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Fat body remodeling in *Spodoptera litura* F. (Lepidoptera: Noctuidae) during postembryonic development

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ABSTRACT: During insect metamorphosis, larval structures including fat body are replaced by the adult ones. This process involves lysosomal enzyme-mediated remodeling of fat body. The objective of this study is to characterize the events leading to fat body remodeling during postembryonic development in an important agricultural pest, *Spodoptera litura*. Present study showed that the fat body undergoes significant changes in its morphology as well as histology. During the larval stage the tissue is primarily synthetic and secretory in nature and releases large amount of macromolecules including hexamerins in the heamolyph. While at pre-pupal and pupal stages it acts as a storage tissue and accumulates number of protein granules. Radiolabelling and DNA analysis studies revealed higher content of DNA in the larval fat body. The decline seen in pre-pupae corroborated well with disintegration of nuclei which were remodeled during pupal and adult stages. Further, the role of an insect morphogenetic hormone, 20-hydroxyecdysone (20E) in fat body reorganization has also been elucidated. This study enables us to understand the basic mechanism and altered micro-environment of the dynamic fat body tissue during larval-pupal-adult transition and metamorphosis.

KEY WORDS: *Spodoptera litura,* fat body, tissue remodeling, metamorphosis, 20 -hydroxyecdysone, postembryonic development

INTRODUCTION

Holometabolous insect life cycle is characterized by the presence of four distinct stages: egg, larva, pupa and adult. Larvae that are hatched out from the eggs develop (grow in size) in stages called instars followed by the dramatic transformation into quiescent, non-feeding pupal stages which then eclose into adults. As the transformation involves alterations in the feeding habits and physiology with each developmental stage, a balanced acquisition and utilization of resources is of high significance in their life cycle (Truman *et al*., 2002). Therefore, insects have developed the ability to store large quantities of protein which are called as hexamerins in their fat body, which serve as a source of amino acids and energy needed for the development of adult tissues and transformation (Haunerland *et al*., 1996).

Fat body, is a vital multi-functional tissue found in the visceral cavity of insect life stages (Telfer and

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Kunkel, 1991; Arrese *et al*., 2010; Hoshizaki *et al*., 2012). It performs diverse functions that include the maintenance of bacterial endosymbionts (Costa-Leonardo *et al*., 2013) , storage of urate during development (Park *et al*., 2013), synthesis, release and storage of a variety of macromolecules (Costa-Leonardo et al., 2013; Roma *et al*., 2013), source of humoral factors and role in immune functions (Gillespie *et al*.,1997) as well. During metamorphosis, the larval fat body being major site of biosynthetic activity, undergoes a chronologically ordered sequence of alterations and is completely remodeled by the time adult emerges (Dean *et al*.,1985; Lakshmi and Dutta-Gupta,1990; Wang and Haunerland,1992) . The histolysis and histogenesis of fat body cells during metamorphosis is preceded by quantitative changes in DNA content (Edgar and Orr-Weaver, 2001). However, owing to structural complexity and pleomorphism of the fat body, elucidation of its postembryonic remodeling has been limited so far. The distribution of stage-specific functions to various cell types of the fat body has been readily acceptable in dipterans due to the well-established underlying mechanism of adult fat body generation and precise stage-specific differences in the functions of fat body in these insects (Jansen and Borgesen, 2000). On the contrary, lepidopteran insect fat body cell types are mixed and integrated into a unified tissue thereby making it difficult to correlate a specific functional activity with a cell type (Haunerland and Shirk, 1995). Moreover, the change of function of fat body during metamorphosis was attributed to the transformation of cellular activity during the reorganization (Dean *et al*., 1985). Nevertheless, studies pertaining to fat body histology of few lepidopteran moths such as Indian meal moth, *Plodiainter punctella* (Shirk and Malone, 1989), *Heliothis zea* (Haunerland *et al*., 1990) have directed a reconsideration of the above perspective. The above studies have reported some striking differences in the fat body remodeling within the same order i.e., Lepidoptera indicating that this phenomena is not identical and thereby suggesting an independent study for every given insect.This paper documents in detail the changes that lead to the remodeling of the fat body in an important lepidopteran agricultural pest, *Spodoptera litura* F., commonly known as tobacco cutworm, during the postembryonic development. The role of major insect morphogenetic hormone, 20-hydroxy ecdysone (20E) in the process has been evaluated and discussed.

