Hexamerins expression during larval development of *Corcyra cephalonica*: Regulation by ecdysteroids

Thesis submitted to University of Hyderabad
for the award of Ph.D. degree in
Department of Animal Biology

By

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08LAPH04

DEPARTMENT OF ANIMAL BIOLOGY

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

HYDERABAD- 500 046

November 2014

University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

Department of Animal Biology
School of Life Sciences
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Hyderabad-500 046

DECLARATION

I, Venkat Rao Vantaku, hereby declare that this thesis entitled **"Hexamerins expression during larval development of *Corcyra cephalonica*: Regulation by ecdysteroids"** submitted by me under the guidance and supervision of Prof. Aparna Dutta Gupta is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date:

Venkata Rao Vantaku

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CERTIFICATE

This is to certify that the thesis entitled **"Hexamerins expression during larval development of *Corcyra cephalonica*: Regulation by ecdysteroids"** is a record of bonafide work done by Mr. Venkata Rao Vantaku, for the Ph.D. programme in the Department of Animal Biology, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Prof. Aparna Dutta Gupta
(Supervisor)

Head of the Department

Dean of the School

Dedicated to my friend Naresh.

You left your fingerprints on our lives. You will not be forgotten.

Acknowledgements

First and foremost, my deepest thanks to my supervisor Prof. Aparna Dutta-Gupta. Her patience, encouragement, and immense knowledge were key motivations throughout my PhD. I had a great freedom to plan and execute my ideas in research without any pressure. This made me to identify my own strength and drawbacks, and particularly boosted my self-confidence. It was great working with you Madam and my heartfelt thanks to you.

I am grateful to the Doctoral committee members, Prof. B. Senthilkumaran and Dr. Brahamanandam for their constant help, valuable comments and suggestions throughout my work.

I am thankful to the present and former Deans Prof .A.S Raghavendra, Prof. R.P.Sharma and Prof. M Ramanadham of School of Life Sciences for allowing me to use the school facilities.

I would like to extend my sincere thanks to the present head Prof. B.Senthilkumaran and former heads Prof. Manjula Sritharan and Prof. S. Dayananda for allowing me to use the departmental facilities.

I would also like to thank all the faculty of School of Life Sciences.

Huge thanks to the fantabulous crew! What made these years great was working alongside of you! those of you who were here when I started and now have moved on (Dr.Manohar, Dr. Damodar, Dr. Jacob) those who have been here throughout most of my PhD duration (Dr. Roy, Dr. Chaitayna, Dr. Suneel, Dr. Sridevi, Dr. Balu, Pavani, Vinod) and two of you have joined the last year (Brahmaiah and Tulasi). Thanks for providing an intellectually inspiring environment to work in.

An extra thank you to all the juniors' Suresh, Ravinder, Elsin, Trinadh and Rambabu.

A massive thanks to the colleagues from Prof. Senthilkumaran's, Prof. Dayanada's Dr. Suresh's and Dr. K. Arun Kumar's lab.

But the support I have leaned on, quite possibly the most, comes from my friends Kranti, Sashi, Yasin, Kishore, Vincent, Pavan, Sumit, Narmadha, Chaitu, Bimo, Jyoti, Prasanna, Madhu, Rajesh, Shiven, Naresh, Geetika, Shyma and Trishla who have kept me afloat when times were hard or crazy.

There are few people I need to mention especially, Mrs. Pavani, Tirshla, Kranthi, Narmadha and Surendra, their friendship and unselfish help enabled me to present my presentation and dissertation more fluently and clearly in English.

Many thanks to all the research scholars from School of Life Sciences and other departments, too many to name individually. Everyone has been useful, in one way or another, in getting this PhD done.

Several people helped in maintain the insect cultures through all years for my work in this regard, I would like to thank Babyya, Mallesh and Nagesh. I would also like to thank the non-teaching staff Jagan and Gopi for their support in using common facilities. Also, thanks to all the administrative staff, especially Ankinaidu and Jyoti.

Now life as a student, isn't all milk and honey, And I'm very thankful to the sources that have supported me financially! Funding from the UGC-SAP, UPE, DBT-CREBB, and DST-FIST to School and Department, CSIR, DST & DBT for lab and fellowship by DBT is greatly acknowledged.

Special thanks to Prof. ABK Bhuyan for allowing to use his lab instrumentation and chemicals.

A special mention to Mrs. Leena Bhashyam for her support for facilitating genomics instrumentation facility.

I thank my parents, my sister and my brother-in-law who have bought great joy to my life. Your support and encouragement was worth more than I can express on paper.

Contents

List of abbreviations

°C	Degree centigrade / Celsius
20E	20-Hydroxyecdysone
ALP	Alkaline phosphatase
ATP	Adenosine 5` triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
cpm	Count per minute
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	1, 4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N`,N`-tetraacetic acid
ELI	Early-last instar larvae
EMSA	Electrophoretic mobility shift assay
HEPES	N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
IPTG	Isopropyl β-D-thiogalactoside
JH	Juvenile hormone
kDa	Kilo Dalton
LB medium	Luria-Bertani medium
LLI	Late-last instar larvae
MARG	Male accessory reproductive gland
mg	Milligram
MLI	Mid-last instar larvae
mM	Millimolar
MOPS	3-Morpholinopropanesulfonic acid
mRNA	Messenger ribonucleic acid
NBT	Nitrotetrazolium blue
ng	Nanogram
nM	Nanomolar

NP-40	Nonidet P-40 (Nonylphenyl polyethylene glycol)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PP	Prepupae
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TAE buffer	Tris-Acetate-EDTA buffer
TCA	Trichloroacetic acid
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N, N, N`, N`, tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
v/v	Volume/volume
w/v	Weight/volume
μg	Microgram
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside

INTRODUCTION

Insects:

Insects are the most diverse group of animals which belong to the phylum Arthropoda. On earth they represent over 90% of the life forms and found in terrestrial as well as in aquatic habitats. They could be herbivorous, carnivorous or detrivorous. They are either predators, parasites or pests. They have a hard outer covering or exoskeleton, a segmented body and jointed legs. In order to grow, arthropods shed their exoskeleton at different intervals in their life cycle, which is generally called as molting.

Metamorphosis:

Majority of insects undergo metamorphosis, during which immature larval or nymphal forms undergo transformation and give rise to adults, which have reproductive capacity. Metamorphosis is generally classified into:

i. Incomplete metamorphosis
ii. Complete metamorphosis

Incomplete metamorphosis: Insects showing incomplete metamorphosis have three life stages. The life cycle starts with egg (embryo) that hatches out into a nymph. The nymph resembles adult, but it is smaller in size, may have different colouration and does not have wings. The nymph grows through stages called instars primarily by shedding its exoskeleton at each stage (ecdysis). Finally, it metamorphoses into a mature adult with wings, which has reproductive activity. Insects belonging to the taxonomical orders Orthoptera (cockroaches and grass hoppers), Odonata (dragonfly) and Hemiptera (bugs) undergo incomplete metamorphosis and are generally called as hemimetabolus insects.

Complete metamorphosis: The insects which undergo complete metamorphosis have four life stages. The life cycle begins with egg, from which first instar larva hatches. The

larva undergoes extensive feeding, molts 3 to 7 times and grows into a large instar, which metsmorphoses into a non-feeding stage called pupa. During pupal stage, extensive remodeling/histolysis of larval organs occur followed by histogenesis of adult organs such as wings, legs, eyes etc. Finally, eclosion takes place and an adult emerges out from the pupa. Such insects that undergo complete metamorphosis are termed as holometabolus insects. Examples of such insects are from taxonomical orders of Lepidoptera (butterflies and moths), Hymenaptera (bees and wasps), Coleoptera (beetles) etc.

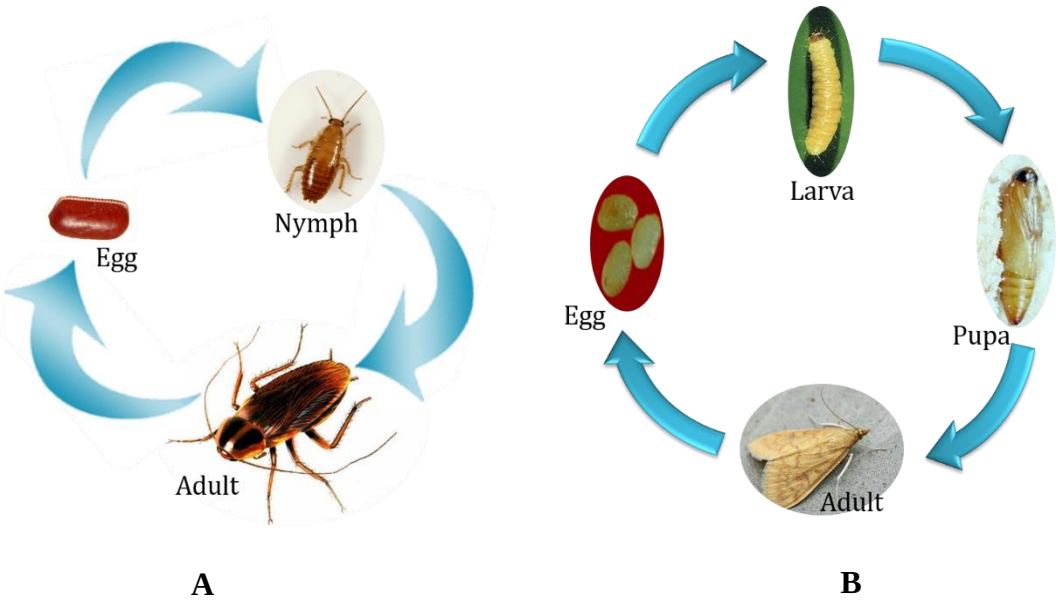


Figure 1: Schematic representation of two types of metamorphosis in insects. A: Incomplete metamorphosis in *Periplaneta americana* (Orthoptera). B: Complete metamorphosis in rice moth *Corcyra cephalonica* (Lepidoptera).

Metamorphosis and its physiological significance:

In a broad sense, metamorphic development is the manifestation of sequential polymorphism which is the differential expression of variety of genes present in the same genome at different stages of development (Highnam, 1981; Nijhout and Wheeler, 1982; Gilbert *et al.,* 1996). This is primarily controlled by an interplay of group of metamorphic hormones, which through several cascade of events decide whether a cell remains at a given stage or advances to the next one or undergo programmed cell death (Wigglesworth, 1954; Nijhout, 1994; Sehnal *et al*., 1996; Gilbert *et al*., 2000; Truman and Riddiford, 2002). One such example is the deposition of cuticle and its pattern during successive larval, pupal and adult stages governed by hormones. The larval cuticular proteins are produced under high titers of juvenile hormone (JH), whereas in the presence of low JH titer, ecdysteroids facilitate pupal cuticular protein synthesis and in the absence of JH, ecdysteroids govern the expression of imaginal cuticular proteins (Willis, 1996). Further, under normal conditions during postembryonic and adult development, the fate of each stage is strictly determined and is neither omitted nor mixed with other stages (Slama, 1975). However, the deficiency of metamorphic hormones and/or alteration in their titer leads to defective development and formation of supernumerary stages as well as non-viable intermediates.

Hormones and their role in development and metamorphosis:

Choreographic precision of differential titers of the morphogenetic hormones, mainly the juvenile hormones (JHs) and ecdysteroids and their interaction is required not only for the growth and development, but also for the molting and metamorphic events to occur normally (Gilbert *et al*., 1996; Riddiford, 2012; Jindra *et al*., 2013). Literature survey clearly shows that several studies have been carried out to unravel the regulation of insect

postembryonic development (Sehnal *et al*., 1996; Henrich *et al*., 1999; Tissot and Stocker, 2000; Truman and Riddiford, 2002; Gilbert *et al*., 2002; Riddiford, 2012; Yamanaka *et al*., 2013). However, knowledge regarding the exact mechanism of regulation of hormone dependent functions including expression of various genes is limited and the field remains largely unexplored with a demand for further research.

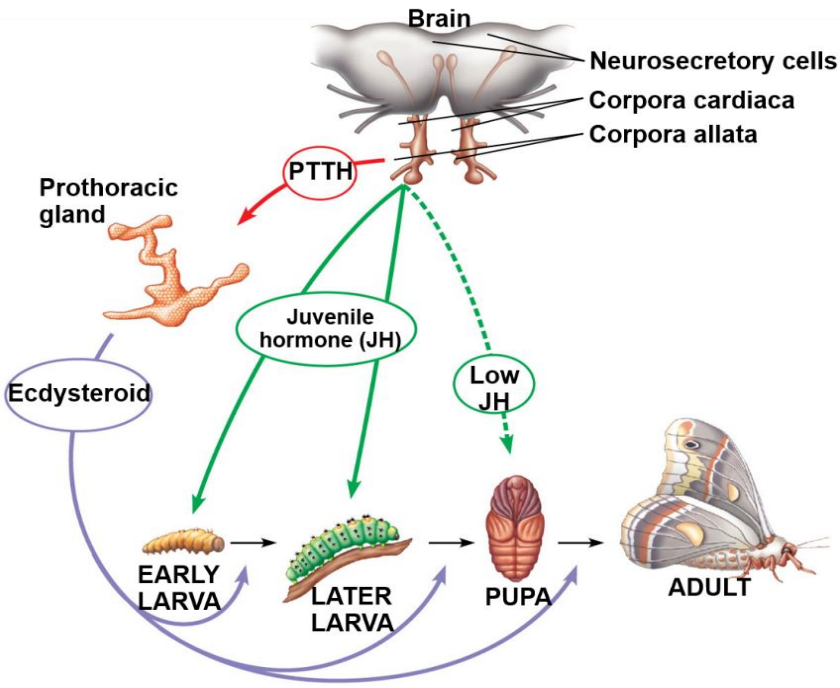


Figure 2: Hormonal control of metamorphosis. The titers of juvenile hormone and ecdysteroids play crucial role in insect development.

(Source- http://bio1152.nicerweb.com/Locked/media/ch45/metamorphosis.html)

Juvenile hormone:

Juvenile hormones (JHs) are group of lipophilic sesquiterpenes produced by the corpora allata (CA) during larval/nymphal and adult stages. Several forms of JHs have been identified so far from various tissues and hemolymph of different insects. All known JHs are either methyl or ethyl esters of epoxy farnesoic acid or its homologs, i.e. with one or several methyl and/or ethyl side chains. The simplest and most ubiquitous one is JHIII found in majority of insects (Meyer *et al*., 1970; Judy *et al*., 1973). However, in

Lepidopteran insects, five of them have been described, including JHIII and other homologs.

JHs have two important roles in insect physiology and its life cycle, one is to prevent metamorphosis and the second is to regulate reproduction (Riddiford, 2012). During larval development, the presence of JH in the hemolymph prevents the precocious metamorphosis (Liu *et al*., 2009) of an insect larva into pupa or adult when passing through different molts, hence this hormone is often called as 'status quo hormone' (Riddiford , 1994; Chaitanya *et al.,* 2013). In the larval and pupal stages of insect life cycle, JHs are known to modify the expression patterns of ecdysteroid regulated genes, making it possibile for an insect to express different types of cuticles, and in particular to develop from a larval form to an adult form through a complex metamorphosis (Willis *et al*., 1996). In females, it stimulates production of yolk for the egg development (Soller and Mala, 1999; Engelmann, 2005), while in adult males, it stimulates the accessory glands to produce proteins needed for seminal fluid and spermatophore formation (Wilson *et al*., 2003; Parthasarathy *et al*., 2009). In the absence of JH production, the adult remains sexually sterile.

Ecdysteroids and mechanism of action:

Ecdysteroids regulate a wide variety of cellular processes in the life cycle of insects during the postembryonic development. Pulses of ecdysteroids coordinate the complex events of growth, molting and metamorphosis, which is essential in the life of the insects (Nijhout, 1994). Two major and widely explored ecdysteroids are ecdysone (α-ecdysone) and 20-hydroxyecdysone (β-ecdysone) (Horn and Bergamasco, 1985). 20-Hydroxyecdysone (20E), which is regarded in most arthropods as the predominant active hormone is primarily derived from α-ecdysone in the peripheral tissues by hydroxylase action.

Ecdysteroids like many other vertebrate steroids are known to regulate variety of physiological functions in insects during embryonic, postembryonic as well as adult development including embryogenesis, coloration, melanization, metabolism, cell death, diapause, spermatogenesis, oogenesis, behavior etc. Evidence from the previous studies suggests that ecdysteroids regulate the expression/repression of various genes in a tissue specific as well as in temporal manner during the insect development (Woodard *et al*, 1994; Thummel, 1996; Yamada *et al*., 2000; Ali *et al.*, 2012; 2013 Schwedes and Carney, 2012; Yamanaka *et al*., 2013).

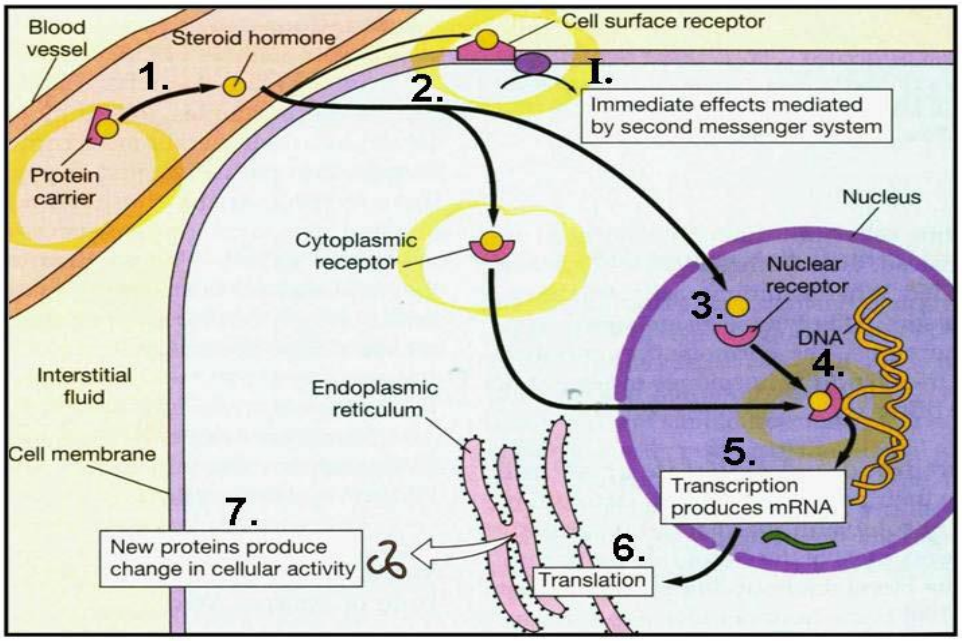


Figure 3: Schematic representation of the mechanism of steroid hormone action. Steroid hormones regulate the biological response in the following steps- 1**.** delivery of hormones to an appropriate target and its dissociation from the carrier protein, 2**.** transport of hormone into cytoplasm or nucleus, 3. & 4**.** binding of activated hormone receptor complex to hormone responsive elements as homo or heterodimers, 5**.** gene activation and transcription, 6**.** release of mRNA from nucleus and its translation into protein and 7**.** changes in the cellular activity due to the regulation of newly synthesized protein. In addition to this the steroid hormones may also exert a direct rapid effect possibly through a cell surface receptor mediated by a second messenger system(I).

(source- http://www.zoo.utoronto.ca/zoo344s/2003Group2/mechanism_steroid.html).

Ecdysteroids mediate their action by two distinct pathways, genomic and non-genomic. The relatively simple ecdysone-inducible genes of fat body larval serum proteins or salivary gland glue proteins for example, have a single transcript. However, other ecdysteroid non-inducible genes show a complex pattern of transcription and stretch over regions of 50 kb or more (Cherbas, 1993; Thummel, 2002). An increasing body of evidence suggests that ecdysteroids like vertebrate steroids (estrogen, androgen, and progesterone) can exert rapid, non-genomic effects (Wehling, 1997; Arif *et al*., 2004; Nakagawa and Henrich, 2009). It typically involves rapid induction of signal transduction cascade, dependent on conventional second messengers as well as kinases. The two modes of ecdysteroid action eventually converge and give rise to the integral cellular response. Fast effects are generally traced back to the cell membrane targets often termed as non-conventional receptors. The genomic pathway effect involves an intracellular receptor, namely the ecdysteroid receptor (EcR) (Koelle *et al.,* 1991; Truss and Beato, 1993; Freedman, 1997; Riddiford *et al*., 2000; Grebe *et al.*, 2004; Fahrbach *et al*., 2012; Hill *et al*., 2013). Like other members of the nuclear receptor superfamily, EcR exhibits the modular structure and is composed of the N-terminal (A/B) domain, the DNA binding domain (C), the hinge region (D), the ligand-binding domain (E), and the C-terminal domain (F). Like vertebrate steroid receptors, the ligand-binding domain of EcR is multifunctional and undergoes ligand-dependent dimerization and transactivation. This induces conformational change and allows the translocation of hormone receptor complex to the nucleus. On the other hand, ligand-independent transactivation and dimerization functions are found in the terminal domains as well as in the region spanning the DNA binding domain and the N-terminal region of the hinge respectively. The EcR is known to heterodimerize with other members of the nuclear receptor superfamily, primarily with the ultraspiracle protein (USP) (Yao *et al.,* 1992; (Bender *et al*., 1997, Jones *et al*., 2013),

which is an orthologue of the vertebrate retinoic acid X receptor (RXR). Ligand bound steroid receptor (EcR-USP heterodimer) binds to specific DNA sequence i.e. the ecdysteroid response elements (ERE) present in the promoter regions of ecdysteroid responsive genes and initiates their transcription (Oro *et al*., 1990; Koelle *et al*., 1991; Yao *et al*., 1992; Yao *et al*., 1993; Mangelsdorf *et al*., 1995; Przibilla *et al*., 2004; Fahrbach *et al*., 2012; Hill *et al*., 2013; Yamanaka *et al*., 2013). The binding of steroid hormone receptor complex to DNA is the first step in the assembly of a protein complex on the DNA, which facilitates recruitment of co-activator proteins that amplify signal and initiate RNA polymerase activity. Most of the nuclear hormone receptors including EcR, function as ligand-controlled transcription factors, a characteristic that renders these receptors or their key regions (i.e. the ligand- and DNA-binding domains) especially suitable as constituents of gene switches (Palli *et al*., 1992; Allgood and Eastman, 1997; Thummel, 1996, 2002; Zheng *et al*., 2003).

Fat body - structure and function:

The fat body in insect is an important multifunctional tissue which occupies major part of the visceral cavity and perivisceral space in thorax as well as abdomen and plays vital role during various stages of insect life (Keeley, 1985, Arrese and Soulages, 2010; Hoshizaki *et al*., 2012). Although historically it is considered as a single tissue with variety of functions, detailed analysis revealed that in different insect orders, it has structural diversity and is made up of variety of cell types (Haunerland and Shirk, 1995). For example, in holometabolus insects the trophocytes (adipocytes) are the major cell types, which perform diverse functions (Poiani and Cruz-Landim, 2012). Other cell types that are found include mycetocytes which maintain bacterial endosymbionts (Costa-Leonardo, 2013) and urocytes which are responsible for the storage of urate during development

(Park *et al*., 2013). Trophocytes are the seat for multimetabolic activity, including synthesis and storage of variety of proteins, carbohydrates, lipids, and recycling of nitrogen (Keeley, 1985; Roma *et al*., 2010; Costa-Leonardo, 2013). It was also demonstrated as a possible source of humoral factors (Gray e*t al.*, 1987). In view of the complex function performed by fat body, it is often compared with vertebrate liver. The fat body undergoes growth and development along with other tissues and its function changes in accordance with the developmental stage of the insect (Vanishree *et al*., 2005; Poiani and Cruz-Landim, 2012).

The fat body tissue is structurally organized to provide maximal exposure to the hemolymph and due to its changing metabolic role and integral position in maintaining metabolic homeostasis, it serves as an ideal model for endocrine regulation related studies. Furthermore, the fat body is a known target for several hormones including ecdysteroids. Hence, it is exploited extensively for the studies of the stage, tissue and sex specific expression of genes, post-transcriptional regulation of RNA and post-translational control of proteins (Hansen *et al*., 2002; Wu *et al*., 2014). Most of the studies with protein expression and sequestration by fat body have been carried out with the entire tissue. However, there are evidences in both Diptera and Lepidoptera suggesting regional differences in activity and function of fat body tissue. In case of *Helicoverpa zea*, the storage proteins are synthesized by peripheral fat body fraction but are sequestered and stored only by perivisceral fat body (Wang and Haunerland, 1994 a, b). In the silkworm *Bombyx mori*, it has been shown that the dorsal and ventral perivisceral fat body contains the most competent cells for sequestering hemolymph proteins as compared to the peripheral and hindgut associated fat body tissue (Vanishree *et al*., 2005). Organ cultures of various insect tissues /organs have been powerful tool and facilitate analysis of *in vitro*

effects of hormones and other molecules. Studies with fat body have further demonstrated similar expression pattern of various genes under *in vivo* and cultured condition (Nakanishi and Garen, 1983; Damara *et al*., 2010a). Hence, the fat body becomes a valuable model system for analyzing the hormonal and developmental changes which take place during the postembryonic and adult stages, under isolated conditions which could be influenced by addition of either single or multiple hormones and their analogs.

The fat body is known to be the major site of biosynthetic activity in insects and undergoes a chronologically ordered sequence of alteration during postembryonic development (Dean and Collins, 1985; Yamashita, 1986). In actively feeding larval stages, the fat body synthesizes various macromolecules including fatty acids, lipids, carbohydrates and a wide range of proteins, which are released in to the hemolymph (Telfer and Kunkel, 1991; Haunerland, 1996; Arrese and Soulages, 2010). Through the hemolymph, these macromolecules are transported to different tissues, where they are sequestered to perform various physiological functions. At the end of the larval development and during pupal-adult transformation there is considerable change in morphology as well as physiology of fat body cells (Roma *et al*., 2010). The tissue's intermediary metabolic and protein synthetic activities diminish and from a synthetic organ it primarily becomes a storage tissue (Price, 1973; Dean and Collins, 1985). This switch is characterized by the appearance of large number of electron dense storage granules and coated vesicles inside the cytoplasm of the cell. Extensive studies show that fat body is the major organ responsible for nutritional homeostasis, the stored macromolecules are mobilized and used as metabolic precursors as well as building blocks for pupal-adult transformation mainly in holometabolous insects including the order Lepidoptera (Leevenbook, 1985; Inagaki

and Yamashita, 1986; Bean and Silhacek, 1989; Kanost *et al*., 1990; Telfer and Kunkel, 1991; Haunerland, 1996; Burmester, 1999; Bond *et al*., 2010).

Larval hemolymph contains a diverse array of peptides and proteins, such as immune response proteins (Shahbuddin *et al*., 1998; Vizoli *et al*., 2000; Nakajima *et al*., 2001; Roy *et al*., 2004; Yoe *et al*., 2006), lipophorins (Burks *et al*., 1992; Kanost *et al*., 1995; Rimoldi *et al*., 1996), hormone-binding and ion-binding proteins (Koopsmanchap and de Kort, 1992; Braun and Wyatt, 1996; Maya-Monteiro *et al*., 2000; Gudderra *et al*., 2002), hemocyanins (Van Holde and Miller, 1995; Burmester, 2002; Zacharieva *et al*., 2008) and hexamerins (Telfer and Kunkel, 1991; Haunerland, 1996; Martins *et al*., 2008). Presence of these proteins/peptides clearly reflects diversified roles played by hemolymph in mediating intercellular communication, transporting metabolites and imposing a defensive barrier against micro-organisms. Among all the proteins synthesized by the fat body and secreted in to the hemolymph, storage proteins like hexamerins are the most important which play a vital role in the postembryonic development of insects.

Fat body: synthesis and storage of nutrients and other macromolecules:

Recently, Arrese and Soulages (2010) have reviewed and shown that insect fat body performs functions analogous to vertebrate liver as well as adipose tissue and serves not only as synthetic organ but also as a site for nutrient storage and energy metabolism. The holometabolus insects including the order Lepidoptera do not feed during pre-pupal and pupal stages thereby depending wholly on the nutrient storage built during feeding phase of larva, which are stored in the fat body tissue as granules during the post-feeding phase. Later the stored nutrients are mobilized to meet the demands of energy as well as macro molecules not only during the molting and metamorphosis but also during diapause, reproduction, environmental starvation etc. (Wheeler and Buck, 1996; Wheeler *et al.*,

2000; Hahn and Denlinger 2007; Hossain *et al.*, 2013). Literature survey reveals that the fat body of insects synthesize and secrete large quantities of storage proteins primarily hexamerins and hemocyanins during active feeding stage, which circulate in haemolymph, later they are sequestered by the fat body during non-feeding pre-pupal and pupal stages (Haunerland, 1996; Burmester and Scheller, 1997) and form distinct protein granules in the cytosol . When amino acids are required for *de novo* synthesis of proteins in non-feeding pupal stage during histogenesis, storage proteins are hydrolyzed and released amino acids are used for synthesis of pupal proteins.

Hexamerins:

A major group of the proteins present in the larval hemolymph belong to hexamerin protein family and are often called as larval serum proteins (LSP) or larval hemolymph proteins (LHP). They are high molecular proteins with native molecular mass around 500 kDa (Telfer and Kunkel, 1991; Burmester and Scheller, 1999), which are either homo- or hetero- hexamers, with subunits in the mass range of 70-90 kDa. This characteristic feature seems to be consistent in the several orders of holometabolous insects including Lepidoptera and in some orders of hemimetabolous insects too (Rehn and Rolim, 1990; Telfer and Kunkel, 1991; Tojo and Yoshiga, 1994; Haunerland, 1996; Martinez *et al*., 2000). In *C. cephalonica*, the native hexamerin protein in final instar larva is a hexamer (500 kDa) composed of three subunits with masses 86 kDa (Hex 86), 84 kDa (Hex 84) and 82 kDa (Hex 82) (KiranKumar *et al*., 1997; Nagamanju *et al*., 2003). The hexamerins are synthesized predominantly by the fat body during the actively feeding larval period and are released into the hemolymph. During the last larval instars these proteins in hemolymph nearly account for 70-80% of the total soluble protein by weight (Kanost *et al*., 1990; Telfer and Kunkel, 1991). Shortly before pupation, they are sequestered by the

fat body through a receptor mediated endocytic process and accumulate as dense protein granules (Bean and Silhacek, 1989; Chrysanthis *et al*., 1994; Haunerland, 1996; Burmester and Scheller, 1999).

