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Quantitative and qualitative changes in proteins and shift in the utilization of amino acids for cuticle sclerotisation and energy release during development in *Culex quinquefasciatus*

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ABSTRACT: Whole body protein of *Culex quinquefasciatus* showed a gradual increase during development and the content in 1st instar larvae was 3% and in adults it was 5% on fresh weight basis. The amount of total free amino acids showed a gradual increase up to 4th instar larvae but a sharp decline in pupae and adults, because of the mobilization of amino acids for the formation of new proteins in pupae. Appearance of new protein bands was not prominent during larval development but pupation resulted in origin of new protein bands. Protein profile of adult male and female did not exhibit marked difference in SDS-PAGE. Activity of alanine aminotransferase showed a gradual elevation during larval development but an antiparallel pattern was shown by another related enzyme aspartate aminotransferase. The ratio of activity of AlAT: AsAT, which is an index of utilization of amino acids in Krebs cycle via keto acids, was always below 0.3 in larvae, elevated to 0.6 in adults, which may be a part of flight adaptation. Maintenance of high activity of glutamate dehydrogenase (GDH) by adult mosquitoes in comparison with their larvae is also a part of flight adaptation because GDH- Transaminase system is responsible for supplying pyruvic acid and oxaloacetic acid from amino acid pool to Krebs cycle. Activity of phenol oxidase (tyrosinase), which is an index of melanization of cuticle and defense against parasites, showed a sharp increase from first instar to adults. As the melanization and sclerotisation of *C. quinquefasciatus* were practically completed by the 48 hour of eclosion, the activity of phenol oxidase showed a gradual retardation.

KEY WORDS: *Culex quinquefasciatus*, alanine aminotransferase, glutamate dehydrogenase, phenol oxidase, melanization.

INTRODUCTION

The holometabolous group of insects has distinct larval and pupal stages and undergoes some of the most complex transformations seen in animal kingdom(Sehnal *et al*., 1996, Truman and Riddiford,1999).The major morphogenetic events, such as determination of organ systems during embryogenesis, growth and moulting during larval life,as well as transformation from larva to pupa at the time of metamorphosis, are accompanied by characteristic variations in the patterns of amino acids, peptides and proteins (Chen, 1966). Mosquitoes are very peculiar in having highly mobile pupae with very short pupal life of 48-60 hours within which the aquatic detritivore is converted into a sanguivorous disease vector.Information on the metabolism of amino acids in insects like

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Periplaneta (Sacktor, 1978), *Schistocerca* and *Drosophila* (Schneider and Chen, 1981) is known, but that of mosquito and its developing stages is not clearly understood so far. Studies in other insects such as *Oecophylla smaragdina* (Vidhu and Evans, 2015) and *Oryctes rhinoceros* (Adhira and Evans, 2015) showed that glucose, the most important energy releaser of vertebrates, the content of which remained unaltered in extra energy necessitating stressed states such as continuous biting on the intruder till death (Vidhu and Evans, 2015) and exposure to hypothermia (Adhira and Evans, 2015). Contrary to it, the enzymes related to conversion of amino acids to keto acids and mobilization of keto acids into Krebs cycle became greatly elevated in stressed states. Also, the activity of phenol oxidase enzyme has been elucidated during the various stages of life cycle. Physiological and biochemical studies on insect development can provide a better understanding of the mechanisms of hormone action, protein synthesis, growth and differentiation.So it was proposed to study the protein profile and biochemical changes in amino acid metabolism during course of development using*Culex quinquefasciatus* as a model system.

MATERIALS AND METHODS

Collection of Culex egg rafts: Egg rafts of *Culex* mosquito were collected by keeping 3 litres of water containing 2 eggs of domestic fowl in plasticbuckets (5L), placed at damp corners of the College Campus. Mosquitoes started laying eggs after 2-3 days, once foul smell emanatedfrom them and egg rafts were collected

Rearing of Culex larvae, pupae and adults: Collected egg rafts were transferred to plastic pans (20 cm diameter and 8.5 cm depth), containing 500 ml of the same medium in which the eggs were laid, kept at insectarium in the department. Water was changed once every 3 days. Hatched out different instars and pupae were collected using droppers. Emerging pupae were transferred to mosquito cages for their development into adults. Adults were provided with 10% sucrose solution ad libitum.

Experimental organism: *Culex quinquefasciatus* was identified by the methods of Barraud (1934) and Harbach (2014). Adults and developing stages of *C. quinquefasciatus* reared in the insectarium of the department was used for the biochemical studies. The temperature of the insectariums ranged between 26-30°C. The different larval instars were separated as described by Tripathy and Dash (1988).Mosquito larvae and pupae were separated from the rearing colony and washed four times using dechlorinated tap water and finally by distilled water. With respect to developing stages, the whole body was processed for the assays. In case of adults, appendages, wings, legs, antennae and mouth parts were removed before homogenization by keeping them in ice cold condition. Three day old mosquitoes fed with 10% Sucrose were used for the studies.