MATERIALS AND METHODS

Spodoptera litura (Noctuidae: Lepidoptera) is a polyphagous pest throughout India on economically important crops like tobacco, castor and groundnut. The insects were reared in a culture room at 70±5% relative humidity, 14:10 light and dark period. The temperature was maintained at 26±1°C. Freshly hatched larvae were fed on castor leaves. After three to four days the larvae were transferred to sterile glass vials and fed on artificial diet (Gupta et al., 2005). The pupae were collected and disinfected with 0.02% formaldehyde and kept in plastic troughs on moist sponge for adult emergence. For the current study early-last, late-last larval instars, pre-pupa, pupa and adult stages were collected (Budatha et al.,2011).

Morphological and histological studies: Morphological changes occurring in the fat body during metamorphosis were visualized under a stereo zoom binocular microscope [Olympus]. For histological studies, the fat body was dissected out in insect Ringer and fixed in Bouin's fluid. Tissue was dehydrated in series of alcohol, cleared in xylene and embedded in paraffin. Paraffin sections of 6µm thickness were cut and stained with Hiedenhain's iron alum hematoxylin eosin stain (Godwin Avwioro, 2011). Histological preparations were analyzed using Zeiss photomicroscope.

DNA extraction and estimation: DNA was extracted from fat body tissue using DNA isolation kit (Qiagen). The quality of the isolated DNA was analyzed by agarose gel electrophoresis and the concentration was estimated using NanoDrop-1000 spectrophotometer (Thermo Scientific Nanodrop 2000).

Thymidine incorporation studies: For autoradiographic studies, early-last instars were

injected with 4µCi of [H^3] thymidine and incubated for different durations. A batch of the injected larvae was incubated till they attained the pre-pupal and adult stages. The anterior and the posterior fat body were dissected out separately and fixed in Carnoy's fixative. Paraffin sections (5µm thickness) were cut and processed for autoradiography using Ilford K2 emulsion. The emulsion coated slides were developed in Kodak D 198 developer and autoradiograms were photographed under Zeiss photomicroscope, using phase optics.

Surgical procedures and hormone treatments: Thorax ligation was carried out using silk suture thread to deplete or reduce the endogenous hormone titer (Dutta-Gupta and Ashok, 1998). The larvae were anesthetized on ice, a loop of the silk thread was made and the position of the loop was adjusted behind the prolegs of the larva and the knot was tightened. The anterior part was cut and the wound was sealed using bee- wax after application of streptomycin sulphate - penicillin mixture (1:1).

Hormone 20E (Sigma, USA) was dissolved in ethanol and then diluted in insect Ringer solution (130 mMNaCl, 0.5 mMKCl, 0.1 mM $CaCl_2$) to obtain a final concentration of 80 nM per insect. The final concentration of ethanol in working 20E solution never exceeded 0.05% in any of the experiments (Arif *et al*., 2004). Experimental insects were thorax ligated 24 h prior to the hormone treatment. Control insects received equal volumes of the carrier.

Analysis of protein content, protein profile and identification of hexamerins: Fat body tissue was dissected from early-last, late-last instar larvae and pre-pupa, homogenized in insect Ringer solution to which cocktail of protease inhibitors was added. Protein content was estimated using Bradford's method (Bradford, 1976) while profiling was done using SDS-PAGE analysis (Laemmli, 1970). Identity of high molecular weight proteins present in the fat body was established using immunoblotting (Towbin *et al*., 1979) with polyclonal antibodies generated against purified larval hexamerins from the hemolymph. The antibody-bound to protein was detected using ALP conjugated anti-rabbit IgG. The visualization of the specific cross-reactivity was carried with BCIP-NBT.

Statistical analysis: All the experiments were repeated thrice and the results were expressed as mean ± SEM of three replicates. Statistical significance between control and treated groups were assessed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' post hoc test. Significant changes at $p<0.05$) are indicated.