Hexamerins have been biochemically purified and characterized from a number of insects belonging to orders: Lepidoptera (Telfer and Kunkel, 1991; KiranKumar *et al*., 1997; Korochkina *et al*., 1997; Gudderra *et al*., 2002); Diptera (Hagner-Holler, 2007), Coleoptera (Koopmanschap *et al*., 1992) as well as in a number of hemimetabolous insects (Rehn and Rolim, 1990; Faria *et al*., 1994; Ancsin and Wyatt, 1996; Jamroj *et al*., 1996; Pick and Burmester, 2009)

Based on the amino acid composition, the larval hexamerins are generally classified into four distinct categories. The first category of hexamerin is characterized by exceptionally high content of aromatic amino acids (18-26%) and low methionine content (2%) and is generally called as arylphorin. This class includes prototype storage protein calliphorin, from *C. erythrocephala* (Munn *et al*., 1971). These are the major hemolymph proteins in Diptera (Naumann and Scheller, 1991; Burmester *et al.*, 1998). The second category is the Lepidopteran glycoprotein with high aromatic amino acid and low methionine content and is named as Lepidopteran arylphorins (Palli and Locke, 1987; Webb and Riddiford, 1988; Kunkel *et al*., 1991). The third group of hexamerin is found in many Dipteran insects, which is neither rich in aromatic amino acids nor in methionine (Haunerland, 1996; Braun and Wyatt, 1996; Korochkina *et al*., 1997; Crampton *et al*., 1999). The fourth group has relatively high methionine content (about 6%) and low aromatic amino acid content and they are predominantly expressed in the female larvae of Lepidoptera, and serve as yolk protein precursors (Webb and Riddiford, 1988; Bean and Silhacek, 1989; Rehn and Rolim, 1990; Wang *et al*., 1993; Jamroj *et al*., 1996; Pan and Telfer, 1996; Mi *et al*., 1998; Hwang *et al*., 2001; Zhu *et al*., 2002). Majority of hexamerins have been shown to undergo post-

translational modifications like glycosylation and lipidation and are reported either as glycoprotein or lipoglycoprotein. Few studies reveal that hexamerins are not only synthesized during larval/nymphal but also expressed during pre-pupal as well as pupal stages (Martins *et al*., 2008). In honey bees, four types of hexamerins are found in larval hemolymph, whereas one of the hexamerin protein Hex70a was also reported in adults. These reports interestingly show that adult Hex70a has more than 15% aromatic amino acid content and is expressed in developing gonads of drones, workers and queens (Martins *et al*., 2010).

To date, there are many hexamerins, whose gene and amino acid sequences have been established (Memmel *et al*., 1994; Burmester *et al*., 1998; Hwang *et al*., 2001; Zhu *et al*., 2002). Our research group primarily focuses on the purification, characterization of hexamerins and their genes from stored grain as well as agricultural pests, belonging to order Lepidoptera. Previously, our group has cloned and characterized an arylphorin hexamerin cDNA (Nagamanju *et al.*, 2003), as well as methionine rich hexamerin cDNA (Damara and Dutta-Gupta, 2010) from larval form of rice moth, *C. cephalonica*. Multiple alignment analysis of the deduced amino acid sequence derived from the cDNA sequences showed their homology to most of the reported insect hexamerins. It is interesting to note that arthropod tyrosinase, arthropod hemocyanin, insect hexamerin (from Lepidoptera and Diptera) and Dipteran arylphorin receptor share a common origin in phylogenetic tree (Burmester and Scheller, 1997; Burmester *et al*., 1998). This further suggests a common ancestral molecule, which during evolution gave rise to diverse proteins with different functions.

Hexamerin receptor and role of 20E in its activation for hexamerin uptake:

The transport of hexamerins across the fat body cell membrane by the non-feeding pre-pupal and pupal stages requires the existence of a specific receptor. These receptors have been recognized in Dipteran as well as Lepidopteran insects but the sequences of the receptors are known only from two Dipterans, notably from flesh fly *S. peregrina* (Chung *et al*., 1995), blow fly *Calliphora vicina* (Burmester and Scheller, 1995) and fruit fly *D. melanogaster* (Burmester *et al*., 1999) and a Lepidopteran *C. cephalonica* (Damara and Dutta-Gupta, 2010). Detailed analysis of receptor sequence revealed their similarity with the ligands i.e. hexamerins, which clearly indicate that the receptors have evolved from their own ligands even before the divergence of winged insects (Burmester and Scheller, 1997; Burmester, 2002). In *S. peregrina*, a 120 kDa receptor was identified which under the influence of 20E, acquires hexamerin sequestration activity (Ueno and Natori, 1984; Chung *et al*., 1995). Studies in *C. vicina* revealed that the hexamerin receptor is synthesized as a precursor (130 kDa), which is subjected to a three-fold post-translational cleavage that gives rise to the active receptor (Burmester and Scheller, 1997). Later, presence of an anterior fat body protein in *C. vicina*, which interacts with the hexamerin receptor and regulates hexamerin uptake by the fat body cells in the posterior part of the organ was reported. Further interaction studies using yeast-two-hybrid system validated the role of the afore said protein (Hansen *et al*., 2002).

Literature survey, clearly reveals identification of plasma membrane bound protein receptors in Lepidoptera which facilitate uptake of hexamerins. In *Helicoverpa zea*, a single 80 kDa receptor protein was reported to facilitate the sequesteration of storage proteins as well as VHDL (Wang and Haunerland, 1994a). Our group has not only identified hexamerins in *C. cephalonica* and *Chilo partellus* but also focused on the uptake

of these proteins by the fat body and also by the male accessory reproductive glands (MARG) (Bajaj *et al.,* 1990; Ismail and Dutta-Gupta, 1991). Using ligand binding studies KiranKumar *et al*., (1997) demonstrated the presence of 120 kDa hexamerin receptor in the larval fat body membrane of *C. cephalonica*. The receptor was found to be present in the last larval instar and at maximal concentration in the pre-pupal stage. Unlike other Lepidopteran insects sequesteration of hexamerin was not observed during the larval stage but was seen only during pre-pupal stage in *C. cephalonica*. (Bajaj *et al.,* 1990). However, 20E treatment induced a precocious uptake of hexamerins in the late last instar larval fat body (Ismail and Dutta-Gupta, 1991; Burmester *et al.,* 1995). These studies suggested that ecdysteroids activate the hexamerin receptor prior to hexamerin uptake. (Arif *et al.,* 2003; 2008) later demonstrated that 120 kDa hexamerin recptor undergoes tyrosine kinase dependent phosphorylation for its activation.

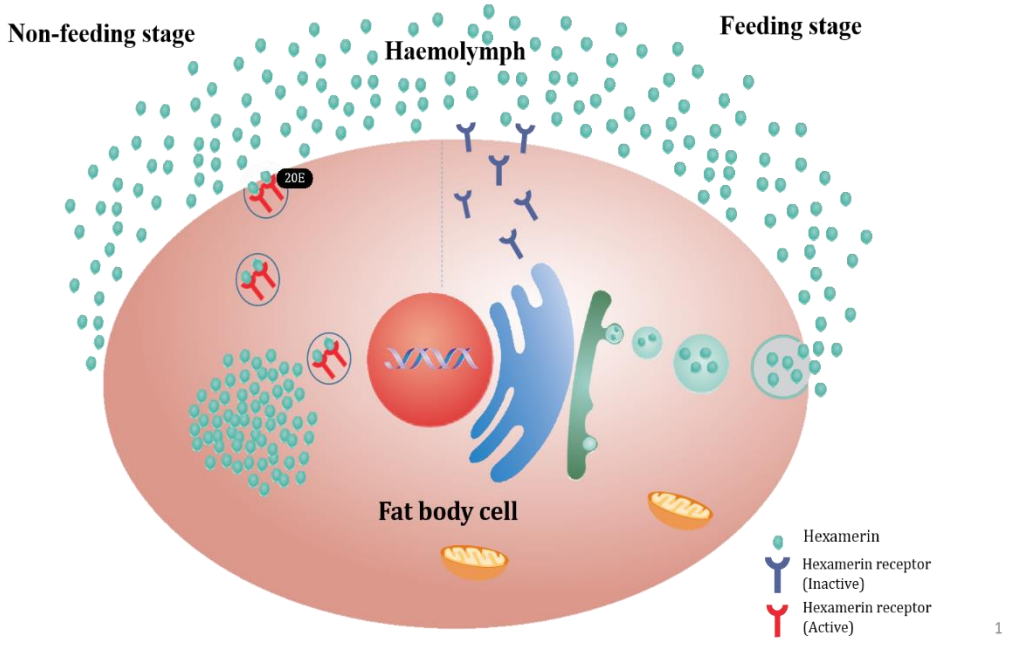


Figure 4: Schematic representation of hexamerin synthesis by the actively feeding larval fat body cells, its release into hemolymph and its uptake by the pupal fat body cells (shown in same cell) through a 20E dependent receptor mediated endocytosis in *C. cephalonica.*

Finally existence of a number of genes encoding hexamerins in different insect species clearly supports the idea that they evolved slowly to perform distinct roles as a result of physiological adaptations. Detailed genetic analysis reveals that all the hexamerin genes evolved from hemocyanin related genes, and the proteins have three distinct N, M and C domains like hemocyanins. Hexamerin genes share a common pattern of developmental expression in holometabolous insects, with a concomitant accumulation of transcripts and protein synthesis in late larval stages, followed by fast depletion after pupal ecdysis. This expression pattern is consistent with activation of *hex* genes for hexamerin synthesis and accumulation at the expense of the bulk of nutrients ingested by the feeding larvae. When the larvae stop feeding, *Hex* genes are turned off and the hexamerins are then sequestered from the hemolymph to attend amino acid needs during pupal and pharate adult stages.

Role of hexamerins in the pupal–adult transformation, reproduction, caste regulation and immunity is well demonstrated in various Lepidopteran insects. Further, the role of the morphogenetic hormones both JHs and 20E in the regulation of the hexamerins protein synthesis and their uptake is well documented. To the best of our knowledge a comprehensive study on the developmental as well as endocrine regulation of various hexamerins gene (s) expression is lacking in a specific Lepidopteran model. In the present study an attempt has been made to characterize a new hexamerin and analyse the expression of various hexamerin gene(s) in a stored grain pest *C. cephalonica* (rice moth) which belongs to order Lepidoptera. Further, efforts have also been made to identify the mechanism by which steroids regulate the expression of *Hex* genes in this model organism during postembryonic development.

Keeping the above in view following objectives were framed:

1. Cloning and characterization of *Hex 82* from *Corcyra cephalonica* last instar larval fat body.
2. Validation of reference gene(s) as internal control for quantitative polymerase chain reaction (qRT-PCR) analysis in *C. cephalonica*
3. Ontogeny and hormonal regulation of hexamerins in *C. cephalonica*; *in vivo* and *in vitro* studies
4. Identification and characterization of ecdysone regulatory element(s) in the upstream sequence of *Hex 84* gene of *C. cephalonica*

MATERIALS AND METHODS

Chemicals, kits used:

[γ^{32}P] dATP (3000 Ci/mmol) and [^{35}S] methionine (1000 Ci/mmol) were purchased from Board of Radiation Isotope and Technology (BRIT), India. Sephadex G-25, G-50 and G-100 and DEAE Sephacel were procured from Amersham Biosciences (USA). Agarose from Life Technologies (USA), 20-Hydroxyecdysone, Methoprene, Commassie brilliant blue G-250 and R-250, BSA, Bromophenol blue, DTT, EDTA, HEPES, TRI reagent, TEMED, and Triton X-100 were obtained from Sigma Chemical Co. USA. Insect culture medium TC-100 and Grace's cell culture medium were purchased from JRH Biosciences and Gibco Inc. USA respectively. ALP conjugated secondary antibodies, NBT/BCIP mix were purchased from Bangalore Genei, India. Acrylamide, N-N`-methylene bisacrylamide, 2-mercaptoethanol, glycerol, SDS, glycine, silver nitrate, Tris base and other chemicals were purchased from Sisco Research Laboratories Pvt. Ltd. India. Agar, ampicillin, MOPS, LB broth, LB Agar and skim milk powder were obtained from HiMedia Laboratories, India. SMARTer™ RACE cDNA Amplification Kit for rapid amplification of cDNA ends from Clone Tech, Superscript III® first strand synthesis system for RT PCR from Invitrogen, InsTAclone™ PCR cloning kit, DNA, RNA and protein markers were obtained from Thermo Scientific. QIAprep® spin miniprep kit for plasmid isolation and QIAquick® gel extraction kit were supplied by Qiagen. Genomewalker™ universal kit was procured from Clontech. Dual luciferase reporter system was bought from Promega. Supersignal® western blot chemiluminiscence HRP substrate was obtained from Takara Bio Inc. Nitrocellulose membrane from Amersham Biosciences, SYBR Green master mix and other real-time PCR reagents from Applied Biosystems were procured. DME medium, FBS, Bleocin and Geneticin were supplied by Gibco-BRL. All other chemicals used were of analytical grade and were obtained from local sources in India

Primers used for the study:

For the present study, various primers (degenerate, gene specific, RACE, qRT-PCR, genome walking, promoter mapping, and EMSA) were designed using primer 3 and oligo analyzer softwares and all the primers were synthesized by Integrated DNA Technologies, Inc. (IDT). The length of the primers used ranged between 22 to 30 nucleotides.

i) Primers used for cloning of *Hex 82*:

S.No	Name of the primer	Sequence (5' ⟶ 3')
Cloning of partial fragment		
1	Hex 82 PF FP	CAR MTN BTN RCH CGY TAY TAY ATG G
2	Hex 82 PF RP	CTG TCR TAY TTR GGN GCY ARG AAG A
3	Hex 82 GS FP	CTC GCT ACT ACA TGG CGC GTC
4	Hex 82 GS RP	GCT TGG CCG CTT CGC TCT TAA C
RACE		
5	Hex82-Hex5' GSP1	TGA AGC CAA GTT CTG GAA TCT CAC C
6	Hex82-Hex5' GSP2	CAG ACG CGC CAT GTA GTA GCG AG
7	Hex82-Hex3' GSP1	AGA GCG TGT TAG TAC AAC GTC CG
8	Hex82-Hex3' GSP2	GTT AAG AGC GAA GCG GCC AAG C
Cloning of full length cDNA		
9	Hex 82 full FP	ATG GGT CGA ATT GGA CTT CCT G
10	Hex 82 full RP	TTC CGT ATA TGC TGG TAC CTG TG

ii) Primers used for cloning and validation of reference genes:

Degenerate primers		
S.No.	**Name of the primer**	**Sequence (5' ⟶ 3')**
1	18S rRNA FP	GAG AAA CGG CTA CCA CAT CCA AG
2	18S rRNA RP	TAC GGT TAG AAC TAG GGC GGT ATC
3	Act FP	GAT CTG GCA CCA CAC CTT CTA C
4	Act RP	TTC TGC ATC CTG TCG GCG ATA C
5	βtub FP	TAY CCY GAC AGA ATH ATG AAC AC
6	βtub RP	GCC TGA ACA TAG CGG TGA ACT
7	EF1α FP	GCC TGA ACA TAG CGG TGA ACT
8	EF1α RP	CAA TGT GRG CTG TGT GGC AAT C
9	GAPDH FP	WGC WAT RTT TTC HCK RTT CCA WG
10	GAPDH RP	GCR GCR TCR AAG ATK GAM GAG TG
11	rS7 FP	GAR ACM AAC TCH GAC CTB AAR GC
12	rS7 RP	ACT TCR CGN CCY GTY ARC TTC
13	TBP FP	CWT TRC ATC AAC CWG AAG ARG AYC
14	TBP RP	CCR TGT GTW AGT ACT AAR CCT TC

Gene specific primers for reference genes		
S.No.	**Name of the primer**	**Sequence (5' ⟶ 3')**
1	18S rRNA GS FP	AAC GGC TAC CAC ATC CAA GGA AG
2	18S rRNA GSRP	TAC GGT TAG AAC TAG GGC GGT ATC
3	Act GS FP	GAT CTG GCA CCA CAC CTT CTA C
4	Act GSRP	TTC TGC ATC CTG TCG GCG ATA C
5	βtub GS FP	GGT GTT CAT GAT TCT GTC AGG ATA
6	βtub GSRP	GCC TGA ACA TAG CGG TGA ACT G

7	EF1α GS FP	CTG GTG AGT TTG AGG CCG GTA TC
8	EF1α GSRP	CAA TGT GGG CTG TGT GGC AAT CG
9	GAPDH GS FP	TCT CTG AGA GGG ACC CCA AGG CTA
10	GAPDH GS RP	CGG CGT CGA AGA TGG ACG AGT GG
11	rS7 GS FP	GAG ACA AAC TCT GAC CTG AAG GCC
12	rS7 GS RP	ACT TCG CGG CCT GTT AGC TTC TTG
13	TBP GSFP	CTT TAC ATC AAC CAG AAG AGG ACC
14	TBP GS RP	CCG TGT GTT AGT ACT AAG CCT TC

qRT-PCR primers		
S.No.	**Name of the primer**	**Sequence (5' ⟶ 3')**
1	18S rRNA rt FP	ATT AGA GTG CTC AAA GCG GGC TCA
2	18S rRNA rtRP	TGC TTT CGC TGA TGT TCG TCT TGC
3	Act rt FP	ACT CTG CTA TGT TGC CCT CGA CTT
4	Act rtRP	TGG AGT TGT ACG TCG TTT CGT GGA
5	βtub rt FP	GCT TCC GCA CAC TGA AAT TGT CCA
6	βtub rtRP	TTG ACC AGG GAA CCT AAG GCA AGT
7	EF1α rt FP	CAT TGA GGC CCT TGA TGC CAT CTT
8	EF1α rtRP	TGG CAC TGT ACC AAT ACC ACC GAT
9	GAPDH rt FP	TCG ACG GAC CTT CTG GCA AAC TAT
10	GAPDH rt RP	ACG GAA GGC CAT ACC TGT TAG CTT
11	rS7 rt FP	AGC TGG ACG GTT CAC AAC TCA TCA
12	rS7 rt RP	TTC GCG GCC TGT TAG CTT CTT GTA
13	TBP rtFP	AGG CTC TTC ACC ACT CGT TGG ATT
14	TBP rt RP	TGA CTA AGC ATT TGT TGC CCT GCC

iii) Primers used for developmental profile and hormonal regulation studies:

Hexamerins qRT-PCR primers		
S.No.	**Name of the primer**	**Sequence (5' ⟶3')**
1	Hex 82 rt FP	ATT CCCT GTG AGA CCC GAC CAT TT
2	Hex 82 rt RP	TTG AAT CCG GGC TGG TAA TGC TGA
3	Hex 84 rt FP	TGT ACA TTC CCG CTG CTT TGG ACT
4	Hex 84 rt RP	ACC TTG ACG CCA ACG TAA TGG AGA
5	Hex 86 rt FP	TCA CCC TTC ACC TTG AAC GTC ACT
6	Hex 86 rt RP	TGG TGG AGG TAA GAC GAT GCC TTT

iv) Primers used for genome walking, promoter mapping and electrophoretic mobility shift assay (EMSA):

S.No	**Name of the primer**	**Sequence (5'⟶ 3')**
Genome walking		
1	Hex84 GW-GSP1	GTA TTG TGG ATA GGA TCC AAC AAC
2	Hex84 GW-GSP2	GCT AAG ATC AGG ACA GTC TTC ATT GTC
Promoter mapping		
5	E74a	GCT AGC CCA ATA TTC CGG GAT CTG C
6	Ecr	GCT AGC AAT TCA TTT TAA ATA AAT AAA ACA TAG GAG
7	SGF1	GCT AGC GGA GAA TAT AAC GTA TTT ATT ATA ATA ATA TCA
8	CREB	GCT AGC ACC TCC AGC ACG TCA TCG GAG
9	RXR	GCT AGC ACT CTC ACC TGG TAT GAC CTT GCA G
10	Zeste	GCT AGC GTA GGC ACT CAC CAA GTG A

11	Fox1a	GCT AGC TTA CCC TTA TTT ATA AAT TAT GGT ATA CAG
12	TATA Box	GCT AGC GCG GAG ATA ATG AGC GGT ATA TA
13	PRM RP	CTC GAG CAG TCT TCA CCT GTC ACC AT
14	ECRlucFP	GCT AGC ATT CAT TTT AAA TAA ATA AAA CAT AGG A
15	ECRMUT lucFP	ATT CAG CGC CGG CGC GGC CGC TGT AGG AG
EMSA		
16	ECR FP	ATT CAT TTT AAA TAA ATA AAA CAT AGG AGG TC
17	ECR RP	CTC CTA TGT TTT ATT TAT TTA AAA TGA ATG TC
18	ECRMUT FP	ATT CAG CGC CGG CGC GGC CGC TGT AGG AGG TC
19	ECRMUTRP	CTC CTA CAG CGG CCG CGC CGG CGC TGA ATG TC

Experimental insects:

***Corcyra cephalonica* (Stainton):**

Corcyra cephalonica is commonly called as rice moth and it belongs to the order Lepidoptera and family Galleridae. It is a serious pest of stored cereals such as wheat, rice, sorghum, maize, millet, etc. in tropical and sub-tropical regions of the world (Osman, 1984, 1986)

The eggs of *C. cephalonica* were purchased from National Bureau of Agriculturally Important Insects (NBAII, ICAR), Bengaluru. The eggs were hatched in culture troughs containing coarsely crushed sorghum seeds and multi vitamin tablets. The cultures were maintained at 26±1 ^{0}C, 60±5 % relative humidity (RH) and 14:10 h light:dark (L:D) photoperiod. The larval development proceeds through five instars and is completed in about 45-50 days followed by the pre-pupal stage extended over 4-5 days which is then followed by a pupal stage which lasted for 7-8 days. The adult moths normally survived for 8-10 days.

Stages of insect used for experiments:

For the present study, all the stages of insect i.e. embryo, larva, pre-pupa, pupa, and adult were used. Further, the last instar larva used were distinguished based on their body weight and the size of the head capsule (Lakshmi and Dutta-Gupta, 1990). The larvae weighing 56-65 mg with head capsule size of 0.8-0.95 mm were categorized as early last instar (ELI). Larvae weighing 66-75 mg with a head capsule size of 0.96-1.03 mm were classified as mid last instar (MLI) and those weighing 76-85 mg with a head capsule size of 1.03-1.08 mm were classified as late last instar (LLI) (Lakshmi and Dutta Gupta, 1990). Insects collected during the wandering stage were designated as pre-pupae (PP).

Surgical manipulations:

(i) Thorax-ligation: It was carried out using the LLI larva to deplete or reduce the endogenous hormone titer (Dutta-Gupta and Ashok, 1998). The LLI larvae to be ligated were narcotized for 10 min by placing them on ice. Ligation was done behind the first pair of prolegs by slipping a loop of silk thread around the head of the larvae (Ashok and Dutta-Gupta, 1991). The loop was adjusted behind the first pair of prolegs and gradually tightened. The region anterior to ligature was cut with sterile scissors and wound was dressed with traces of antibiotic mixture (1:1, penicillin and streptomycin) and phenylthiourea (0.025%). Finally it was sealed with wax (paraffin and beeswax, 10:0.1). These ligatures (isolated abdomen) were kept in petridishes covered with moist filter paper to maintain humidity and to prevent desiccation.

(ii) Microinjection: The appropriate larvae to be microinjected were placed on ice for narcotization and were injected with hormone or radioisotope methionine [^{35}S] or carrier solvent using a microsyringe (5 µl). The wound was dressed with traces of antibiotic mixture (penicillin and streptomycin) and phenylthiourea (0.025%). It was sealed using wax (paraffin and beeswax, 10:0.1).

Hormone treatment:

A stock solution of 20E was prepared by dissolving 1 mg of hormone in 100 µl of ethanol, which was finally diluted to 1 ml with distilled water. This was aliquoted into small volume and stored at -20^{0}C until use. This stock solution was further diluted as per requirement. The final concentration of ethanol in working 20E solution never exceeded 0.05% in any of the experiments. The LLI larvae were injected with 80 nM 20E, after the required period of thorax-ligation. Control insects received equal volume of carrier solvent (0.05%

ethanol). The insects were sacrificed after the required period of hormone treatment and the fat body was dissected in cold insect Ringer solution. Homogenates were prepared and used for protein estimation and other studies.

Organ culture:

Fat body dissected out under sterile condition in ice cold insect Ringer and were rinsed in 200 µl of TC-100 insect culture medium (JRH Biosciences, Inc. USA) with traces of streptomycin sulfate and penicillin (1:1). The fat body was rinsed once again in sterile TC-100 medium without antibiotics and then cultured in 200 µl of fresh medium at 27ºC for 1 h preconditioning prior to the required experimental set up. In hormonal studies 20E (80nM) was added to the *in vitro* cultures of fat body for various time periods. For radio-labeling studies, 10µCi of [^{35}S] methionine was added to the experimental sample during the last 1 h of each incubation period, whereas to the control samples an equal volume of the solvent carrier and isotope had been added. Depending on the requirement of a specific experiment, the tissue was incubated in the culture medium for various time periods ranging from 1-12 h at 27ºC. After required incubation time, tissues were rinsed in ice cold insect Ringer and processed for homogenate preparation.

Collection and preparation of hemolymph sample:

The larval stage of interest was narcotized on ice. The proleg was cut with a fine scissors and the oozing hemolymph was collected with the help of 20 µl micro pipette, into microfuge tube pre-coated with 0.025% phenylthiourea in order to prevent tyrosinase activity and melanization. These hemolymph samples were diluted (1:50) with 10 mM Tris-Cl (pH 7.4) and were centrifuged at 1,000 g for 5 min at 4°C to sediment the hemocytes. The supernatant i.e. the cell free hemolymph samples were used either immediately or stored at -20ºC.

Preparation of tissue homogenate:

Desired tissues were dissected from the appropriate developmental stage or the ligated/unligated larva treated/injected with hormones, isotopes etc., in cold Ringer solution. Homogenization was carried out in 200 µl of homogenization buffer (10 mM Tris-Cl pH 7.4, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT) and homogenates were centrifuged at 10,000 rpm for 5 min for removal of larger debris. The aliquots of the supernatant were used for various studies such as protein estimation, protein profiling etc.

Genomic DNA isolation:

The genomic DNA from total body of LLI larvae was isolated as described in Birren *et al*., (1997), with slight modifications. The narcotized larvae (~1.0 g) pulverized in a mortar half filled with liquid nitrogen. The pulverized tissue was transferred to a centrifuge tube. To this, sucrose-proteinase K cell lysis buffer (27% sucrose, 1X SSC, 1 mM EDTA, 1% SDS and 200 µg/ml proteinase K) was added (1 ml/100 mg tissue) and mixed thoroughly. The lysate was incubated overnight at 37 ºC for the release of chromosomal DNA. The viscous lysate was transferred to a fresh tube to which, 10 ml of equilibrated phenol, chloroform and iso-amyl alcohol (25:24:1) mix was added, followed by centrifugation at 6,000 g for 10 min at room temperature. The upper aqueous layer was carefully collected and re-extracted with 5 ml of equilibrated phenol, chloroform and iso-amyl alcohol (25:24:1) mix. The upper aqueous phase was again collected, to which equal volume of chloroform was added, mixed thoroughly and centrifuged at 5,000 g for 10 min at room temperature to remove the residual phenol from the aqueous phase. To the aqueous phase, $1/10^{th}$ volume of 3 M sodium acetate was added and mixed thoroughly. Equal volume of iso-propanol was added to this mixture and incubated for 30 min at -20°C. The DNA was

collected after centrifugation at 12,000 g for 15 min at room temperature. The DNA pellet was washed twice with 70% ethanol and air dried. The genomic DNA was dissolved in 200 µl of TE buffer [10 mM Tris Cl (pH 8.0) with 1 mM EDTA] and stored at 4ºC until use.

RNA isolation:

The desired tissue was dissected under sterile condition in RNase free insect Ringer rinsed in the same solution and homogenized (100 mg tissue) in 1 ml of TRI reagent (Sigma Chemical co). The homogenate was allowed to settle for 5 min at room temperature followed by addition of 200 µl chloroform and incubated for 10 min at room temperature. The mixture was centrifuged at 13,000 rpm for 15 min at 4°C. The upper aqueous phase that contained RNA was collected carefully, to which equal volume of isopropanol added, mixed thoroughly and incubated at -80°C for 1 h. The RNA pellet was collected after centrifugation at 12,000 g for 20 min at 4ºC. The pellet was washed thrice with 70 % ethanol prepared with DEPC water. The pellet was air dried and dissolved in 20 µl of diethyl pyrocarbonate (DEPC) treated water and stored at -70ºC until further use. During the entire preparation, care was taken to avoid RNase contamination by treating the solutions, plastic as well as glassware with DEPC followed by sterilization. This protocol was adopted from the manufacturer (Sigma Chemical Co.). The purity and quantity of RNA was checked using Nanodrop (ND-1000) spectrophotometer.

Agarose gel electrophoresis:

i) DNA electrophoresis: An estimated amount of plasmid DNA or genomic DNA (1-2 µg), was mixed with 6X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) and loaded into the 0.8 or 1% agarose gel for electrophoretic separations. The gel was polymerized using 1X TAE. The electrophoresis was carried out

using the same buffer at voltage 5V/cm^2 until the dye reached 3/4th of the length of the gel. After running, ethidium bromide gels were visualized using UV-transilluminator.

ii) RNA electrophoresis: The RNA sample (1-2 µg) was prepared by mixing 12.5 µl formamide, 2.5 µl 10X formaldehyde gel buffer and 4 µl formaldehyde in a total volume of 25 µl. The mix was heated at 65 °C for 5 min followed by cooling on ice for 5 min. To this, 2.5 µl of gel loading dye (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was added and used. The RNA samples were electrophoresed on 1.2% agarose formaldehyde denaturing gel (Sambrook *et al*., 2001). The agarose was dissolved in DEPC treated sterile distilled water. It was boiled at 100 °C and cooled till 55-60 °C. To it, 1X formaldehyde gel buffer and formaldehyde (17 ml/100 ml) were added along with ethidium bromide (0.5 µg/ml). This was followed by polymerization of 10 cm long gel on horizontal gel electrophoresis system. The electrophoresis was carried using 1X formaldehyde gel buffer at voltage 5 V/cm^2 until the dye reached the 3/4th of the gel. The ethidium bromide stained gels were visualized using UV-transilluminator.

First strand cDNA synthesis:

The first strand cDNA was synthesized using 3 µg of total RNA. Total RNA, 1 µl of oligo(dT) (50 µM), 1 µl of dNTP (10 mM) mix and nuclease free water were added to make a final volume of 10 µl in a PCR tube. The tube was incubated at 65 ºC for 5 min and was then placed on ice for 2 min. To this tube, a mix of 2 µl 10 X RT buffer, 4 µl of $MgCl_2$ (25 mM), 2 µl of DTT (0.1 mM), 1 µl of RNase OUT (40 U/µl) and 1 µl of Superscript III RT (200U/µl) were added to make a 20 µl reaction mixture. The mixture was incubated at 50°C for 50 min and later the reaction was terminated at 85ºC for 5 min. To this, 1 µl of RNaseH (2 U/µl) was added and incubated at 37ºC for 20 min. The

synthesized cDNA was stored at -20ºC until further use. This protocol was adopted from the manufacturer (Invitrogen Life Technologies).

