70 sequences were up regulated during early (D0), middle (D75) and late age (D135 to D165) of diapause period. The presence of Hsp23 was obtained during preparatory phase of diapause (IV instar) and pupae of early and mid aged diapause period (D0 to D75) and late age of diapause (D135 to D165). ESTs of Hsp22 were seen during preparatory phase of diapause (IV & V instar), throughout diapause period and even after diapause period was over. ESTs of Hsps90 were seen during preparatory stage (IV instar) and middle and late age of diapause period. Est of PCNA were down regulated throughout diapause period, their up regulation was seen at the time of diapause termination. Another group of ESTs obtained from different sets of Hsps 70 primers were up regulated intermittently through out the diapause period. Hsps90 were upregulated during middle and late age of true diapause period. At the fag end of true diapause period, ESTs disappeared when pupae became older than 165 days as no ESTs were seen when pupae were 195 days old indicating the actual age of diapause termination. It was also evidenced by the up regulation of PCNA.ESTs whose concentration remained very low through out the diapause period but its intensity increased at 195 days which further increased at 210 days. The pupae of Daba BV of 195 days and older can be further exploited for low temperature treatment to delay the moth emergence in adverse summer season so as to produce dfls matching with the actual cropping schedule.*

Key Words: *Antheraea mylitta,* Diapause specific expressed sequence tags, Diapause termination state

Introduction

Diapause is an arrested state of development which is pre-programmed, that allows animals to save themselves from the harsh environmental conditions the expression of which sets in during unfavourable environmental conditions, such as winter, extreme summer, periods of drought and season in which appropriate food is not available (Tauber *et al.,* 1986; Denlinger, 1986; Hairston and Kearns, 1995, Denlinger, 2002) Diapausing insects become highly tolerant to cold, heat, desiccation and starvation, but these abilities are adaptively significant only when diapause starts at an appropriate time (Masaki, 2002). This timing in most cases is controlled by the response to seasonal clues such as day length and temperature and its geographic adjustment is accomplished by a photoperiodic clue.

Email: pkmishra99@gmail.com

Diapause occurs in genetically determined stage (s) of metamorphosis which are species specific and is represented by low metabolic activity. It has been divided into three phases: diapause induction or pre-diapause (in the sensitive stage of insects), diapause maintenance (responsive stage) and diapause termination or post diapause (Tommasini, and Van Lenteren, 2003). It is widely documented that photoperiod plays a major role in diapause induction in insects and temperature is typically seen as one of the possible modifiers of the photoperiod responses (Mc Nell and Fields, 1985). Low temperature is reported to be the reason for diapause induction in tropical insects, high temperatures blocks initiation of development and the individual remain in quiescence stage and the diapause can be terminated only when temperature is lowered (Denlinger, 1986). This mechanism is used to maintain diapause during the hot dry season and development is triggered with cool rainy season. The onset of rains is frequently linked to diapause termination and may account for the rapid increase of insects (Bowden, 1976). In tropical insects termination of diapause is temperature dependent. Lower temperature results in longer diapause and in some cases diapause can be terminated rapidly by a four-day exposure to 25°C in some flesh flies (Denlinger, 1986).

Insects prepare for the molting, metamorphosis and reproduction by accumulating hexamerins in their fat body and blood (Pan and Telfer, 2001) and their occurrence and activity can differ during course of development in a single species (Telfer and Kunkle, 1991). The storage proteins are accumulated when amino acids intake is greater than demand and are utilized when amino acids demand exceeds intake (Daniel and Wheeler, 2003). Insect storage proteins are heamocynin - related group of proteins composed of six identical or similar subunits in 70-90 kDa range (Telfer and Kunkle, 1991; Burmester, 2002). These hexameric proteins generally referred to as storage proteins have high content of aromatic amino acids and are classified as arylphorins. When diapause is terminated, they quickly disappear from the haemolymph. It is reported that in both group of insects (holometabolous and hemimetabolous) these proteins also provide amino acids for egg production and rebuilding tissue after diapause (Lewis *et al.,* 2002; Hahn and Wheeler, 2003; Chandrashekar *et al.,* 2008). These proteins are specific to different states of diapause (pre-diapause, diapause, and diapause termination) and post diapause growth period. All are controlled by their specific genes. Genes from storage hexamerins have been cloned from insects, *Spodoptera litura* (Zheng *et al.,* 2000), *Musca domestica* (Moreira *et al.,* 2004), *Drosophila melanogaster* (Arrese *et al.,* 2008), *Corcyra cephalonica* (Nagmanju *et al.,* 2003), *Manduca sexta* (Telfer and Pan, 2003), *Helicoverpa zea* (Suma and Haunerland, 2007), *Omphisa fuscidentalis* (Tungjitwitayakul *et al.,* 2008), *Sesamia nonagrioides* (Spyliotopoulos *et al.,* 2007), *Spodoptera exigua* (Tang *et al.,* 2010). Further, diapause entails molecular, physiological and morphological remodeling of living animals, culminating in a dormant state characterized by enhanced stress tolerance which is supported by the gene expression, differential mRNA and protein accumulation and protein modifications (MacRae, 2010). The understanding of the molecular basis of diapause has advanced considerably during the last decade (Flannagan *et al.,* 1998; Denlinger, 2002; Robich and Denlinger*.,* 2005; Robich *et al.,* 2007). Based on their expression patterns, diapause-associated genes can be classified into (i)genes up regulated throughout diapause, including the 70 kDa heat shock protein (Hsp70) (Rinehart *et al.,* 2000a) and Hsp23 (Yocum *et al.,* 1998), (ii) genes down regulated throughout diapause, including proliferating cell nuclear antigen (PCNA) (Tammariello and Denlinger, 1998) and Hsp90 (Rinehart and Denlinger 2000b); (iii) unchanged genes during diapause including ecdysone receptor (Rinehart *et al.,*

2001), heat shock 70 cognate, and 28S ribosomal protein (Reinhert *et al.,* 2000b); (iv) early diapause genes such as one encoding cystatin, acysteine proteinase inhibitor (v) late diapause genes such as ultraspiracle (USP) (Rinehart *et al.,* 2001); and (vi) those expressed intermittently through diapause such as a gene encoding 60S ribosomal protein PO, an apurinic/apyrimidinic endonuclease (Craig and Denlinger, 2000). Among these diapause-associated genes, Hsps are a well-known family of proteins up-regulated in response to a variety of stresses. However, not all of the Hsps are upregulated in the flesh fly during diapause (Rinehart *et al .,* 2007). Two members of Hsp70 family and at least four members of the small Hsp family are upregulated, while Hsp90 is down regulated during diapause. Up-regulation of Hsps and during diapuse is not specific to the flesh fly, but is rather common in many other insect orders with different diapausing stages (Li, 2008). Diapause-related genes also can be categorized into different functional groups such as genes with regulatory functions, metabolically-related genes, stress response genes, cytoskeletal genes, ibosomal genes, transposable elements, as represented in the northern house mosquito, *Culex pipiens pipiens* (Robich *et al.,* 2007). Up-regulation of Hsp70 and Hsp23 has been reported during diapause development (Parsell and Lindquist, 1993; Fedre and Hofmann, 1999; Sun and MacRae, 2005). Low expression of proliferating cell nuclear antigen (PCNA), a gene involved in cell proliferation, is associated with cell cycle arrest during the pupal diapause of *S. crassipalpis* and its level increases after termination of diapause (Tammariello and Denlinger, 1998). Its down regulation during diapause has also been reported in *Chymomyza costata* (Kostal *et al,* 2009).Therefore the role of PCNA, Hsps and hexamerins is important during course of diapause development.