RESULTS

Fat body reorganization during larval-pupal-adult transition:

a) *Morphological alteration in S. litura fat body during larval-pupal-adult development:*

Microscopic examination of *S. litura* fat body during larval, pre-pupal, pupal and adult stages revealed notable changes in the morphology (Fig. 1a-d). At larval stage, fat body appears as thin ribbon-like sheet being composed of large number of adipocytes (a). During the larval pupal transformation the fat body undergoes significant metamorphic changes,

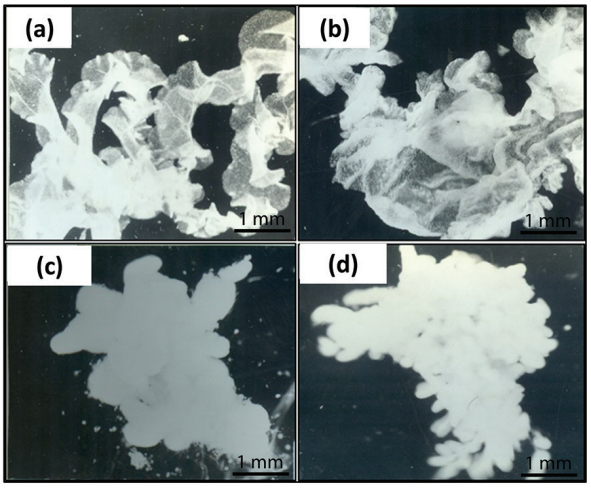

Fig.1. Progressive changes in the fat body morphology during larval-pupal-adult development. (a) Thin ribbon like sheets in late-last instar larvae; (b) Beginning of metamorphic changes where fat body cells appear more dense in pre-pupa; (c) Compact fat body in freshly molted pupa and (d) Reorganized finger lobed fat body in adult.

it gradually becomes more compact in pre-pupa (b) and it is a fairly dense structure in freshly molted pupa (c). In adult stage, fat body cells are further reorganized into compact finger-like lobular structures (d).

b) Histological changes:

Histological changes in the fat body during the postembryonic development are presented in figure 2. In the late-last larval instar, the fat body is composed of large cuboidal cells commonly known as adipocytes with centrally located nucleus. The cytoplasm consists of large number of lipid vacuoles. Based on the morphometric analysis of the lipid granules, it can be inferred that the macromolecular storage increases during development from the late-last larval instar to the pupal stage. During pre-pupal stage, the cytoplasm of the fat body cells shows accumulation of densely stained membrane bound granules interspersed with lipid vacuoles. The nuclear volume declines and the chromatin appears fairly condensed. In freshly molted pupae, the density of cytoplasmic granules increases markedly and the cell membrane becomes indistinct. In newly emerged adults, the fat body cells undergo extensive re-organization showing prominent nucleus and large number of vacuoles in the cytoplasm however the density of cytoplasmic granules declined.

c) Autoradiographic studies:

For this study, the early-last instar larvae were injected with [H^3]thymidine and incubated for varying time points i.e., 48, 72 h and several days till they attain pre-pupal, and pharate adult stages (Fig. 3). With 48 and 72 h incubation periods, the fat body cells of anterior and the posterior regions show different degree of incorporation of radioactivity in their nuclei. The anterior fat body cell nuclei show a lower level of incorporation of [H^3]-thymidine with 48 h incubation period and the intensity of labeling in the nuclei increases at 72 h (Fig. 3a and 3b). The posterior fat body cell nuclei show intense labeling within 48 h which tends to increase further at 72 h (Fig. 3c and 3d). The amount of radioactivity observed in the posterior fat body cells is much higher as compared to that of anterior fat body. This incorporation of [H^3] thymidine is primarily due to endopolyploidy in fat body which is fairly well known phenomenon in holometabolous insects.

