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METABOLIC TURNOVER OF CARBOHYDRATES DURING PUPAL-ADULT TRANSITION STAGE IN THE SILK WORM, *BOMBYX MORI*

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ABSTRACT

The present study has been focused on determination of various constituents of carbohydrate's metabolism during pupal-adult transition stage in the silk worm, *Bombyx mori.* Vth instar larvae of the silkworm, *Bombyx mori* (DFLs), obtained from the local grainages were maintained under laboratory conditions of 26^0C-28^0 C temperature range according to the guidelines of Krishnaswamy (1978).The pupae and adult male & female moths were sacrificed on selected days viz.1st, 3rd, 5th, 7thand 9thdays and two selected tissues such as fat body and haemolymph were collectedand stored at -70^0C. At the time of biochemical analysis, the tissues were thawed and used. Various constituents of the Carbohydrate metabolism viz. Total carbohydrates, glycogen and Trehalosealong with their associated enzymes such as Alpha-amylase and Trehalase activity were estimated in the Fat body and Haemolymph as per the standard methods. Biochemical variations, observed in carbohydrates during the 9 day duration of pupal life and also during pupal-adult transition period indicated utilization of carbohydrates for energy production. Present finding clearly established the obsorption and also exchange relationship of biochemical constituents between fat body and haemolymph, of course a significant differences in male and female moths. These Sex-specific variations in biochemical constituents of carbohydrate metabolism correlated well with the energy demands of male and female adults.

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INTRODUCTION

The dietary Carbohydrates, the major sources of fuel are reserved in two forms i.e. glycogen and trehalose in silkworm, which meets the energy demand during their growth and metamorphosis (Shivakumar and Shamitha, 2011; Anandkumar and Sandhya, 2012). Trehalose, the major blood sugar in insects rely its functions as an intermediate form between glycogen and glucose and its level varies in different stages of development. Since trehalose is found as membrane-bound impermeable form, its turnover among fat body and haemolymph was home statistically regulated by trehalase and trehalosetransportors. Always the changes in trehalase activity levels are closely related to shifts in physiological conditions and developmental events, thus trehalase is effectively involved both in the maintenance of homeostasis in the body and to ensure the energy supply for development by hydrolyzing the reserve trehalose in the metabolically active tissues such as fat body and haemolymph (Silva *et al.,* 2009). Like trehalase, the trehalose transporters are actively expressed during pupal and adult stages in silkworm and incorporate the levels of trehalose among different tissues (Kanamori, 2010). In silkworm *Bombyx mori,* two types of trehalases were identified; membrane bound and soluble forms (Thompson, 2003; Mitsumasu *et al*., 2005). The trehalase activity changes during the growth and development of silkworm and their optimal activity were identified in the mid gut of the larval stage, fat body and haemolymph of pupal and adult stage (Tatun*et al*., 2008). The recent report shows that fat body, not only controls the synthesis and utilization of energy reserves such as trehalose and glycogen but also synthesize most of the heamolymph proteins (Arrese and Soulages, 2010). Despite the availability of voluminous data on characterization of trehalase, amylase and the metabolism of carbohydrates in insects, their metabolic turnover during pupal-adult transition of *Bombyx mori* is not clearly elucidated so far. Hence, the present study is focused on establishing the biochemical

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relationship between fat body and haemolymph by analyzing the changes in the levels of total carbohydrates, glycogen, trehalose and the associated enzymes viz. amylase and trehalase in the above selected tissues in *Bombyx mori.*

MATERIALS AND METHODS

Experimental protocol

Test Species: PM X CSR2 hybrid variety of the silkworm, *Bombyx mori.*

Rearing: Reared under standard environmental conditions of 28ºC and 85% RH.

After hatching, the larvae were fed with M4 Varietyof Mulberry leaves five times a day at 6 AM, 10 Am, 2 PM, 6 PM and 10 PM.

Stages selected: Alternate days of Pupal stage (Day-1, 3, 5, 7, 9) and Adult stages.

Tissues selected: Fat body and Haemolymph.

Tissue collection: Fat body extracted by mid dorsal dissection of pupa of silkworm in Bombyx ringer. Haemolymph was collected from pupae bymaking puncture on their dorsal surface with fine needle, collected with syringe and diluted as per requirement.