Tropical tasar silkworm, *A. mylitta* is an important sericigenous insect of tropical India which produces tasar silk. Its Daba bivoltine ecorace is commercially exploited in Central India. It shows facultative pupal diapause. Its seed cocoons are preserved in pupal diapause stage for about 7 to 8 months (November-June). During course of preservation of seed cocoons, pupal mortality occurs due to erratic emergence and unseasonal emergence of moths. Loss is estimated to be 20-30% of the total seed cocoon stock preserved. The loss becomes more when diapause is set to terminate at the fag end of June when a larger proportion of pupae die due to uncongenial weather (high temperature). The phases of diapause in this insect have been explored based on the haemolymph biochemical markers and qualitative brain protein profile of *A. mylitta* which comprises three phases *viz.*, induction, maintenance, termination and the termination stage of pupae is temperature sensitive and as per need. This stage can be exploited for regulation/delaying of moth emergence in grainages (Mishra *et al.,* 2008).

The role of PCNA, Hsps and hexamerins gene in cell development and induction, maintenance and termination of diapause on the basis of differential gene expression has been considerably studied in other insects, however, such studies are completely lacking in tropical tasar silkworm *A. mylitta.* Therefore, the present study has been undertaken to record the presence of diapause specific expressed sequence tags in *A. mylitta* applying molecular biology techniques to find out the actual period of diapause termination.

Materials and Methods

Maintenance of *A. mylitta* stock: *Antheraea mylitta* Drury [Lepidoptera: Saturniidae] stocks used in this experiment were maintained at Central Tasar Research and Training Institute, Field Laboratory, Piska-Nagri (23.21°N, 85.20°E, 654.16 meters AMSL), Ranchi, India. The non-diapausing stock was raised during July-August

whereas the diapausing stock was raised during September to November and the diapausing pupae were preserved till next June from the harvest of diapausing generation.

Surgical operation and sample collection: Based on the information generated from our laboratory we have successfully identified the specific stages of diapause at physiological and biochemical level. Accordingly, the brain samples from the non-diapausing generation (i) Brain of IV and V Instar larval instars, (ii) Brain of Day 0 to day 2 pupae and (iii) Brain of Day 8-10 pupae) and diapausing generation (iv) Brain of IV and V Instar larval instars-preparatory for diapause, (v) Brain of Day 0 to day 30 pupae - the time when diapause sets in, (vi) Brain of Day 90 to 175 days pupae at 15 to 30 days interval - the refractory period of pupal diapause and (vii) Brain of 195 – 210 days pupae -when diapause terminates) were collected in sterile conditions.

Insects were dissected in cold condition (Tris buffer pH 6.8) and kept in RNA stabilization solution and stored at -80°C till utilisation. These cold stored samples were further homogenized in cold and sterilized by using RNAase free plastic (Tarson) homogenizer. Before use, all glassware and plastic wares were sterilized with DEPC treated water and autoclaved twice. Total RNA was isolated by using standard procedure of Himedia protocol with slight modification. Different stage/ age samples of *A. mylitta* brain cDNA were synthesized by using standard Fermentas protocol with slight modification. In a nuclease free tube 0.5 µg specific RNA + Random hexamer primer (0.2 µg/µl) + DEPC - treated water to a final volume of 11.5 µl were added. This whole mixture was shaken gently, centrifuged at 6000 rpm and incubated at 65°C for 5 min, chilled on ice and briefly centrifuged. Tubes were again placed on ice. Reaction mixture, 5X reaction buffer for reverse transcriptase 4 µl, RiboLock™ RNase inhibitor 0.5 µl (20µl), dNTP mix, 10 mM each 2 µl (1 mM final concentration), RevertAid™ H Minus M- MµIV Reverse Transcriptase 1 µl (200 ul) was added to make the total volume 20 µl. and gently mixed well before it was centrifuged briefly and kept for incubation for 10 minutes at 25°C followed by 60 minutes at 42°C. For reverse transcription of GC-rich RNA reaction temperature was increased up to 45°C. The reaction was terminated by heating at 70°C for 10 min. The reverse transcription reaction product was directly used for second strand cDNA synthesis using synthesized gene specific primers, storing rest of the first strand cDNA at -20°C.The cDNA was prepared following protocol available at http://www.fermentas.com/ en/ home and info@ genetixbiotech.com www.genetixbiotech. com

Diapause Gene Specific Primers designing and synthesis: Extensive literature surveys were carried out for designing the diapause specific primers using online software as shown in Table 1 and it was synthesized by Hysel India Pvt. Ltd., New Delhi.

Post-PCR Steps: Extraction of DNA from PCR product was done with the help of Agarose Gel extraction kit, details of which is shown at http:/ /www.iwai-chem. co. jp/ products/ 5prime/ gelelute.pdf. PCR amplified products of different samples of brain were run and DNA band was excised from the agarose gel with a clean, sharp scalpel. Gel slice was weighed in a colorless 1.5 ml microfuge tube for processing of up to 250 mg agarose. Appropriate volume of Buffer GX1 was added to 1.5 - ml microfuge tube and 30µl Gel Elute was added to the sample mixed/ suspended by vortexing for 30 sec. Suspended sample was incubated at 50°C for 10 min to solubilize the agarose and bind the DNA and mixed properly by vortexing every 2 min to keep Gel Elute in suspension at pH 7.5. Sample was incubated for an additional 5 minutes and centrifuged for 30 sec. The supernatant was carefully removed with a