Autoradiograms obtained with larvae incubated for long duration till pre-pupal stage, clearly show a lower degree of dispersed labeling in the anterior as well as posterior fat body cells as compared to the 48 and 72 h time points (Fig. 3e and 3f). It is interesting to note that the radioactivity although

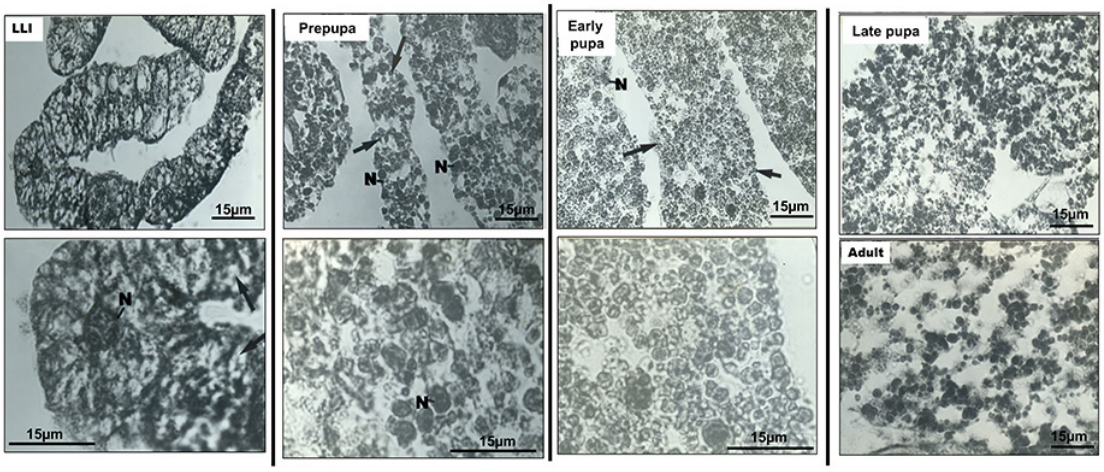

Fig.2. Cytological changes in the fat body during larval-pupal-adult transformation. Large cuboidal cells with fine granular cytoplasm with lipid vacuoles are seen in late-last instar larvae (LLI). Please note the accumulation of densely stained membrane bound granules in pre-pupal fat body. Freshly molted pupal (early pupa) fat body cells show increased density of cytoplasmic granules. Please note the disintegration of cytoplasmic granules at late-pupal (5-6 day old) stage while newly emerged adult fat body cells show large number of vacuoles and few granules in cytoplasm. (N- Nucleus, → membrane bound protein granules)

higher than anterior fat body, it was more diffused in the posterior fat body. The early-last instars injected with [H^3]thymidine and incubated till the pharate adult stage of development once again show localized labeling in the fat body cell nuclei which is once again higher in posterior fat body than anterior fat body (Fig. 3g and 3h).