Biochemical analysis and SDS- PAGE:100mg body weight of tissue was weighed out for all the stages of development. They were blotted using filter paper and transferred into a glass homogenizer containing appropriate volumes of ice cold buffer. For each enzyme assay, the buffer mentioned in the standard procedure was used for extracting the enzyme from the tissue. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4ºC (Eppendorf) and supernatant was taken. The pH and molarity of the buffer used was in accordance with standard procedure. Total protein was estimated by the method of Lowry *et al*.,1951 using BSA as standard. Estimation of total free amino acids was done according to Spies (1957).SDS-PAGE electrophoresis of whole body homogenate was carried out by the method devised by Laemmli (1970). It was carried out using 12% Polyacrylamide gel, pH 8.8. Acrylamide: Bisacrylamide ratio was 30:1. All protein samples contained 100µg of protein and were pretreated with 10% SDS and 1% mercaptoethanol at 95ºC for 3-5 minutes. The gel was run at 120V until the tracking dye (Bromophenol blue) was leaving the gel. Gel was stained in Coomassie Brilliant blue R250 overnight and destained in 7% acetic acid and photographed using Transilluminator (Biotech, Yercaud, India).

Assay of Aspartate aminotransferase (AsAT, EC. 2.6.1.1) and Alanine aminotransferase (AlAT, EC. 2.6.1.2) was done according to the method of Reitman and Frankel, 1957. Glutamate dehydrogenase (GDH, EC.1.4.1.4)and phenoloxidase (EC. 1.14.18.1) were assayed according to methods of Strecker (1955) and Lerch (1987) respectively. All chemicals were purchased from Sigma Aldrich Co.

Statistical analysis: Data obtained was subjected to statistical analysis using SPSS 22.0 software for Windows. One way analysis of Variance (ANOVA) was done followed by Duncan's Multiple Range test (DMRT). Data was considered to be statistically significant, if $P \leq 0.05$.

RESULTS

Total protein and total free amino acids:

Total protein during course of development ranged between 3% and 5% of fresh body weight,showing a gradual increase from 1st instar to adult mosquitoes. Adult females had the maximum content of total protein among the different stages of life cycle.The amount of total free amino acids on the other hand showed a sharp increase up to 4th instar larvae. There was a decline from pupa to adultsand the results are also shown in Fig.1.

SDS- PAGE Analysis:

Elevation of protein content from first instar to adults was substantiated through a significant change in the protein profile in 1D gel electropherogram (Fig. 2). The protein profile of pupa was conspicuously different from the rest with a protein band within 66-97.4 kDa molecular weight of large intensity and volume which was absent in different larval instars and adults. The protein profiles of males and females resolved into 21 bands and bands were of the same molecular weight range.

Transaminases:

The activity of alanine aminotransferase (AlAT)showed a significant elevation from 1st instar to 4th instar, declined in pupa and reached maximum in males and females.aspartate aminotransferase (AsAT) activity showed antagonistic response from first instar to pupae and an elevation in males and females. The AlAT/AsAT ratio upto pupae was always ≤ 0.3, but the ratio in adult males and