Table 1. Diapause specific primers designed or utilized along with the source

Sl. No.	Primers for the Diapause Specific Genes	Source
1.	aatttgggcagtgtggtagc cctcatcctgcactgtcaga	PCNA gene from *Drosophila yakubi* Accession (Acc.) No. XM_002095658
2.	tctgacagtgcaggatgagg gcggtttaggttgctgagag	
3.	ctttgccatcgtcaccacta tcacgggctttctcttcaat	9D11 18 kDa *Sarcophaga rassipalpis* Acc. No.EF1103578/686
4.	ccagttggcaaagatggttt tgagctggaccagtttgttg	9D11 23 kDa *Sarcophaga crassipalpis* Acc. No U96099
5.	acatcaagttgctcgtcgtg cctcttgaggcttgggtaca	
6.	ctacggttggcaaagatggt ctttttctgcaccgttagcc	7H7 25 kDa *Sarcophaga crassipalpis* Acc. No EF1103577
7.	acgattgggatcgttttctg gaatgaggccgtgttcatct	
8.	caggtggtgtgatgaccaag aacgacccttgtcgtttttg	HSP 70 kDa *Sarcophaga crassipalpis* Acc. No AF107338
9.	caagcactgaggcgacaata gagagaggagccacatcgac	
10.	actgcagcagcacttgctta acgtgctcgtgtgatctttg	*Chironomous yoshimatsui* HSP 70 mRNA Acc. No AB162946
11.	tcaaaggctgacattgatcg tccatgcatctttgccatta	
12.	actgcagcagcacttgctta tcaaaacgtgctcgtgtgat	
13.	gctgaacagtatgccgatga3' acagttgggccaccatagtt	5B11 Heat Shock Protein 70, *Sarcophaga crassipalpis* Acc. No EF103580
14.	catagctgcacgcaatcaac aggtcagctttttccgctaa	
15.	tgaggccgaagaagaaaaga gtacggcgaggaatgaagag	5B11 Heat Shock Protein 70, *Sarcophaga crassipalpis* Acc. No AF 261773
16.	tgaggccgaagaagaaaaga gagtacggcgaggaatgaag	
17.	ccttgcccaaggactatgat gatgaatgtgaacgaggttaagg	7 H 10 Small Heat Shock Protein mRNA, *Sarcophaga crassipalpis* Acc. No EF 103579
18.	ccttgcccaaggactatgat tgaatgtgaacgaggttaagg	
19.	Ccagttggcaaagatggttt tgagctggaccagtttgttg	23 kDa heat shock protein, *Sarcophaga crassipalpis* Acc. No U9 96099
20.	tggattgggattgtctccat tcatagcctttgggcaaaac	
21.	gattgcagttcaagcgaggctgc cggagatcaatccttggttctgg	Non-diapause specific gene designed from the sequences of different Lepidopteron sequences
22.	ggaatgcaactaacgccatt gaagtggtaaggcacgggta	Locus AF 294808 2 a hexamerin *cephalonica*

23.	caaaggccggaaaactacaa gagtcgtggttgacgaacct	
24.	cccaagccgttcgataagta cggagtcatccttgaagagc	LOCUS AJ 249471 *Spodoptera litura* mRNA
25.	gcattcaagggtgtcaaggt cgaagaactggaatgggaaa	LOCUS MOTARYBB *Manduca sexta* beta subunit mRNA
26.	acgttaacgagggcatgtt ggcctttgagcaaatggata	

pipette. The pellet was washed with 500 µl of Buffer GX1 and further centrifuged for 30 sec to remove all traces of supernatant with a pipette. The pellet was washed twice with 500 µl of Buffer GE and resuspended by vortexing. Pellet was air-dried for 10–15 min or until the pellet becomes white. To elute DNA, 20 µl of 10 mM Tris-Cl, pH 8.5 or H_2O was added and the pellet resuspended by vortexing and incubated 5 min at room temperature. As per requirement sample was also eluted in water and the DNA stored at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA was also eluted in TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 8.0). Centrifuged for 30 sec. and carefully pipetted the supernatant into a clean tube. The supernatant contained the purified DNA which was stored at -20°C till utilized. All centrifugation steps were done at maximum speed (10,000 x g, 13,000 rpm) in a conventional, table-top micro centrifuge. The gel extraction kit from 5-prime gel-elute extraction kit mentioned at www.5Prime.com was used.

Sequencing of Agarose gel extracted PCR amplican: The gel eluted sample were collected from different age/stage group of *A. mylitta* in sterilized conditions were sent for sequencing to Genei Merck Specialties Private Limited, Bangalore. Different sequences of *A. mylitta* Brain were subjected to NCBI blast analysis and submitted to http://www.ncbi.nlm.nih.gov/ for obtaining gene bank expressed sequence tag numbers. Based on the presence of these expressed sequence tags through out the diapause and non-diapause state the actual state of diapause termination was worked out. NCBI blast analysis revealed that diapause specific of Accession (Acc.) No.HO348172.1 of *A. mylitta* had a maximum score of 42.8 with mRNA sequence of sea anemone, *Anemonia viridis,* an invertebrate. Acc. No. HO348173.1) had maximum matching score of 462 with the mRNA sequences of *Escheria coli* challenged fat body of fifth instar larvae of eri silkworm, *Samia cynthia ricini* (DC 860126.1), mulberry silkworm *Bombyx mori* cDNA (DC571932.1 and AV401659.1), *Manduca sexta* bacteria induces sequences (DC870552.1) and its fat body mRNA sequence (BI262519.1), *Spodoptera frugiperda* (FP364396.1 and FP362966.1) mRNA. Acc. No.H03481714.1 matched to some extent with mRNA sequences of *A. mylitta* (EB742974.1, EB 743516.1, EB 7403050.1) with maximum score of 49.6, *A. pernyi* pupal tissue mRNA (GH334854.1). Acc. No. HO348175.1 matched with silk gland of fifth instar mRNA of *A. assama* (EG592266.1, EB743205.1); larval testes (FG222882.1); ovary (FG217142.1); 96 h embryo (FE962005.1); brain (FG203515.1), *S. cynthia ricini* (DC861008.1). Acc. No. HO348176.1 had homology with the mRNA sequence of *S. littoralis* (FQ0204331.1). Acc. No. HO348177.1 matched with a maximum score of 42.8 with mRNA sequences of *D. auraria* obtained from whole body adults (DK298279.1 & DK290469.1). Acc. No. HO348180.1 matched to some extent (score 48.2) with the sequences of *Drosophila melanogaster* (BI 586581.1, BI 584228.1), *Glossina moristans* (FM960111.1). Acc. No. HO348181.1 matched with Acc. No. DY792975.1 of *S.frugiperda*. Acc. No.