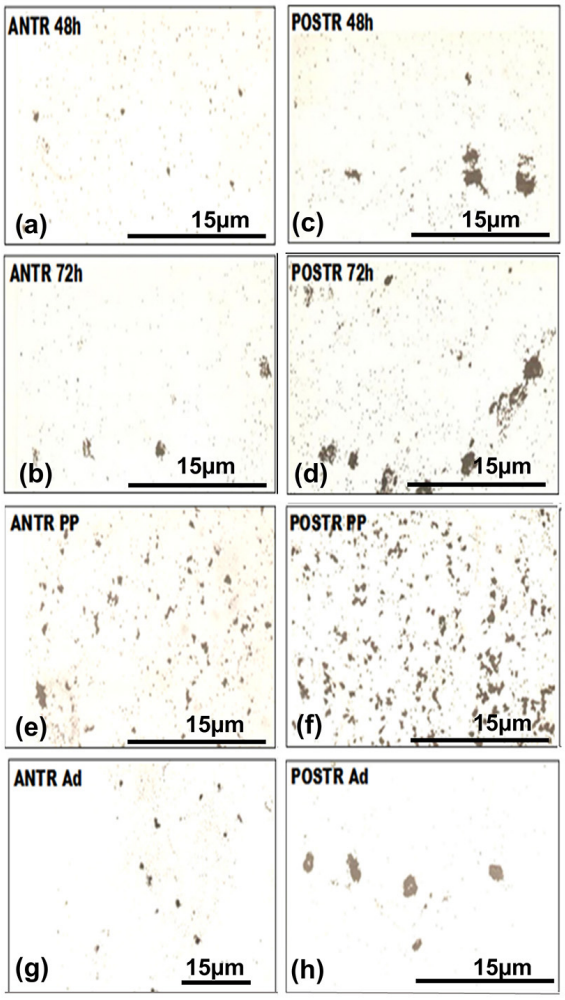

Fig. 3.Autoradiograph showing [H^3] thymidine incorporation in fat body cells of early-last instar larva for various incubation periods. (a) Incubation time 48 hours – anterior fat body cell nuclei show poor incorporation; (b) Incubation time 72 hours – showing moderate incorporation into the anterior fat body cell nuclei (which suggests synthesis of DNA in fat body cells); (c) and (d) Incubation time 48 hours and 72 hours respectively – posterior fat body cell nuclei showing higher degree of incorporation than the anterior fat body; (e) and (f) Incubation time was extended till the attainment of pre-pupal stage. Please note moderate labelling is seen in the anterior fat body cells which is dispersed type (e) as compared to the posteriorfat body where higher but dispersed labelling is observed (f); (g) and (h) Incubation time was extended till pharate adult stage of development is reached. Intense labelling of fat body cell nuclei is noticeable. Anterior: ANTR; Posterior: POSTR; Adult: Ad.

Changes in the DNA content of fat body:

a) *Changes in the DNA content during postembryonic development:*

For this study, fat body was carefully dissected from 4thinstar larvae till adult. Total DNA content of the fat body was found to be low in the 4th instar which increased significantly in early-last instar larvae and declined thereafter during late-last instar and

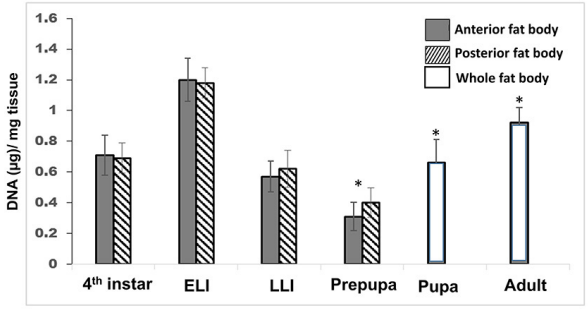

Fig. 4. DNA content in the anterior and posterior fat body during postembryonic development. DNA was isolated from the anterior and posterior fat body of different developmental stages of *S. litura* and estimated. For the pupal and adult stages, it is not possible to differentiate the anterior and posterior regions of fat body. Hence, the whole fat body was used.

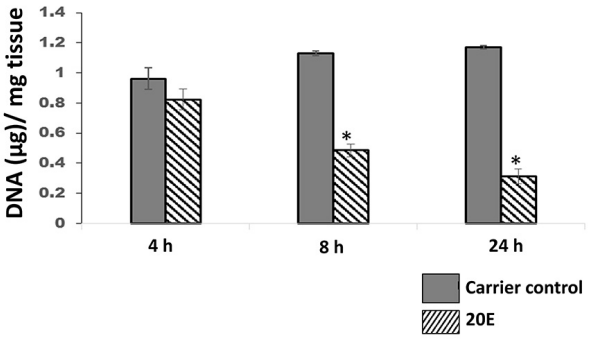

Fig. 5. Effect of 20E on the posterior fat body DNA content of ligated early-last instar larvae. 24h thorax-ligated early-last instars larvae were injected with 20E (1µg/ insect in 10µl of 10% ethanol) and incubated for 24h. The values represent mean ± standard deviation of 4 determinations. For each determination, fat body tissue was pooled from 3 insects. Significance was calculated using Student Newman-Keul's test and values were considered significant at $p<0.05$.

reaching to a fairly low value in the pre-pupal stages which is most likely due to the accumulation of hemolymph proteins in the fat body as well as remodeling seen during the pre-pupal stage and fairly evident from our histological as well as DNA synthesis studies reported above. However, it increased again during pupal development and in freshly emerged adults it was high (Fig.4).

b) ***Effect of 20E on DNA content in the posterior fat body:***

Significant reduction was observed in the DNA content of posterior fat body after 8 h of 20E administration to thorax ligated abdomens and the effect lasted till 24 h post injection (Fig. 5). Please note a moderate but gradual increase in age matched unligated control insects during this period.