Purification of hexamerins:

The hexamerins were purified primarily from the hemolymph collected from the last instar larva of C. *cephalonica* however for two experiments they were also purified from the hemolymph collected from early larval instars. The hemocyte free diluted hemolymph (1 mg protein/50 µl) was passed through a Sephadex G-100 column (1.5 x 60 cm) equilibrated with 10 mM Tris-HCl (pH 7.4) at room temperature. The protein was eluted with the same buffer at a flow rate of 1 ml/2 min till the absorbance of the elutants at 280 nm reached 0.002. Fractions having protein was checked by SDS-PAGE. The fractions that contained hexamerins were pooled and loaded on to ion-exchange DEAE Sephacel column (1.25 x 25 cm) pre-equilibrated with 10 mM Tris-HCl (pH 7.4). The bound hexamerins were eluted with a linear gradient of 0-0.5 M NaCl. The peak fractions were pooled and analyzed on 7.5% resolving SDS-PAGE for purity.

Immunoprecipitation:

For this study 24 h post thorax ligated LLI larva were treated with 20E (80nm) for different time periods (6 or 12 h) and they were injected with 10 µCi of [^{35}S] methionine during the last 2 h. Controls received equal volume of carrier and incubated for same period (either 6 or 12 h). Fat body was dissected from injected insects and rinsed thoroughly in insect Ringer containing 0.1 % Triton X-100, for removal of any residual proteins that were attached to it. It was then homogenized, quantified and equal amount of protein was loaded on to the gel. The gel was electrophoresed, stained, and vacuum dried and exposed to the X-ray film and autoradiogram was developed. In the continuing experiment, the hemolymph samples collected from above set of insects were immunopreicipitated with

the hexamerin antibodies and immunoprecipate was analyzed using SDS-PAGE and autoradiography.

Protein estimation by Bradford assay method:

Protein concentration of various samples was estimated using the micro-protein assay method of Bradford (1976). Bovine serum albumin was used for preparation of standard.

(i) Preparation of protein reagent: Brilliant blue G-250 (10 mg) was completely dissolved in 5 ml of 95% ethanol. To this solution, 10 ml of 85% (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with double distilled water, filtered through Whatman No. 1 filter paper and stored in an amber colored bottle at 4 $^{\circ}$C till further use.

(ii) Procedure for protein estimation: An aliquot of the sample was taken into a tube and the volume was adjusted to 0.1 ml with 10 mM TrisCl (pH 7.4). To this 1 ml of protein reagent was added and mixed. After 10 min, absorbance at 595 nm was measured spectrophotometrically against blank solution. The protein content in the sample was calculated using a standard curve prepared using BSA.

Protein profile:

(i) Polyacrylamide gel electrophoresis (SDS-PAGE): Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure of Laemmli (1970), using 1 cm long, 2.1 % stacking gel (pH 6.8) followed by a 7.5 cm long, 10% resolving gel (pH 8.8) for hexamerins. Tris-glycine (25 mM Tris and 192 mM glycine, pH 8.3) with 0.1% SDS was used as the electrode buffer. Electrophoresis was carried out at 80 V for stacking and 100 V for resolving gel until the tracking dye reached 1 cm above the base of the resolving gel. The samples were prepared by mixing

an aliquot of the protein sample with sample buffer containing 0.125 M TrisCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue followed by boiling at 100ºC for 3 min.

(ii) Visualization of electrophoretically separated proteins on polyacrylamide gels:

(a) Coomassie staining: This was carried according to the method of Wilson, (1983). The gel was incubated for staining in commassie solution (0.025% Brilliant blue- R 250 in 40% methanol and 7% acetic acid) for 1 h. To visualize the reversible binding of stain to proteins, destaining with 5% methanol and 7.5% acetic acid was done to remove background staining.

(b) Silver staining: This was carried according to the procedure of Blum *et al.*, (1987). The gel was incubated in fixative (50% methanol, 12% acetic acid and 0.05% of formaldehyde) for 1 h to 4 h followed by three washes in 50% ethanol. Subsequently the gel was sensitized with sodium thiosulphate (20 mg/100 ml) for 1 min and rinsed thrice (30 sec each) with distilled water. The gel was incubated in silver nitrate solution [0.2% $Ag(NO)_3$ with 0.076% of formaldehyde] with gentle agitation for 30 min. The incubated gel was rinsed with distilled water and developed with 6% sodium carbonate (w/v) containing 0.05% of formaldehyde (v/v). Once the proteins were visualized on the gel, staining reaction was stopped by adding 12% acetic acid. Finally, the stained gel was thoroughly rinsed with distilled water and stored in 50% methanol.

Western blotting:

The electrophoretically separated proteins were transferred to nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad) according to the procedure of Towbin *et al.*, (1979). In this, the gel was first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 10 min, followed by transfer to the membrane for overnight at 30 V

with 250 mA current limit. The transfer of protein to membrane was checked by reversible Ponceau S staining (1 mg/ ml of 5% acetic acid). The stain was removed by 3-4 washes with TBS [Tris buffered saline, 10 mM Tris-Cl (pH 7.4), 150 mM NaCl]. For immunostaining, the protein blot was incubated with 5% skim milk powder (w/v) in TBS for 4 h at room temperature to block the non-specific binding sites followed by washing (15 min x 3 changes) with TBST (TBS with 0.02% Tween 20). The blot was then incubated with the primary antibody diluted in TBS containing 5% skim milk powder (w/v) for 4 h to overnight. This was again followed by a thorough wash with TBST (15 min x 3 changes). Thereafter, the blot was incubated with alkaline phosphatase (ALP)/ Horse radish peroxidase (HRP) conjugated anti-mouse or antirabbit IgG for 2 h. Once again, the blot was washed using TBST (15 min x 3 changes). The blot was further processed with BCIP/NBT (ALP) or enhanced chemiluminiscence (ECL) substrate (HRP). The blots were imaged with Kodak Photo Imager.

Real-time PCR analysis:

The purity and quantity of RNA was checked by using nanodrop spectrophotometer (ND-1000). 3 µg of total RNA was used for cDNA preparation using Superscript III^{TM} first strand synthesis kit (Invitrogen). PCR reactions (20µl) were performed using Power SYBR Green Master mix (Applied Biosystems). Reactions were set up using respective primer sets and real-time PCR was carried out using Fast 7500 Real time PCR system (Applied Biosystems). PCR conditions were optimized to generate >95 % PCR efficiency. Dissociation curve analysis was performed after the last cycle to confirm amplification of a single product. The real time results are expressed as change in expression relative to control using target gene. C_t values were normalized to that of internal control gene, Ct values based on the $2^{(-\Delta\Delta C(T)}$ method (Livak and Schmittgen, 2001).

Genome walking for isolation of 5' upstream elements:

Genomic DNA was isolated from whole body of LLI larva and four separate aliquots of genomic DNA (25 µg) were completely digested with four different restriction enzymes *Eco*RV, *Pvu*II, *Dra*I and *Stu*I that leave blunt ends. Digested DNA was purified using phenol chloroform method. Each digested genomic DNA was then ligated separately to the Genome Walker Adaptor. These uncloned adaptor ligated genomic DNA fragments were referred as Genome Walker "libraries." After the library construction, the primary PCR was carried out using adaptor primer (AP1) provided in the kit and a gene-specific primer 1 (GSP1). The primary PCR mixture was then diluted to 50 times and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specific primer 2 (GSP2). The amplified DNA fragments were cloned into pTZ57R/T vector and sequenced.

Genome walking library was constructed using Universal Genome Walker kit (Clontech) following manufacturer's protocol. *Hex 84* upstream region was isolated using gene specific primer 1 (Hex 84 GW-GSP1) and the adapter primer-1 [provided in the kit (AP1)]. Touchdown PCR was used for the amplification. Cycling conditions were 94ºC for 30 sec, 72ºC for 3 min, 5 cycles, 94ºC for 30 sec, 67ºC for 30 sec, 72ºC for 3 min for 30 cycles. A nested PCR was carried out using gene specific primer 2 (Hex 84 GW-GSP2) and adaptor primer-2 [provided in the kit (AP2)]. All the amplified 5' fragments were cloned into pTZ57R/T vector (Fermentas) and sequenced bi-directionally.

Preparation of PCR based progressive deletion constructs:

Progressive 5' deletion constructs (TATA box, SGF, ECR, FOXL1a, CREB, E74a, ZESTE, RXR and E-BOX) were amplified with Q5 high fidelity enzyme (NEB). Resultant constructs were cloned in *Nhe* 1 and *Xho* 1 sites of pGL3 basic firefly luciferase vector

(Promega). Presence of each construct was confirmed by double digestion and the absence of mutation was determined by nucleotide sequencing.

Transient transfection and luciferase assay:

Sf9 cells (derived from ovarian tissue of *Spodoptera frugiperda*) were maintained at low passage number (n<10) to have 70-80% confluence at the time of transfection. Cells were plated 24 h before transfection in 6 well plates and transfection was carried out with Escort^{TM} IV reagent (Sigma Chemical Co.) following manufacturer's protocol. Reporter gene constructs (Firefly luciferase) and the pRL-TK plasmids (Renilla luciferase) were co-transfected into 80-90 % confluent cell cultures. After 24 h of transfection promoter was induced with hormone 20E. Promoter activation lead to expression of secreted luciferase protein. After 24 h of hormone treatment, cells were collected and washed with PBS (phosphate buffered saline). The cells were lysed by addition of 100 µl passive lysis buffer (provided by Promega). Luciferase reporter activity was determined with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luciferase activity was measured using GloMax®-96 Microplate Luminometer and the promoter activity was expressed in relative luciferase units.

Electrophoretic Mobility shift assay (EMSA):

(i) Nuclear extract preparation: Fat body was excised and homogenized gently in ice-cold PBS. The homogenate was incubated in 0.5 ml of HEPES buffer [10 mM HEPES (pH 7.9)] containing 10 mM KCl, 0.1 mM EDTA, 0.1mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/ml Leupeptin, 2 µg/ml Aprotinin and 0.5 mg/ml Benzamidine for 20 min on ice. Then, 10 % NP-40 was added for lysis of the cells. The sample was vortexed vigorously for 10 sec and was centrifuged for 1 min at 2,000 rpm. The supernatant (cytoplasmic extract) was collected and stored at -70ºC. The nuclear pellet was resuspended in 50 µl of

ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml Leupeptin, 2 µg/ml Aprotinin and 0.5 mg/ml Benzamidine]. The tubes were then incubated on ice for 30 min. resultant nuclear extract was centrifuged for 5 min at 2,000 rpm and was stored at -70ºC for further use.

(ii) Probe preparation: The custom made wild type as well as mutated oligonucleotides which have a - OH group at their 5'-end, were labeled using [γ^{32} P] dATP and catalyzed by T4 polynucleotide kinase. The forward and reverse primers were mixed, boiled for 5 min in boiling water bath and left at room temperature for overnight for the formation of double strand oligo, which was used for [γ^{32} P] dATP labeling. A reaction mixture containing 2 µl of oligonucleotides (final concentration of 2 pmoles), 5 µl of 10 X polynucleotide kinase buffer, 2 µl of [γ^{32} P] dATP (100 µCi/µl) and 4 µl of T4 polynucleotide kinase (10 U/µl) was set to make the final volume of 50 µl with nuclease free water. The reaction mixture was incubated at 37ºC for 30 min. The reaction was stopped by addition of 2 µl of 0.5 M EDTA. The radiolabeled mix was then purified using G-25 Sephadex column to remove any unlabeled oligos. The radioactivity in sample was detected (Cerenkov counting).

(iii) Polyacrylamide gel preparation: A 6 % gel was casted by mixing 6.25 ml of acrylamide:bis acrylamide (29.2:0.80%), 2.5 ml of 10X TBE , 0.4 ml of 10 % ammonium persulphate, 0.05 ml TEMED and 15.85 ml of water, to make up to a final volume of 25 ml. It was allowed to polymerize for 1 h. The gel was pre-run using 1 X TBE buffer at 150 V and later the samples were loaded and separated.

Gel shift assay:

Gel shift assay was performed by incubating 20 µg of nuclear extract with 15 femto moles of [γ^{32} P] end labeled double stranded wild or mutated ERE oligonucleotides in the presence of 0.5 µg of poly dI:dC in a binding buffer [20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1mM DTT, 50 mM KCl, 5 % glycerol, 200 µg/ml of BSA] for 20 min at 37ºC. The formed DNA-protein complex was separated from free oligonucleotide on a 6 % native polyacrylamide gel. 80 nM of 20E was used for induction. For supershift, 1 µg of sheep anti *Chironomous*-ecdysone receptor antibody was incubated with the nuclear extract at room temperature for 20 min prior to addition of radio-labeled probe and was detected using goat anti-rabbit IgG-HRP conjugate. The gel was exposed to film and developed for detection of radioactivity.

Computer assisted analysis:

BankIt tool was used to submit nucleotide sequences of *Hex 82, 18S rRNA, GAPDH, rS7, Actin, β-tubulin, TBP* and *Ef1α*. ClustalW and Multalin programs were used to perform multiple alignment analysis of DNA and protein sequences. BLAST (Basic Alignment Search Tool) algorithm was primarily used for similarity searches in sequence databases. Pymol and Phylip was used for construction and analysis of phylogenetic tree. NetPhos 2.01, NetNglyo 3.1 and NetOglyo 1.0 were used for prediction of phosphorylation and glycosylation sites. geNorm, Normfinder, Bestkeeper tools were used for validation of reference genes. Core Promoter program was used for prediction of the transcriptional start sites and localizing them into 50-100 bp core-promoter regions. MatInspector program was used in finding potential transcription factor binding sites in DNA sequences in addition to that TESS (Transcription Element Search System) and Transfac was also used. ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of

Bioinformatics was used to analyse the protein sequences and structures. VecScreen system from NCBI was used for identification of vector contamination in nucleic acid sequences.

Statistical analysis:

Data were expressed as mean ± SEM (n=3). Significance between groups was analyzed by ANOVA followed by Student New-man-Keuls test using Sigmastat. Values were considered significant at $P<0.05$.

CHAPTER 1

Cloning and characterization of *Hex 82* from *Corcyra cephalonica* last instar larval fat body

Plasma serum proteins in various animals including insects are known to bind variety of molecules varying from hormones to other physiologically important macro- and micro-molecules. In this chapter, we attempt to demonstrate that hexamerins not only act as storage proteins but probably also are carrier proteins, which bind to small molecules like riboflavin.

Cloning of *Hex 82* full length cDNA from fat body *Corcyra cephalonica* LLI larva*:*

The degenerate primers were designed based on other Lepidopteran insect hexamerin sequences present in the data bank, which were neither arylphorin nor methionine rich hexamerins. A partial fragment of 800 bp cDNA was obtained using first strand cDNA prepared from total RNA isolated from LLI larval fat body of *C. cephalonica*. The partial fragment obtained was further confirmed using gene specific primers (Fig. 5A). This partial sequence was used to isolate full length cDNA using RACE (rapid amplification of cDNA ends) strategy with a kit from Clontech Laboratories Inc, USA. The 5' end was amplified using the adaptor specific forward primer provided by the manufacturer and the gene specific reverse primer designed from the 800 bp *Hex 82* cDNA clone. Figure 5B shows the 850 bp amplicon which was cloned and confirmed by PCR. The 3' end was amplified using the adaptor specific reverse primer provided by the manufacturer and the gene specific forward primer designed from the 850 bp partial *Hex 82* cDNA clone. Figure 5C shows the amplification of 760 bp fragment, which was amplified by 3'RACE reaction. After obtaining the three different fragments, attempt was made to clone full length cDNA from LLI larval fat body, using a new set of forward and reverse primers and figure 5D shows the full length cDNA obtained in the present study which was sequenced.

Detailed analysis of sequence (Fig. 6) revealed that the total length of the cDNA is 2.4 kb with an ORF of 2.2kb, which has ATG initiation codon and TAA stop codon. The 5' UTR region consist of 128 bp while 3' UTR region consist of 256 bp with TAATATATA poly-

adenylation signal. *In silico* analysis of deduced amino acid sequence of Hex 82 shows that it has 706 amino acids with molecular weight of 82 kDa. Further Hex 82 deduced sequence also shows the conservation of N, M and C domains of hemocyanin like all other hexamerins. It also has putative phosphorylation and glycosylation sites with an N-terminal signal peptide of 1-18 amino acids. The theoretical pI of deduced Hex 82 protein is 6.04. *Hex 82* nucleotide sequence was submitted as riboflavin binding hexamerin of *C. cephalonica* to the GenBank (Accession No. KF984196). The clustalW analysis of the deduced amino acid sequence of Hex 82 cloned in the present study with two other hexamerins Hex 84 and Hex 86 of *C. cephalonica* reported earlier shows that it has 62% homology with Hex 84 and 54% homology with Hex 86 (Fig. 7). Based on the phylogenetic tree analysis presented in figure 8, we suggest that the Hex 82 from *C. cephalonica* is closely related to hexmerin from *Galleria mellonella.*

Ontogeny and tissue distribution of *Hex* 82:

Ontogeny and tissue distribution analysis was carried out by semi-quantitative and quantitative PCR. Results shows the *Hex 82* is a last larval specific gene (Fig. 9 A, B) and is exclusively expressed in fat body (Fig. 9 C). The results further show that *Hex 82* expression begins during early last instar larval development and reaches its highest during the mid-as well as late-last instar development and declines in prepupal stage and reaching a low value of pupal (Fig. 9 B).

Purification of hexamerins from hemolymph:

The hexamerin proteins were purified from hemolymph using two step purification protocol that includes gel filtration and anion exchange chromatography. The crude hemolymph when subjected to gel filtration yielded a fraction which was enriched and contained predominantly hexamerins (Fig. 10A). It was loaded on to DEAE- Sephacel ion

exchanger and eluted using a salt gradient, pure hexamerin proteins obtained (Fig. 10B) and purity was further confirmed by immunoblotting with hexamerin antibodies (Fig. 10C). For purification of the proteins from different developmental stages, the hemolymph collected from fourth and fifth instar larva was used.

Qualitative release of bound riboflavin from hexamerins, by fluorescence spectroscopy:

Based on the sequence similarity of *Hex 82* with riboflavin binding hexamerin precursor protein from *Hyalophora cecropia*, in the present study riboflavin binding properties of larval hexamerins from *C. cephalonica* were analyzed. Heating of purified hexamerin mixture at 85ºC for 5 min resulted in unfolding and precipitation of the protein simultaneously. The released riboflavin in supernatant was separated from the precipitate by centrifuging the solution at 10,000 rpm for 10 min. The supernatant thus obtained was subjected to qualitative estimation of riboflavin by collecting the fluorescence spectra of riboflavin. It has an excitation maxima of 450 nm and emission maxima of 525 nm. Figure 11A shows the release of riboflavin from purified larval hexamerins obtained from LLI larva only, while the hexamerins purified from 4^{th} instar larva failed to release riboflavin (Fig. 11B) suggesting that this property is only associated with hexamerins present primarily in the last instar larval hemolymph.

Homology modeling:

The amino acid sequence alignment revealed 29% identity between the *C. cephalonica* Hex 82 and *Antheraea pernyi* arylphorin (APA). The Ramachandran plot for Hex 82 (Fig. 12A) showed that approximately 97.6% of all amino acids residues were within the generously allowed region and 1.4% of residues were in the disallowed region.

Sequence identity scores and Ramachandran plot statistics for generated Hex 82 model. Scores obtained clearly indicate identity with amino acid sequence of the template, APA used in the present analysis.

Ramachandran plot statistics

	Sequence Identity	Core	Allowed	Generously allowed	Disallowed
Hex 82	29%	89%	8.6%	1.1%	1.4%

Molecular docking:

We used the program Autodock to examine the binding mode of Hex 82 with riboflavin (Fig. 12C). The binding constant and free energy change revealed riboflavin as a potential substrate due to the tight-fit in to the active site of Hex 82. The riboflavin molecule was located within the binding pocket, and adjacent to hydrophobic residues Asp 117, Lys 644, Ile 647 contributed to the formation of hydrogen bonds between the Hex 82-riboflavin complexes (Fig. 12D).

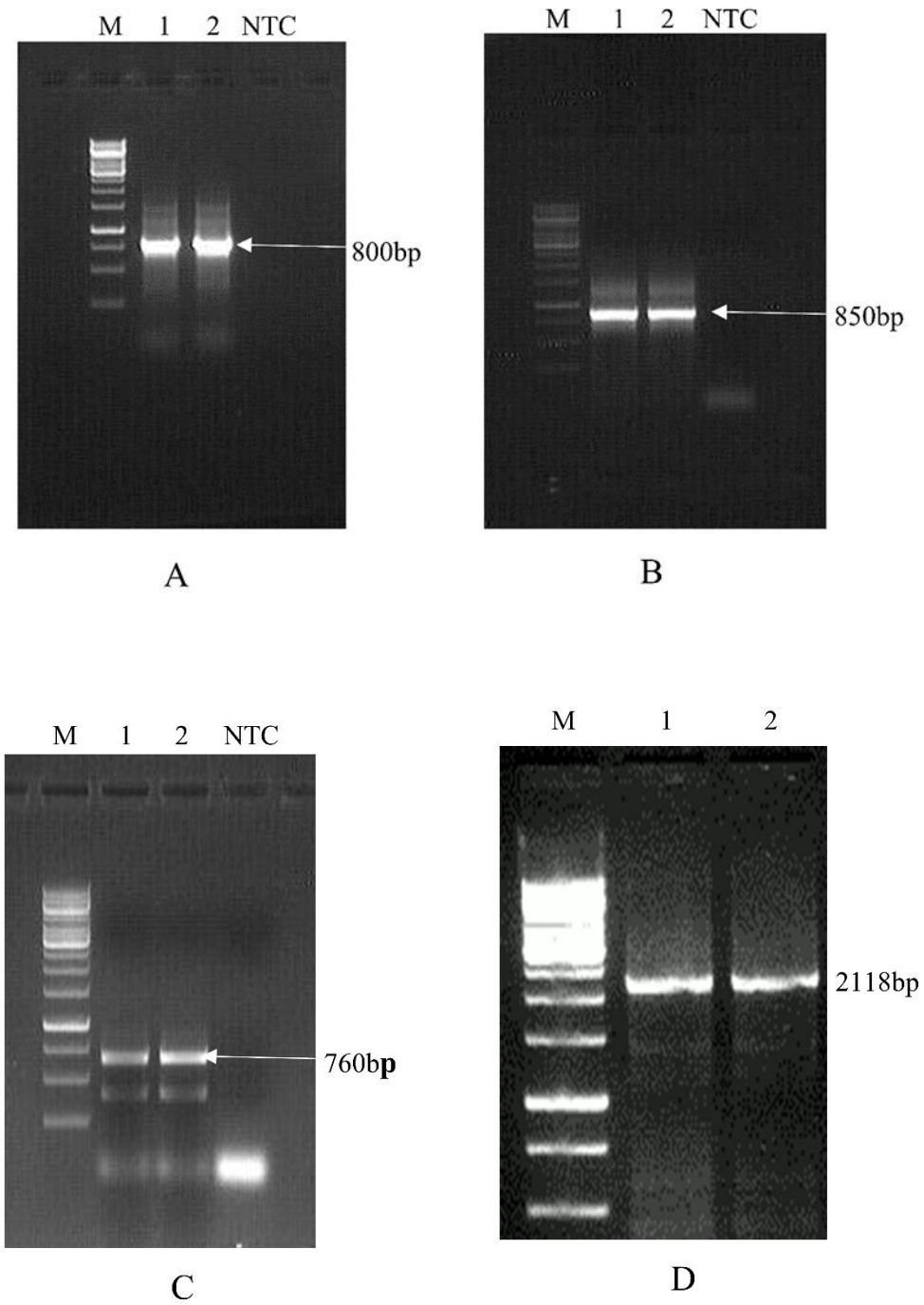


Figure 5: Isolation of *Hex 82* full length cDNA: A: Shows the PCR amplification of 800 bp *Hex 82* partial cDNA fragment with degenerate primers. LLI larva whole body cDNA was used as template. **B:** Amplification of 850 bp fragment using 5' RACE. **C:** 3' RACE amplification of 760 bp fragment **D:** Amplification of 2.1 kb full length nucleotide sequence of *Hex 82*.

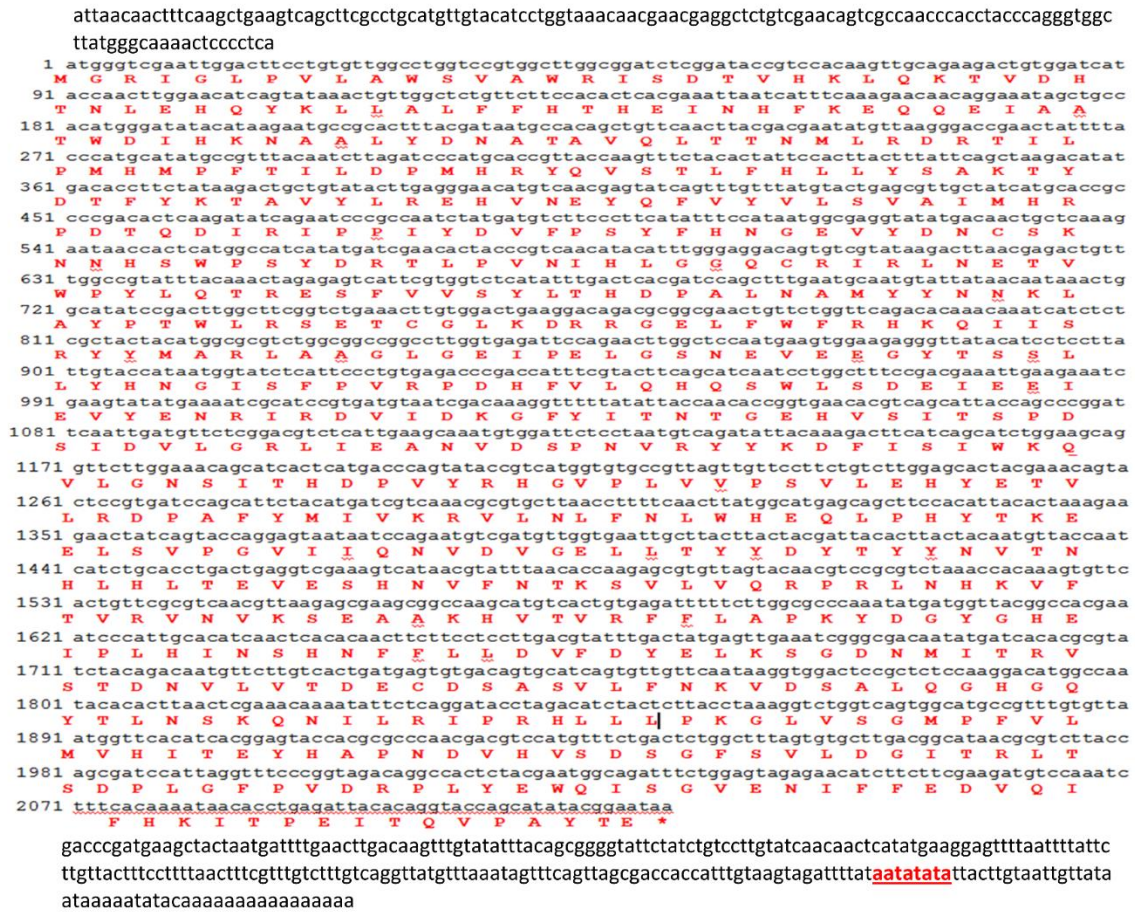


Figure 6: Full length cDNA sequence and deduced amino acid sequence of Hex 82. The 5' UTR and 3'UTR regions are represented in black color, top and bottom. The coding sequence and the deduced amino acid sequence are represented in black and red colors respectively. The poly-adenylation signal sequence (AATATATA) in the 3' UTR region is underlined, submitted to gen bank (Accession No. KF984196).