Table 2. Details of diapause specific ESTs of *A. mylitta*

dbEST_Id	User_Id	Gen Bank_Ac	Primers	Sequences
70774952	PKM001	HO348172	tctgacagtgcaggatgagg gcggtttaggttgctgagag	TATGCTGATTATTGTGTGATTACATGAGCCGCCT TTGACTGCCTCGATTAATTCTGATGAAAAAATTTC TGATGATCTTTTTCAGCCAAAACATGTCGAGAGA TTTGTGACATAGTGGATTGTGTTGACCCATAATG GACACTGATTTACATGTTCGAATTCTCATCATCAT CTCGGAATGAGCAGCCTTCTTCTCTAAAAAGTTC TATAACTGGGGACACCTGTTTTGCTGCCCAATGC AAACTATTGCCTCCACTGAGTCTATGCCATGAAT GACTTACCCCACTTACTATACACATATTTGCTGA CTGCTCACAGGTACTTTATCAAACACCCCACCAC CA
70774953	PKM002	HO348173	ctacggttggcaaagatggt ctttttctgcaccgttagcc	CGAATAAGGTGATAAGGAACTCAGGTTCTCCGG GCTATGGCTTACCTGGAGTTCCTGGCTTTAAAG GAGACACGGGAATGCCAGGTTTAGATGGATCG CCAGGATTACAGGGGCAAAAGGGAGATCGCGG TTTCCCAGGCTTAATAGGGCAGAAAGGTAATAC AGGCTTGCCTGGTGTATCAGGGCGACCCGGAG AACCAGGTTTGGATGGTGCTCCAGGTTTACCAG GAGAAATAGGACTACCCGGCTTACAAGGTGAAA AAGGCGATAATGGAGACATAGGACTTCCTGGTA GAGATGGCTTTGATGGTCAAAAAGGTGATCAAG GCCCAATGGGTCCCGTTGGGTTGACAGGACCC TCAGGTTTTCCAGGCCTAAAAGGAGATCGAGGT CTTCCTGGTTTGTCTATAAACGTAAAAGGAGAT AAAGGAGAAGTTGGTCCACCGGGAATAATTGG GGCTCAAGGCCAAAAAGGAGAAAGAGGCTTGG AGGGAGCTCAAGGATTCCAGGGTGAAAAAGGT GATCGTGGTTTCACCGGAGCTAAAGGAGAAGC AGGACGCATAGGACTTACAGGAGAAAAAGGTA ATTACAAAATTTTAAATGTTTATCTAATTTATGTA ATAATTCTAATTATTACTTATAGTTAATATTATTT TGTTGTTTATATATTAAATACTCAGATAGTCTTA GCCATTATATTTATGTACTAGCTTCTACATGCGA CTTCGTTCACGTGGAATAAGTTCTATGGGGCTA ACGGGGCAAAAAAGG
70774954	PKM003	HO348174	caggtggtgtgatgaccaag aacgacccttgtcgtttttg	AGGATACTTTGGTTGTGTTGGCATGCCGTTTCTC TGTCCTGCTTTTCTATTCTCTCTTTGCTCAGTCGT TTCTGATACTTGCTTTGACAAGGTTTCGTTGTTAC TGGAGCTCGCCCGAGGCTCCGGATAGGTATCTT TCTTACGCAAGTGATTGACGCTCACCCTTATCGA GAGATCACAAAATTCGCAGCCGAACGGCGAGGC CCTGTACCGCATTGACCACCGCTGTACAGGGGC TCCGCATTCGCCATTTTGCTCTGTTGTCGCGCTC GTTGGGTAGATTAGGTTACACTTGCGTCGGAGG TCACCATATCCCGACTACCGACGGACCTCCATTA TCAAGTCAACCTCTTCGGTTAGCTCCTCAGAAAC TTACTGCTGCATAGAGCGAATTTGATAGCTCGGT AAGGAACGGAATTGCCCGCCCGTCTCAGATTGC TTGGTCTCACACCACCCGGA

70774955	PKM004	HO348175	actgcagcagcacttgctta tcaaaacgtgctcgtgtgat	CTTTTGCCCCTCAAACATTCAAAGGAAGTTTTTG TGGCATTTCAGAATTTCTTGCTCCGTGGTACTAT TTTCCATTTACTTCACTTATGCACAATAAACTAAC TATATTACGTAAAACACCACCAAGTTTACAAAATA TAGAGTCAAATAACACAAGATTATGAATTAAATAT AAAATCCACTACATGACATACTAAAATCAAGCAA AAAACTCCTTTAACGTTGTTTTTATACTTTTTATAA CGTGTCGTGAATGTTACACTAAAAAGGTTAATAG TTCTTATAAAAAGAGAAGACAGTTCCTACACGAA TATAAAACTAAATACGAATAAAATAGAATACTAGT TTTTGCCAGCGGCTTCGGTCCTATTAATAATATA GACTCGACTTATTTTATTTATTTTTTTATTTTATAT ATTTAGCGCACCAACATAAATATTCACATAGTTAA AATTACATATGGTCACTTACAAAATAAAATTACAG TGTAATAATTACAATCACAGGCATGCATGACATT AATAGATGACAAAAAAAACAACTAATTACAAACTA CTTTGAAAGAATAATATCTTAATTTGCACAAATAC GCCATCAAGCTATGTTTTTAAAGCAAGGGGCTGC TGCAAATAAA
70774956	PKM005	HO348176	actgcagcagcacttgctta tcaaaacgtgctcgtgtgat	ATTCTTTACAAGGCCTCGTAAGCTCAAAATTCAT TAATCTTCAGTCGACCTTCCACTAAGCTCGGTTG TCTGGGTTAAAGCACCAATGAATCGAAAACCGA TAAAATTCTATTAATGTTTGCGTTGAAAAAAAAAC GTGACAGCGCTCGTTTCGCTCTCATGATAGATT GAAATTTCTTTGTTCGAATACACAATGCGAAGTA TTTAACTAGCTACTGGATCATAAATCACGTTAAC TACGAGTATGTAAGTTGTTTATATTAGTGAGTTTA TCACAGTCACTCAAAATTGCTACTGAGTTGATCA AAAATCTAAGTAACTCATTCTATTGAGCGATTCA TTTATTACACACATATCACACGAGCACGTTTTGA AAAAAA
70774957	PKM006	HO348177	gctgaacagtatgccgatga acagttgggccaccatagtt	TAATTTGTCGCTTCTTTCCGTCTGTTTCGTATGC ATTGGCTCGGATTTCTTCTGCCCTATCATTCTTC TCACACTGGCTTGATGCCCGTGTTTTGCTTTGTT ACATACCCATTTCCTGCACAGTCCTATTCTCCAT GCTCCGGAATGCACCTGATTTCGAACTACTATAT TATGAATAACCTTCTCCGGAGAAGGATAAGCAC CCCTTCCTAGGTGTATTATTGTTCTTAAAACTATT ATTGATTTTTCGCATTTCTCTTGTCGAAATCAAAG TGAGGAACACCGGTTAGCTTACTATCCCTTAATA CACCGTCAGATTTTACCTTCTCCTTCACCGGAAT AGGTTCTTCATTATATAGTATTTCTATATATGGTA CATGCCTTTTTTTGGAGAATATGACTGCTGAAGA TTTGGGTATGGCCAATGACAAACCATGGTCATC AAGCCACTGTCCTAAGTATGATAAGGCAGTATTT AACTGAGCCACTGCATAAGAAATAGACGGATTA GAAGCGTACACTACTAAATCATCGGATTACTGTT CAGCAA