Biochemical parameters studied

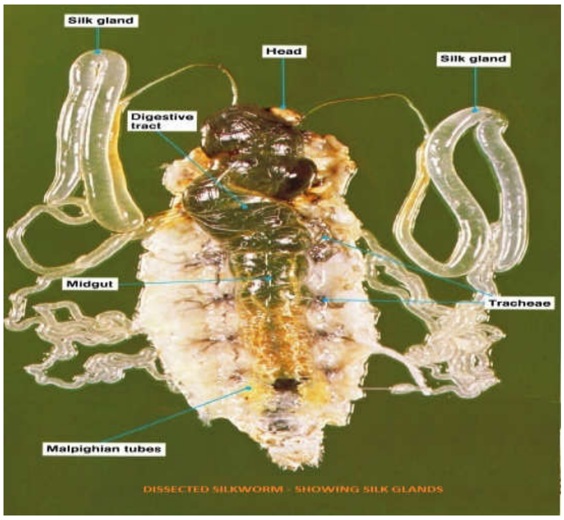
a) Total carbohydrates;
b) Glycogen ;
c) Trehalose;
d) Alpha-amylase;
e) Trehalase

Methodology employed for estimation of biochemical constituents and their associated enzymes

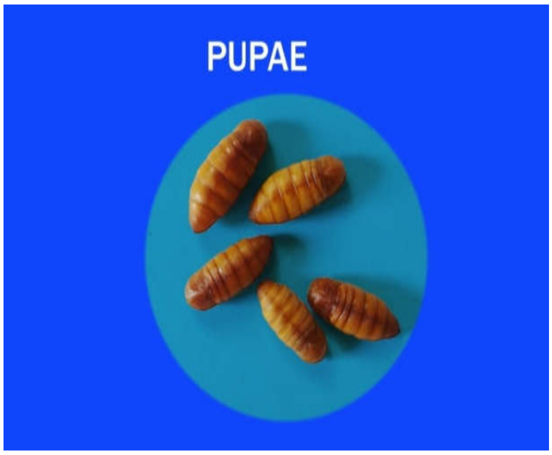
Biochemical constituent	Name of the tissue	% of homogenate	Method
Total carbohydrate	Fat body Haemolymph	2% 1:9 dilution in 5% TCA	Carroll*et al.,* (1956)
Glycogen	Fat body Haemolymph	1% 1:9 dilution in 5% TCA	Carroll*et al.,* (1956)
Trehalose	Fat body Haemolymph	1% 1:9 dilution in ice cold distilled water.	Roe(1955)
Alpha amylase	Fat body Haemolymph	2% 1:9 dilution in 0.5M acetate buffer	Bernfeld (1995)
Trehalase	Fat body Haemolymph	5% 1:19 dilution in 0.05 M phosphate buffer	Dalhman (1971)

Statistical analysis

The experimental data was statistically analyzed in terms of mean, standard deviation (SD) and test of significance and per cent changes. While the mean and SD were computed using M.S. Excel, the test of significance and percent changes were calculated online, using the Graphpad (www.graphpad.com/ quickcalcs/indexcfm/) and Percent Change (www.percent – change com/index php) software's. In order to draw meaningful conclusions, the data was also interpreted in terms of Compound Periodical Growth Rates (CPGR's) wherever possible, as given by Sivaprasad (2012).



Dissected Vth Stage Larva showing Silk Glands



Adult Male

Adult Female

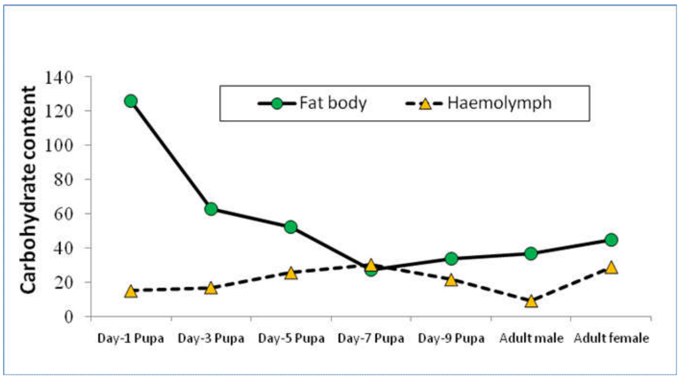
Picture showing the dissected Vth stage larva, pupa, adult male and female moths of the silkworm, Bombyx mori

RESULTS

Total carbohydrates: (Graph.1.)