Alteration in fat body protein profile and identification of hexamerins:

Results presented in figure 6 show that the fat body protein concentration increased significantly during different developmental stages (Fig. 6a) and SDS profile of proteins revealed presence of large molecular weight proteins in hemolymph of last instar larvae (Fig. 6b, lane 2). Using ammonium sulphate fractionation, hexamerins were partially purified from hemolymph of last-instar larvae of *S. litura* (Fig. 6b, lanes 2-6) and these hexamerins with molecular weight of 82-86 kDa, present in fairly pure form (Fig. 6b, lane 7) were used for the generation of polyclonal antibodies. The antisera showed selective cross reactivity with high molecular weight hexamerins (82-86 kDa) alone present in the hemolymph (Fig. 6c). SDS-PAGE profile of fat body proteins during the early- and late-larval instar and pre-pupal stages clearly show a significant increase in protein content during pre-pupal stages (data not presented). The presence of hexamerins in the fat body was also detected by western blotting (Fig. 6d). It was found to be fairly high in pre-pupal fat body when compared to early-last and late-last instar larval stages which is most likely due to sequestration of hexamerins from hemolymph at this stage and its widely reported phenomenon in various lepidopteran insects.

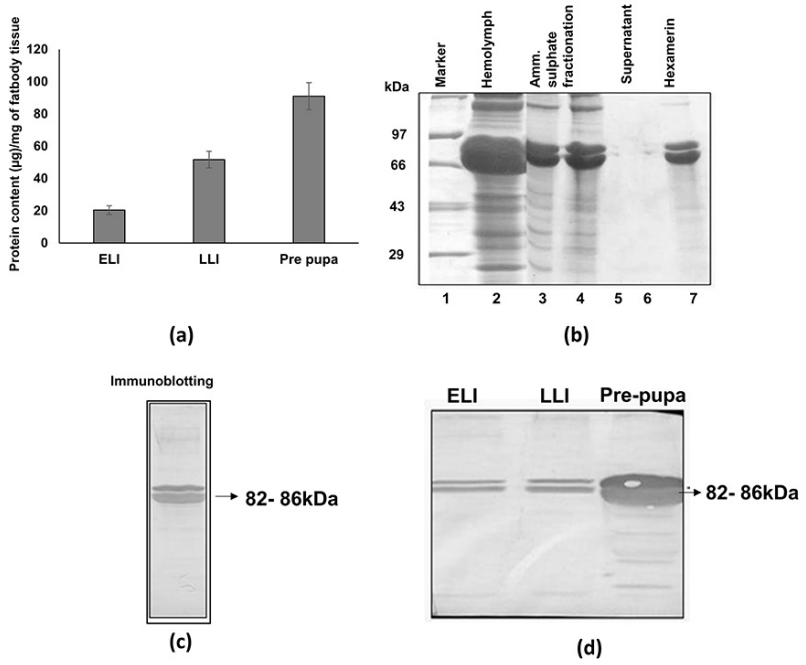

Fig. 6. Hexamerin profile in the fat body tissue during last larval and pre-pupal stages of development. Please note a significant increase in protein content of the fat body tissue from early-last to late-last instar larvae, which further increased during pre-pupal stage (a); partial purification of hexamerins from the hemolymph by ammonium sulphate fractionation (b); western blotting to show the detection of hemolymph hexamerins (c) and immuno-detection of hexamerins in the fat body of early-last (ELI), late-last (LLI) larvae and pre-pupa. Equal quantity of total protein was loaded in all the lanes (d).