70774958	PKM007	HO348178	tgaggccgaagaagaaaaga gtacggcgaggaatgaagag	CATGATCACCGACCGACAGGCATTGATAAATAA ACTATTCCGAGGTTAGTGGTAGATTAACACTGAA TTTATTATAGCATTAGTCAATAAACGCGAGTAATT CACCGAATTTTGTCGTAAATTTACGTTGGGATGC ATAGAGTTTACCAAAGCACAATATTGTTTTTACAA GTGCCATGCCTTGACTTATGACTCAACTTGAAAT ATCGATACAGATTATTTTAAAATTTATTAAGAAAT TAGGTAAGGTAGTTATAGGTAGTGTATATATAGA TAGGTAAGGAGACTAAATGTTCTTTGTAAAAAAA AACAAAATTATTTTTATTTAATTTATTTTTTCGAGA TTTATTTTACTTATTTCAATTATTTTTAATATTCCA AAGGCAGAATCATAATTAAGCTCAAATCCTGTAC AAAGGGGATGCTTATATTTTTTGGACCTCGAAAT TCATTGGAAATCAACCGAGCAAAATTATCTTATA TTAATGGAATTTAAATGGAAACTCTGCACTCAAC CCTATGAAAACCTCTTCATTCCTCGCCGTACATT CCGGTGAAACCCCCTCCCCCTCCTTCCTCCCCC GAAAAAAAAAAAA
70774959	PKM008	HO348179	gattgcagttcaagcgaggctgc cggagatcaatccttggttctgg	TTTTTTTTACTGTTGACACTTTTCCATAATCTACC CCACTGCCAGCCTCCGCTTGGATTGTCAGCACT ATGGCTGCCGAGCGTGGGATAACAAGCTTAGCA GGTGTCATCTTGGGTAACGCCTTCTTTGTCTTTG CTGGGGGTTTTTTCTTGGCCCCACGACTTGCCA CCACGGCCCAGCTTTCTTTCGTAATAGGCGCTT GTACAGAAGAAGTTTTTTGTGAGGTTGAAGCCTC CTGTGCTGCCATCGATTTCTTTTTCCCCTTTTTTT TCTTTCCAGCTGTATCAAAGGAAATAGCGGCTG GAGGCGCTACTGTCTCAGGGGCTACAGTGCTAT CTGTCACTTGAGCCGACCGTTTATCCCCGGCTA GTGAAGGACGCAAACGTTGTCCTTCAAGGGCCT CGAAATAAGTTTTCATCTTATTCCCCATCTCGGC CATTAGGTTGCCCATTGCCTCATTCAGCATATCG CTGGTCACCACAAAGGAGCCATCGCGCTGAGA GAATGATCTACGTGGCTTTGGTAGCTGAGGGAA ATCATTCTTCCTCTGTTTTCGTGGAGGAGAGCCA GCATCTTCCATGCTTGGGCCAGCCGGAGCATTG CAATCCAAAGCAGGGGCCACAATTCGACTGTTT TCCAGACTGGCTCTCAACTCAGCCAGCTCCTTG CGGAGCTCATCCATCTCTGCAGACCTTTTTGCCT CATTGGCCTTCAGGGTGTTATAGGCATCTTGAG CCTGTTGACCTCCTCTGAGGCAGTCCTGTTGGC CAGCTCGACGACTTCTCTGCGATCGAGTCGCAG CTCCCCTTTGAACTGCATCACA
70774960	PKM009	HO348180	gcattcaagggtgtcaaggt cgaagaactggaatgggaaa	CTGTTCGATTACTAGATTGACGGCACATTTCCTG TTCTGATCCATTATACTATCCAGCTGAATGCTTT GTGAAAATATTAATTCTCTTATATTAACCATTACT TAACTGAAACTCTCTATTTTTTCAGGAAATATTTT CACCATAGCTATTATTAAATTGTTTATACAGTACA TTCAAATAAACTGTAAAGAACAAAGGTTATGAAT TACAAAGTTATATAAACGTTGAGAAGAAAATTTC CCTCCAAAAAACTAATTGGATAATTACTACCGAC TGTGTTGTGCAAAGTATTGTAAAGAAAATTGAGA TTGATTGTAAAAAACAGTAAACAAAAACATGCTG ATTGTAATCTGCAATTAGTCACATAATCTGTACCT TACTTCTAATTTGTAGTTAAAAAAAAGCACATATG TAAATTGTAAATACATTTTGGACCACGTGAGGCG TAAAATTTTTTGGTCAAACAACGAATCTATGCAT CGATTTTTCGATGGCAAATGTAATCTACAAACAA ATAAAAAAACTATGAACAATACTAAATGCACCCA ACATTCCCATTTGATTAACTAATGAATCATGAAC AGCAAATCTGCCGTCATCTCTTAATATATCGAAC TAACTTGACACCCTTGAATGCA

70774961	PKM010	HO348181	gcattcaagggtgtcaaggt cgaagaactggaatgggaaa	CGCTCAGGTATATTCCATGTCTTCAGCGATTGG TAAAATGCGTGACGGACTGGACTGCGACACGC GACGCGTGCCCTGGCTAGTTTCAGCCTCGGGC ACACGTGGTTCCTCATGTTAGGTTTTCAGTCTTT TATGACCCCTTTTTGACACACGCTCTTTAGCCTT ATCGAGTAATGGAAGTAGCTTCTTATCCTTTGCT AATATTTGCAGGAGCGTTATTATAAACGGCATG TAGTTATGGCGTCTGCGTGCCAATTCCAACTTA TATTTTCCCATTCCAGTTCTTC
70774962	PKM011	HO348182	tgaggccgaagaagaaaaga gagtacggcgaggaatgaag	CCGGAACGATGCGGTATCTGCGCTTCGTCTGCC GGAGGTGGATTGAGCCTTTGGTAAGAAATTTGG TTTGACCCCTGAACATTTGCTGAAACGACGGAC AACTACCAGAGGCATGAGGTCGAGCAGCAACCG GTTCCGCCCCTAGAGGCCGCCGCTGAATATGCA GGAGCATGGGTTCAGCGGCCGAAGTGGCCGCC G
70774963	PKM012	HO348183	ccagttggcaaagatggttt tgagctggaccagtttgttg	AAAGGTGAGGTCGAGTAGACCGTCTGACGCGG TACTATCGTTTAGCCCTAGATATATGGCTATGTC TAAGACGTCTTGTGTTGTGGGGCCGTAGTATGT AGGCTCGTCTGGCCCACAAATCTCGTAACCAGC GCTCTCCGCATGTTGTTGCAAGAGCCTACCTGC TGTAGTGGTAGTCGAGGAGTTC
70774964	PKM013	HO348184	actgcagcagcacttgctta tcaaaacgtgctcgtgtgat	AAAAATAATTTACAAGGCCTCGTAAAGCTCAAAA ATTCATCTAATCTTCAGTCGACCTTCCACTAAGC TCGGTTGTCTGGGTTAAAGCACCAATGAATCGA AAACCGATAAAATTCTATTAATGTTTGCGTTGAAA AAAAAACGTGACAGCGCTCGTTTCGCTCTCATG ATAGATTGAAATTTCTTTGTTCGAATACACAATGC GAAGTATTTAACTAGCTACTGGATCATAAATCAC GTTAACTACGAGTATGTAAGTTGTTTATATTAGT GAGTTTATCACAGTCACTCAAAATTGCTACTGAG TTGATCAAAAATCTAAGTAACTCATTCTATTGAGC GATTCATTTATTACACACATATCACACGGGCACG TTTTGAAAAAA
70774965	PKM014	HO348185	ccagttggcaaagatggttt tgagctggaccagtttgttg	GGAGGGGGCCGGGTCGAGTAGACCGTCTGACG CGGTACTACGTTCAGCCCTGAATATATGGCTATG TCTAAGACGTCTTGTGTTGTGGGGCCGTAGTAT GTAGGCTCGTCTGGCCCACAAATCTCGTAACCA GCGCTCTCCGCATGTTGTTGCAAGAGCCTACCT GCTGTAGTGGTAGTCGAGGAGTTCCAGTAGGCG TTTTTAATGTTGAAGTCGCCTGCCAAGATTGTAG GCGTAGGCGAGTCCAACAAACTGGTCCAGCTCA AGAA
70774968	PKM015	HO348188	ggaatgcaactaacgccatt gaagtggtaaggcacgggta	AAAACAGGGGAAAAGCGTGCCATGGTTGAGGCA GGCCAATACCTAGGAGTTAACATCGACAGCCTA CTGAGGTTCAAGAACCACACCGATTACTTAGTG GGTCGAGTCCGAGCGAAACGAGCTAAACTTAAG CCCGTGCTGTCATCATCGCTCCCTCTAAGAACG AAGCTCGGGATTTACAAAACTTATATTAGATCTC GCCTAACATACGCGGCACCGGTTTGTTACGCAT ACCTGTTC GAAACTCAAAAGAGGACTCAAGTAAGACGCTCA TGCCTCAATTTCAAGATCACAATGCAA