Fat body: The observations showed that the total carbohydrate levels in the Fat body of pupa recorded a steady decreased from day-1 to day -7 with a negative Compound Periodical Growth Rate (CPGR) of 22.57%. Later, from day -7 to day- 9, the total carbohydrate content showed elevatory trend and recorded a positive CPGR of 11.66% during last three days of pupal development. Carbohydrates in the Fat body increased further during pupal-adult transition. One interesting observation was that the registered positive CPGR in males was less (1.01%) than in females (32.5%).

Haemolymph: In contrast with the fat body, the total carbohydrates content in haemolymph increased from day-1 to day-7 during pupal development with a positive CPGR of 12.7% during 7 days of pupal development. Later, the total carbohydrates were decreased from day-7 to day-9, with a negative CPGR of15.99% during last 3 days of pupal development. Contrasting trends were observed in male and female fat bodies during pupal adult transition, wherein males have registered negative CPGR of 59%, while the females have registered positive CPGR of 35.74%.

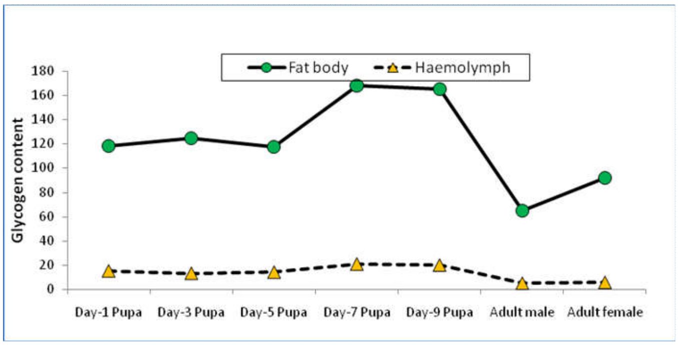


Graph.1. Carbohydrates in Fat body (mg/g) and Haemolymph (mg/ml) in metamorphosing silkworm

Glycogen levels: (Graph.2.)

Fat body: The results showed that Fat body glycogen levels followed an elevatory trend up to day 3 of pupal development with a positive CPGR of 2.66% during first three days of pupal development. Here after, the glycogen level showed decreasing trend upto day 5 of pupal development with a negative CPGR of 2.9% during the middle 3 days of pupal development. Again, there was an elevation in the level of glycogen up to day-7, which was ~43% with a positive CPGR of 19.49%. Thereafter declining trend was observed which recorded a negative CPGR of 0.81%. The decline was continued upto adult stage, but the decline was more in adult female with a negative CPGR of 60.58%, and 44.43% in males during pupal- adult transition.

Haemolymph: As against the Fat body, the glycogen level in the Haemolymph declined from day-1 today-3 and recorded a negative CPGR of 6.94% during first 3 days of pupation. After 5th day, elevation was observed up to 7th day of pupal development and registered a positive CPGR of 12.26% from day- 7 to day- 9 followed by again depletion in glycogen levels and showed a negative CPGR of 2.13%. Further, the glycogen levels were depleted during pupal-adult transition, of course in both sexes but the decline in adult male was more with a negative CPGR of 76.1% and comparatively less in adult female with a negative CPGR of 72.53%.

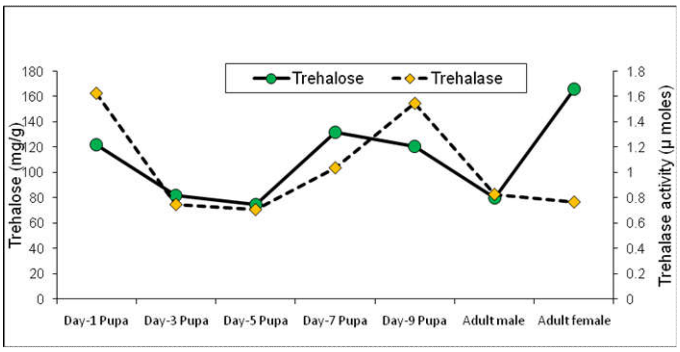


Graph 2. Glycogen in Fat body (mg/g) and Haemolymph (mg/ml) in metamorphosing silkworm