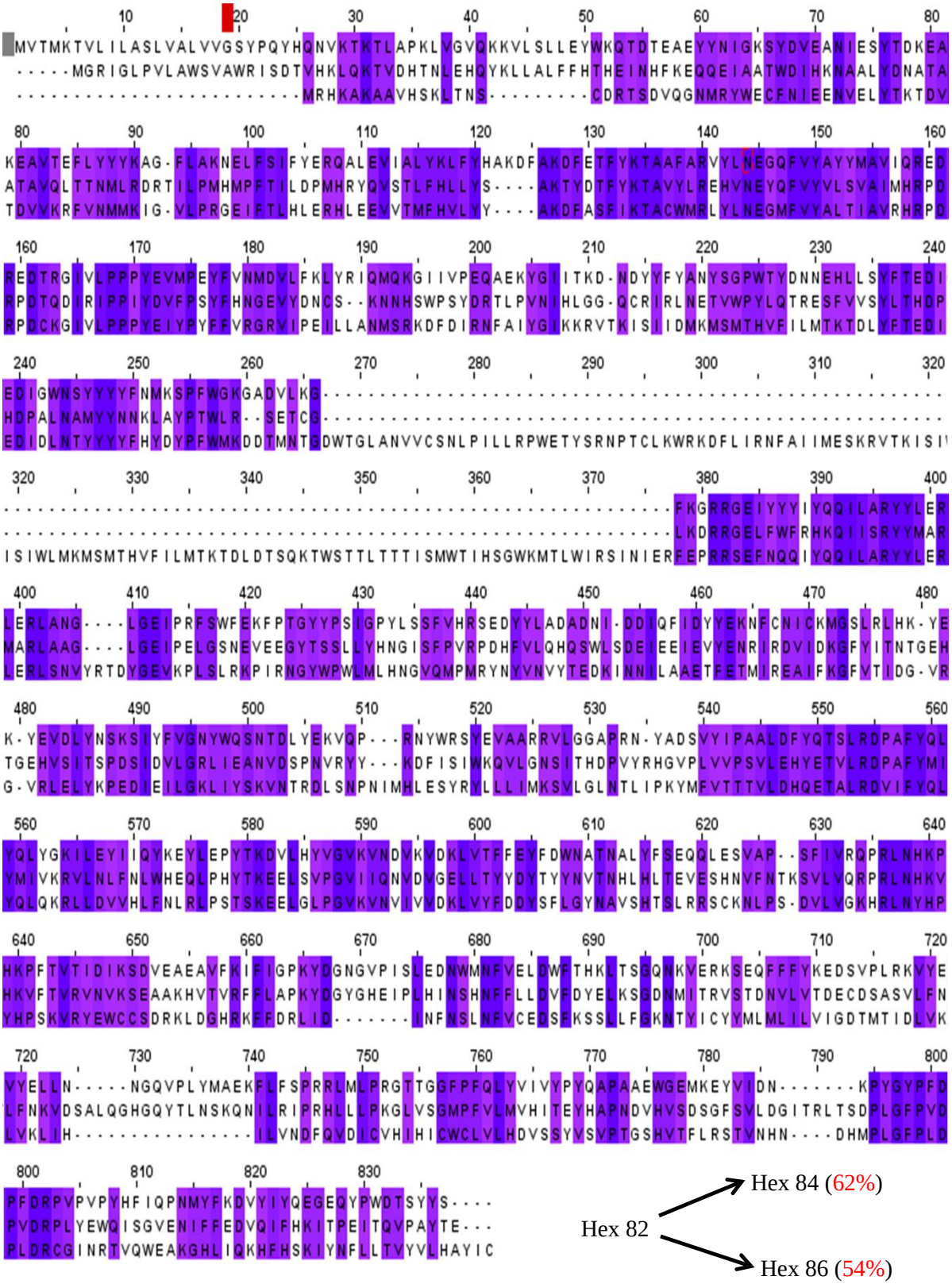


Figure 7: ClustalW alignment of the deduced amino acid sequence of Hex 82 with other two hexamerins (Hex 84 and Hex 86) present in *C. cephalonica*. It shows 62% similarity with Hex 84 and 54% similarity with Hex 86. Row 1: Hex 82, Row 2: Hex 84 and Row 3: Hex 86

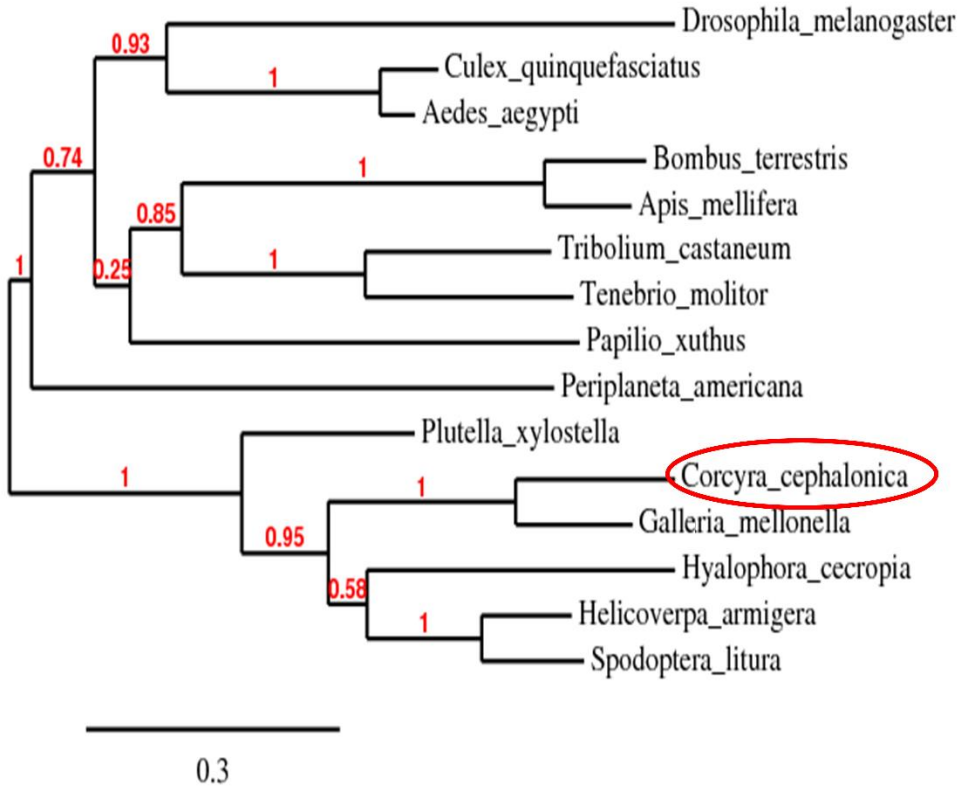
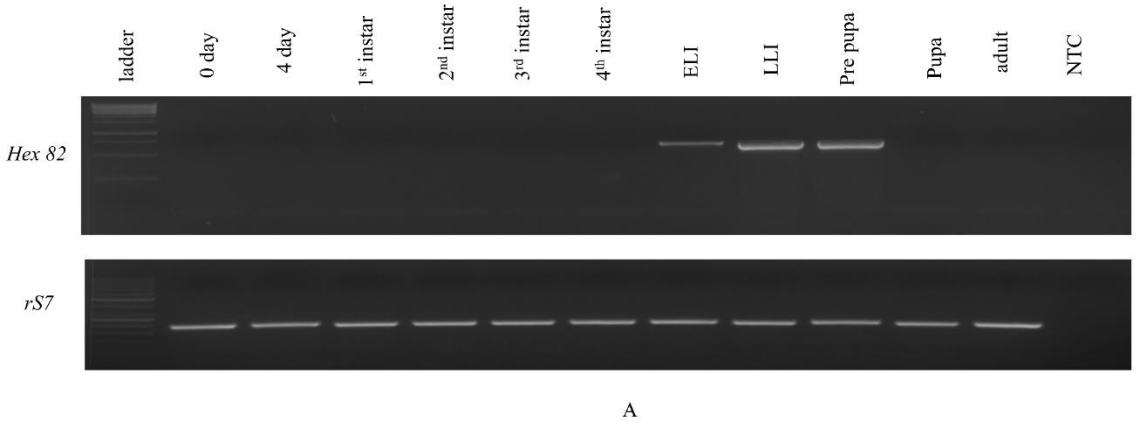
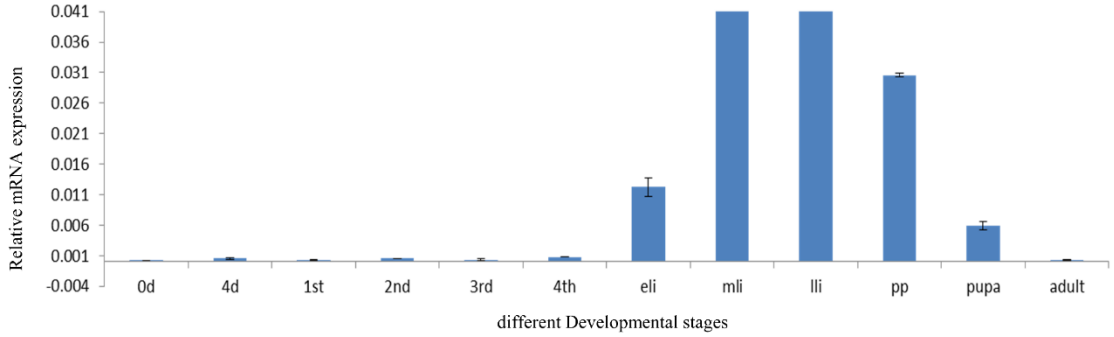


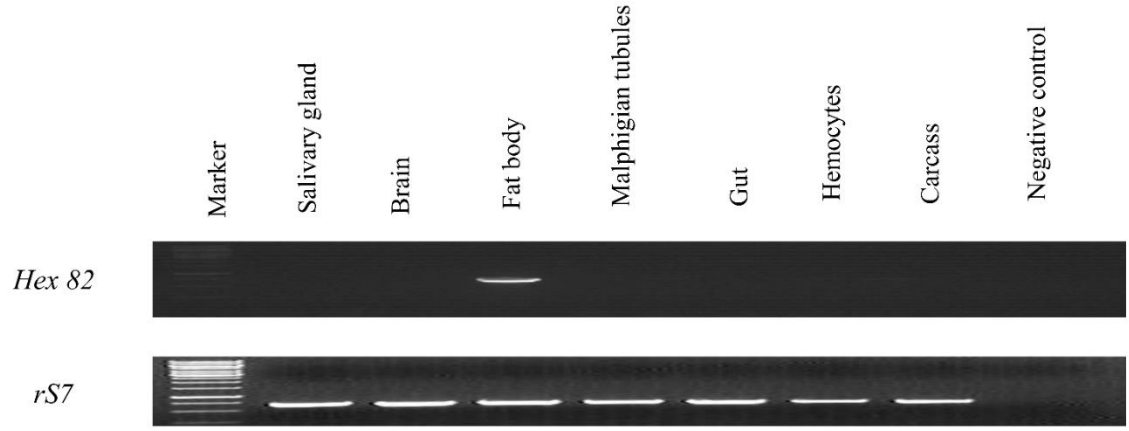
Figure 8: Phylogenetic tree showing evolutionary relationship of non-methionine rich and non-arylphorin hexamerins from various Lepidopteran insects. Hex 82 of *C. cephalonica* sequence has highest homology with *Galleria mellonella* (Accession No. 449954) and both fall in the same clad.



A



B



C

Figure 9: Ontogeny and tissue distribution of *Hex 82*: **A**: Semi quantitative RT-PCR of ontogeny of *Hex 82* confirms the LLI specific expression of *Hex 82*. **B**: qRT-PCR analysis showing expression of *Hex 82* at various stages of last instar larval developement **C:** Semi quantitative PCR shows that fat body is the only tissue which expresses *Hex 82*. *rS7* was used as internal control.

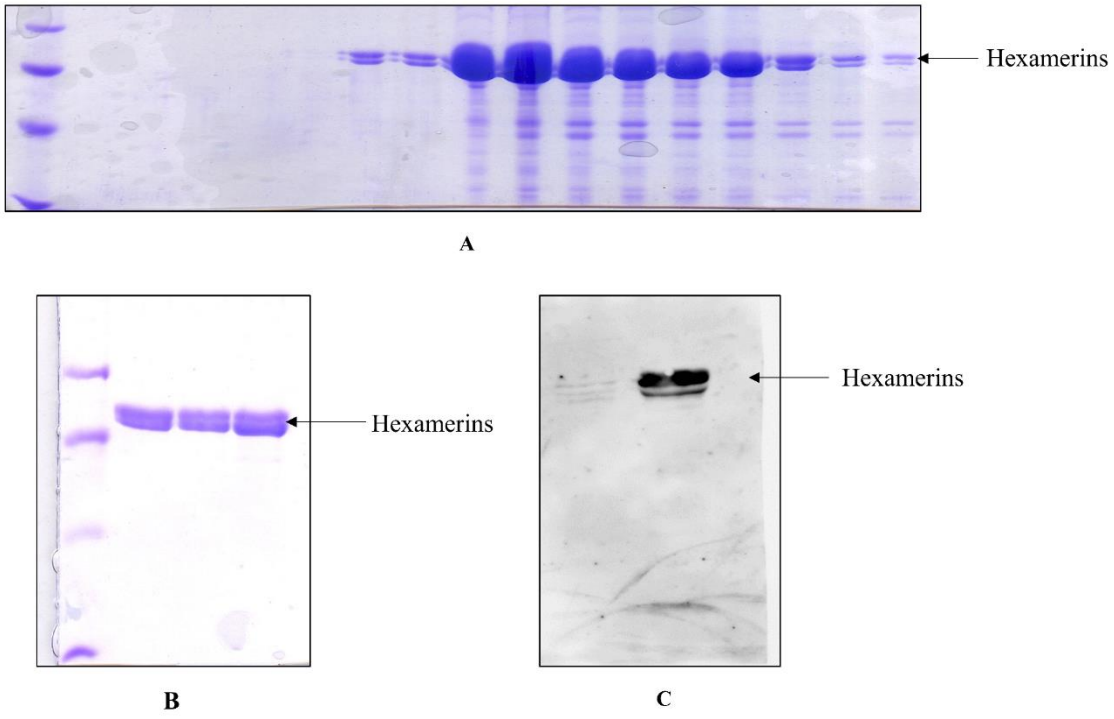


Figure 10: Purification of hexamerins. **A**: Shows the gel filtration fractions having predominantly the hexamerins with other minor contaminats. **B**: DEAE-Sephacel ion exchange elutes having pure hexamerins. **C**: Confirmation of purified hexamerins by immunoblotting using anti hexamerin antibody generated against *C. cephalonica* hexamerin mix.

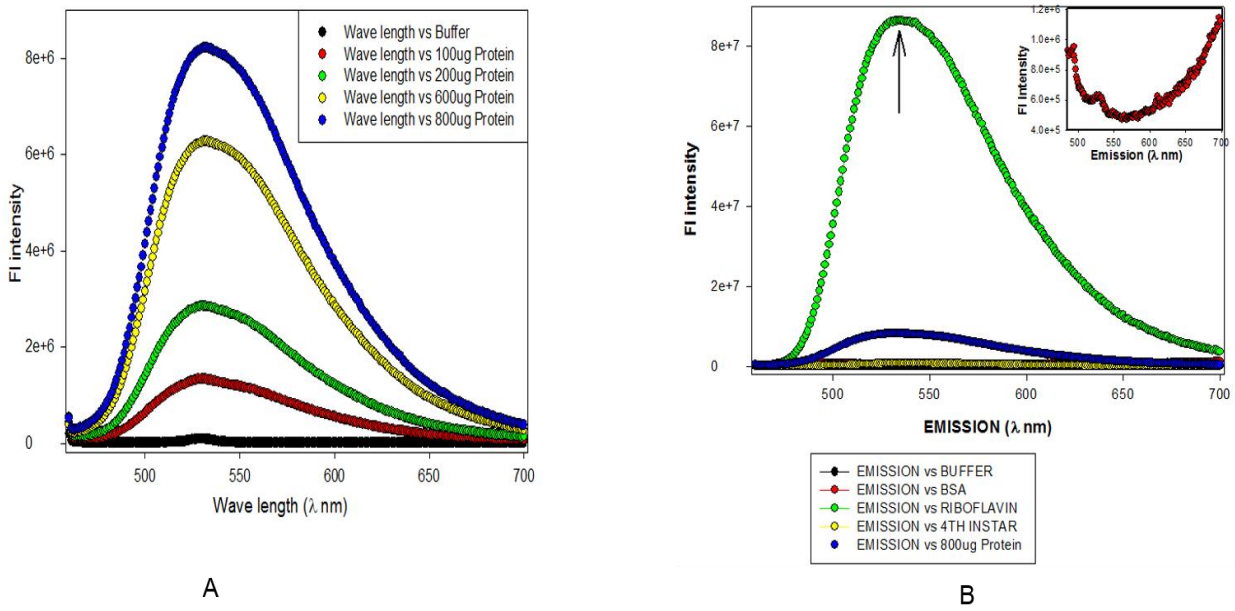


Figure 11: Release of riboflavin from hexamerins (Hex 82). **A:** Shows the qualitative release of riboflavin from purified hexamerins. Different color curves indicate different concentrations of purified hexamerins. **B:** Shows release of riboflavin from hexamerins purified from specific stages (i.e. 4th instar larva and LLI larva). Blue color curve shows the release of riboflavin from purified hexamerins of LLI larva. Yellow color curve shows that no riboflavin is released from purified hexamerins obtained from 4th instar larva.

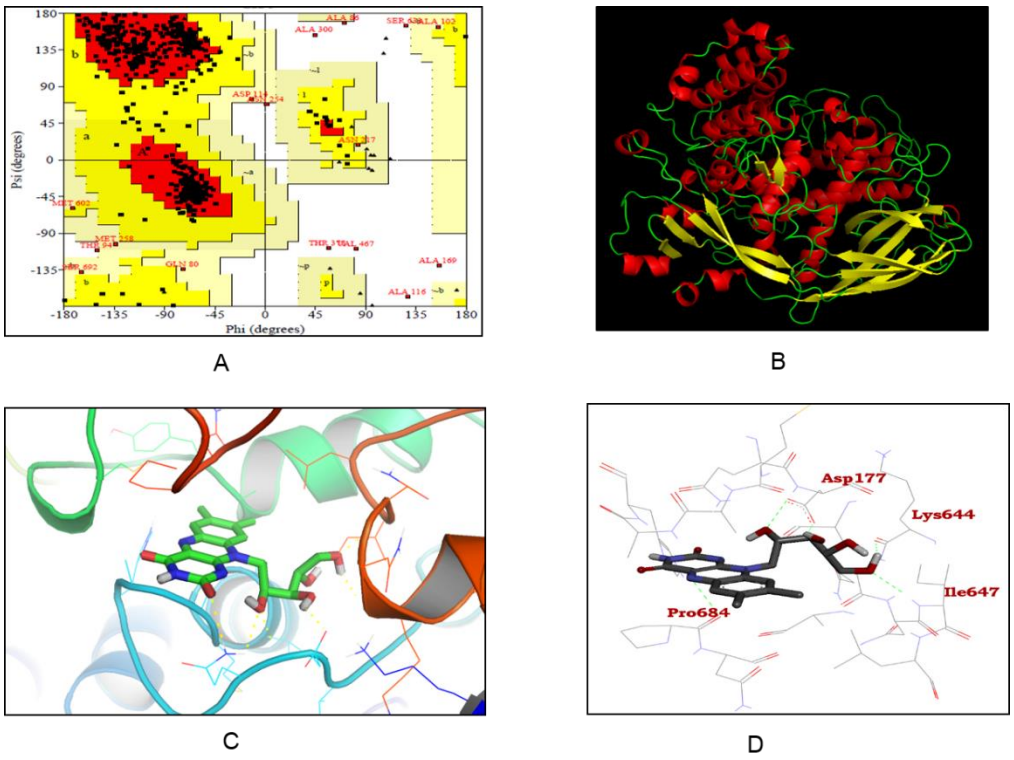


Figure 12: Homology modeling and molecular docking analysis with hexamerins **A:** Ramachandran plot of Hex 82 shows that approximately 97.6% of all residues were within the generously allowed region. **B**: Shows the 3D model of Hex 82**. C:** shows the Hex 82 and riboflavin complex and **D:** shows predicted active site in Hex 82 for riboflavin binding.

CHAPTER 2

Validation of reference gene(s) as internal control for quantitative polymerase chain reaction (qRT-PCR) analysis in *C. cephalonica*

During gene expression analysis, quantification of specific mRNA is carried out using northern blot, microarray and quantitative real time PCR (qRT-PCR). These are the most widely used tools in present day molecular biology for quantification of specific mRNA. qRT-PCR is commonly used because of its accuracy and cost effectiveness; whereas microarray being too expensive and northern blot posing radioactive risks are less preferred. However, this method requires stringent selection of appropriate normalization factors, to account for any errors and differences generated through the multi-step process involved in the generation of cDNA. The use of constitutively expressed genes (house-keeping genes, HKGs) as an internal control or reference genes is widely accepted method for qRT-PCR data normalization. These house-keeping genes are essential for the basic cellular functions and are constitutively expressed in all cells of an organism. Selection of one or more reference genes, also called as internal controls is very important to eliminate the inter-sample variations in analyzing qRT-PCR data. There are two ways to choose a reference gene. One is to use house-keeping genes as reference genes that were commonly used in previous studies by many groups; another is to use the homologous gene of a widely used reference gene in other model species. However few studies have suggested that house-keeping genes are not always expressed stably under all experimental conditions. The α-actin gene which is commonly used as a reference gene, was reported to be highly regulated by ecdysone (Selvey *et al*. 2001) and therefore unsuitable as an internal control in the present study. Other house-keeping genes, like *α-tubulin* and *GAPDH*, were also reported to have varied expression levels in some instances such as different tissues or different developmental stages etc., (Brunner *et al*., 2004; Trivedi and Arasu 2005; Pei *et al*., 2007; Dhar *et al*., 2009; Dong *et al*., 2010; Lord *et al*., 2010; Wang *et al*., 2010; 2012). Jian *et al*., (2008) reported that inappropriate choice of reference genes

produced low precision and misleading results. Therefore, it is necessary to validate the expression stabilities of HKGs, while selecting them as reference genes in qPCR analysis.

The larval forms of holometabolus insects undergo repeated molting primarily to shed rigid cuticular exoskeleton which doesn't allow continuous growth of body size followed by metamorphic development to give rise to pupae and finally transform into adults. Metamorphic hormones (JH and 20E) are known to regulate the molting and metamorphosis in Lepidopteran insects including *C. cephalonica*. Although cytoskeletal proteins actin and tubulin play a major role during postembryonic development, their genes are hormonally modulated during molting and metamorphosis, hence they do not serve as good internal reference controls for quantitative PCR analysis in Lepidopteran insects.

In this chapter, we chose seven distinct constitutive genes (*tubulin, 18S rRNA, TATA binding protein (TBP), actin, rS7, GAPDH,* and *EF1α)* to evaluate their stability during different developmental stages, in different tissues and after metamorphic hormone(s) treatment.

Results:

Cloning of partial fragments of selected house-keeping genes:

Various developmental stages of insects from egg (embryo) to adults were collected and total RNA was isolated. For tissue distribution and hormonal treatment analysis last instar larva was used. The quality and integrity of RNA was checked by Nanodrop and 2% agarose gel electrophoresis. The RNA samples were treated with DNase I prior to first strand cDNA synthesis. Three micrograms of RNA was used for 1st strand cDNA synthesis and the same was used for amplifying partial fragments of *18SrRNA* (680bp), *α-actin* (690 bp), *β-tubulin* (686 bp), *TBP* (TATA binding protein 700 bp), *rS7* (460 bp),

EF1α (720 bp) and *glyceraldehyde -3- phosphate dehydrogenase (GAPDH* 664 bp) (Fig. 13), using the degenerate primers designed based on the reported sequence present in NCBI database. The amplified partial fragment was cloned into pTZ57R/T vector and was sequenced with M13 primers. Once again, the identity of the fragment was confirmed by sequencing using gene specific primers and the sequences were submitted to GenBank (Table 1).

qRT-PCR for selected reference genes:

Based on the partial cDNA fragment sequence cloned from *C. cephalonica*, qRT-PCR primers were designed, and the reactions were carried out using SYBR Green Premix Ex Taq (Takara Bio Inc.) following the manufacturer's protocol using an ABI-7500 fast real-time PCR system (Applied Biosystems). The PCR was carried out in triplicates with 10 µl reaction. The qPCR protocol included an initial step of 95ºC for 30 sec, followed by 40 cycles of 95ºC for 5 sec and then annealed at 60ºC for 30 sec, followed by one cycle of 95ºC for 15 sec, 60°C for 30 sec, and 95°C for 15 sec. A 40-cycle PCR was carried out. Experimental C_t values were used as inputs for geNorm, Normfinder and Bestkeeper softwares analysis for gene stability. The C_t values in qRT-PCR gave an overview of the gene expression variation in the samples. All the seven analyzed house-keeping genes had C_t values ranging between 8-25 cycles, at different developmental stages of *C. cephalonica*.

Expression levels and validation of reference genes during different developmental stages:

Gene expression profiles across different developmental stages of *C. cephalonica* were investigated by qRT-PCR. C_t values were used as inputs for geNorm, NormFinder and Bestkeeper software analysis, which provided the expression stability of given house-

keeping gene. These softwares estimate the gene expression stability by calculating stability measure *M* for a house-keeping gene as the average pair-wise variation for that gene with all other tested house-keeping genes. The genes with low *M* value are the stably expressed genes. In the present study, the stabilities of selected house-keeping genes obtained were: *rS7* (M=1.18) > *EF1α* (M=2.21) > *GAPDH* (M=3.31) >*18S rRNA* (M=4.16) > *TBP* (M=4.30) > *α-actin* (M=4.5) > *β-tubulin* (M=6.0) (Fig. 14). These results suggest that during normal embryonic, postembryonic and adult development, *rS7* is one of the most stable gene, while *β-tubulin* which is frequently used by several groups is the least stable gene in the insect model *C. cephalonica* used in the present study.

Gene expression levels and validation of reference genes for tissue based analysis:

Gene expression pattern of different tissues (fat body, salivary gland, brain, Malphigian tubules, gut, hemocytes and carcass) were studied using LLI larvae of *C. cephalonica*. Expression stability of various genes were analyzed using the above mentioned softwares. Gene stability analysis various tissues were in various tissues were once again carried out using selected house-keeping genes as reference genes. The present study once again suggested that *α-actin* as well as *β-tubulin* are not stable and not suitable to be used as internal control genes, although they are frequently used as internal references in various insect studies.

Although the ranking orders of different genes differed in various tissues, the most stable gene was found to be rS7, while 18S rRNA gene was found to be least stable. M= *rS7* (1.32) > *TBP* (1.86) > *EF1α* (2.49) > *GAPDH* (3.36) >*α-actin* (5.47) > *β-tubulin* (5.985) > *18S rRNA* (6.435) (Fig. 15).

Reference gene stability analysis upon 20E treatment:

To study the effect of 20E on the expression pattern of selected house-keeping genes, we injected 80 nM of 20E to 24 h post thorax-ligated abdomen preparation from LLI larva (as mentioned in materials and methods) and insects were incubated. Treated samples were collected at different time periods, 1, 3, 6 and 12 h of 20E hormone treatment, this was primarily based on the reported circulatory half-life of 20E in various Lepidopteran insect. The results obtained from this analysis surprisingly revealed that 18S rRNA has the least M value i.e. highest stability. The order of gene stability upon hormone treatment was found to be *18S rRNA* (M=1.31) > *TBP* (M=1.41) > *α-actin* (M=3.32) > *β-tubulin* (M=3.36) > *GAPDH* (M=5.00) > *EF1α* (M=6.23) > *rS7* (M=6.73). The present study clearly showed that there is a significant alteration in gene stability order after 20E treatment, suggesting that appropriate reference gene should be selected as the may not be stable in expression in different conditions.

Reference gene stability analysis upon JH analog methoprene treatment:

Methoprene is a widely used JH analog, which mimics the action of naturally occurring hormones in insects. Thorax-ligated isolated abdomen of LLI larva (after 24 h) were topically applied with 10 µl of 0.4 pg/µl methoprene. Treated insects were collected at different time periods (1, 3, 6 and 12 h) and qRT-PCR was carried out to determine the C_t values of the reference genes. Gene stability order after methoprene treatment was found to be fairly different, when compared with untreated normal as well as 20 E treated insects. The stability order obtained was *TBP* (M=1.41) >*β-tubulin* (M=2.11) > *rS7* (M=3.08) > *GAPDH* (M=3.63) > *α-actin* (M=3.72) > *18S rRNA* (M=5.04) > *EF1α* (M=6.73). Further *TBP* showed the lowest M value i.e. highest gene stability upon methoprene application,

suggesting that the expression profile of genes are differentially altered by different hormones.

For a gene to be a good reference gene, the house-keeping gene should fulfill the following criteria: (i) the amplification efficiency should be similar to that of target genes; (ii) the gene should have moderate level of expression; (iii) the expression of the gene should be fairly stable in the given sets. However, it is impossible to identify a house keeping gene that would serve as universal reference gene during various experimental conditions in a given species (Bustin, 2000). Hence, it becomes essential to identify suitable house-keeping genes which could be used as internal reference control for expression analysis of various genes. In the present study, suitable reference gene for *C. cephalonica* under normal and hormone treated conditions were identified and validated. Under normal insect developmental conditions *rS7* is most suitable gene, whereas upon 20E treatment *18S rRNA* was found to be most stable gene, while for methoprene treatment studies, *TBP* was found to be the suitable candidate gene.

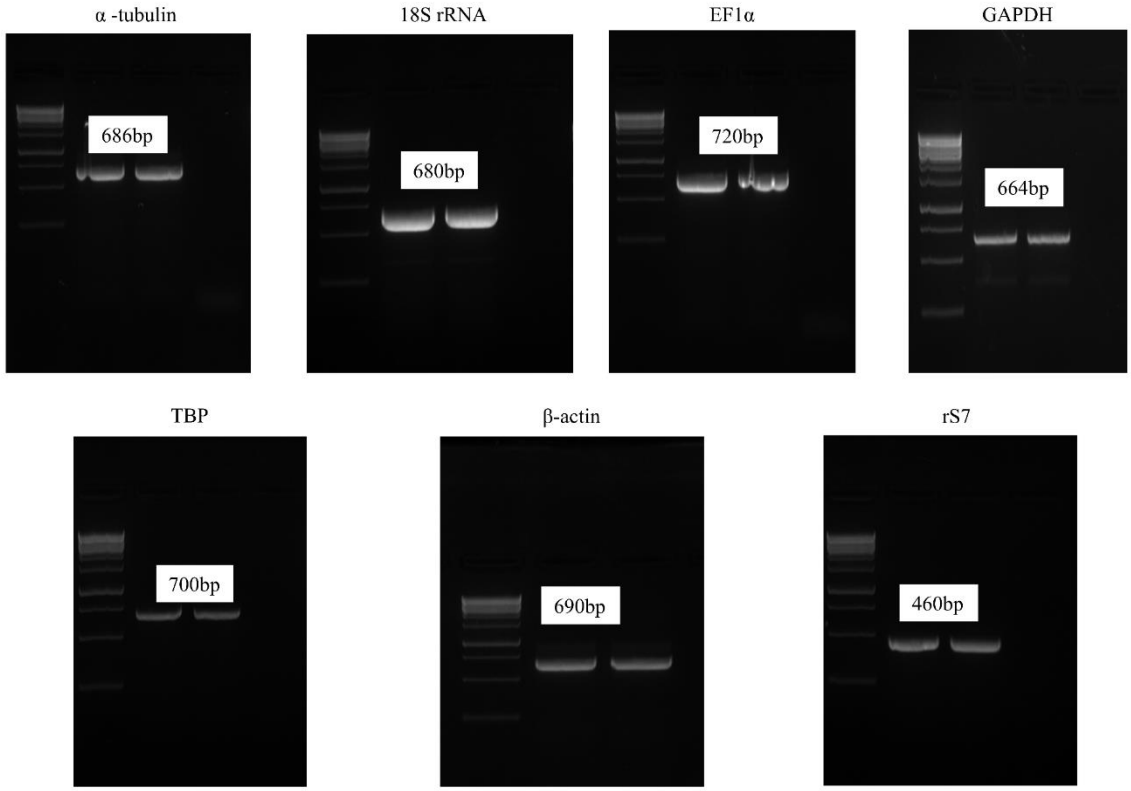


Figure 13: Shows separation of partial fragments of the selected house-keeping genes of expected sizes on agarose gel (1.2%).

S.NO	Gene	Acc no.	Function
1	*Tubulin*	KF984199	Cytoskeletal structural protein
2	*18S rRNA*	KF984197	Ribosomal RNA
3	*GAPDH*	KF984200	Glycolysis enzyme
4	*EF1α*	KF984203	Translational elongation
5	*rS7*	KF984201	Ribosomal protein
6	*TBP*	KF984202	Transcriptional initiation protein
7	*Actin*	KF984198	Cytoskeletal structural protein

Table 1: GenBank accession numbers of partial cDNA sequences of *C. cephalonica* generated in the present study and their functions.