70774966	PKM016	HO348186	ggaatgcaactaacgccatt gaagtggtaaggcacgggta	AAGGTAGTCTTGATATTACTATTCCATGAAAAGC AACTACAGCAATATTGCGTAGTCGAAACAGCGA CCACATTCAAATAGTAGCCGGCAAAATATGGAAA CAACCTGTTCTATTAAAATAAAATAAATAATTGAG TCGTCACGAGACGCCTGGCAGATATGAAGCATC GAAATTGTTATCATCGTACAAATATTAAATTATAC TATAGGTACGCGAGCTCAGTGCGGCAAGAGAAA TTACGCACGGTCGCTTGCGCATGCCGTCGTCGC GTCGTCGCAACACCAACAGTCAGGTTTAACCGC GCCTATAGATGTTTTACATCAATAAAAAATTGTTC GAATCGAACGATTCTCAAATTACACTATAGGTAT GCAAGCTCAATGCGGCAAAAGGAGATCGCGCAC AATCGCTTGCGCATGGCATCGCGTCACGTCTCA CACACTCGCCACGCCACTAATTACGTTTACCCGT GCCTTAACCACTTCAAAAAAAAA
70774967	PKM017	HO348187	actgcagcagcacttgctta acgtgctcgtgtgatctttg	ACGAAAACTGAATTAATCCATCTCTTTATTCTATT CATATAGTTCGTAAATTCGTTTTTTTTGTCATCTA TTAATGTCATGAGCACGCCTGTGATTGTAATTAT TACACTGTAATTTTATTTTGTAAGTGACCATATGT AATTTTAACTATGTGTAATATTTATGTTGGTGCGC TAAATATATAAAATAAAAATGAAATAAAATATCTT AAATTAATATGCGCCACTGTCAATTATTGTATCTC AATGTGTAATCGGGTATTAGGCTTCCATTAGACA GGTATTGACTAGTTGTCTAATTTGAAAGAAAGAA AGAAGATACATTTATTCATCACATACAACACAGA ATTATATGTACATAAACAAAAAGCACAAATTAAAG TAAGACGAGATGTGTTGCATGAGGTTAAAAGGA TTTTGTATCAGCATTAGCTGCAGGCTTGCATGTA GGAGCATTGCAGCGCTGGTTTTCAGACAAAGCC ATTGTATTATTATGACATACACAAAATTACAAATA CACGGACGTATTTAAATTACGAATCCAAATTAAT ATTATACACGCCCTAAATTCTTAAGTAGTAAAGT GTAATCTAGTACTGCCTACACATCATACAGCACA CTAGCTCAAAGATCAACACGAGCACGTAA

HO348182.1 had little homology with mRNA sequence of *Aedes aegypti* (DV409461.1 and DV409459.1). Acc. No. HO348183.1, HO348185.1 and HO348188.2 did not match with any sequence of insects available. Acc. No. HO348184.1 showed little matching (score 41.0) with *S. litura* sequence Accession No. GW415437.17. Acc. No. HO348186.1 had little matching with sequences of *S. cynthia ricini* (DC862805.1), *A. mylitta* (EB742615.1), *B. mori* (BY923721.1), *S. littoralis* (FQ021141.1). Acc. No. HO34817.1 matched (score - 244.1) with sequences of *Choristoneura fumifera* (FC964358.1), *Aphis gossypii* (GW559870.1). None of the sequences which showed some matching in EST data base are reported in relation with diapause in any one of the insect species mentioned in the preceding lines. Although the sequences obtained under the present study matched with the sericigenous insects as well as wild insects' species belonging to Lepidoptera and Diptera. These sequences can be used as good marker for monitoring the diapause in *A. mylitta.*

Based on the presence of these expressed sequence tags (ESTs) throughout pupal diapause period a chart was prepared as shown in Table 3. It may be seen that most of these ESTs were only present during actual period of diapause. The ESTs obtained with the help of diapause specific primers designed from the different diapause specific sequences of Hsp23, Hsp70, Hsp22 and Hexamerins were abundantly present during course of pupal diapause. Some of the ESTs were also prevalent during preparatory period of diapause. The ESTs obtained form the primers of

Table 3. Expression pattern of diapause specific expressed sequence tags in non-diapausing and diapausing generations

GENE SPECIFIC PRIMERS/ STAGE	LARVA						PUPA												
	III		IV		V		NDD				DD								
	NDD	DD	NDD	DD	NDD	DD	D0	D4	D6	D12	D0	D30	D45	D75	D105	D135	D165	D195	D210
AmPCNA																			
AmHP23																			
Am25H																			
Am70HA1																			
Am70HSA																			
Am70HS Am70HA2 Am70HA3																			
Am70HA5B Am70HA5B																			
Am90HA																			
AmAF90A																			
Am23HSD																			
Am70HA5A																			
AmHEX1 AmHEX2																			
AmA22A AmA22B																			

Note: Colour intensity denotes the band intensity in a specific stage

Abbreviations: NDD=Non-diapause destined generation; DD=Diapause destined generation; D = Days (the age of diapausing pupae); III=Third instar larva; IV= Fifth instar larva

Hexamerins were only seen when pupae were 65 and 165 days old. ESTs obtained form the primers designed from Hsps 70 sequences were upregulated during early (D0), middle (D75) and late age (D135 to D165) of diapause period. The presence of Hsp23 was obtained during preparatory phase of diapause (IV instar) and pupae of early and mid aged diapause period (D0 to D75) and late age of diapause (D135 to D165). ESTs of Hsp22 were seen during preparatory phase of diapause (IV and V instar), through out diapause period and even after diapause period was over. ESTs of Hsps90 were seen during preparatory stage (IV instar) and middle and late age of diapause period. ESTs of PCNA were down regulated through out diapause period but their upregulation was seen at the time of diapause termination. Another group of ESTs obtained form different set of Hsps 70 primers were up regulated intermittently through out the diapause period. Hsps90 were up regulated during middle and late age of true diapause period. At the fag end of true diapause period, these ESTs disappeared when pupae became older than 165 days because they were not seen when pupae were 195 days old indicating that actual state of diapause termination starts when pupae are 190-195 days old. It was also evidenced by the upregulation of AmPCNA ESTs whose concentration remained very low through out the diapause period but its intensity increased at 195 days showing an increasing trend up to 210 days. The ESTs from PCNA sequences were also seen when diapause had terminated. Most of the ESTs disappeared when pupae were more than 195 days old. Thus the pupae of 195 days and older can be further exploited for low

temperature treatment to delay the moth emergence in adverse summer season so as to produce dfls matching with the actual cropping schedule. The sequences obtained from these ESTs were observed to be unique and *A. mylitta* specific as they did not entirely match with the original sequences from which the primers were designed.