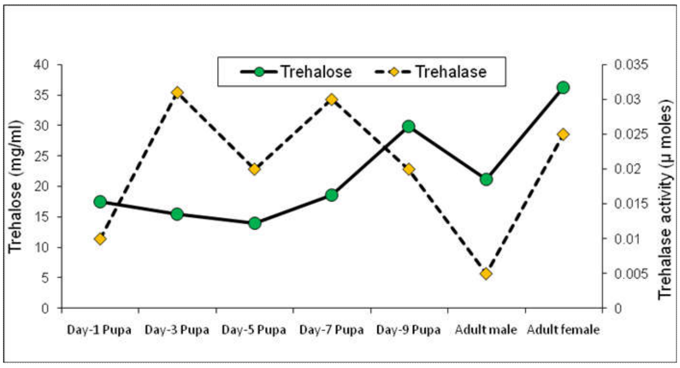
Trehalose (Graphs. 3&4)

Fat body: As a corollary to the glycogen, the trehalose levels of the Fat body showed a declining trend from day 1 to day 5 of pupal development and recorded a negative CPGR of 11.56% during first 5 days of pupal development. Later, it showed an elevatory trend from day 5 to day 7, with ~76.5% elevation on day 7 and with a positive CPGR of~ 32.86% during 3 days of mid-pupal development. Thence, it followed declining trend upto day 9 of pupa, and recorded a negative CPGR of 4.17% during last 3 days of pupal development. During pupal-adult transition, the trehalose levels declined in males with a negative CPGR of 33.95% while in females, elevation in trehalose was observed with a positive CPGR of 32.27%.

Haemolymph: As in the case of Fat body, a similar trend was observed in Trehalose levels upto day- 5. The decline was ~12% on day-3 and ~ 10% on day-5 and recorded a negative CPGR of 5.68% during first 5 days of pupal development. There after, trehalose levels were increased upto day- 9 and recorded a CPGR of 21.06% during last 5 days of pupal development. During pupal-adult transition, the trehalose levels showed opposing trends in males and females. In males a declining trend was observed with a negative CPGR of 29.08% and in the case of females, the increase has recorded with a positive CPGR of 21.6%. One significant observation here was that eventhough trehalose levels of Fat body and Haemolymph followed more or less a similar trend but trehalose level was lower in Haemolymph when compared with that of the Fat body.



Graph 3. Fat body: Trehalose v/s trehalase activity in metamorphosing silkworm

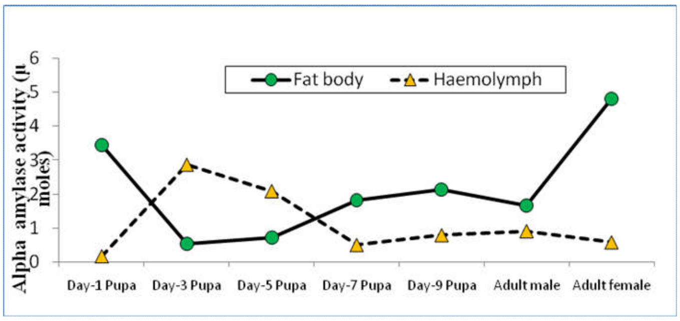


Graph 4. Haemolymph: Trehalose v/s Trehalase activity in metamorphosing silkworm

Alpha-Amylase Activity (Graph 5)

Fat body: From the results, it was clear that the Alpha-amylase activity in fat body showed a declining trend from day 1 to day 3, 3 with a negative CPGR of 60.75% during pupal development. Later, the alpha-amylase activity followed an increasing trend upto day 9. The increase was 34% on day 5 and 156% on day 7 and 18% on day 9 with a positive CPGR of 26.19% during last four days of pupal development. During pupal-adult transformation, the amylase activity showed contrasting trends in adult males with a negative CPGR of 22.43% and in females with a positive CPGR of 125%.

Haemolymph: Contrary to the Fat body, α - amylase activity in haemolymph exhibited several fluctuations in its levels during the pupal duration. For example, α - amylase activity recorded an elevatary trend up to day-3 with an elevation of ~ 323%. Thereafter, it showed a declining trend up to day-7 with a negative CPGR of 33.66% during mid-pupal stage i.e. from day 3 to day7 of pupal development. Subsequently, it showed an increasing trend from day 7 to day 9, and recorded a positive CPGR of 26.17% during last three days of pupal development. During pupal-adult transition, the alpha amylase activity showed contrasting trends in males and females, wherein increasing trend was observed in males with a positive CPGR of 15.38% , in females a decline was recorded with a negative CPGR of 26.92%.