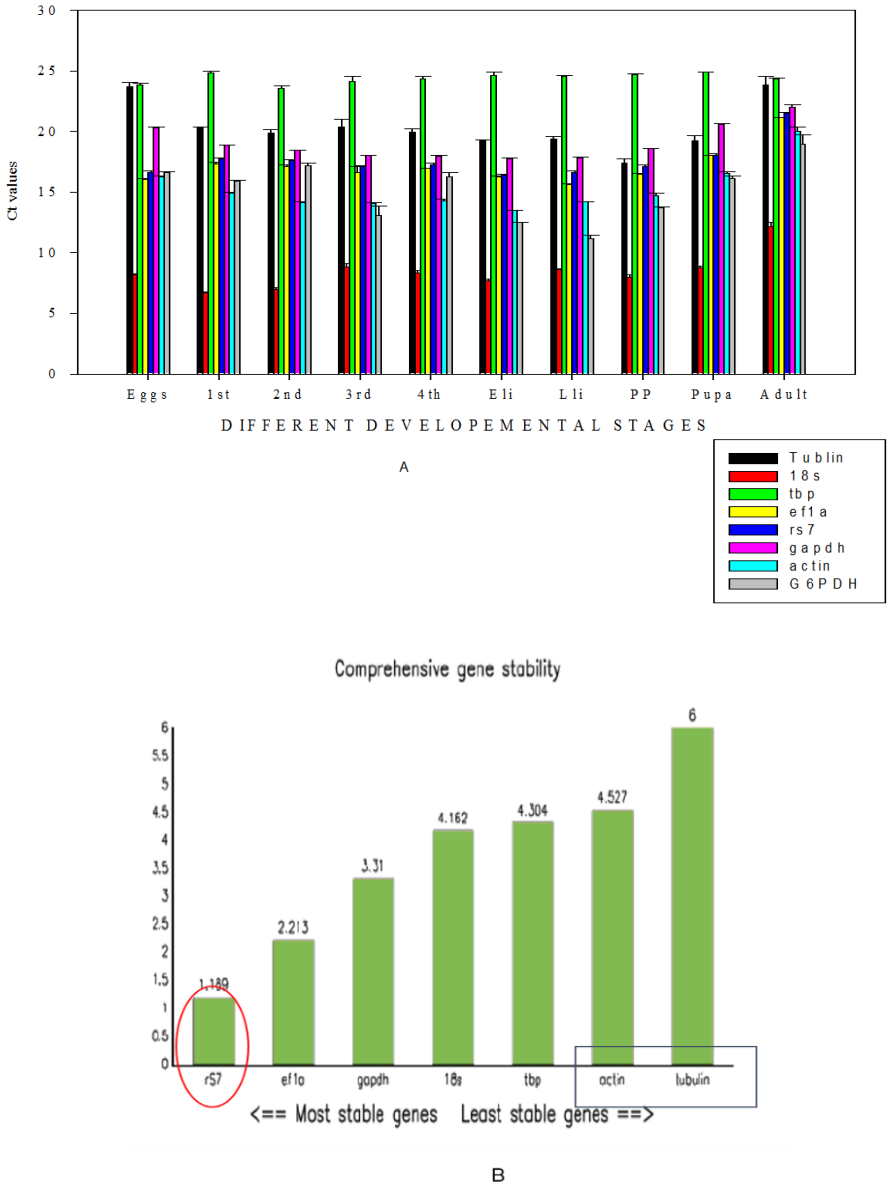
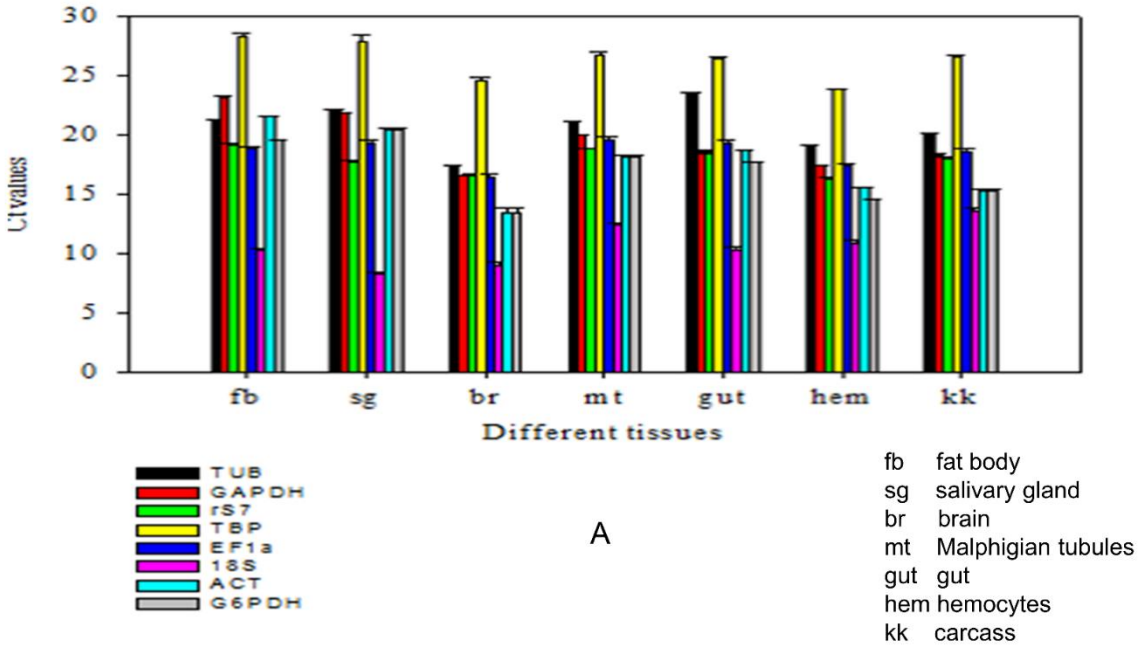
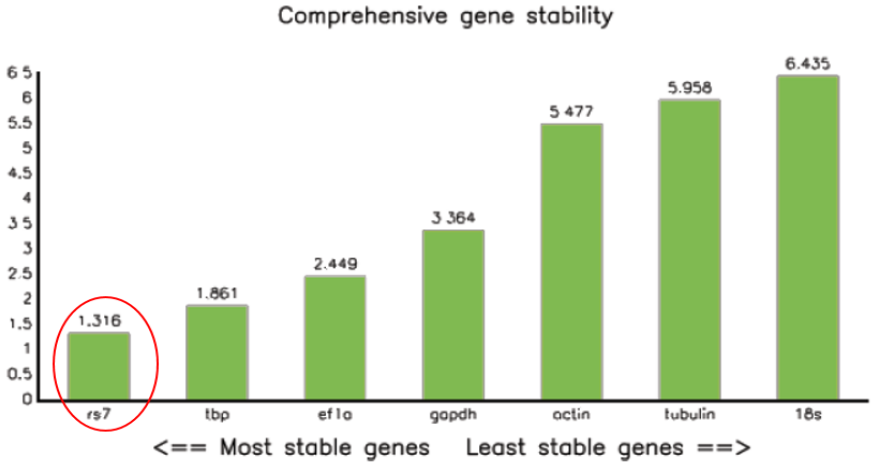


Figure 14: C_t values **A:** and the expression stability **B:** of selected house-keeping genes in different developmental stages of *C. cephalonica.*



A



B

Figure 15: C_t values **A:** and the expression stability **B:** of selected house-keeping genes in different tissues of LLI larvae.

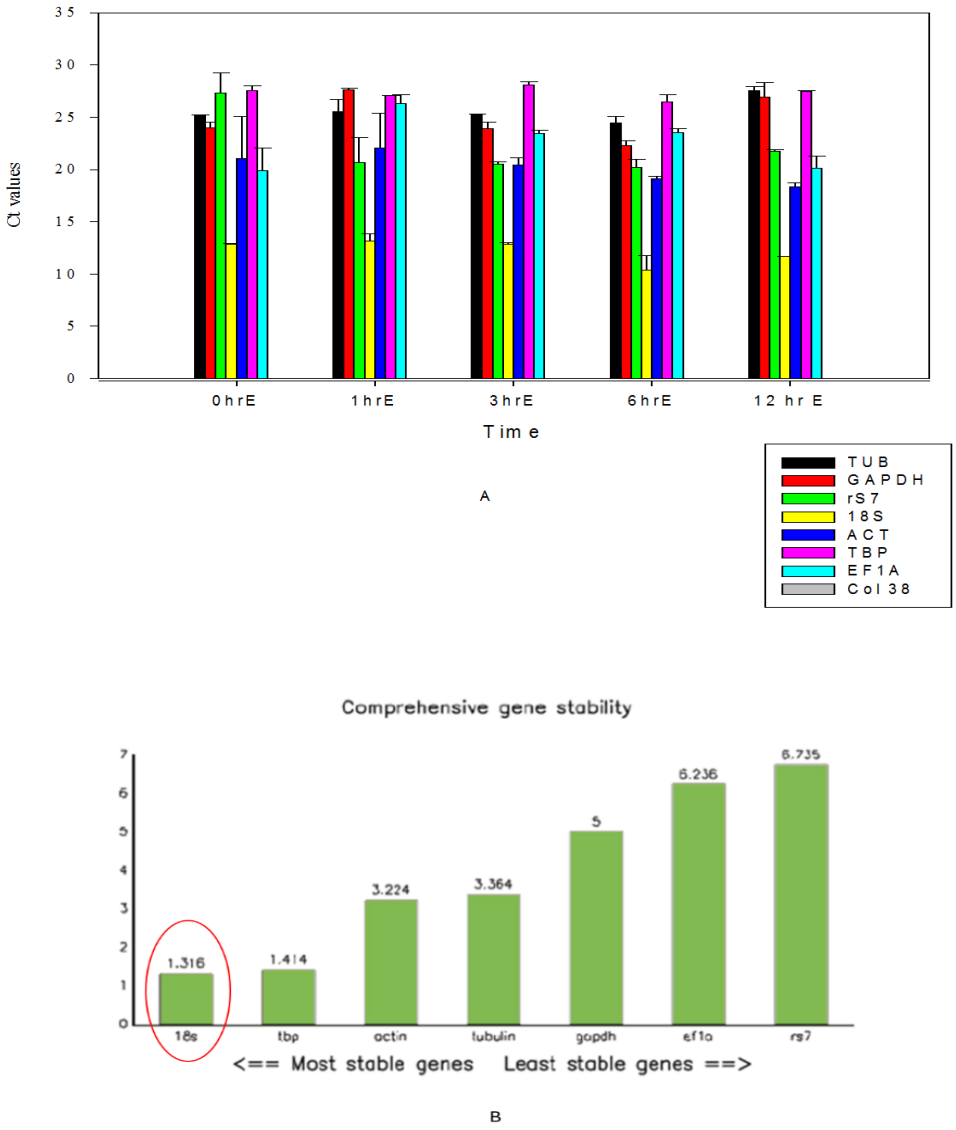
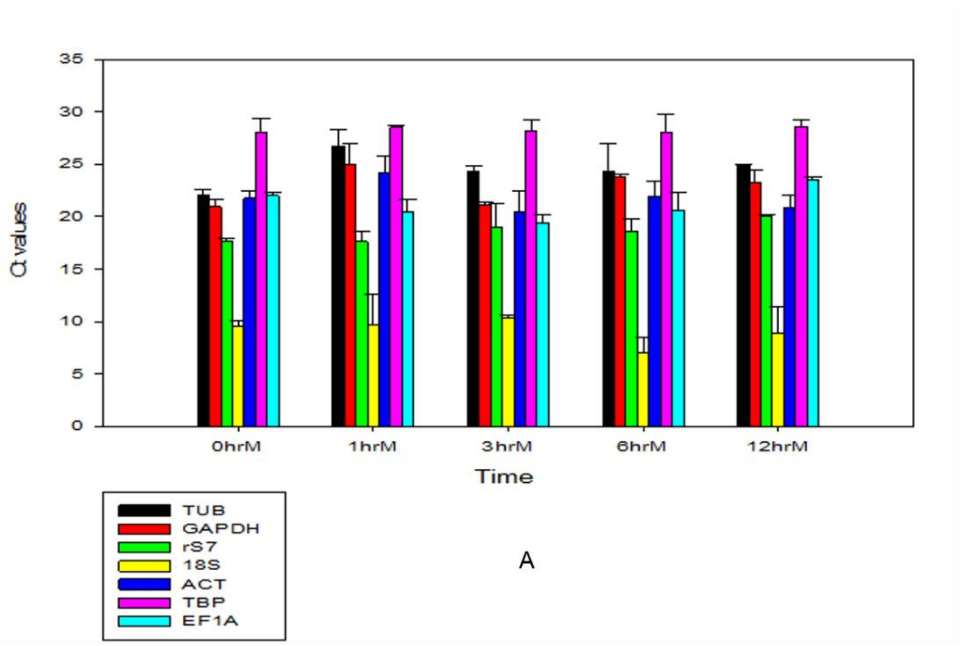


Figure 16: C_t values **A:** and the expression stability **B:** of selected house-keeping genes after 20E treatment to the thorax ligated isolated abdomen.



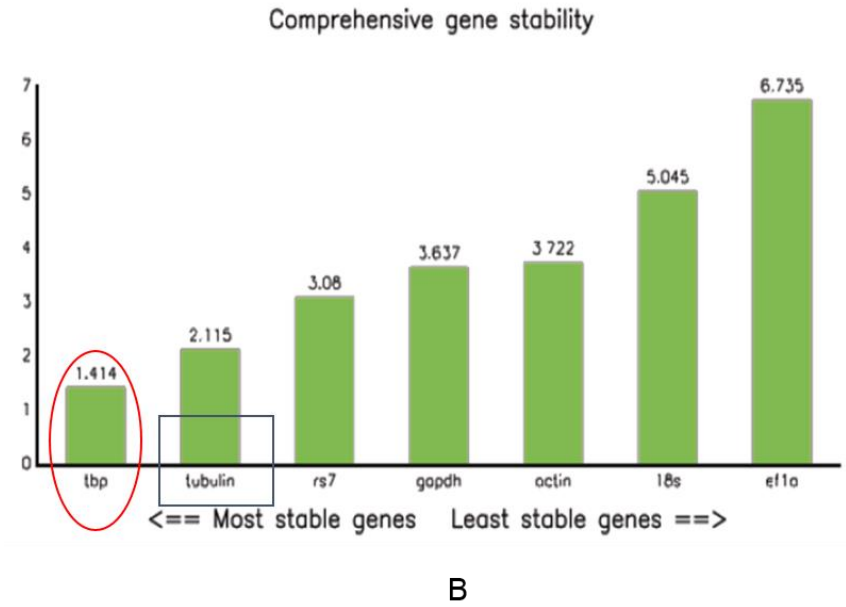


Figure 17: C_t values **A:** and the expression stability **B:** of selected house-keeping genes after application of methoprene to the thorax ligated isolated abdomen.

CHAPTER 3

Ontogeny and hormonal regulation of hexamerins in *C. cephalonica*; *in vivo* and *in vitro* studies

Ontogeny (developmental profile) of a gene is normally correlated with its function and is indicative of the physiological status of the organism. Differential expression of various genes during larval development of *C. cephalonica* suggest that their expression might be hormonally regulated, which is in line with similar results reported for holometabolus insects as their developmental stages are precisely controlled by varying titers of hormones (JH and 20E) as well as precise interplay between them. As hexamerins are major proteins that play an array of roles during postembryonic development, their expression could also be under the regulation of these metamorphic hormones.

Results:

Developmental profile of *Hexamerin* gene expression in *C. cephalonica*:

Various stages of insect from embryo to adult were collected and used. Whole organism was used for RNA isolation. Equal concentration of RNA from each stage was used for 1st strand cDNA synthesis. Semi quantitative PCR (Fig. 18A) and qRT-PCR (Fig. 18B) were performed using primer sets mentioned in the material and methods section. Results obtained demonstrate that stage dependent expression of different hexamerins gene(s) in *C. cephalonica*. Expression of *Hex 84* was seen from 2nd larval instar, while for *Hex 86* it was from 4th larval instar, however *Hex 82* was exclusively expressed in LLI larval stage. Furthermore, the overall expression of hexamerins which was seen from 2nd larval instar, increased gradually and reached its maximum at MLI larva, remained more or less same in LLI larval stages, but declined gradually further on.

Effect of 20E on fat body total protein synthesis:

To make hormone free environment LLI larva was thorax ligated and maintained in moist environment for 24 h to deplete endogenous hormones. They were then used for [^{35}S] methionine incorporation and exogenous hormone application studies after 24 h. Autoradiographs show that [^{35}S] methionine is incorporated into the fat body cells of LLI larva, which were treated with 20E (80 nM/insect) or its carrier solvent (ethanol). After 6 h of incubation, control insects showed fairly low incorporation of radiolabel (Fig. 19A), while upon 20E treatment the cells showed higher radioactivity (Fig. 19C). With an increase in incubation period to 12 h, the hormone treated insects showed still higher incorporation of [^{35}S] methionine (Fig. 19D), while the increase in carrier treated control was only moderate (Fig. 19B). This study clearly suggests that 20E stimulates protein synthesis in the larval fat body of *C. cephalonica.*

Effect of 20E on hexamerin synthesis and their release during LLI stage- *in vivo* studies:

[^{35}S] Methionine incorporation study reported in the above section clearly indicated that the fat body protein synthesis was upregulated by 20E. To further confirm whether the hexamerin synthesis and release under *in vivo* condition was also influenced by 20E, [^{35}S] methionine incorporation study was carried out. For this 24 h post ligated LLI larva were injected with 80 nM of 20E, while controls were injected with equal volume of carrier. The insects were incubated for a period of either 6 or 12 h and 2 h prior to dissection the insects were injected with 10 µCi of [^{35}S] methionine, and fat body was dissected and homogenized. Further the hemolymph was also collected and diluted as mentioned in materials, and methods section and used for immune precipitation experiment. Protein was quantified and equal concentration as it was separated on 10% SDS-PAGE. After

electrophoresis the gel was stained, vacuum dried and exposed to X-ray film. The results presented (Fig. 20A) clearly show that 20E treatment promotes the synthesis of hexamerins and the intensity of the commassie stained hexamerins protein band is fairly high in hormone treated insects. Autoradiogram presented (Fig. 20B) further shows that 20E treatment for a period of 6 and 12 h caused a significant increase in hexamerin synthesis when compared to respective controls. As fat body is known to synthesize and release hexamerins actively in the hemolymph during active feeding phase, an attempt was made to analyze the release of hexamerins after 20E treatment. The hemolymph proteins collected from hormone treated as well as controls were immunoprecipitated with hexamerin antibodies, electrophoresed and analyzed by autoradiography (Fig. 20C). Results clearly showed that the radioactivity seen in hexamerin band was much higher in the hemolymph of 20E treated insects as compared to the control. Furthermore, there was a time dependent increase in radioactivity of hexamerin band confirming that 20E not only promotes the synthesis of hexamerins, but also stimulates the release of hexamerins into the hemolymph during larval development of *C. cephalonica*.

Effect of 20E on hexamerin synthesis and their release during last larval instar stage *in vitro* studies:

The fat body from LLI larvae was dissected under sterile condition and rinsed thoroughly in insect Ringer for removal of adhered hexamerins. The tissue was then cultured in the TC-100 insect culture medium for 6 h as a preconditioning step for the depletion of endogenous hormone. After this tissue was transferred to fresh medium (200 µl) and 20E at a final concentration of 80 nM was added in the experimental cultures, while equal volume of solvent (0.05% ethanol) was added to the control. The samples were incubated at 27°C with agitation for different time periods. After the incubation tissue as well as

culture media were collected and processed for protein quantification and profiling. Equal quantity of protein (20 µg) was separated using SDS- PAGE (Fig. 21A) and immunodetected using hexamerin antibodies (Fig. 21B). These results correlated well with *in vivo* studies, i.e. there was a significant increase in the release of hexamerins and the concentration was high in the medium of 20E treated fat body cultures when compared with the controls. Further the effect seen was time dependent.

Effect of 20E on *hexamerin* transcription:

The results presented in previous section clearly show that hexamerins in *C. cephalonica* show stage specific expression as well as 20E promotes hexamerin synthesis. An attempt was made to analyze the probable regulation of genes at the transcriptional level. 24 h post thorax ligated LLI larva was injected with 80 nM of 20E, after different time periods i.e. 1 h, 3 h, 6 h, 12 h and 24 h, the fat body was dissected from 20E treated as well as control insects. Total RNA was isolated, cDNA was synthesized and hexamerin expression was quantified. Results obtained (Fig. 22) clearly show that transcription of all the three hexamerins was up-regulated upon 20E treatment. Figs. 22 A, B and C are the results of semi-quantitative PCR of *Hex 82*, *Hex 84,* and *Hex 86* respectively showing the expression pattern at different time points, in control and hormone treated insects, while D, E, and F are qRT-PCR showing the same. This increase expression was gradual and time dependent up to12 h, after which it declined. This might be due to the depletion of exogenous 20E for which reported half-life is about 6-9 h in thorax ligated insects which do not have a known source of ecdysteroids.

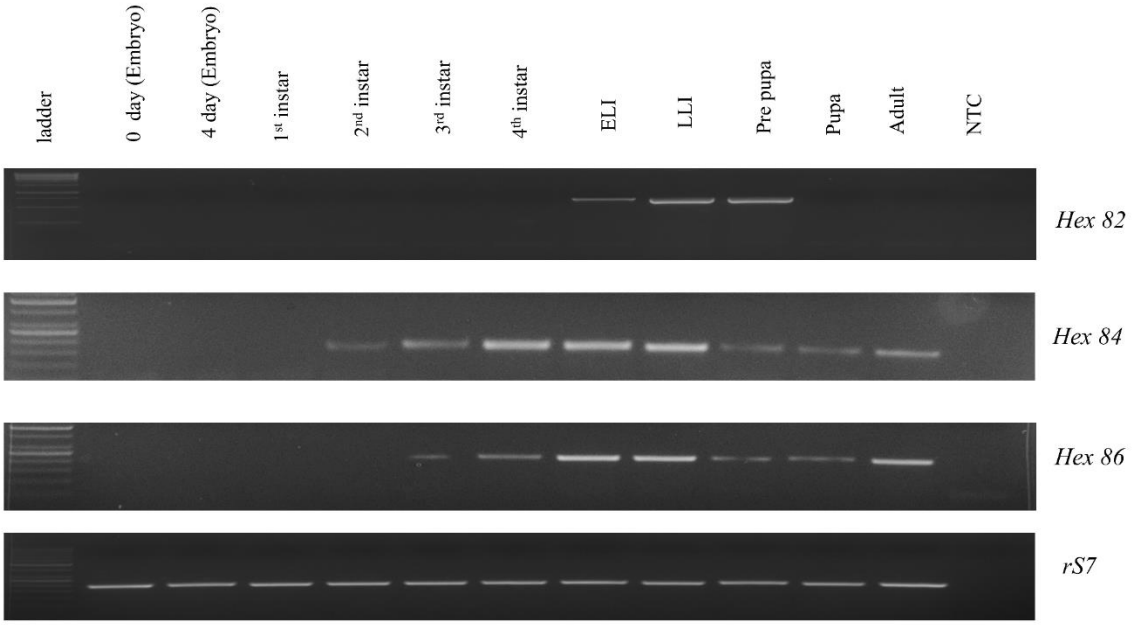
Effect of methoprene on *hexamerin(s)* transcription:

During postembryonic development, not only 20E but also the JH plays an important role. Hence, the effect of methoprene which mimics the action of naturally occurring JHs was

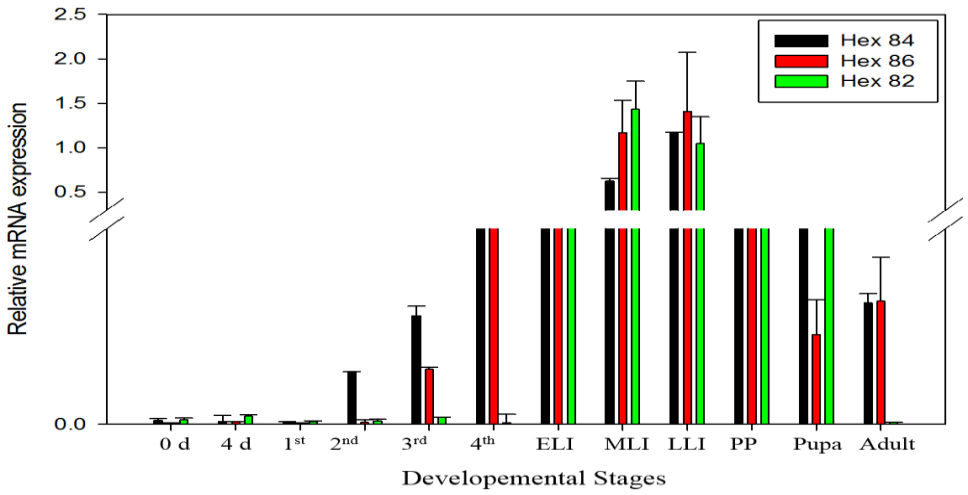
analyzed on *hexamerin(s)* expression. 24 h post thorax ligated LLI larvae were topical applied with 0.4 pg of methoprene, and dissected after different time periods to collect fat body i.e. 1, 3 , 6 , 12 and 24 h. Total RNA was isolated and cDNA was synthesized and used for the expression analysis of hexamerin genes. The semi quantitative PCR and qRT-PCR results, (Fig. 23 A) clearly revealed that methoprene had no influence on the expression of hexamerin genes. Fig. 23 A, B and C represent semi-quantitative PCR results at different time points in control and methoprene applied insects, while D, E, and F are qRT-PCR results of the same.

Effect of simultaneous application of 20E and methoprene on *hexamerin(s)* transcription:

For this study, 24 hours post thorax ligated LLI larvae were treated with both 20E and methoprene, as mentioned in materials & methods for different time periods i.e. 1, 3, 6, 12 and 24 h. The fat body was dissected from the hormone treated as well as the control insects. RNA was isolated and used for 1st strand cDNA synthesis for expression analysis of *hexamerins.* The results of semi quantitative PCR and quantitative PCR (Fig. 24) show that transcription of all three hexamerins were up-regulated (Fig. 24) and matched with results obtained were comparable with the results of 20E treatment alone (Fig. 22). These findings suggests that methoprene probably acts as 'status *quo*' factor, which maintains and stabilizes the expressed transcripts that are induced and expressed upon 20E treatment.



A



B

Figure 18: Ontogeny of hexamerins genes: **A:** Semi-quantitative PCR showing the stage dependent expression of three hexamerins genes (*Hex 82*, *Hex 84* and *Hex 86*) and rS7 was used as internal control. **B:** similar expression pattern of hexamerins was obtained with semi quantitative PCR. Hexamerins expression is reported as fold change relative to zero day embryo control.

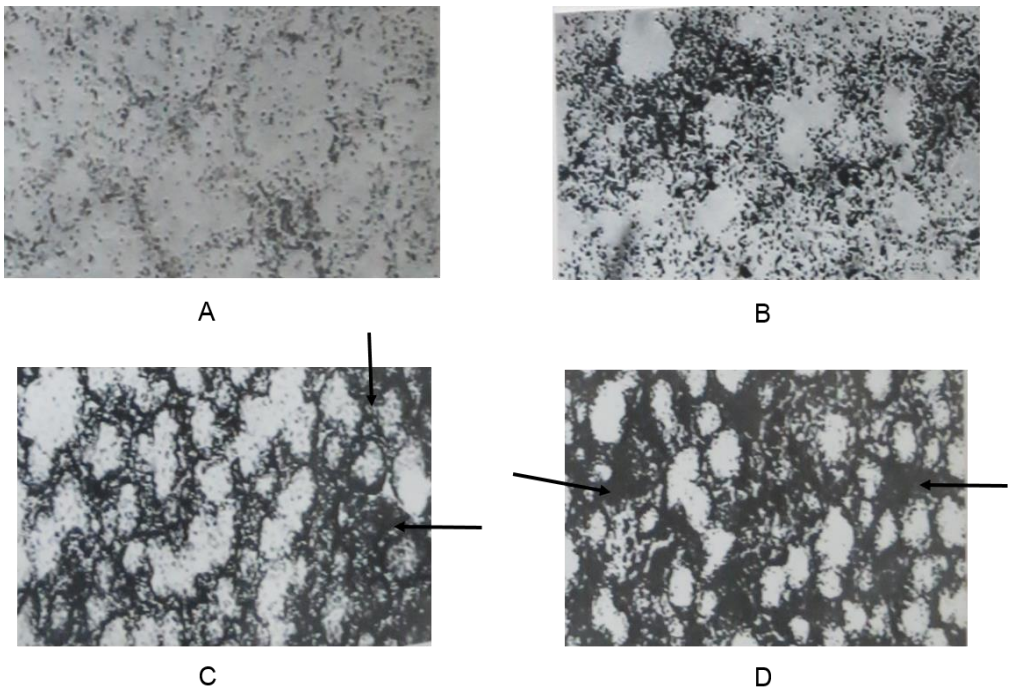


Figure 19: Autoradiograps showing the pattern of [^{35}S] methionine incorporation into the fat body of LLI larva of *C. cephalonica*. **A** and **B** are controls for 6 h and 12 h. While **C** and **D** are from 6 h and 12 h 20E treated fat body samples. Please note the increase in radiolabel incorporation after hormone treatment (20E), (⟶) when compared with respective controls

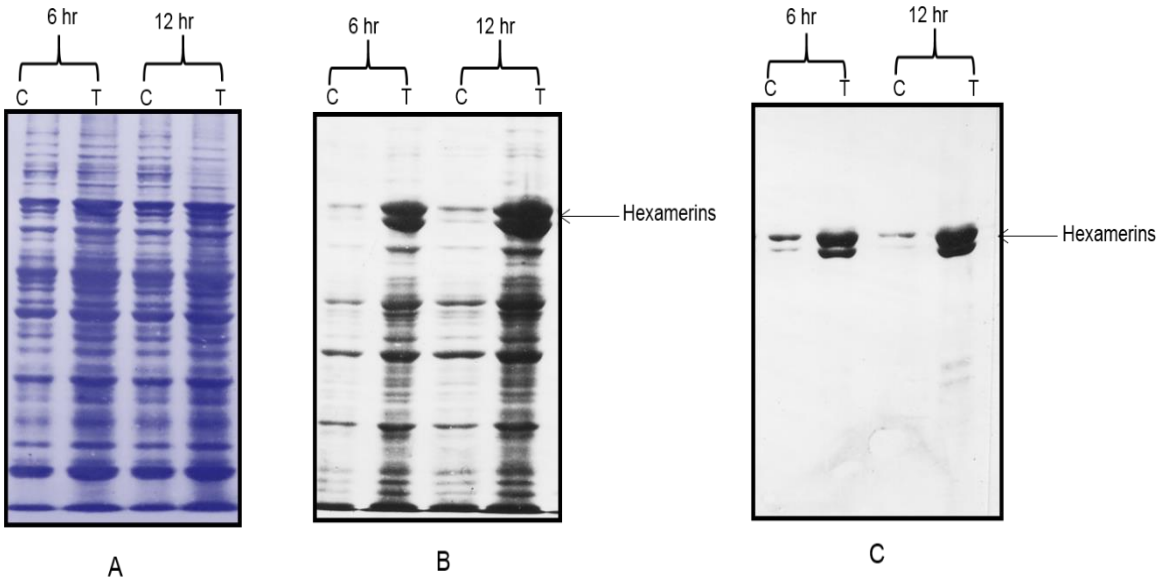


Figure 20: Effect of 20E on hexamerin synthesis (*in vivo* studies). For the above experiment 24 hr post- thorax ligated late last instar larvae were injected with 80 nM 20E either for 6 or 12 hr and 2 hr prior to collection of tissue samples they were injected with 10 µCi of [^{35}S] methionine. **A:** showing the effect of 20E on LLI larval fat body protein synthesis after 6 hr and 12hr time periods (C= control, T= treated). **B:** Autoradiogram of the SDS-PAGE. **C:** Autoradiogram showing the immuno-predicated radio-labeled hexamerins from hemolymph with the hexamerin antibodies. In this, equal concentration of protein was taken for the precipitation reaction. The 20E treated insects showed the presence of higher quantity of radiolabelled hexamerins in the hemolymph when compared with the respective control.