Diapausing animals switch between favouring some metabolic substrates early in diapause and then favouring others later in diapause (Yocum *et al.,* 2005; Zhou and Miesfeld, 2009). Deciphering proteomic signatures in early diapause of *Nasonia vitripennis,* a metabolic shift from reconstruction to maintenance occurs which is evidenced by high levels of proteins involved in replication/transcription (histones) and translation (ribosomal protein, translation factor) followed by an increase of metabolic enzymes and maintenance proteins (ferritin, some hexamerins) results into induction and further maintenance of diapause (Wolschin and Gadau, 2009). The expression of a hexamerin protein, AgSP-1, changed throughout diapause development (Lewis *et al.,* 2002). The PCNA ESTs were up-regulated at the fag end of diapause when pupae attained the age of 195 days and same trend continued up to 210 days of pupae.

The enrichment of diapause gene sets have been reported in the pupae of flesh fly and the lists of these genes overlap in between diapause and oxidative stress responses in three major areas; metabolism genes involved in glycolysis/ gluconeogenesis, general stress response elements including HSPs and antioxidants/ detoxification genes, and mechanism that suppress anabolic synthetic activity (Ragland *et al.,* 2010). Pupal diapause has been reported to be in the form of reduced cellular growth (Tammariello and Denlinger, 1998). Heat shock proteins (Hsps) are up regulated by diverse stresses (Parsell and Lindquist, 1993; Fedre and Hofmann, 1999; Sun and MacRae, 2005).The Hsps sequences have been divided into two groups based on their expression pattern: those with low expression under non-stress conditions, but can quickly be induced under stress conditions (Hsp70) and as second group consisting of sequences that are constitutively expressed under non-stress conditions, but with little or no induced expression after shock, Hsc70 (Michael *et al.,* 2003). Both act as molecular chaperones helping to stabilize protein during folding, and both participate in reactions to remove abnormal cellular proteins (Terlecky *et al.,* 1992) and Hsps are correlated with thermal tolerance (Li and Werb, 1982). A small Hsp (Hsp23) and Hsp70 are highly up-regulated during pupal diapause (Yocum *et al.,* 1998; Flannagan *et al.,* 1998; Reinhert *et al.,* 2000). During diapause Hsp70, Hsp60 and the small Hsps are all up-regulated but Hsp90 is actually down regulated and show an interesting dichotomy of function under these two different circumstances in insects (Reinhert *et al.,* 2007). In case of *A. mylitta* also the up regulation of small heat shock proteins and primer designed from Hsp 70 expressed in through out pupal diapause, in some cases a primer of Hsp 90 also expressed. This indicates that *A. mylitta* may also have a similar mechanism for surviving harsh conditions prevailing during diapause. However, there may be differences in the level of expression as this species is a tropical species and passes both winter and summer during pupal diapause. A tentative period of diapause termination is found when all these expressed proteins disappear from the brain tissues when pupae attain the age of 195 to 200 days. This particular age can be exploited for consigning diapausing pupae to low temperature for getting delayed and synchronized emergence during adverse seasons. The partial clones of Hsp70 are also reported from other insects by using PCR primers designed from conserved insect sequences following established protocol such as in walnut husk maggot (Gene bank Acc. No.

EF103585), European corn borer, *Ostrinia nubilais* (Gene Bank Acc. No. EF103583) and the apple maggot, *Rhagoletis pommenella* (Gene bank Acc. No. EF103584). In the present study too the partial clones of Hsp70, Hsp 23, Hsp 90 and Hexamerins obtained in the brain tissue of *A. mylitta* are up-regulated during diapause and playing a major role in maintenance of diapause state in *A. mylitta*.Their up regulation at a specific age of diapause indicates that the there was demand of more amino acids during course of diapause development in *A. mylitta*. Upregulation of PCNA gene fragments occurs at the fag end of diapause when pupae were 195 days old. This indicates the stage of diapause termination. Therefore 195 days and older pupae can be exploited for working out a low temperature treatment schedule for delaying moth emergence matching with cropping schedule in unfavourable climatic conditions during summer.

References

Arrrese, E. L., Rivera, L., Hamada, M. and Soulages, J. L. (2008) Purification and characterization of recombinant lipid storage protein-2 from *Drosophila melanogaster*. *Protein Pep. Lett.,* **15,** 61-72.

Bowden, J. (1976) Weather and phenology of some African Tabanidae. *J. Entomol. Soc. South Afr.,* **39,** 207-245.

Burmester, T. (2002) Origin and evolution of arthropods hemocyanins. *Eur. J. Enytomol.,* **96,** 213-235.

Chandrasekar, R., Sumithra, P., Seo S. J. and Krishnan, M. (2008) Sequestration of storage protein 1(SP1) in differentiated fat body tissues of the female groundnut pest *Amsacta albistriga* (Lepidoptera: Arctiidae). *Int. J. Trop. Insect Sci.,* **28,** 78-87

Craig, T.L. and Denlinger, D.L. (2000) Sequence and transcription patterns of 60s ribosomal protein PO, a diapause-related AP endonuclease in the flesh fly *Sarcophaga crassipalpis*. Gene, **255,** 381 - 388.

Daniel, A.H. and Wheeler, D. E. (2003) Presence of a single abundant storage hexamerins in both larvae and adults of the grasshopper, *Schistocerca americana*. *J. Insect Physiol.,* **49,** 1189-1197.

Denlinger, D.L. (1986) Dormancies in tropical insects. *Ann. Rev. Entomol.,* **31,** 239-264.

Denlinger, D.L. (2002) Regulation of Diapause, *Ann. Rev. Entomol.*, 47, 93-122.

Fedre, M. E. and Hofmann, G. E. (1999) Heat shock proteins molecular chaperones, and the stress response. Evolutionary and Ecological Physiology. *Ann. Rev. Physiol.*, **61,** 243-282.

Flannngan, R. D. Tammariello, S. P., Joplin, K. H., Circa-Ireland, R. A. , Yocum, G. D. and Denlinger, D. L. (1998) Diapause-Specific Gene Expression in Pupae of the Flesh Fly *Sarcophaga crassipalpis*. *Proc. Natl. Acad. Sci. USA,* **95,** 5616-5620.

Goto, S.G., and Denlinger, D.L. (2002) Genes encoding two cystatins in the flesh fly *Sarcophaga crassipalpis* and their distinct expression patterns in relation to pupal diapause. *Gene,* **292,** 121-127.

Hahn, D. A. and Wheeler D. E. (2003) Presence of a single abundant storage hexamerin in both larvae and adults of the grasshopper, *Schistocerca. Americana. J. Insect Physiol.,* **49,**1189-1197

Hairston, N. G., Jr. and Kearns (1995) The interaction of photoperiod and temperature in diapause timing: a copepod example. *Boil. Bull.,* **189,** 42-48.

Koštál , V., Šimùnková, P., Kobelková, A. and Shimada, K. (2009) Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*, *Insect Biochem. Mol. Biol.*, **39,** 875-883.

Lewis, D. K. ,Spurgeon, D., Sappington T. W., and Keeley ,L. L. (2002) A hexamerin protein, AgSP-1, is associated with diapause in the boll weevil. *J. Insect Physiol.,* **48,** 887-901.

Li, G.C. and Werb, Z. (1982) Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc. Natl. Acad. Sci. USA,* **79**, 3218-3222

Li, A. (2008). Identification of proteins associated with insect diapause and stress tolerance dissertation.Ph. D.Thesis. Graduate School of The Ohio State University, USA pp 196.