Graph 5. Alpha amylase activity in fat body and Haemolymph in metamorphosing silkworm

Trehalase Activity (Graphs 3& 4)

Fat body: From the results, it was obvious that Fat body trehalase activity was declined upto day 5 of pupal development as that of the trehalose. The decline was ~54% on day 3 and ~5% on day 5 and recorded a negative CPGR of 18.67% during first 5 days of pupal development. Thereafter, it followed an elevatory trend up to day- 9. The elevation was ~ 46% on day- 7 and ~ 49% on day 9 and recorded a positive CPGR of 21% during last four days of pupal development. During pupal-adult transformation trehalase activity was declined both in males and females. The decline has recorded a negative CPGR of 46.45% in males and 50.32% in females.

Haemolymph: On the contrary, the Trehalase activity levels of haemolymph followed elevatory trend from day 1 to day 3, and recorded a positive CPGR of 40.59%. Thereafter from day 3 to day 5, the trehalase activity levels followed declining trend, the decline was ~35 on day 5 with a negative CPGR of 19.6. Later on, it was increased upto day 7 and the elevation was 50% on day 7 and again it was declined on day 9 by 33% and recorded a CPGR of 18.35%. Trehalase activity was decreased in both male and female adults during pupal adult transition. The registered negative CPGR in males was 75% and in females, 99.5%.

DISCUSSION

In the present study, the results clearly demonstrated that there was a significant decline in the levels of the total carbohydrates in the fat body of pupa from day 1 to day 7 with a negative CPGR of 22.57%. Fat body, a dynamic tissue undergoes not only structural changes but also in their biochemical constituents during metamorphosis since it needs energy which leads to decreased level of total carbohydrates. In addition to this, stored glycogen can be readily degraded on demand and can be used as a glycolytic fuel to generate energy to drive the metabolic functions. The positive CPGR of 11.66% in the last 3 days of pupal development might be because of accumulation of energy reserves, by moving from other histolytic organs such as gut and fat body or due to gluconeogenesis. Contrary to the above, the haemolymph carbohydrate levels showed increasing trend to that of fat body during pupal development with a positive CPGR of 12.77% which may be due to histolysis and mobilization of carbohydrates into haemolymph from other organs where they are utilized for energy production. The fat body arrangement bathed by haemolymph facilitates close contact between the haemolymph and the fat body cell, a well suited system highly adapted to the unique physiology of insects.

Further, the total carbohydrate levels showed contrasting trends in females and males during pupal adult transition period which may be attributed to their reproductive behaviour where the males require more energy for courtship and mating behaviour, hence the declined level was observed. Whereas in females, increasing trend was due to less utilization for their energy production and also due to release of more trehalose into haemolymph from gut wall cells (Bhuvaneswari and Sivaprasad, 2013). An interesting observation, that the total carbohydrates, both in male and female adult increased significantly but more in females than in males demonstrated that the energy demand for male is more than in female. The reason might be that utilization of glucose for the synthesis of lipids is more in females than in males or males utilize more glucose for flight, copulation etc. while the females accumulate glucose for lipogenesis (Zhao *et al*., 2004; Shiao *et al*., 2008). Results of the present study showed that the levels of glycogen were increased upto day-3 of pupa which indicates accumulation of glycogen in the pupal fat body in the early pupal stage which signified the storage of food reserves to be used as an immediate source of energy in the early pupal period. The pupa is a quiescent stage and is in a stage of spontaneous metamorphosis for the formation of the imago from the fasting pupa; the higher glycogen content indicates that the pupal fat body is metabolically more active in deposition and utilization. Decreased level of glycogen in the Fat body later in mid-pupal stage is because of histolysis or remodeling of fat body. Fat body glycogen then was added up from day-5 to day-7 of pupa.may be due to re-association of fat body cells, redeposit of glycogen into the metamorphosing fat body cells. Again after day-7, there is a decline in the level of glycogen may be due to histogenesis of adult tissues, which needs energy.(Malik and Malik, 2009) reported that pupal development involves significant synthetic activity in relation to reorganization of larval tissues into imaginal structures, mainly from fat body glycogenolysis. The decrease continues upto adult stage but the decline was more in adult female than the adult male. This may be due to mobilization of glycogen to the eggs via haemolymph. Haemolymph glycogen levels showed contrasting trend to that of fat body during first 3 days of pupal development, indicating mobilization of haemolymph glycogen into the fat body for storage. The decline from day-7 onwards indicates diffusion of haemolymph glycogen to other tissues such as gut and imaginal structures. Stage specific changes in the haemolymph glycogen levels are also observed (Malik and Malik, 2009). Like fat body, the haemolymph glycogen level also declined during pupal adult transition but significantly observed in adult males than in females. The contrasting trend between the haemolymph and fat body's glycogen levels in the adult males and females established a reciprocal relationship between them. Glycogen in the fat body and haemolymph decreases towards pupal-adult ecdysis, in association with chitin synthesis, development of the imaginal organs and synthesis of trehalose as a flight fuel in the newly ecdysed adult. In the adult moth, glycogen serves to provide the necessary substrates for the development and maturation of the egg and to meet the energy requirements for mating and egg laying activity.