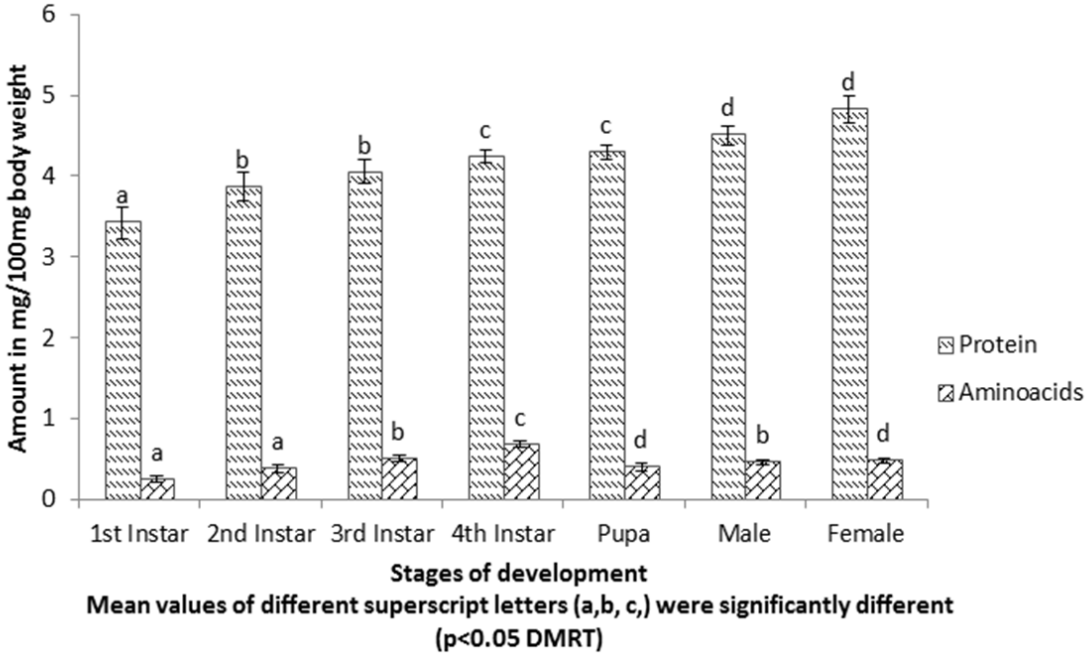


Fig.1 Total protein content and total free amino acids in *Culex quinquefasciatus* during course of development

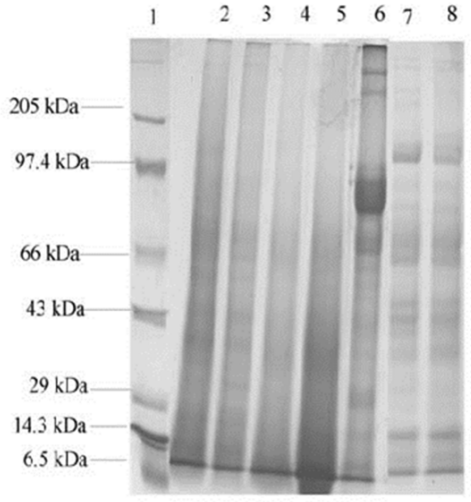
females was 0.6. Maximum ratio of transaminase activity was shown by adult females i.e 0.65 (Table 1).

Glutamate dehydrogenase:

Activity of glutamate dehydrogenase (GDH) also showed a sharp decreasefrom 1st larval instar to pupal stage, where it had the minimum activity and showed an exponential elevation in adult males and females (Fig.3). Maximum activity of glutamate dehydrogenase was shown by adult females.

Phenol oxidase:

Activity of phenol oxidase exhibited significant



. Lane 1- Marker, Lane 2- 1st instar larvae, Lane 3- 2nd instar larvae , Lane 4 – 3rd instar larvae, Lane 5- 4th instar larvae, Lane 6 – Pupae, Lane 7- Adult Male and Lane 8- Adult female.

Table 1. Activity of alanine aminotransferase and aspartate aminotransferase during the course of development of *Culex quinquefasciatus.*

Stage of development	Activity of Transaminases		AlAT/AsAT Ratio
	AlAT/GPT*	AsAT/GOT**	
1st Instar	0.2903 ± 0.0585[a]	3.8517± 0.1354[a]	0.08
2nd Instar	0.518 ± 0.1427[b]	3.6953± 0.1276[b]	0.14
3rd Instar	0.754 ± 0.1424[c]	3.2651± 0.1287[c]	0.23
4th Instar	1.0512 ± 0.1286[d]	3.0922± 0.1667[c]	0.33
Pupa	0.7282 ± 0.0698[a]	2..8333±0.0973[d]	0.25
Male	1.8052± 0.1483[c]	2.9027± 0.2001[c]	0.6
Female	2.011 ± 0.1449[e]	3.1017± 0.1943[c]	0.65

*** Activity is expressed in micromoles of Pyruvate liberated/min/mg of protein.**
**** Activity is expressed in micromoles of Oxaloacetate liberated/min/mg of protein.**
All values are Mean ± SE; n=6.
In Columns, Mean values of different superscript letters (a, b, c,) were significantly different (p<0.05 DMRT)