Mac Rae, T. H. (2010) Gene expression, metabolic regulation and stress tolerance, Cell. Mol. Life Sci. DOI. 10.1007/s00018-010-0311-0, Online, March, 2010

Masaki, S. (2002) Ecophysiological consequences of variability in diapause intensity. *Euro. J. Entomol.,* **99,**143-154.

Mc Nell, N. J. and Fields, P. G. (1985) Seasonal diapause development and diapause termination in the European skipper, *Thymelicus lineola* (Ochs.) *J. Insect. Physiol.,* **31,** 467-470.

Michael, Q., W., G.T., Wu, B. S. Walker, V. K. and Roberston, R. M. (2003) Cloning and characterization of a member of the Hsp 70 gene family from *Locusta migratoria*, a highly thermo-tolerant insect. *Cell Str. Chap.,* **8,** 144-152.

Mishra, P. K., Sharan, S. K., Kumar, D. Singh, B. M. K., Subramanayam, B. and Suryanarayana, N. (2008) Phases of diapause in tasar silkworm *Antheraea mylitta* Drury. *J. Adv. Zool.,* **29,** 38-45

Moreira C. K., Capurro, M. L., Walter, M., Pavlove, E., Biessmann, H., James, A. A., de Bianchi, A. G. and Marinotti, O. (2004) Primary characteridsation and basal propmoter activity of two hexamerin gene of *Musca domestica*. *J. Insect Physiol.,* **4,** 1-10.

Nagmanju, P., Hansen, I. A., Bumester. T., Meyer, S. R., Scheller, K. and Dutta-Gupta, A. (2003) Complete sequence, expression and evolution of two members of the hexamerin protein family during larval development of the rice moth, *Corcyra cepahlonica*. *Insect Biochem Mol. Biol.,* **33,** 73-80.

Pan, M. L. and Telfer, W. H. (2001) Storage hexamerin utilization in ytwo lepidopterarans; differences correlated with the timing of egg formation. *J. Insect Sci.,* 1.2. Available online insect science.org/1.2

Parsell, D. A. and Lindquist, S. (1993) The functions of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Ann. Rev.Genet.,* **27,** 437-496.

Ragland, G. J., Dellinger, D. L. and Daniel A. Hahn (2010) Mechanism of suspended animation are revealed by transcript profiling of diapause in flesh fly. PNAS, see www.pnas.org /cgi/doi/ 10.1073/pnas.1007075107

Reinhert, J. P. , Yocum, G. D. and Denlinger, D. L. (2000a) Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem. Mole. Biol.,* **30,** 515-521.

Reinhert, J. P., Li, A., Yocum, G. D., Robich, R. M. and Hayward Scott, A. L.(2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *PNAS*, July, 11130-11137.

Reinhert, J.P. and Denlinger, D.L.(2000b) Heat shock protein 90 is down regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. Insect Mol. Biol., **9,** 641-645.

Rinehart J. P., Rebecca A., Cikra-Ireland, Flannagan R. D. and Denlinger D. L. (2001) Expression of ecdysone receptor is unaffected by pupal diapause in the flesh fly, *Sarcophaga crassipalpis,* while its dimerization partner, USP, is down regulated. *J. Insect Physiol.,* **47,** 915-921.

Robich, R. M. and Denlinger, D. L. (2005) Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony PNAS, **102,** 15912–15917.

Robich, R. M., Rinehart, J. P., Kitchen, L. J. and Denlinger, D. L. (2007) Diapause specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. *J. Insect Physiol.,* **53,** 235-245.

Spyliotopoulos, A., Gkouvitsas, T., Fantinou, a. and Kourti, A. (2007) Expression of a CDNA encoding a member of the hxamerin storage proteins from the other *Sesamia nonagrioides* (Lef.). during diapause. *Comp. Biochem Physiol.,* **148,** 44-54.

Suma, H. and Haunerland, N. H. (2007) VHDL, a larval storage protein from the corn earworm, *Helicoverpa zea*, is member of vitellogenin gene family. *Insect Biochem. Mol. Biol.,* **37,** 1086-1093.

Sun, Y. and Mac Rae, T. H. (2005) The small heat shock proteins and their role in human disease. *FEBS J.,* **272,** 2613-2627.

Tamareillo, S.P. and Denlinger, D.L.(1998) G0/G1 cell cycle arrest in the brain of Sarcophaga crassipalpis during pupal diapause and the expression pattern of the cell cycle regulator, proliferating cell nuclear antigen. *Insect Biochem. Mol. Biol.,* **28,** 83-89.

Tang, B., Wang, S. and Zhang, F. (2010) Two storage hexamerins from the beet armyworm Spodoptera exigua: cloning and characterization and the effect of gene silencing on survival. *BMC Mol. Biol.,* **11,** 65-77.

Tauber, M. J., C. A. Tauber, and Masaki, S. (1986) Seasonal adaptations of Insects. Oxford University Press, Oxford.

Telfer, W. and Kunkle, J. (1991) The function and evolution of insect storage hexmers. *Ann. Rev. Entomol.,* **36.** 205-228.

Telfer, W. H. and Pan M. L. (2003) storage utilization in *Manduca sexta*. *J. Insect. Physiol.,* **3,** 1-6.

Terlecky, S. R., Chiang, H. L., Olson, T. L. and Dice, J. F. (1992) Protein and peptide binding and stimulation of in vitro lysosoma proteolysis by 73kDa heat shock protein. *J. Biol. Chem.,* **267,** 9202-9209.

Tommasini, M. G. and Van Lenteren, J. C. (2003) Occurence of diapause in *Orius laevigatus*. *Bulletin of Insectology,* **56,** 225-251.

Tungjitwitayakul, J., Sinftripop, T., Nettagul, A., Oda, Y., Tatun, N., Sekimoto, T. and Sakurai, S. (2008) Identification, Characterization and developmental regulation of two storage regulation proteins in the bamboo borer, *Omphisa fuscidentalis*. *J. Insect Physiol.* **54,** 62-76.

Wolschin, F., and Gadau, J.(2009). Deciphering Proteomic Signatures of Early Diapause in Nasonia. PLoS ONE 4: e6394 www.plosone.org 1

Yocum, G. D., Joplin, K. H. and Denlinger, D. L. (1998) Upregulation of a 23kDa small heat shock protein transcript during pupal diapsue in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem. Mol. Biol.,* **28,** 677-682.

Yocum, G. D., Kemp, W. P., Bosc, J. and Knoblett, J. N. (2005) Temporal variation in over wintering gene expression and respiration in the solitary bee *Megachile rotundata*. *J. Insect Physiol.,* **51,** 621-629.

Zheng, X., Yoshiga, T. and Tojo, S. (2000) cDNA cloning and deduced amino acid sequences of three storage proteins in the common cutworm, *Spodoptera litura* (Lepidoptera:Noctuindae). *Appl. Entomol. Zool.,* **35,** 31-39.

Zhou, G. and Miesfled, R. L. (2009) Energy metabolism during diapause in *Culex pipiens* mosquitoes. *J. Insect Physiol.*, **55,** 40-46.