Present study clearly demonstrated a strong link between the fat body and haemolymph of silkworm Bombyx mori, where these two tissues together act as a reservoir of nutrients. Our study further revealed the function of fat body as a nutrient sensing organ i.e. whenever there is depletion in fat body energy reserves, it should be compensated though the mobilization of carbohydrates from haemolymph. Especially during the glycogen synthesis, the mobilization of carbohydrates occurs towards the ovaries via haemolymph from fat body (Attardo *et al*., 2005). Present study also showed a decline in the levels of trehalose in the fat body in adult male thus demonstrating the fact that here trehalose was converted into glucose so the activity of trehalase is more. Long-term regulation of trehalose metabolism during metamorphosis seems to be achieved by quantitative and qualitative changes in the key enzyme trehalase synthase. Trehalose, the most common blood sugar in insects is synthesized from the glucose derived from the dietary carbohydrates such as pectin, xylan, cellulose, sucrose and starch present in the mulberry leaf and later transported to the gut wall, and then it is stored either in the same form or in the form of glycogen in the gut wall cells, fat body and muscle (Anand *et al.,* 2010). The digestive system of *Bombyx mori* is principally concerned with the digestion and absorption of dietary nutrients present in the mulberry leaf. The principle nutrients of mulberry leaf include carbohydrates which are digested and absorbed as glucose molecules and finally assimilated in the form of storage sugar called trehalose in the fat body and gut wall cells (Shivakumar and Shamitha 2011; Lokesh *et al*., 2012).The trehalose, so derived is degraded to glucose by an enzyme called trehalase. The metabolic utilization of trehalose is dependent on trehalase. Previous studies on carbohydrate metabolism during larval and pupal development of silkworms and blowfly have shown that glycogen which is stored in the fat body was released into the haemolymph in the form of trehalose. Studies on developmental changes in trehalose synthase activity in fat body also revealed that increased enzyme activity during first 3 days of larval development and high level was maintained during larval maturation. After cessation of feeding, the activity began to decline markedly and only trace activity was found on the day of pupal ecdysis. This low level remained throughout the pupal-adult development, and there was a slight increase in the last phase of adult development. Decrease in the trehalose synthase activity is one of the reasons for the declining trend of trehalose concentration of fat body. Trehalose is involved in cell metabolism, so before this, it has to be reconverted into glucose.

This was achieved by the enzyme trehalase.Trehalase activity in the mid gut increases at the onset of prepupal period, which accelerates the conversion of trehalose in haemolymph to glucose, which is incorporated into the fat body where glucose is converted to glycogen as an energy storage form. The physiological stress in the body of silkworm ensures the conversion of glycogen rapidly by activating the glycogen phosphorylase to release glucose later to synthesis trehalose in the fat body and haemolymph (Satake *et al*., 2000). The fact that the Enzyme activity level depends on substrate concentration was well correlated with the results of present study wherein during the first 5 days of pupal development, a decline in trehalose concentration had led to depletion in the activity levels of trehalase. Another interesting finding i.e. increased level of Trehalose in fat body from day 5 to day 7 of pupal development was due to synthesis of trehalose from glucose, which is mobilized to fat body from haemolymph and also due to maintenance of a steep concentration gradient by the fat body which removes glucose or other sugars from the haemolymph, converts it to trehalose and then returns it to the haemolymph (Thomson, 2003). Increase in trehalose concentration leads to increase in the reactive activity of trehalase, which continues upto 9th day of pupal development.Present study establishes a relationship between fat body and haemolymph, which supports that there is rapid exchange of trehalose between haemolymph and fat body.