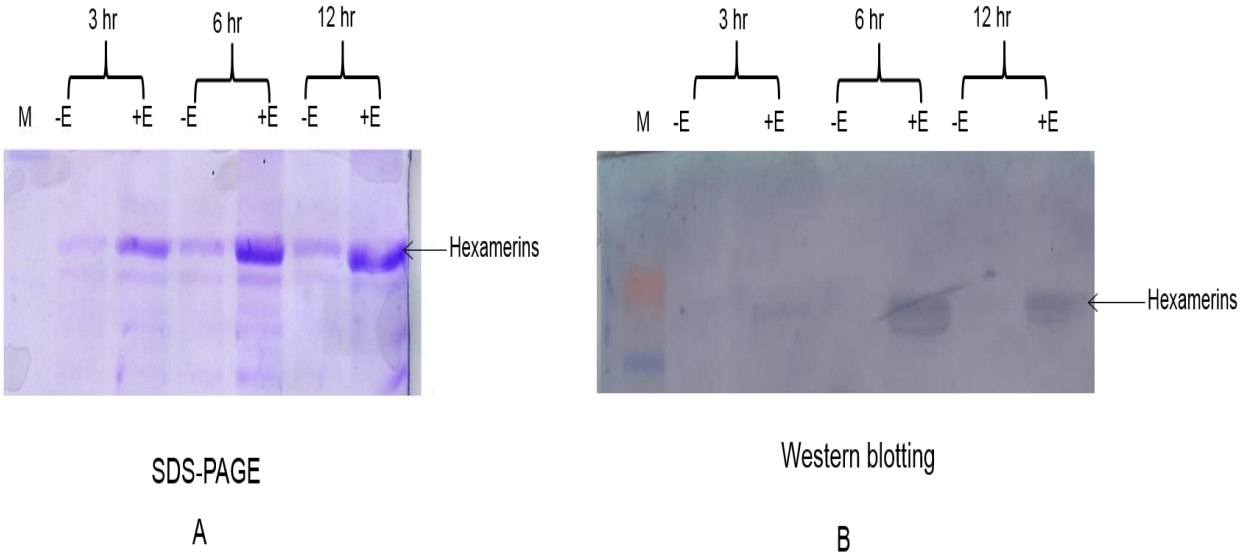


Figure 21: Effect of 20E on hexamerin synthesis and release in organ culture (*in vitro)* study. **A:** SDS-PAGE showing the released proteins into media after 20E and ethanol (carrier) treatment after 3 hr, 6 hr and 12 hr time periods. **B:** Western blot of released proteins with anti-hexamerin antibodies. (-E= carrier treated, +E= 20E treated)

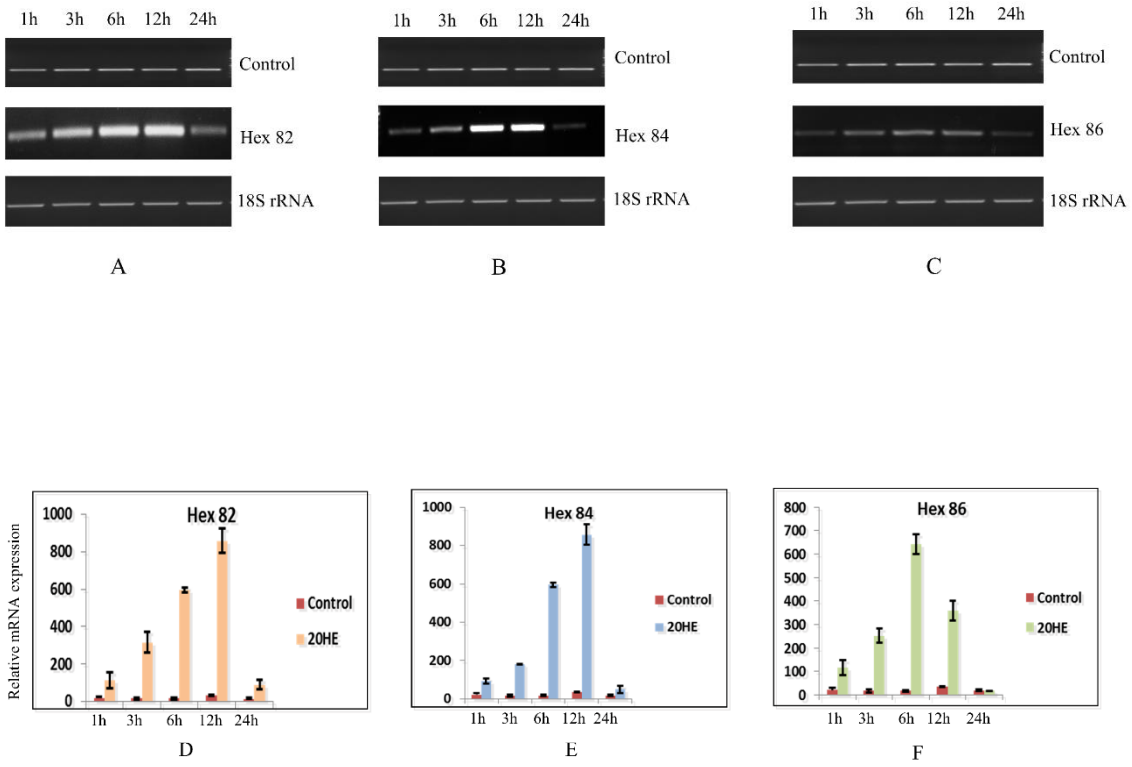


Figure 22: Effect of 20E on *hexamerins (Hex* 82, *Hex* 84, *Hex* 86*)* expression in the fat body of 24 h post-thorax-ligated LLI larvae which were injected with 20E for required time periods. **A**, **B** and **C** are results obtained from semi-quantitative PCR of three genes showing the expression at time points 1 , 3, 6, 12 and 24 h in control (ethanol treated) and hormone treated insects. 18SrRNA was used as loading control. **D, E,** and **F** are results obtained from Real-time PCR, showing the expression at 1, 3, 6, 12 and 24 h time points in control (ethanol treated) and hormone treated insects. *Hexamerin* expression is reported as fold change relative to internal control.

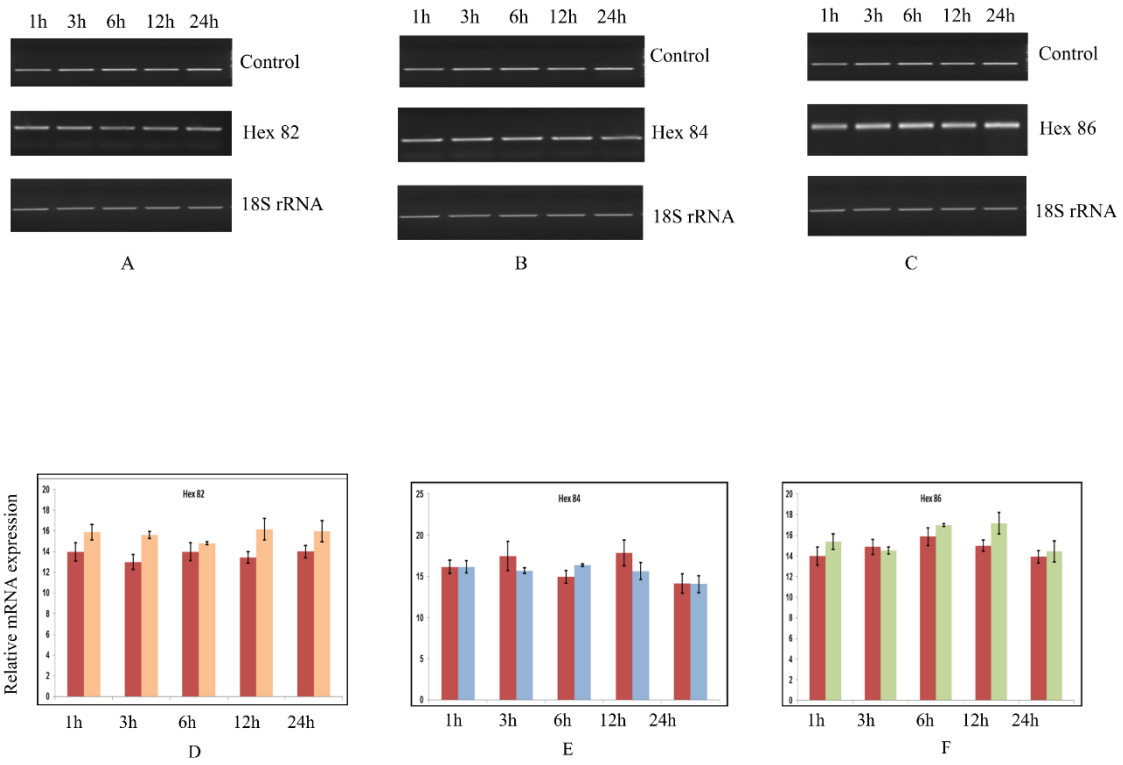


Figure 23: Effect of methoprene on *hexamerins (Hex* 82, *Hex* 84, *Hex* 86*)* expression in the fat body of 24 h post-thorax-ligated LLI larva that was topically applied (0.4 pg/larva) with methoprene for required time periods. **A**, **B** and **C** are Semi-quantitative PCR results from three genes showing the expression at time points 1, 3, 6, 12 and 24 h in control (acetone) and hormone treated insects. TBP (TAT binding protein) was used as loading control. **D**, **E**, and **F** are qRT-PCR results showing the expression at 1, 3, 6, 12 and 24 h time points in control (acetone applied) and hormone applied insects. *Hexamerins* expression is reported as fold change relative to internal control.

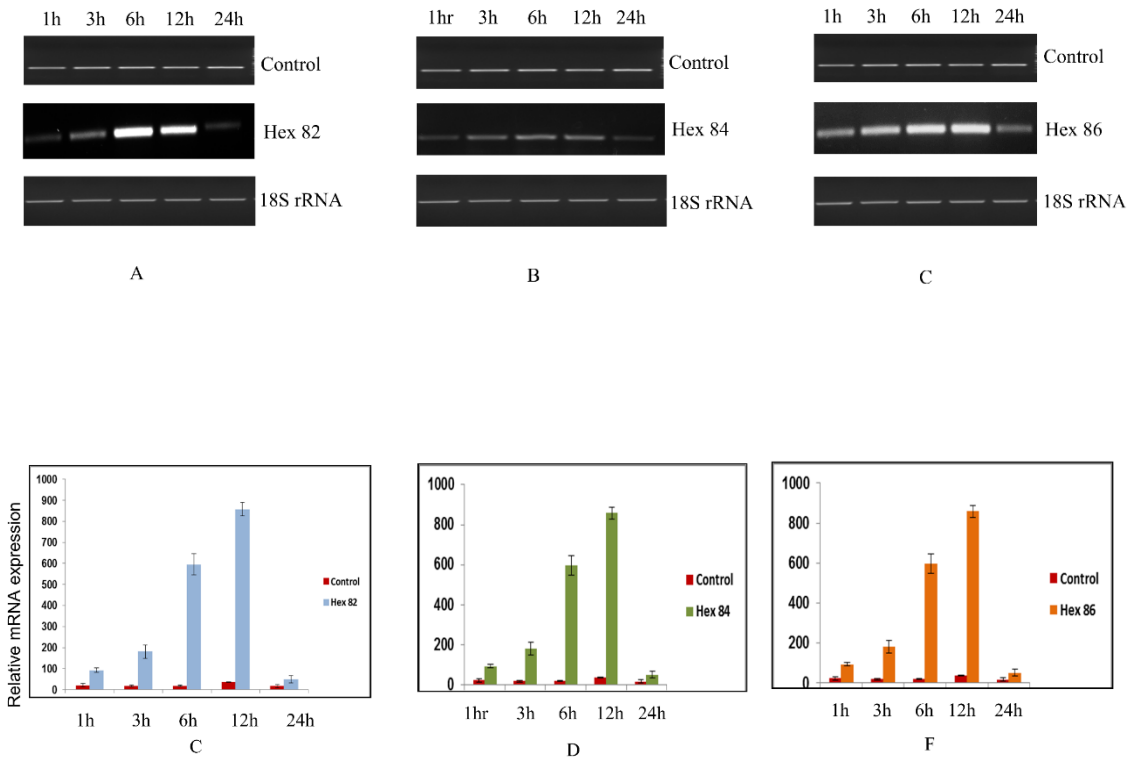


Figure 24: Effect of 20E and methoprene on *hexamerins (Hex 82, Hex 84, Hex 86)* expression of fat body of 24h post thorax ligated LLI larva that were treated with both hormones for required time periods. **A**, **B** and **C** are semi-quantitative PCR of three genes showing the expression at time points 1, 3 , 6 , 12 and 24 h in control (ethanol and acetone) and hormones treated insects. TBP (TAT binding protein) was used as loading control. **D**, **E**, and **F** are Real-time PCR showing the expression at 1, 3, 6, 12 and 24 h time points in control (ethanol and acetone) and hormone treated insects. *Hexamerins* expression is reported as fold change relative to internal control.

CHAPTER 4

Identification and characterization of ecdysteroid regulatory element(s) in the upstream region of *Hex 84* gene of *C. cephalonica*

Work presented chapters 2 and 3 show that transcription of hexamerin genes are hormonally regulated. Further, 20E is a major hormone that upregulates the hexamerins transcription in the larval fat body. Steroid hormones including ecdysteroids are known to pass through cell as well as nuclear membrane and bind to the receptor proteins, promote dimerization and form the hormone receptor complex, which interacts with hormone responsive elements primarily present in upstream sequence of variety of genes and modulate gene transcription. Various reports show that in insects the ecdysteroids receptor (EcR) and ultraspiracle (USP) are functional ecdysteroids receptors, which mediate the hormonal response by binding to the regulatory elements present in the 5' region of the responsive target genes. As 20E stimulated hexamerins gene expression in *C.cephalonica* larvae, an attempt was made to identify the regulatory elements in the gene.

Results:

Genome walking analysis of hexamerins:

Genomic DNA was isolated from whole body of LLI larva of *C. cephalonica.* Four aliquots of DNA were digested individually using *Stu 1, Dra 1, PuvII, EcoRV* and four different libraries were constructed. Each library was used for primary amplification using AP1 and Hex GSP1 primers. Out of four, only two, *Stu I* and *Puv II* libraries gave amplification (Fig. 25A). The amplification was further reconfirmed using AP2 and Hex GSP2 primers (Fig. 25B). The 3 kb amplicons obtained from the *Stu I* and *Puv II* libraries were cloned into pTZ57R/T vector and sequenced. Details of AP1, AP2, and GSP1and GSP2 primers used for present study are in materials and methods section.

In silico analysis of 5'flanking sequence of _Hex 84_:

The 5' flanking sequence obtained was analyzed by using TESS master, Matinspector and Transfec version 2.2 to predict transcriptional factor binding sites. Upstream sequence analysis revealed presence of several putative transcription binding sites, TATA sequence at -82 relative to the ATG sequence, SGF (silk gland factor) sequence at -256, ERE (ecdysteroid response element) sequence at -280, FOXL1a sequence at -617, E74a sequence at -1071, CREB (cAMP response element-binding**)** sequence at -1167, ZESTE sequence at -1243 and RXR sequence at -1618. (Fig. 26)

Generation of deletion constructs for luciferase assay:

For this analysis *Hex* 84 5' flanking region progressive deletion constructs were amplified by PCR, using gene specific primers which were designed based on the sequence of specific restriction sites (Fig. 27A). Resultant constructs were cloned in *Nhe 1* and *Xho I* sites of pGL3 basic firefly luciferase vector (Promega). The length of each construct was verified by double digestion and the absence of sequence artifacts was determined by nucleotide sequencing.

Identification of functional binding sites in the upstream region of _Hex 84_:

To assess the functionality of binding sites, a series of PCR based deletion constructs were generated, cloned into pGL3 vector (Fig. 27B) and were transfected into Sf9 (*Spodoptera frugiperda*) cells. Transfected cells were further induced with 20E (1µg/ml) and after 24 h of induction, luciferase activity was measured. The constructs that contained up to EcR binding sequence ERE at -280 showed a significant promoter activity, when compared with the control (Fig. 28). The constructs with SGF and TATA box as well as TATA box alone revealed negligible promoter activity. The findings of this experiment suggest the

presence of a functional ERE in the *Hex 84* 5' flanking region or promoter region. These results further demonstrate that the effect of 20E on *Hex 84* expression in *C. cephalonica* larvae is probably mediated through ecdysone receptor binding element (ERE).

Confirmation of ERE functionality in *Hex 84* promoter:

EMSA (electrophoretic mobility shift assay) was carried out to further confirm the ERE functionality. It was performed using normal and mutated probes designed according to the EcR binding element sequence. The nuclear extract was prepared from the hormone induced fat body of 24 h post thorax ligated LLI larva. Upon 20E treatment, EcR/USP (ecdysteroid receptor/ultraspiracle protein) complex present in the nuclear extract bound to the [γ^{32}P] dATP labeled oligomeric sequence containing ERE motif and resulted in the formation of specific protein/DNA complex, which has altered mobility (Fig. 29). However this was not seen when the mutated probe was used.

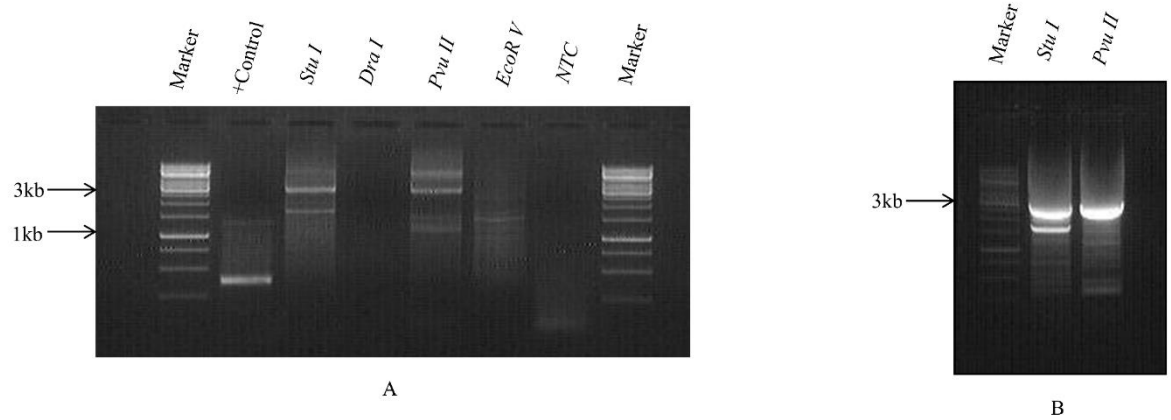
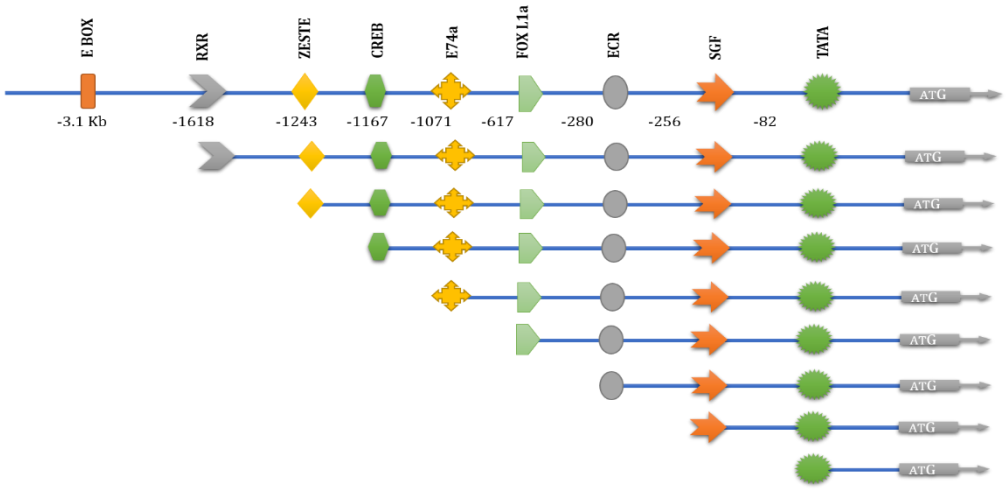
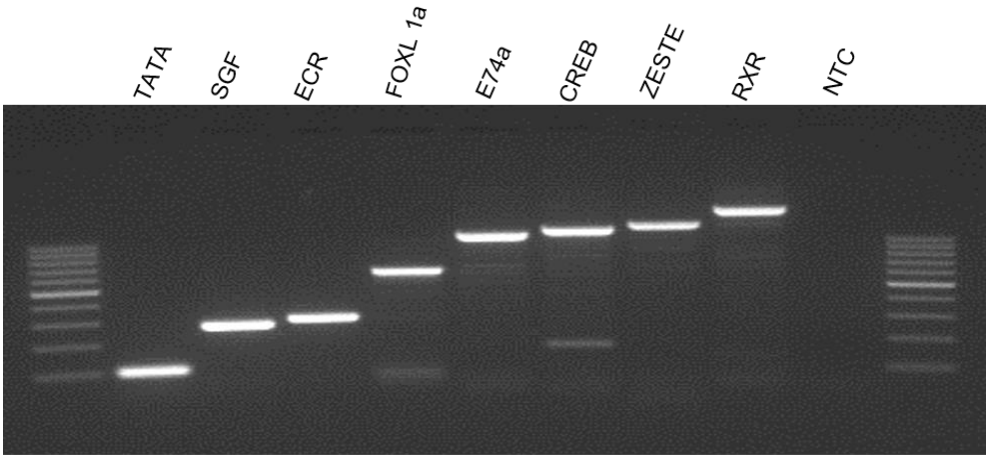


Figure 25: PCR amplification using *Hex 84* gene specific primers from *C. cephalonica* genome walking library. **A:** Primary PCR shows the amplification with different restriction enzyme libraries with AP1 and GSP1; **B:** Shows the secondary PCR of 3 kb fragment with AP2 and GSP2 (AP: Adapter primer, GSP: Gene specific primer)

Figure 26: Nucleotide sequence of the 5' flanking region of the *Hex 84* obtained from sequencing. The putative transcription factor binding sites are underlined and labeled.



A



B

Figure 27: A: Schematic representation of cloning of deletion constructs from 5' flanking region of *Hex 84*, **B:** Cloning of different deletion constructs into PGL3 vector.

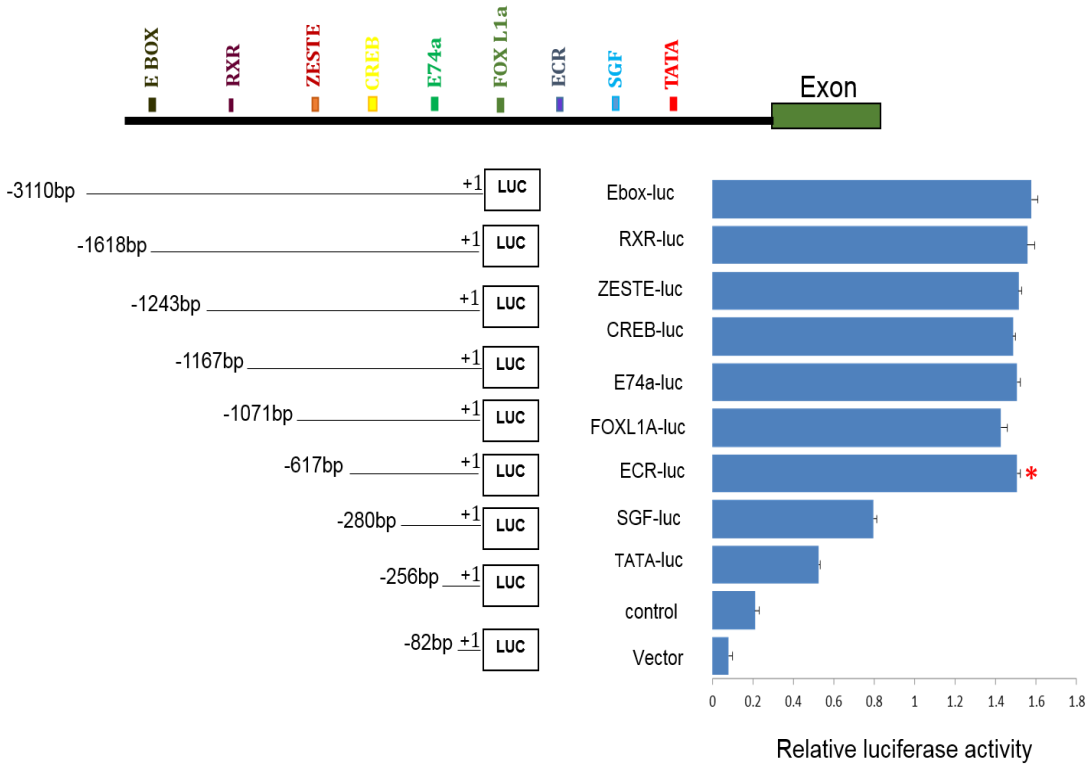


Figure 28: Functional analysis of different *Hex 84* promoter constructs co-transfected with pRL-TK into *Sf9* cells. Luciferase activity is presented as relative to the activities measured for *Renilla* luciferase.

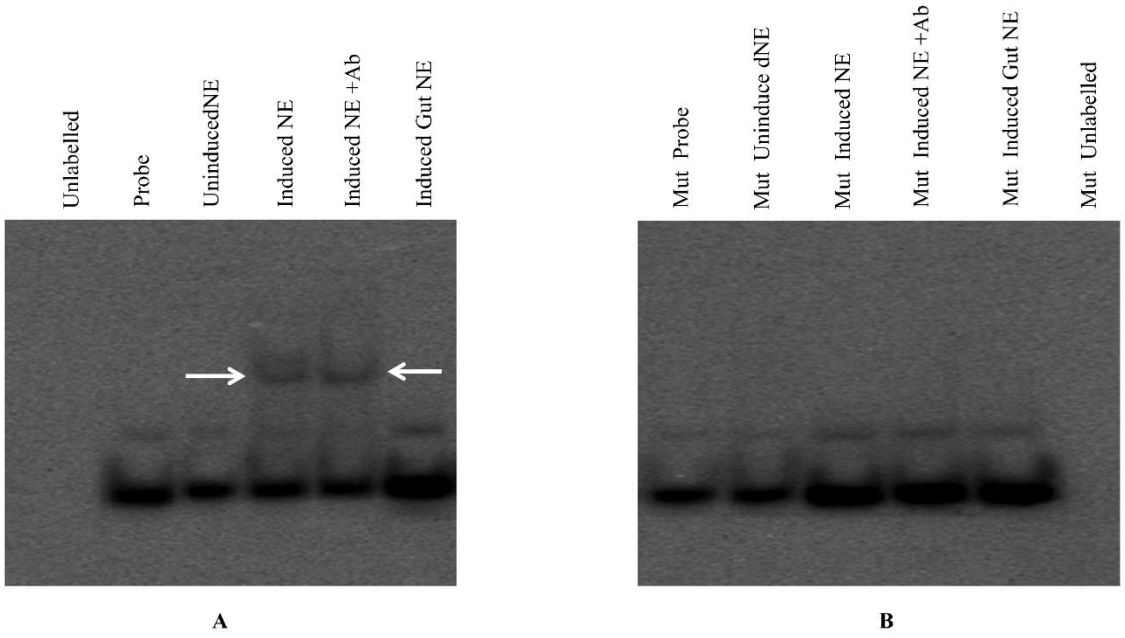


Figure 29: Auto-radiograms showing electrophoretic mobility gel shift of 20E induced fat body nuclear extracts. **A**: ERE element shows the gel shift after induction with 20E. **B**: Shows the mutated ERE element not shows any gel shift. The shift is shown with the arrows (⟶)

DISCUSSION

Corcyra cephalonica, commonly known as rice moth is a key pest of grains, cereals and seeds. The larvae primarily feed on broken grains. It is one of the most common destructive pest of store –ware houses, where the larvae not only feed on grains but also cause webbing by secreting silk thread, which renders infested products unacceptable for human consumption (Osman, 1984; Alisha Coleman, USDA report, 2010,). During the last three decades our laboratory has primarily focused on deciphering the role of various genes/their proteins including hexamerins (KiranKumar *et al*., 1997; Nagamanju *et al*., 2003; Arif *et al*., 2004; Damara *et al.*, 2010a).

Hexamerins are hemocyanin derived proteins that serve as storage proteins (Burmester *et al.*, 2002). They are actively synthesized and secreted by the larval fat body of holometabolus insects including Lepidoptera and stored in the hemolymph during the active feeding stage (Keeley, 1985; Dean and Collins, 1985; Arrese *et al*., 2010). By the end of the larval stages, all the hexamerins are sequestered back into the fat body and processed. Earlier, these proteins were thought to exclusively function as an amino acid source for tissue remodeling during the pupal-adult transformation. However, recent reports including work from our group demonstrate that hexamerins might have other functions in addition to being merely storage proteins. In grasshoppers, they play a role as hemolymph juvenile hormone transporters (Braun *et al*., 1996). It was also demonstrated that hexamerins interact with other proteins, in a multiprotein complex engaged in sequestration and transport of juvenile hormone, thus regulating its levels and actions (Zalewska *et al*., 2009), including the action on caste determination in social insects. In the termite *Reticulitermes flavipes*, a hexamerin was seen to be associated with the regulation of the juvenile hormone-dependent soldier caste phenotype (Zhou *et al*., 2006). Hexamerins were shown to play important role in adult reproduction of holometabolus insects, where the insects have a quicent nonfeeding pupal stage and depend heavily on

the protein reserves accumulated during larval phase, which actively feeds. Hexamerins support egg formation primarily by providing precursors for the synthesis of yolk proteins by the fat body and chorion proteins by the follicle cells (Wyatt, 1991; O'Brien, *et al.*, 2002; Cristino *et al*., 2010). Earlier studies from our laboratory have clearly demonstrated that hexamerins play a role not only in female reproduction but also in male reproduction of Lepidopteran insects and make a major component of seminal secretion (Bajaj *et al*., 1990). Our group further demonstrated the presence of hexamerins(s) receptor in the plasma membrane of various tissues of *C. cephalonica* including male accessory reproductive glands (MARG), ovary, and salivary glands along with fat body (KiranKumar *et al*., 1997; Arif *et al*., 2003; Damara *et al.,* 2010b). Further this 120 kDa receptor was shown be involved in hexamerin sequestration (KiranKumar *et al*., 1997; Arif *et al*., 2003). Incorporation of hexamerins in cuticle during pupal adult development was seen in many insects (Marinotti *et al.*, 1988; Peter and Scheller, 1991). In the ant *Componatus festinates*, hexamerins were shown to play a crucial role in brood nourishment and colony founding (Martinez *et al*., 2000). Hexamerins also bind to potentially toxic xenobiotics (Haunerland and Bowers, 1986) as well as modulate microbial toxicity in Lepidopteran insects (Budatha *et al*., 2007). This feature could be exploited for the site directed delivery of bio-pesticides, toxins and growth regulators for management of insect pests.