DISCUSSION

Present study clearly shows that the fat body undergoes a gradual but significant alteration which is morphological as well as histological during postembryonic development of *S. litura*. The larval fat body appears fairly synthetic which releases proteins and other macromolecules synthesized by it into the hemolymph, thereafter it gradually changes into a dense structure which is primarily a storage tissue. These findings corroborate well with earlier reports of lepidopteran as well as dipteran insects (Levenbook, 1985). This study reveals considerable increase in DNA concentration of the fat body during penultimate to early-last instar larval development which is associated with DNA synthesis as shown by incorporation of [H^3] thymidine. Autoradiographic studies further suggest that this DNA synthesis occurs in the absence of nuclear division and results from polyploidy (Dean *et al*., 1985). Usually in fat body cells, this DNA synthesis often precedes the synthesis of storage proteins (hexamerins) in larval stages of holometabolous insects (Dean *et al*.,1985; Lakshmi and Dutta-Gupta,1990) and vitellogenin synthesis in adult insects (Klowden, 2013) which is stimulated by juvenile hormone (JH) (Ramaswamy *et al*.,1997). Furthermore, present autoradiographic studies show differential degree of polyploidy in anterior and posterior fat body cells, and it is higher in the posterior fat body of *S. litura* larvae. During the late-last larval instar, DNA content of the fat body declines and is most likely due to extensive increase in the protein content seen at this stage (Wang and Haunerland ,1991). During pre-pupal stage not only the DNA content of the fat body declines but one can see the fragmentation of radiolabeled nuclei, which were injected with [H^3] thymidine at early-last instar larval stage.

The onset of wandering behavior during non-feeding stage marks a switch over from larval to pupal program. At this time the JH titer drops and large quantity of ecdysteroids are produced in lepidopteran insects (Bollenbacher, 1988; Nijhout, 1998). Present results suggest that the ecdysteroids promote remodeling of the fat body and stimulate degradation of DNA which was synthesized during larval development. Furthermore, 20E injection to thorax-ligated larvae, which were deprived of endogenous hormone caused substantial decline in the DNA content of the fat body in time dependent manner, suggesting that dissociation and remodeling of the fat body cells is promoted by ecdysteroids. The DNA content of the fat body increases during the pupal development and reaches a high level in adults. Earlier studies showed that the new DNA synthesized during pupal-adult metamorphosis is primarily supported by nucleotides which are released from the larval tissue DNA (Dean *et al*., 1985). Our present autoradiographic studies also support this finding where early-last instar larvae injected with [H^3] thymidine showed incorporation of radiolabel in pupal fat body further suggesting that salvage pathway of DNA synthesis might be operative during pupal development of *S. litura*.

In *S. litura* protein content of the fat body gradually increases during postembryonic development from early-last instar larval stage to pre-pupal stage. The result corroborates well with earlier reports of other lepidopteran insects (Levenbook, 1985; Kiran Kumar *et al*., 1997). Further our electrophoretic studies reveal presence of high molecular weight hexamerin proteins (82-86 kDa) in the fat body. Previous experimental studies from our laboratory in *Corcyra cephalonica* has already demonstrated that arylphorin hexamerin (84kDa) a multifunctional protein, is expressed in tissue specific manner by the larval fat body during postembryonic development and its gene is transcriptionally regulated by 20E (Manohar *et al*., 2010; Venkat Rao *et al*., 2015). However, the hexamerins are known to be synthesized by the fat body and released immediately into the hemolymph during the active feeding phase of insect; hence they do not accumulate in the fat body cells (Burmester, 2002). Interestingly, the non-feeding pre-pupal stage fat body of *S. litura* in the present study not only shows increase in total protein content but also abundance of high molecular weight protein (82-86kDa) which cross-reacted intensely with the polyclonal antibodies generated against purified haemolymph hexamerins. Earlier studies from our as well as other groups have already demonstrated that fat body undergoes functional transition during

larval-pupal development from a synthetic to storage organ. The fat bodies of *C. cephalonica* pre-pupa as well as pupae were shown to actively incorporate remarkable amounts of injected radiolabeled hexamerins from the haemolymph (Ismail and Dutta-Gupta, 1990). Further, this uptake was shown to be mediated by plasma membrane bound receptor (KiranKumar *et al*.,1997), which gets activated under the influence of 20E (Arif *et al*.,2003).

The present study unambiguously demonstrates that the morphological alteration in fat body structure and its compaction during the larval-pupal transformation seen in *S. litura* is associated with the massive sequestration and accumulation of hexamerins in protein granules most likely act as amino acid resources for metamorphosis.

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