Observation in the present study also revealed that trehalose level recorded a declining trend of from day-7 to day-9 of pupa, which might be due to hyper trehalosemic hormones as well as octopamine (insect hormone), which decreases the synthesis of trehalose from glycogen and they act to provide substrate, glucose or fatty acids for supporting aerobic respiration (Berg *et al*., 2002). Pupal adult transition begins on 9th day, so that this decline in trehalose continues in adult male, while in adult female increasing trend was observed which might be due to increased conversion of glycogen into trehalose in the fat body again to supply trehalose to ovaries for egg maturation (Arresse and Soulages, 2010). Eventhough trehalose plays a relatively minor role as energy reserve during adult development, it may be more significant in carbohydrate transport, perhaps conveying glucose units from fat body glycogen to sites of metabolism in other tissues via haemolymph. In a similar way, the Haemolymph trehalose showed a declining trend upto day-5 of pupal development later on it increased upto day-9. After that during pupal-adult transition, it showed declining trend in adult males and increasing trend in adult females. Trehalose is a major haemolymph sugar in all insects dynamically controlled during post embryonic development and also it is involved in survival in the face of environmental extremes. Decreasing trehalose concentration in haemolymph during first 5 days of pupal development is due to rise in ecdysteroid concentration, in haemolymph, which was responsible for larval-pupal ecdyis (Tatum *et al*., 2008a). Regardig Alpha-amylase activity level, a down trend from day 1 to day 3 of pupal development followed by elevator trend from day 3 to day 9 showing a maximum increase on day 5 may be due to various reasons viz. differential expression of *Bm Amy* gene in the gut (Ngernyuang *et al.,* 2011), indicates the onset of histolysis, changed environment as histogenesis starts, reorganization of fat body which starts on day -5 and continues further to build up the adult fat body etc. Since this entire process needs energy, so that the activity of amylase, i.e., the conversion of complex carbohydrates into simple carbohydrates may start on day-5 of pupal development.

Our results also revealed that haemolymph α-amylase activity reached a peak level on day 3 (~323) which was due to release of more quantity of enzyme from gut into haeamolymph, which acts as a flowing reservoir for all biomolecules. This may be one of the reasons for contrasting trends observed in haemolymph and fat body amylase activity(Rajitha and Savithri, 2014).Even though the pupa is the non-feeding stage, it showed decreasing trend in haemolymph amylase activity thus demonstrating that the changes in the biochemical composition in haemolymph reflectthe morphological characters. During last three days of pupal development, high level of amylase in the haemolymph may be due to its important physiological role behind its digestive function. Even though both male and female adults are non-feeding, stages, there is re-entry of α-amylase from fat body into haemolymph, which is later supplied to the flight muscles for degradation of muscle glycogen, which releases energy for vigorous wing beating in males. In females depletion may be due to degradation of energy reserves and more accumulation in the form of yolk in the eggs. In parallel with trehalose, the activity level of trehalase of fat body was also decreased upto day-5 of pupal development, later on its activity increased upto day-9. Since Trehalose is involved in cell metabolism, it has to be reconverted into glucose which is aided by trehalase enzyme activity which in turn is substrate-dependent process. Present study clearly showed that whether decline or elevation trehalose and trehalase go parallel during pupal developmentwhich suggest that physiological control of haemolymph trehalase activity is of crucial importance in regulating the supply of glucose to the tissues from haemolymph. During pupal-adult transition, the declining trend of trehalase in both sexes was in conisidence with fat body trehalose of adult male but in females, contrasting tend to that of fat body trehalose may be due to release of the enzyme into haemolymph which acts as a flowing reservoir for various biochemical components. Further the decline is more in the fat body of females than in males, may be due to mobilization of reserves towards egg production via haemolymph.

Conclusion

Present study clearly demonstrated that there was rapid exchange of substances in between haemolymph and fat body during pupal development and also during pupal-adult transition to serve many functions as already mentioned.

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