Based on the electrophoretic mobility of larval hemolymph proteins our group has identified three different hexamerins Hex 82 (82 kDa), Hex 84 (84 kDa), and Hex 86 (86 kDa) proteins in *C. cephalonica* (KiranKumar *et al.*, 1997), like other many other lepidopteran insects. Larval hexamerins of *C. cephalonica* were further purified and characterized from *C. cephalonica* (KiranKumar *et al*., 1997; Arif *et al.*, 2001) and were shown to be glyco-lipoproteins. Nagamanju *et al.*, (2003) cloned full length cDNA for 84

kDa hexamerin (Hex 84) of *C. cephalonica* and based on its deduced amino acid sequence, characterized it as an arylophorin. Later 86 kDa hexamerin of *C. cephalonica* was cloned and characterized as methionine rich hexamerin (Damara *et al.*, 2010). However Hex 82 protein has not yet been characterized from *C. cephalonica.* Although studies from our laboratory show precise developemental regulation of hexamerins but the exact mechanism of regulation of hexamerin synthesis during postembryonic development of *C. cephalonica* is not fully understood.

The present study primarily focuses on the cloning and characterization of Hex 82 (i.e riboflavin binding hexamerin) from the larvae of *C. cephalonica.* The full length cds of *Hex 82* was cloned using degenerate primers based amplification and RACE strategies. Degenerate primers that were designed to pick specific hexamerin, other than *Hex 84* and *Hex 86* in *C. cephalonica* resulted in amplification of 800 bp fragment. Sequence analysis of fragment showed that it is different from already reported hexamerins i.e. Hex 84 and Hex 86, suggesting the presence of an additional hexamerin in *C. cephalonica*. RACE reaction was carried using the 800 bp sequence information for obtaining the 5′ and 3′ ends. The PCR amplification using RACE adapter specific universal primer and gene specific primers of 5′ and 3′ (designed from 800 bp fragment) resulted in the amplification of 850 bp and 760 bp fragments respectively. After obtaining the three fragments, they were aligned to obtain the complete *Hex 82* sequence. The total length of the obtained cDNA is of 2.3 kb with an ORF of 2.1 kb suggesting that it is slightly smaller than reported sizes of *Hex 84* and *Hex 86*, where *Hex 84* ORF is 2.2 kb (Nagamanju *et al.*, 2003) and *Hex 86* ORF is 2.3 kb (Damara *et al.*, 2010) respectively. Translation analysis of the obtained ORF suggested that it encodes an 82 kDa protein, hence we designated it as *Corcyra cephalonica* hexamerin 82 (Hex 82). *In silico* analysis of *Hex 82* deduced amino acid sequence indicated the presence of putative glycosylation as well as phosphorylation

sites, suggesting that the protein might undergo post-translational modifications similar to other reported hexamerins of *C. cephalonica* (Nagamanju *et al.*, 2003; Damara *et al.*, 2010b). Moreover, signal peptide of 16 amino acids was also observed to be present in Hex 82 suggesting that this is also a secretory protein like other hexamerins. Homology comparison results show that Hex 8*2* has 62% sequence similarity with Hex 84 and 54% similarity with Hex 86. This also suggests that it's neither arylporin nor methionine rich hexamerin and is likely to be new class of protein. The isoelectric point of the deduced amino acid sequence of Hex 82 was 6.04, which differs from Hex 84 (5.05) and Hex 86 (9.01), which further confirms that Hex 82 does not belong to either of the classes reported earlier for *C. cephalonica* (Nagamanju *et al.*, 2003; Damara and Dutta-Gupta, 2010). Phylogenetic analysis of Hex 82 further indicated that Hex 82 shares substantial amino acid sequence identity with *Galleria mellonella*, non-arylphorin, non-methionine rich hexamerin (Memmel *et al*., 1994). Present analysis shows that both the sequences, fall under the same clad, and this is most likely due to the close evolutionary relation, as both of them belong to same family Pyralidae. Ontogeny (developmental profile) result obtained in the present study clearly reveals that *Hex 82* is last larval instar stage specific gene. Although, the other two hexamerins *Hex 84* and *Hex 86* also show a precise developmental profile but, *Hex 82* is the only hexamerin which shows exclusive expression during last instar larval development. Further like other two hexamerins of *C. cephalonica*, *Hex 82* shows fat body specific expression in present tissue distribution analysis.

BLASTn results reveal that 2118 bp ORF of *Hex 82* has 65% sequence identity with *Hylophora cercopia* riboflavin binding precursor protein (Magee *et al*., 2004). Based on this information, we tried to test the riboflavin binding property of Hex 82. In initial attempt the hexamerins separated by SDS-PAGE were extracted, refolded and used for

binding studies. However, no riboflavin binding was observed. This could be due to improper refolding of the denatured protein(s). Hence the experiments were repeated using native hexamerins purified from the hemolymph of different larval instars employing chromatographic techniques. Using fluorescence spectrophotometry, riboflavin release was measured instead of binding as native proteins are already bound to riboflavin and further binding might not be possible. Results obtained clearly show the release of bound riboflavin from hexamerins purified from last instar larval hemolymph but not from the hexamerins of earlier stages i.e, 4^{th} instar larvae. Our developemental profile analysis clearly reveals that its only LLI larval hemolymph has all the three hexamerins (Hex 82, 84 and 86) while 2^{nd} instar larva has only Hex 84 and 4^{th} instar larva has two hexamerins (Hex 84 and Hex 86). Based on the developmental profile data and the results obtained from riboflavin release analysis, we suggest that its Hex 82 present in the hemolymph of the late last instar larva, most likely binds to riboflavin and this protein has riboflavin binding activity. This assumption was further confirmed by homology modelling and docking studies. The docking studies of riboflavin with all three hexamerins clearly show that Hex 82 has the highest binding constant, when compared with Hex 84 and Hex 86. Based on the above riboflavin release and docking studies we propose Hex 82 as a putative riboflavin binding hexamerin of *C. cephalonica.* Further the present study also strengthens the concept that hexamerins not only serves as storage proteins, but also bind, carry and release small molecules like riboflavin, which may play vital physiological function during the pupal-adult transformation.

Real-time PCR is widely used as powerful sensitive technique to quantify transcriptional expression of genes in variety of organismsmal systems (Gachon *et al*., 2004; Nolan *et al*., 2006). Although it is a rapid method, requires low concentrations of RNA but reliability of the results heavily depend on, accurate normalization for which appropriate control

should be used. Further control gene should have similar expression regardless of experimental conditions, including different developmental stages, tissues and/or different treatments. qRT-PCR technique is prone to errors in various steps of the experiment like sample-to-sample variation in RNA integrity, differences in the efficiency of reverse transcriptase enzyme and the amount of first strand cDNA used in each PCR reaction. The credibility of the qRT-PCR results therefore depend on the reference genes that are stably expressed across all samples and normalization of the expression level of target gene(s) against a stably expressed internal control gene that compensates for the above variations (Vandesompele *et al.,* 2002; Andewrson e*t al.,* 2004; Pfaffl *et al*., 2004). Although extensive data is available in this area, none of them suggest a single reference gene which shows a stable expression under various experimental conditions. Therefore, for the present study it was necessary to validate and identify stability of a control gene under specific experimental conditions prior to its use for normalization. 18S rRNA, actin and tubulin are common reference genes used for gene normalization. However, experimental evidence show that these genes are not always stably expressed under different experimental conditions (Thellin *et al*., 1999; Ruan and Lai, 2007). In addition the holometabolus insects including Lepidoptera undergo molting as well as metamorphosis, a process highly dependent on morphogenetic hormones (Gilbert *et al*., 2002) which modulate expression of various genes. Among many structural proteins, actin and tubulin whose genes are frequently used as internal control for real time analysis of gene expression, play important role in molting/metamorphosis. Further, the actin as well as tubulin synthesis was shown to be modulated by 20E even in *C. cephalonica* (Shanavas *et al*., 1996).

In the current insect model *C. cephalonica,* the stability of commonly used housekeeping genes such as *tubulin, 18S rRNA, TATA* binding protein (*TBP*), *actin, rS7, GAPDH* and

EF1α was analysed under different experimental conditions. All the listed housekeeping genes, chosen for the study were cloned and the sequence was confirmed by semi-quantitative PCR and sequencing analysis. geNorm, Normfinder and Bestkeeper tools were used for evaluation of results obtained from qRT-PCR of the above genes in different conditions. During different developmental stages i.e. embryo to adult, *rS7* has shown the highest stability as compared to other housekeeping genes. The results obtained from the above analysis further show that *actin* and *tubulin* have poor stability which is due to their altered gene expression in different larval stages, and these results corroborate with our earlier findings (Shanavas *et al*., 1996). Hence neither of these could be good reference/internal control genes for the analysis of gene expression in the ontogeny studies. Gene stability values for various housekeeping genes in different tissues from the last instar larva again support the candidature of *rS7*, as the best reference gene under normal physiological conditions in *C. cephalonica.*

Ecdysone and juvenile hormones are the two major morphogenetic hormones which regulate various physiological functions during insect development (Wigglesworth, 1954; Riddiford, 1980, 1994; Thummel 1996, 2002; Gilbert, 2000). In the present study the gene stability of housekeeping genes was evaluated after depletion of endogenous hormones and/or application of exogenous hormones. The endogenous hormone was depleted primarily by doing thorax-ligation (Hiruma *et al*., 1977). Earlier studies from our laboratory have shown that the last instar larva of *C. cephalonica,* gets depleted of 20E in about 24h after thorax-ligation (Ashok and Dutta-Gupta, 1988) Furthermore circulatory ecdysteroids are known to have maximum circulatory half-life of 9-12 h (Cherbas *et al*., 1991). In the present analysis thorax-ligated insects, which were cleared of endogenous hormones were treated with specific exogenous hormone(s) to ensure that the effect observed was only due to a given hormone(s). Gene stability values obtained show that

18S rRNA is the most stable housekeeping gene under 20E treatment, whereas TBP showed the highest stability under methoprene (JH analog) treatment. It is interesting to note that, tubulin gene under JH treatment shows good stability, similar to that of *TBP*. This could be due to the independent application of the hormones and in absence of 20E, JH has a variable effect on the expression of *tubulin* gene. All the above experiments along with reports from the literature, strongly suggest that reference genes cannot be universal and have to be validated for a given experiment al set up. Since the model used *C. cephalonica* is Lepidopteran stored grain pest, the above results could also be used for the closely related other insects of the same category after validation.

Ontogeny of the three hexamerins in the present study demonstrated their stage specific expression in *C. cephalonica*. Expression of hexamerin genes started from early larval instar, gradually increased towards late larval instars and declined during pupal stage. Expression of *Hex 84* was seen from 2nd larval instar, while for *Hex 86* it was from 4th larval instar and *Hex 82* was expressed exclusively in last larval instar. The ontogeny profile correlates well with their physiological functions reported earlier (Nagamanju *et al.*, 2003; Damara *et al.*, 2010b). Hex 84 is an arylphorin known to be involved in various functions like cuticle deposition (KiranKumar *et al*., 1997, 1998), immune response (Arif *et al*., 2003) and xenobiotic binding (Budatha *et al.,* 2007; Ningshen *et al.,* 2013). Owing to its varied and diverse functions, Hex 84 starts getting expressed from the early larval instar stage. Hex 86 is methionine rich hexamerin and is known to be primarily involved in processes like reproduction. Hence the expression of *Hex 86* gene from fourth larval instar facilitates the synthesis and accumulation of Hex 86, which gradually increases in hemolymph and parallels well with the development of reproductive organs. Further the continuous expression of *Hex 84* and *Hex 86* genes help in expression of large quantity of transcripts which are translated extensively during the larval stages and the proteins are

released from the fat body to hemolymph, where their concentration gradually increases (Ismail *et al*., 1991; KiranKumar *et al.*, 1997; Nagamanju *et al.*, 2003; Damara *et al*., 2010a). The exclusive expression of *Hex 82* in the last larval instar, could be attributed to several functions which are not completely known. However, we hypothesize that being a putative riboflavin binding protein it binds to small molecules (riboflavin or its derivatives) that are important for the larval- pupal-adult development and transformation. However, further studies need to be carried out to decipher the function.

Hormones are known to influence the insect development and various stages of insect have precise titers of JH and ecdysteroids, which modulate various physiological events (Gilbert, 2000; Thummel, 2002). Hence, we evaluated the effect of 20E and methoprene (JH analogue) on transcription as well as translation of hexamerins in *C. cephalonica*. 20E treatment increased the total protein synthesis as well as content in the larval fat body. Immunoprecipitation of total hemolymph protein from control and hormone treated insects with hexamerin antibodies clearly showed that the 20E stimulates the synthesis of hexamerins at protein level. Organ culture studies further confirm the role of 20E on hexamerins at the translational level. Quantitative analysis after hormone treatment demonstrates that 20E induced mRNA expression of all three hexamerins, showing highest effect between 6 to 12 hours. This might be associated with known circulatory half-life of ecdysteroids which is reported to be 9-12 hours in various Lepidopteran insects (Cherbas *et al*., 1991). Methoprene alone did not show any effect on the *Hex* gene expression, but when it was given in combination with 20E, hexamerins expression was induced even more when compared with 20E alone. This could be due to known role of methoprene (JH analogue), which acts as *status quo* factor (Chaitanya *et al*., 2011). Further, it might also facilitate the stability of hexamerin RNA which was earlier reported in *Galleria mellonella* (Ray *et al*., 1987).

The morphogenetic hormones are critical regulators of postembryonic development, larval molting and metamorphosis of insects and interplay between them (JH and 20E) govern not only the induction but also suppression of various genes at specific stages of development (Chaitanya *et al*., 2011; Dubrovsky and Bernard, 2014). The present i*n vitro* and *in vivo* studies mentioned above confirm that 20E upregulates fat body hexamerin gene expression during the larval development and these results corroborate well with the earlier reports for other tissues like epidermis, silk glands, and wing discs of various Lepidopteran insects (Hiruma and Riddiford, 2001; Stilwell *et al*., 2003; Sekimoto *et al*., 2006, 2007; Ali *et al*., 2012, 2013). As *Hex 84* is expressed from early to late larval stages in *C. cephalonica*, this was selected for analysis of hormonal regulation. Ecdysterods like other steroid hormones act through nuclear receptors which bind to the specific DNA sequences i.e. coagnate elements (Crispi *et al*., 2001) and mediate expression of ecdysone regulated genes. Biologically active ecdysteroid, 20E promotes the association of EcR with USP, the insect homologue of retinoid X receptor (RXR) (Thomas *et al.*, 1993; Riddiford *et al*., 2000; Henrich, 2005), facilitates its dimerization, this heterodimer binds and promotes transcription of genes, which have ecdysone response elements (EREs) (Oro *et al*., 1990; Yu *et al*., 2012). In the present study genome walking strategy was used for the identification of 5' flanking sequence of *Hex 84*. Analysis of 5' flanking region of *Hex 84* of *C. cephalonica* showed the presence of various putative steroid regulatory elements like ERE, FOXL1a, E74a, CREB, ZESTE, RXR along with TATA box. To find out the active element, which is important for *Hex 84* expression, luciferase assay was carried out using sequential deletion constructs. Luciferase assay results obtained in the present study revealed that ERE which is present between -256 to -280 upstream region showed the promoter up regulation activity. Further substitution mutation in the identified canonical sequence of *C. cephalonica Hex 84* gene resulted in a total loss of 20E response. Earlier

study from our laboratory has already reported the presence of an ERE in upstream sequence of H-fibroin gene, which negatively regulated H-fibroin expression during postembryonic development of *C. cephalonica* (Chaitanya *et al.*, 2011). EMSA results of present study further demonstrate the activity of ERE element, upon 20E induction by showing shift in probe interaction with 20E induced nuclear extract but not in uninduced extract or with mutated probe. The sequence region (TTTTAAATAAATAAAACA), which is responsible for upregulation of *Hex 84* present in the 5' flanking region, is designated as CcERE. This plays role in transcriptional regulation of hexamerin gene in *C. cephalonica* during the larval development.

SUMMARY

Hexamerins are important proteins which play a vital role in the development as well as reproduction of Lepidopteran insects. Structurally hexamerins are multimeric aggregates of six subunits in the range of 70-90 kDa, which are either homo- or hetero-hexamers with native molecular mass around 450- 500 kDa (Telfer and Kunkel, 1991). Based on amino acid composition hexamerins have been classified into four main classes (Telfer and Kunkel, 1991): **(i)** the arylphorin, is characterized by exceptionally high content of aromatic amino acids (18-26%) and low methionine content (2%), **(ii)** the glycoprotein with high aromatic amino acid and low methionine content and named as Lepidopteran arylphorin, **(iii)** Dipteran hexamerin which is neither rich in aromatic amino acids nor in methionine and **(iv)** the hexamerin with relatively high methionine (about 6%) and low aromatic amino acid content, predominantly expressed in the female larvae of Lepidoptera, where they form the yolk protein precursors. Hexamerins are predominantly synthesized by the larval fat body and released into the haemolymph, their concentration increases gradually from penultimate larval to final larval instar and nearly account for 80% of the total haemolymph protein by weight. Later they are sequestered back by receptor mediated process and accumulated in various tissues including fat body.

Our group has identified three distinct hexamerins Hex 86 (86 kDa), Hex 84 (84 kDa), and Hex 82 (82 kDa) in *C. cephalonica.* Hexamerins have been biochemically purified and characterized from larval hemolymph of *C. cephalonica* (KiranKumar *et al*., 1987). Further, Hex 84 was show as arylphorin (Nagamanju *et al*., 2003), while Hex 86 was characterized as methionine rich hexamerin (Damara and Dutta-Gupta, 2010).

In the present study an attempt was made to clone the third hexamerin (Hex 82) and carryout detailed studies on developemental as well as hormonal regulation of hexamerin(s) synthesis and their release during larval development of *C. cephalonica*.

Chapter 1: Cloning and characterization of *Hex 82* form *Corcyra cephalonica* last instar larval fat body

Full length coding sequence of *Hex 82* was cloned using degenerate primer and RACE strategies. ORF of the *Hex* 82 contain 2118 bp, which gave to deduced protein with molecular weight of 82 kDa. *In silico* analysis of Hex 82 sequence showed that it has putative glycosylation as well as phosphorylation sites, and has an isoelectric point 6.04. Homology comparison with other *C. cephalonica* hexamerins show 62% sequence similarity with H*ex* 84 and 54% similarity with *Hex* 86. Ontogeny results demonstrate that *Hex* 82 is last larval instar specific gene. Tissue distribution studies further indicate its expression in fat body. BLASTn results reveal that *Hex* 82 2118 bp ORF has 65% sequence identity with *Hyalophora cercopia* riboflavin binding precursor protein. Riboflavin binding studies reveal only Hex 82 has riboflavin binding property. This was further confirmed by homology modeling and molecular docking studies, which show that Hex 82 has the highest binding constant when compared with Hex 84 and Hex 86. Based on the above, we propose Hex 82 as a putative riboflavin binding hexamerin of *C. cephalonica.*

Chapter 2: Validation of reference gene(s) as internal control for quantitative polymerase chain reaction (qRT-PCR) analysis in *C. cephalonica*

Identification of good internal reference controls for quantitative PCR analysis is a primary requirement. In this study, we chose seven distinct constitutive genes (*tubulin*, *18SrRNA*, *TATA binding protein* (TBP), *actin*, *rS7*, *GAPDH, EF1α*) and cloned them by degenerate primer strategy. Expression levels of these constitutive genes were measured during different larval stages and under the influence of morphogenetic hormone treatments using qRT-PCR. The Ct values of this qRT-PCR were then analyzed using geNorm (Vandesompele *et al*., 2002), Normfinder (Andersen *et al*., 2004) and Bestkeeper (Pfaffl, *et al.*, 2004) softwares. Gene stability (M) values obtained varied in different conditions.

During different developmental stages *rS7* showed the highest gene stability while upon 20E treatment *18sRNA* revealed highest gene stability. On the other hand *TBP* showed the highest gene stability in methoprene treatment. This analysis also shows that *actin* and *tubulin* have the least stability during hormone treatment in *C. cephalonica* and their expression is modulated by morphogenetic hormones during postembryonic/larval development (Shanavas *et al*., 1996; 2004).

Chapter 3: Ontogeny and role of morphogenetic hormones in transcriptional as well as translational regulation of *Hex 82, Hex 84,* and *Hex 86*: *in vivo* and *in vitro* studies

Ontogeny of the three hexamerins was carried out using qRT-PCR. Results demonstrated that expression of hexamerins is stage specific. Expression started from early larval instar, gradually increased towards late larval instars and declined in pupal stages. Expression of H*ex 84* was seen from 2nd larval instar, while for *Hex 86* it was from 4th larval instar, however *Hex 82* was exclusively expressed in final larval instar. We evaluated the effect of 20E and methoprene (JH analogue) on transcription as well as translation of hexamerins. Results from *in vitro* and *in vivo* studies showed that 20E promoted synthesis of hexamerins at transcriptional as well as translational levels. Immunoprecipitation experiments of hormone treated fat body homogenate with hexamerin antibodies further confirmed the stimulatory role of 20E in the synthesis of hexamerins in *C. cephalonica*. Quantitative PCR analysis after hormone treatment also demonstrated that 20E induced, the mRNA expression of all the three hexamerins. However methoprene alone did not alter hexamerins expression but when it was given along with 20E, there was an increased expression of hexamerin(s) genes. This could be due to the known role of JH which acts as *status quo* factor. Further, it might also promote the stability of hexamerin mRNA, which was earlier reported by Ray *et al*., (1987) in *Galleria.*

Chapter 4: Identification and characterization of ecdysteroid regulatory elements in the upstream sequence of *Hex 84* gene of *C. cephalonica*

Steroid hormones are known to pass through cell as well as nuclear membrane, bind to the receptor proteins and form the hormone receptor complex, which interacts with hormone responsive elements primarily present in the upstream sequence of variety of genes and modulate gene transcription. Genome walking strategy was used for the identification of regulatory elements in the upstream promoter region of hexamerins. The promoter sequence was analyzed by different online tools like TESS Master, Matinspector and Transfac to predict transcriptional factor binding sites. Transient transfection studies of different deletion constructs into S*f*9 insect cell line showed the upstream region between -256 to -280 to be associated with significant promoter activity. We identified an active ERE in this study and named it as CcERE responsible for upregulation of 20E modulated hexamerin expression during postembryonic developement. Electrophoretic mobility shift assay (EMSA) further confirmed its functional activity after hormone treatment.

REFERENCES

Ali, M.S., Iwanaga, M. and Kawasaki, H., 2012. Ecdysone-responsive transcription factors determine the expression region of target cuticular protein genes in the epidermis of *Bombyx mori*. Dev. Gene. Evolution 222, 89-97.

Ali, M.S., Iwanaga, M. and Kawasaki, H., 2013. Ecdysone-responsive transcriptional regulation determines the temporal expression of cuticular protein genes in wing discs of *Bombyx mori*. Gene 512, 337-347.

Allgood, V.E. and Eastman, E.M., 1997. Chimeric receptors as gene switches. Current Opinion in Biotech. 8, 474-479.

Ancsin, J.B. and Wyatt, G.R., 1996. Purification and characterization of two storage proteins from *Locusta migratoria* showing distinct developmental and hormonal regulation. Insect Biochem. Mol. Biol. 26, 501-510.

Andersen, C.L., Jensen, J.L. and Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245-5250.

Arif, A., Scheller, K. and Dutta-Gupta, A., 2003. Tyrosine kinase mediated phosphorylation of the hexamerin receptor in the rice moth *Corcyra cephalonica* by ecdysteroids. Insect Biochem Mol. Biol.. 33, 921-928.

Arif, A., Vasanthi, P., Hansen, I.A., Scheller, K. and Dutta-Gupta, A., 2004. The insect hemolymph protein HP19 mediates the nongenomic effect of ecdysteroids on acid phosphatase activity. The J. Biol. Chem., 279, 28000-28008.

Arif, A., Manohar, D., Gullipalli, D. and Dutta-Gupta, A., 2008. Regulation of hexamerin receptor phosphorylation by hemolymph protein HP19 and 20-hydroxyecdysone directs hexamerin uptake in the rice moth *Corcyra cephalonica*. Insect Biochem Mol. Biol. 38, 307-319.

Arrese, E.L. and Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Ann. Rev. Entomol., 55, 207-225.

Ashok, M. and Dutta-Gupta, A., 1988. Ecdysteroid mediated fat body acid phosphatase activity during larval development of rice moth, *Corcyra cephalonica* (Lepidoptera). Biochem. Intl., 17, 1087-1091.

Bajaj, P., Ismail, S. and Dutta-Gupta, A., 1990. Uptake of Corcyra larval haemolymph proteins by the male accessory reproductive glands of stem borer, *Chilo partellus* (Lepidoptera-Insecta). Biochem. Intl., 20, 549-554.

Bean, D.W. and Silhacek, D.L., 1989. Changes in the titer of the female-predominant storage protein (81K) during larval and pupal development of the waxmoth, *Galleria mellonella*. Arch Insect. Biochem. Physiol 10, 333-348.

Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B. and Hogness, D.S., 1997. *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell 91, 777-788.

Birren, B., Green, E. D., Klapholz, S., Myers, R. M. and Roskams, J. , 1997. Genome analysis. A laboratory manual. Cold Spring Harbour Press, New York.

Blum, H., Beier, H. and Gross, H.J., 1987. Improved silver staining of plant proteins, RNA, DNA in polyacrylamide gels. Electrophoresis, 8, 93-99.

Bond, N.D., Hoshizaki, D.K. and Gibbs, A.G., 2010. The role of 20-hydroxyecdysone signaling in *Drosophila* pupal metabolism. Comp. Biochem.Physiol. Part A, Mol. Integr. Physiol. 157, 398-404.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Braun, R.P. and Wyatt, G.R., 1996. Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. J. Biol. Chem. 271, 31756-31762.

Brunner, A.M., Yakovlev, I.A. and Strauss, S.H., 2004. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol. 4, 14.

Budatha, M., Meur, G., Kirti, P.B. and Dutta Gupta, A., 2007. Characterization of *Bacillus thuringiensis* Cry toxin binding novel GPI anchored aminopeptidase from fat body of the moth *Spodoptera litura*. Biotechnol. Let. 29, 1651-1657.

Burks, C. S., Shelby K. S. and Chippendale G. M, 1992. Characteristics of apolipophorin-III of the southwestern corn borer, *Diatraea grandiosella*. Insect Biochem. Mol. Biol. 22, 905-915.

Burmester, T., 2002. Origin and evolution of arthropod hemocyanins and related proteins. J. Comp. Physiol. B 172, 95-107.

Burmester, T. and Scheller, K., 1997. Developmentally controlled cleavage of the *Calliphora* arylphorin receptor and Posttranslational action of the steroid hormone 20-hydroxyecysone. Euro. J.Biochem. 247, 695-702.

Burmester, T. and Scheller, K., 1999. Ligands and receptors: common theme in insect storage protein transport. Naturwissenschaften 86, 468-474.

Burmester, T., Antoniewski, C. and Lepesant, J.A., 1999. Ecdysone-regulation of synthesis and processing of Fat Body Protein 1, the larval serum protein receptor of *Drosophila melanogaster*. Eur. J. Biochem. 262, 49-55

Burmester, T., Matzner, U. and Scheller, K., 1995. Effect of 20-hydroxyecdysone on synthesis and uptake of arylphorin by the larval fat body of *Calliphora vicina* (Diptera: Calliphoridae). Euro. J. Entomol. 92, 217-217.

Burmester, T., Massey Jr, H.C., Zakharkin, S.O. and Benes, H., 1998. The evolution of hexamerins and the phylogeny of insects. J. Mol. Evol. 47, 93-108.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. j. Mol. Endocrinol 25, 169-193.

Chaitanya, R.K., Sridevi, P., Senthilkumaran, B. and Dutta- Gupta, 2011. 20-Hydroxyecdysone regulation of H-fibroin gene in the stored grain pest *Corcyra cephalonica*, during the last instar larval development. Steroids. 76, 125-134.

Chaitanya, R.K., Sridevi, P., Senthilkumaran, B. and Dutta Gupta, A., 2013. Effect of juvenile hormone analog, methoprene on H-fibroin regulation during the last instar larval development of *Corcyra cephalonica*. Gen.Comp. Endocrinol. 181, 10-17.

Cherbas, P., 1993. Ecdysone-responsive genes. Insect Biochem. Mol. Biol. 23, 3-11.

Cherbas, L., Lee, K. and Cherbas, P., 1991. Identification of ecdysone response elements by analysis of the *Drosophila* Eip28/29 gene. Genes Dev. 5, 120-131.

Chrysanthis G., Marmaras V. J. and Christodoulou C, 1981. Major haemolymph proteins in *Ceratitis capitata*. Biosynthesis and secretion during development. Roux's Arch. Dev. Biol. 33-39.

Chung, S.O., Kubo, T. and Natori, S., 1995. Molecular Cloning and sequencing of arylphorin-binding protein in protein granules of the sarcophaga fat body implication of a post-translational processing mechanism. J.Biol. Chem 270, 4624-4631.

Coleman A, Jensen, M.N., Andrews M, and Carlson S, 2010. Household Food Security in the United States, Economic Research Report.

Costa-Leonardo, A.M., Laranjo, L.T., Janei, V. and Haifig, I., 2013. The fat body of termites: functions and stored materials. J. Insect Physiol. 59, 577-587.

Crampton, J., James, A., Gordadze, A., Korochkina, S., Zakharkin, S., Norton, A. and Beneš, H., 1999. Molecular cloning and expression of two hexamerin cDNAs from the mosquito, *Aedes aegypti*. Insect Mol. Biol. 8, 55-66.

Crispi, S., Giordano, E., D'Avino, P.P., Peluso, I., and Furia, M. 2001. Functional analysis of regulatory elements controlling the expression of the ecdysone-regulated *Drosophila* ng-1 gene. Mech. Dev. 1, 25-35.

Cristino, A.S., Nunes, F.M., Barchuk, A.R., Aguiar-Coelho, V.M., Simoes, Z.L., Bitondi, M.M., 2010. Organization, evolution and transcriptional profile of hexamerin genes of the parasitic wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). Insect Mol. Biol. 19 Suppl 1, 137-146.

Damara, M. and Dutta-Gupta, A., (2010). Identification of 86 kDa protein as methionine rich hexamerin in the rice moth, *Corcyra cephalonica*. Comp. Biochem.Physiol. Part B, Biochem.Mol. Biol. 157, 229-237.

Damara , M., Gullipalli, D. and Dutta-Gupta, A., 2010a. Ecdysteroid-mediated expression of hexamerin (arylphorin) in the rice moth, *Corcyra cephalonica*. J. Insect Physiol. 56, 1224-1231.

Damara, M., Gullipalli, D. and Dutta-Gupta, A., 2010b. Cloning and expression of fat body hexamerin receptor and its identification in other hexamerin sequestering tissue of rice moth, *Corcyra cephalonica*. J. Insect Physiol. 56, 1071-1077.

Dean R., L.M. and Collins J., 1985. Structure of the fat body. Comp. Insect Biochem. Physiol. Pharmacol.. Pergamon Press, Oxford. Vol 3, 155-210.

Dhar, A.K., Bowers, R.M., Licon, K.S., Veazey and Read, G. B., 2009. Validation of reference genes for quantitative measurement of immune gene expression in shrimp. Mol. Immunol. 46, 1688-1695.

Dong, L., Sui, C., Liu, Y., Yang, Y., Wei, J. and Yang, Y., 2011. Validation and application of reference genes for quantitative gene expression analyses in various tissues of *Bupleurum chinense*. Mol. Biol. Reports.38, 5017-5023.

Dubrovsky E. B. and Bernardo, T. J. 2014. The juvenile hormone receptor and molecular mechanisms of juvenile hormone action. Adv. Insect Physiol. 46, 305-388.

Dutta-Gupta, A. and Ashok, M. 1998. A comparative study on the ecdysteroid titre in the normal, decapitated and thorax-ligated larvae of stem borer, Chilo partellus and rice moth, *Corcyra cephalonica*. Entomon. 23. 245-250.

Dutta-Gupta A. and Ismail S.M., (1990) Ecdysteroid mediated differential uptake of larval haemolymph proteins during postembryonic development in rice moth, *Corcyra cephalonica*. Proceedings of Fifth International Working Conference on Stored Product Protection, (Eds. Flcurat-Lessano F. and Ducam P.) 1: 47-59

Engelmann, F.and Mala, J., 2005. The cockroach *Leucophaea maderae* needs more than juvenile hormone, vitellogenin and reserves to make a yolky egg. J. Insect Physiol. 51, 465-472.

Fahrbach, S.E., Smagghe, G. and Velarde, R.A., 2012. Insect nuclear receptors. Ann. Rev. Entomol. 57, 83-106.

Faria, F., Garcia, E. and Goldenberg, S., 1994. Synthesis of a haemolymph hexamerin by the fat body and testis of *Rhodnius prolixus*. Insect biochem. Mol. Biol. 24, 59-67.

Freedman, L.P., 1997. Molecular biology of steroid and nuclear hormone recptors. Birkhauser Press, Boston.

Gade, G., 2004. Regulation of intermediary metabolism and water balance of insects by neuropeptides. Ann. Rev. Entomol. 49, 93-113.

Gilbert, L. I., Granger N. A. and Roe M. R. (2000) The juvenile hormones: Historical facts and speculations on future research durations. Insect. Biochem. Mol. Biol. 30: 617-644.

Gilbert, L.I., Rybczynski, R. and Tobe, S.S. 1996. Endocrine cascade in insect metamorphosis. In Metamorphosis - postembryonic reprogramming of gene expression in amphibian and insect cells". (Gilbert L.I., Tata J.R., Atkinson B.G.), Academic Press, San Diego, 59-107.

Gilbert, L.I., Rybczynski, R. and Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Ann. Rev. Entomol. 47, 883-916.

Gray, R., Meola, R. and Holman, G.M., 1987. Fat body stimulation of ecdysone synthesis in *Heliothis zea*. J. Insect Physiol. 33, 325-331.

Grebe, M., Fauth, T. and Spindler-Barth, M., 2004. Dynamic of ligand binding to *Drosophila melanogaster* ecdysteroid receptor. Insect. Biochem. Mol. Biol. 34, 981-989.

Gudderra, N., Sonenshine, D., Apperson, C. and Roe, R., 2002. Hemolymph proteins in ticks. J. Insect Physiol. 48, 269-278.

Hagner-Holler, S., Pick, C., Girgenrath, S., Marden, J.H. and Burmester, T., 2007. Diversity of stonefly hexamerins and implication for the evolution of insect storage proteins. Insect Biochem. Mol. Biol. 37, 1064-1074.

Hahn, D.A. and Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. J.Insect Physiol. 53, 760-773.

Hansen, I.A., Meyer, S.R., Schafer, I. and Scheller, K., 2002. Interaction of the anterior fat body protein with the hexamerin receptor in the blowfly *Calliphora vicina*. Euro. J. Biochem. 269, 954-960.

Haunerland, N. H., 1996. Insect storage proteins: gene families and receptors. Insect Biochem. Mol. Biol. 26, 755-765.

Haunerland N. H. and Bowers W. S., (1986) Binding of insecticides to lipophorin and arylphorin, two hemolymph proteins of *Heliothis zea.* Arch. Insect. Biochem. Physiol. 3: 87-96.

Haunerland, N. H., and Shirk, P., 1995. Regional and functional differentiation in the insect fact body. Ann. Rev. Entomol . 40, 121-145.

Henrich, V.C., Rybczynski, R. and Gilbert, L.I., 1999. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. Vitamins and Hormones 55, 73-125.

Highnam, K.C., 1981. A survey of invertebrate metamorphosis. In, Metamorphosis. Plenum Press. pp 43-73.

Hill, R.J., Billas, I.M., Bonneton, F., Graham, L.D. and Lawrence, M.C., 2013. Ecdysone receptors: from the Ashburner model to structural biology. Ann. Rev. Entomol. 58, 251-271.

Hiruma, K., Bocking, D., Lafont, R. and Riddiford, L.M., 1997. Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. Gen. Comp. Endocrinol. 107, 84-97.

Hiruma, K. and Riddiford, L.M., 2001. Regulation of transcription factors MHR4 and betaFTZ-F1 by 20-hydroxyecdysone during a larval molt in the tobacco hornworm, *Manduca sexta*. Dev. Biol. 232, 265-274.

Horn, D.H.S. and Bergamasco, R, 1985. Chemistry of ecdysteroids. Comp. Insect Physiol. Biochem. Pharmacol. 185-248. Pergamon Press, New York, Vol. 7, 185-248.

Hoshizaki, D.K., A.G. Gibbs and N.D. Bond, 2012. Fat Body, R.F. Chapman's The Insects: Structure and Function, 5th ed, pp 132-145

Hossain, M.S., Liu, Y., Zhou, S., Li, K., Tian, L. and Li, S., 2013. 20-Hydroxyecdysone-induced transcriptional activity of FoxO upregulates brummer and acid lipase-1 and promotes lipolysis in *Bombyx* fat body. Insect Biochem. Mol. Biol. 43, 829-838.

Hwang, S. J., Cheon, H. M., Kim, H. J., Chae, K. S., Chung, D. H., Kim, M. O., Park, J. S., and Seo, S.J., 2001. cDNA sequence and gene expression of storage protein-2—a juvenile hormone-suppressible hexamerin from the fall webworm, *Hyphantria cunea* Drury. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 129, 97-107.

Inagaki S. and Yamashita O. (1986) Metabolic shift from lipogenesis to glycogenesis in the last instar larval fat body of the silkworm, *Bombyx mori.* Insect Biochem. 16, 327-331.

Ismail, P.M. and Dutta-Gupta, A., 1991. *In vitro* uptake of larval haemolymph proteins by male accessory reproductive glands of the stem borer, *Chilo partellus*. Inv. Rep. Dev. 20, 193-199.

Jamroz, R.C., Beintema, J.J., Stam, W.T. and Bradfield, J.Y., 1996. Aromatic hexamerin subunit from adult female cockroaches *Blaberus discoidalis*: Molecular cloning, suppression by juvenile hormone, and evolutionary perspectives. J. Insect Physiol. 42, 115-124.

Jian, B., Liu, B., Bi, Y., Hou, W., Wu, C. and Han, T., 2008. Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol. Biol. 9, 59.

Jindra, M., Palli, S.R. and Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Ann. Rev. Entomol. 58, 181-204.

Jones, G., Teal, P., Henrich, V.C., Krzywonos, A., Sapa, A., Wozniak, M., Smolka, J. and Jones, D., 2013. Ligand binding pocket function of *Drosophila* USP is necessary for metamorphosis. Gen. Comp. Endocrinol. 182, 73-82.

Judy, K.J., Schooley, D.A., Dunham, L.L., Hall, M.S., Bergot, B.J. and Siddall, J.B., 1973. Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. Proc. Natl. Acad. Sci. U S A 70, 1509-1513.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C. and Ziegler, R., 1990. Insect haemolymph proteins. Adv. Insect Physiol. 22, 299-396.

Keeley, L., 1985. Physiology and biochemistry of the fat body Comp. insect physiol. Biochem Pharmacol. Pergamon press, Oxford, Vol. 3, 211-248.

KiranKumar, N. Ismail, S. M., and Dutta-Gupta, A., 1997. Uptake of storage protein in the rice moth *Corcyra cephalonica*: identification of storage protein binding proteins in the fat body cell membranes. Insect biochem. Mol. Biol. 27, 671-679.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P. and Hogness, D.S., 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67, 59-77.

Koopmanschap, B., Lammers, H. and De Kort, S., 1992. Storage proteins are present in the hemolymph from larvae and adults of the Colorado potato beetle. Arch. Insect Biochem. Physiol. 20, 119-133.

Korochkina, S., Gordadze, A., York, J. and Beneš, H., 1997. Mosquito hexamerins: characterization during larval development. Insect Mol. Biol. 6, 11-21.

Lakshmi, M. and Dutta-Gupta, A., 1990. Juvenile hormone mediated DNA synthesis during larval development of *Corcyra cephalonica* (Insecta). Biochem. Intl. 22, 269-278.

Laemmli U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Levenbook L. (1985) Insect storage proteins. Comp. Insect Biochem Physiol Pharm. Pergamon Press, Oxford. Vol. 10, 307- 347.

Liu, Y., Sheng, Z., Liu, H., Wen, D., He, Q., Wang, S., Shao, W., Jiang, R.J., An, S., Sun, Y., Bendena, W.G., Wang, J., Gilbert, L.I., Wilson, T.G., Song, Q. and Li, S., 2009. Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. Development 136, 2015-2025.

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402-408.

Lord, J.C., Hartzer, K., Toutges, M. and Oppert, B., 2010. Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. J. Microbiol. Methods. 80, 219-221.

Magee, J., Kraynack, N., Massey, H.C., Jr. and Telfer, W.H., 1994. Properties and significance of a riboflavin-binding hexamerin in the hemolymph of *Hyalophora cecropia*. Arch. Insect Biochem. Physiol. 25, 137-157.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M. and Chambon, P., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835-839.

Marinotti, O., Nunes, L.R. and de Bianchi, A.G., 1988. Heterogeneous glycosylation of *Musca domestica* arylphorin. Biochem. Biophys. Res. Commun. 151, 1004-1010.

Martinez, T., Burmester, T., Veenstra, J. and Wheeler, D., 2000. Sequence and evolution of a hexamerin from the ant *Camponotus festinatus*. Insect Mol. Biol. 9, 427-431.

Martins, J.R., Nunes, F.M., Cristino, A.S., Simoes, Z.L. and Bitondi, M.M., 2010. The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. BMC Mol. Biol. 11, 23.

Martins, J.R., Nunes, F.M., Simoes, Z.L. and Bitondi, M.M., 2008. A honeybee storage protein gene, hex 70a, expressed in developing gonads and nutritionally regulated in adult fat body. J. Insect Physiol. 54, 867-877.

Maya-Monteiro, C.M., Daffre, S., Logullo, C., Lara, F.A., Alves, E.W., Capurro, M.L., Zingali, R., Almeida, I.C. and Oliveira, P.L., 2000. HeLp, a heme lipoprotein from the hemolymph of the cattle tick, *Boophilus microplus*. J. Biol. Chem. 275, 36584-36589.

Memmel, N.A., Trewitt, P.M., Grzelak, K., Rajaratnam, V.S. and Kumaran, A.K., 1994. Nucleotide sequence, structure and developmental regulation of LHP82, a juvenile hormone-suppressible hexamerin gene from the waxmoth, *Galleria mellonella*. Insect Biochem. Mol. Biol. 24, 133-144.

Meyer, A.S., Hanzmann, E., Schneiderman, H.A., Gilbert, L.I. and Boyette, M., 1970. The isolation and identification of the two juvenile hormones from the *Cecropia* silk moth. Arch. Biochem. Biophys. 137, 190-213.

Mi, C.H., Hwan, H., Hwa, C.D. and Jae, S., 1998. Sequence analysis and expression of Met-rich storage protein SP-1 of *Hyphantria cunea*. Molec. Cells 8, 219-225.

Munn, E., Feinstein, A. and Greville, G., 1971. The isolation and properties of the protein calliphorin. Biochem. J. 124, 367-374.

Nagamanju, P., Hansen, I.A., Burmester, 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 the larval development of the rice moth, *Corcyra cephalonica*. Insect Biochem. Mol. Biol. 33, 73-80.

Nakagawa, Y. and Henrich, V.C., 2009. Arthropod nuclear receptors and their role in molting. FEBS J. 276, 6128-6157.

Nakajima Y., Van der Goes., van Naters-Yasui A. and Taylor D., 2001. Yamaksin family from the soft tick, *Ornithodoros moubata* (Acar:Argasidae). Insect Biochem. Mol. Biol., 747-751.

Nakanishi, Y. and Garen, A., 1983. Selective gene expression induced by ecdysterone in cultured fat bodies of *Drosophila*. Proc. Natl. Acad. Sci. U S A 80, 2971-2975.

Naumann, U. and Scheller, K., 1991. Complete cDNA and gene sequence of the developmentally regulated arylphorin of *Calliphora vicina* and its homology to insect hemolymph proteins and arthropod hemocyanins. Biochem. Biophys. Res. Com. 177, 963-972.

Nijhout, H.F., 1994. Insect hormones. Princeton University Press.

Nijhout, H.F. and Wheeler, D.E., 1982. Juvenile hormone and the physiological basis of insect polymorphisms. Quart. Rev. Biol. 57, 109-133.

Ningshen, T.J., Chaitanya, R.K., Hari, P.P., Vimala Devi, P.S. and Dutta-Gupta, A., 2013. Characterization and regulation of *Bacillus thuringiensis* Cry toxin binding aminopeptidases N (APNs) from non-gut visceral tissues, Malpighian tubule and salivary gland: Comparison with midgut-specific APN in the moth *Achaea janata*. Com. Biochem. Physiol. Part B, Biochem. Mol. Biol. 166, 194-202.

Nolan, T., Hands, R.E. and Bustin, S.A., 2006. Quantification of mRNA using real-time RT-PCR. Nature Protocols 1, 1559-1582.

O'Brien, D.M., Fogel, M.L. and Boggs, C.L., 2002. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. Proc. Natl. Acad. Sci. U S A 99, 4413-4418.

Oro, A.E., McKeown, M. and Evans, R.M., 1990. Relationship between the product of the *Drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. Nature 347, 298 - 301.

Osman, N., 1984. Assessment of damage by the rice moth, *Corcyra cephalonica* (St.) on different grains at four levels of moisture content. Pertanika 7, 53-58.

Osman, N., 1986. development of the rice moth, *Corcyra cephalonica* (St.) on Different Grains. Pertanika 9, 155-159.

Palli, S.R. and Locke, M., 1987. Purification and characterization of three major hemolymph proteins of an insect, *Calpodes ethlius* (Lepidoptera, Hesperiidae). Arch. Insect Biochem. Physiol. 5, 233-244.

Palli, S.R., Hiruma, K. and Riddiford, L.M., 1992. An ecdysteroid-inducible *Manduca* gene similar to the *Drosophila* DHR3 gene, a member of the steroid hormone receptor superfamily. Dev. Biol. 150, 306-318.

Pan, M and Telfer, W. 1996 Methionine-rich hexamerin and arylphorin as precursor reservoirs for reproduction and metamorphosis in female Luna moths. Arch. Insect. Biochem. Physiol. 32. 149–162.

Park, M.S., Park, P. and Takeda, M., 2013. Roles of fat body trophocytes, mycetocytes and urocytes in the American cockroach, *Periplaneta americana* under starvation conditions: an ultrastructural study. Arthropod Struc. Dev. 42, 287-295.

Parthasarathy, R., Tan, A., Sun, Z., Chen, Z., Rankin, M. and Palli, S.R., 2009. Juvenile hormone regulation of male accessory gland activity in the red flour beetle, *Tribolium castaneum*. Mech Dev. 126, 563-579.

Pei, D.S., Sun, Y.H., Chen, S.P., Wang, Y.P., Hu, W. and Zhu, Z.Y., 2007. Zebrafish GAPDH can be used as a reference gene for expression analysis in cross-subfamily cloned embryos. Anal. Biochem. 363, 291-293.

Peter M. G. and Scheller K. 1991 Arylphorins and integument. In: The Physiology of Insect Epidermeis, Intaka Press, (Retnakaran. A. and Binnington, K. eds.), North Carolina, 115-124.

Pfaffl, M.W., Tichopad, A., Prgomet, C. and Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotech. Let. 26, 509-515.

Pick, C., and Burmester, T., 2009. A putative hexamerin from a *Campodea sp*. suggests an independent origin of haemocyanin-related storage proteins in Hexapoda. Insect. Mol. Biol. 18, 673-679.

Poiani, S.B. and Cruz-Landim, C. 2012 Storaged products and presence of acid phosphatase in fat body cells at pre-pupal worker stage of *Apis mellifera*. Micron. 43, 475-478.

Price, G.M., 1973. Protein and nucleic acid metabolism in insect fat body. Biological Rev. 48, 333-375.

Przibilla, S., Hitchcock, W.W., Szecsi, M., Grebe, M., Beatty, J., Henrich, V.C. and Spindler-Barth, M., 2004. Functional studies on the ligand-binding domain of Ultraspiracle from *Drosophila melanogaster*. J. Biol. Chem. 385, 21-30.

Ray A., Memmel,N.A. and Kumaran A.K, (1987). Developmental regulation of the larval hemolymph protein genes in *Galleria mellonella*. Roux's Arch. Dev. Biol. 196, 414-420.

Rehn, K.G. and Rolim, A.L., 1990. Purification and properties of a storage protein from the hemolymph of *Rhodnius prolixus*. Insect Biochem. 20, 195-201.

Riddiford, L.M., 1980. Insect endocrinology: action of hormones at the cellular level. Ann. Rev. Physiol. 42, 511-528.

Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24, 213-274.

Riddiford, L.M., 2012. How does juvenile hormone control insect metamorphosis and reproduction. Gen. Comp. Endocrinol. 179, 477-484.

Riddiford, L.M., Cherbas, P. and Truman, J.W., 2000. Ecdysone receptors and their biological actions. Vitamins & Hormones 60, 1-73.

Riddiford, L.M., Palli, S.R. and Hiruma, K., 1990. Hormonal control of sequential gene expression in *Manduca* epidermis. Pro. Clin. Biol. Res. 342, 226-231.

Rimoldi, O.J., Corsico, B., Gonzalez, M.S. and Brenner, R.R., 1996. Detection and quantification of a very high density lipoprotein in different tissues of *Triatoma infestans* during the last nymphal and adult stages. Insect biochem. Mol. Biol. 26, 705-713.

Roma, G.C., Bueno, O.C., and Camargo-Mathias., 2010. Morpho-physiological analysis of the insect fat body: a rev. Micron 41, 395-401.

Roy, M.K., Choudhury, A., Guha, A., Yadav, A., Kumari, J. and Unni, B.G., 2004. Induced immunity in *Antheraea assama* Ww larvae against flacherie causing *Pseudomonas aeruginosa* AC-3. Exp. Parasitol. 106, 75-84.

Ruan, W. and Lai, M., 2007. Actin, a reliable marker of internal control? Clinica Chimica Acta; Intl. J. Clin. Chem. 385, 1-5.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 2001. Molecular cloning: A laboratory manual, Vol 3, Cold Spring Harbour Press, New York.

Schwedes, C.C. and Carney, G.E., 2012. Ecdysone signaling in adult *Drosophila melanogaster*. J. Insect Physiol. 58, 293-302.

Sehnal, F., Svacha, P. and Zrzavy, J. 1996. Evolution of insect metamorphosis. In "Metamorphosis:postembryonic reprogramming of gene expression in amphibian and insect cells". (Gilbert L.I., Tata J.R., Atkinson B.G.), Academic Press, San Diego, 3-58.

Sekimoto, T., Iwami, M. and Sakurai, S., 2006. Coordinate responses of transcription factors to ecdysone during programmed cell death in the anterior silk gland of the silkworm, *Bombyx mori*. Insect. Mol. Biol. 15, 281-292.

Sekimoto, T., Iwami, M. and Sakurai, S., 2007. 20-Hydroxyecdysone regulation of two isoforms of the Ets transcription factor E74 gene in programmed cell death in the silkworm anterior silk gland. Insect. Mol. Biol. 16, 581-590.

Shahabuddin, M., Fields, I., Bulet, P., Hoffmann, J.A and Miller, L.H., 1998. *Plasmodium gallinaceum*: differential killing of some mosquito stages of the parasite by insect defensin. Exp. Parasitol 89, 103-112.

Selvey, S., Thompson, EW., Matthaei, K., Lea RA, Irving MG and Griffiths LR., 2001. Beta *actin* an unsuitable internal control for RT- PCR. Mol. Cell. Probes. 15:307–311.

Shanavas, A., Nayak, B.P. and Dutta-Gupta, A., 1996. Ecdysteroid mediated muscle actin synthesis during the larval development of rice moth, *Corcyra cephalonica*. Biochem. Mol. Biol. Intl. 40, 955-963.

Shanavas, A., Arif, A., Murthy, Ch.R.K. and Dutta Gupta, A., 2004. Developmental and hormonal regulation of actin and tubulin in the central nervous system of silkworm, *Bombyx mori* during postembryonic development. Current Science 87, 383-388.

Slama, K. 1975. Some old concepts and new findings on hormonal control of insect metamorphogenesis. J. insect. Physiol. 21, 921-955.

Soller, M., Bownes, M. and Kubli, E. 1999. Control of oocyte maturation in sexually mature *Drosophila* females., Dev. Biol. 208, 337-351.

Stilwell, G.E., Nelson, C.A., Weller, J., Cui, H., Hiruma, K., Truman, J.W. and Riddiford, L.M., 2003. E74 exhibits stage-specific hormonal regulation in the epidermis of the tobacco hornworm, *Manduca sexta*. Dev. Biol. 258, 76-90.

Telfer, W.H. and Kunkel, J.G., 1991. The function and evolution of insect storage hexamers. Ann. Rev. Entomol. 36, 205-228.

Thellin, Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., and Heinen, E., 1999. Housekeeping genes as internal standards: use and limits. J. Biotechnol. 75, 291-295.

Thummel, C.S., 1996. Flies on steroids-*Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends in Genetics. 12, 306-310.

Thummel, C.S., 2002. Ecdysone-regulated puff genes 2000. Insect Biochem. Mol. Biol. 32, 113-120.

Tissot, M. and Stocker, R.F. 2000. Metamorphosis in *Drosophila* and other insects: the fate of neurons throughout the stages. Prog. Neurobiol. 62, 89-111.

Tojo, S. and Yoshiga, T., 1994. Purification and characterization of three storage proteins in the common cutworm, *Spodoptera litura*. Insect Biochem. Mol. Biol. 24, 729-738.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U S A 76, 4350-4354.

Trivedi, S. and Arasu, P., 2005. Evaluation of endogenous reference genes for real-time PCR quantification of gene expression in *Ancylostoma caninum*. Mol. Biochem. Parasitol. 143, 241-244.

Truman, J.W. and Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. Ann. Rev Entomol.47, 467-500.

Truss, M. and Beato, M., 1993. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocrine Rev. 14, 459-479.

Ueno, K. and Natori, S., 1984. Identification of storage protein receptor and its precursor in the fat body membrane of *Sarcophaga peregrina*. J.Biol. Chem. 259, 12107-12111.

Van Holde K. E. and Miller, K. I., 1995. Hemocyanins. Adv. Protein. Chem.47, 1-81.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N. and De Paepe, A. and Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, Research 0034.

Vanishree, V., Nirmala, X., Arul, E. and Krishnan, M., 2005. Differential sequestration of storage proteins by various fat body tissues during post-larval development in silkworm, *Bombyx mori* L. Invert. Rep. Dev. 48, 81-88.

Vizioli, J., Bulet, P., Charlet, M., Lowenberger, C., Blass, C., Müller, H.M., Dimopoulos, G., Hoffmann, J., Kafatos, F. and Richman, A., 2000. Cloning and analysis of a cecropin gene from the malaria vector mosquito, *Anopheles gambiae*. Insect Mol. Biol. 9, 75-84.

Wang, Y., Han, Z., Yan, S., Mao, A., Wang, B., Ren, H., Chi, Y. and Han, Z., 2010. Evaluation of suitable reference gene for real-time PCR in human umbilical cord mesenchymal stem cells with long-term in vitro expansion. In vitro Cell. Dev. Biol. Animal. 46, 595-599.

Wang, Y., Yu, K., Poysa, V., Shi, C. and Zhou, Y., 2012. Selection of reference genes for normalization of qRT-PCR analysis of differentially expressed genes in soybean exposed to cadmium. Mol. Biol. Rep. 39, 1585-1594.

Wang, Z. and Haunerland, N., 1993. Storage protein uptake in *Helicoverpa zea*. Purification of the very high density lipoprotein receptor from perivisceral fat body. J. Biol. Chem. 268, 16673-16678.

Wang, Z. and Haunerland. N. H., 1994a. Storage protein uptake in *Helicoverpa zea*: Arylphorin and VHDL share a single receptor. Arch. Insect Biochem. Physiol. 26, 15-26.

Wang, Z. and Haunerland, N.H., 1994b. Receptor-mediated endocytosis of storage proteins by the fat body of *Helicoverpa zea*. Cell. Tissue Res. 278, 107-115.

Webb, B.A. and Riddiford, L.M., 1988. Synthesis of two storage proteins during larval development of the tobacco hornworm, *Manduca sexta*. Dev. Biol. 130, 671-681.

Wehling, M., 1997. Specific, nongenomic actions of steroid hormones. Ann. Rev. Physiol. 59, 365-393.

Wheeler, D.E. and Buck, N.A., 1996. A role for storage proteins in autogenous reproduction in *Aedes atropalpus*. J. Insect Physiol. 42, 961-966.

Wheeler, D.E., Tuchinskaya, I.I., Buck, N.A. and Tabashnik, B.E., 2000. Hexameric storage proteins during metamorphosis and egg production in the diamondback moth, *Plutella xylostella* (Lepidoptera). J. Insect Physiol. 46, 951-958.

Wigglesworth, V. B. 1954. The physiology of insect metamorphosis. Cambridge University Press.

Willis, J.H. 1996. Metamorphosis of the cuticle, its protein and their genes. In "Metamorphosis – Postembryonic reprogramming of gene expression in Amphibian and Insect cells" (Gilbert L.I., Tata J.R., Atkinson B.G. eds.,), 253-282, Academic Press, New York.

Wilson, C.M., 1983. Staining of proteins on gels: comparisons of dyes and procedures. Methods Enzymol. 91, 236-247.

Wilson, T.G., DeMoor, S. and Lei, J., 2003. Juvenile hormone involvement in *Drosophila melanogaster* male reproduction as suggested by the methoprene-tolerant(27) mutant phenotype. Insect Biochem. Mol. Biol. 33, 1167-1175.

Woodard, C.T., Baehrecke, E.H. and Thummel, C.S., 1994. A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. Cell 79, 607-615.

Wyatt, G., 1991. Gene regulation in insect reproduction. Invert. Rep. Dev. 20, 1-35.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E. and Ueda, H., 2000. Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. Development 127, 5083-5092.

Yamanaka, N., Rewitz, K.F. and O'Connor, M.B., 2013. Ecdysone control of developmental transitions: lessons from *Drosophila* research. Ann. Rev. Entomol. 58, 497-516.

Yamashita, I., 1986. Metabolic shift from lipogenesis to glycogenesis in the last instar larval fat body of the silkworm, *Bombyx mori*. Insect. Biochem. 16, 327-331.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P. and Evans, R.M., 1993. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. Nature 366, 476-479.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M. and Evans, R.M., 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71, 63-72.

Yoe, S.M., Kang, C.S., Han, S.S. and Bang, I.S., 2006. Characterization and cDNA cloning of hinnavin II, a cecropin family antibacterial peptide from the cabbage butterfly, *Artogeia rapae*. Comp. Biochem Physiol. Part B, Biochem. Mol. Biol 144, 199-205.

Yu, J., Wu, F.Y., Zou, F.M., Jia, J.Q., Wang, S.P., Zhang, G.Z., Guo, X.J. and Gui, Z.Z., 2012. Identification of ecdysone response elements (EcREs) in the *Bombyx mori* cathepsin D promoter. Biochem. Biophys. Res. Commun. 425, 113-118.

Zacharieva, S., Kirilov, G., Orbetzova, M., Elenkova, A., Shigarminova, R., Lozanov, V. and Mitev, V., 2008. Homocysteine, renin and aldosterone in patients with Cushing's syndrome. Methods finding Exptl. Clin. Pharmacol. 30, 221-224.

Zalewska, M., Kochman, A., Estève, J.-P., Lopez, F., Chaoui, K., Susini, C., Ożyhar, A. and Kochman, M., 2009. Juvenile hormone binding protein traffic—Interaction with ATP synthase and lipid transfer proteins. Biochim. Biophys. Acta-Biomem 1788, 1695-1705.

Zheng, X., Wang, J., Haerry, T.E., Wu, A.Y., Martin, J., O'Connor, M.B., Lee, C.H. and Lee, T., 2003. TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. Cell 112, 303-315.

Zhou, X., Oi, F.M. and Scharf, M.E., 2006. Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. Proc. Natl. Acad. Sci. U S A 103, 4499-4504.

Zhu, Y.C., Muthukrishnan, S. and Kramer, K.J., 2002. cDNA sequences and mRNA levels of two hexamerin storage proteins PinSP1 and PinSP2 from the Indian meal moth, *Plodia interpunctella*. Insect Biochem. Mol Biol. 32, 525-536.