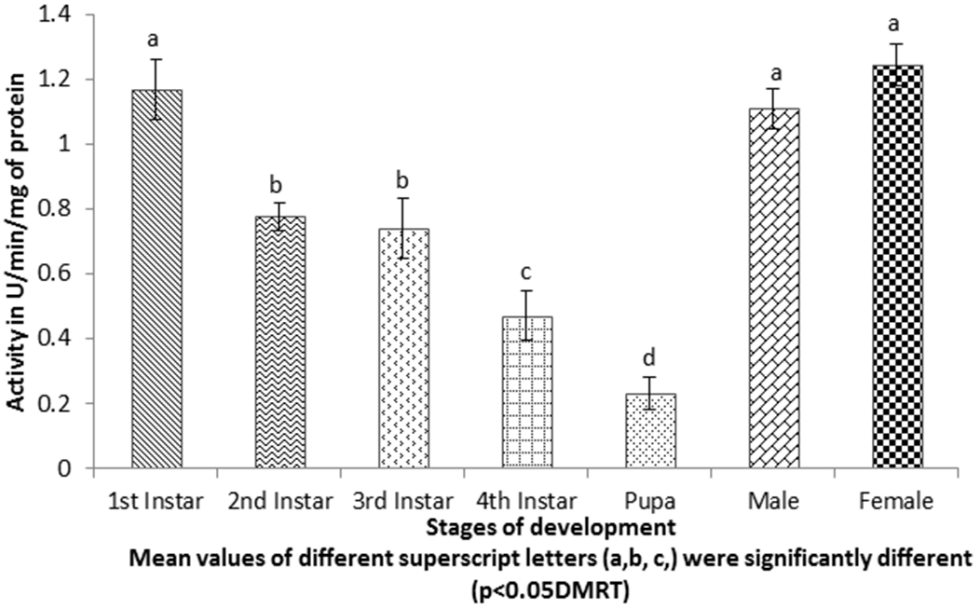


Fig.3. Activity of glutamate dehydrogenase in *Culex quinquefasciatus* during course of development

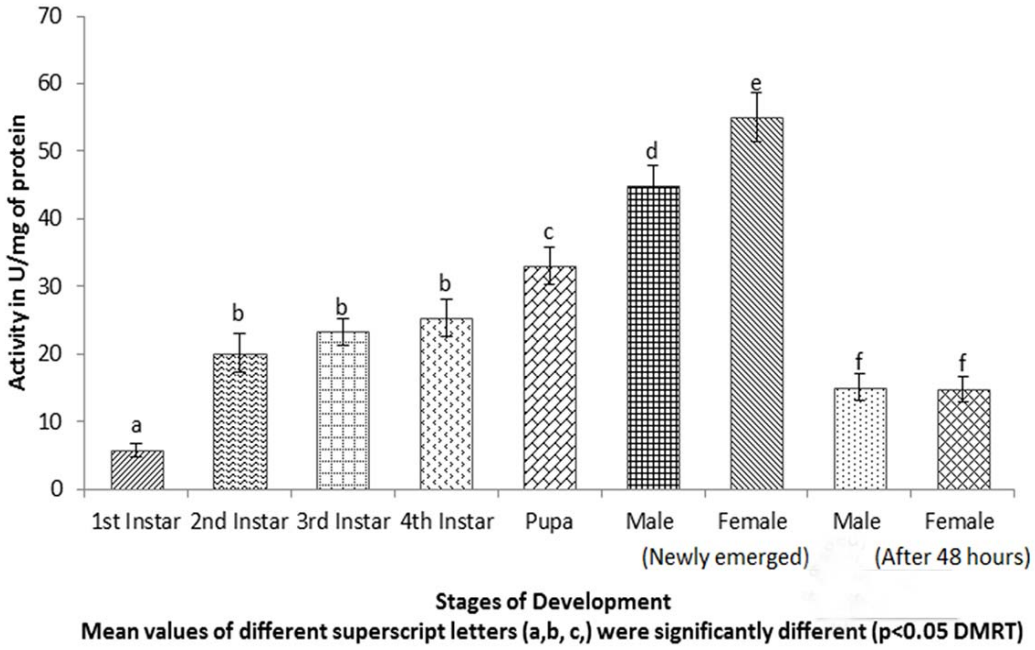


Fig.4. Activity of phenol oxidase in *Culex quinquefasciatus* during development

variation among the different developmental stages and activity showed a sharp elevation from 1st instar to adults, with adult females on emergence showing the maximum activity. However, the trend was reversed in both males and females after 48 hours on emergence where the activity declined sharply (Fig.4).

DISCUSSION

Profound changes in protein turn over and amino acid metabolism take place at various periods during insect development (Chen, 1966). In the present study, qualitative and quantitative changes in the protein and catabolism of amino acids in relation to energy metabolism and activity of phenol oxidase during course of development in filarial vector, *Culex quinquefasciatus* has been investigated.

In *C. quinquefasciatus*, there is a gradual increase in the total protein content from first instar larva to adult mosquitoes, with adult females showing the maximum amount of protein which was identical to the previous reports in *C. quinquefasciatus*The high protein content of fourth instar larvae compared to other instars clearly agreed with the physiology of that stage. This instar has to store large quantities of proteins for pupal life. It is a well established fact that pupae are metabolically very active but they do not feed as they are not provided with functional mouth parts (Mozzelli, 1955). Increase in total protein in pupal stage appears to be directly associated with the conversion of insoluble larval cuticular and other proteins into soluble ones suggesting histolysis and also metabolism of glycogen and chitin.Elevated activity of Cathepsin D, an enzyme directly involved in the internal reorganization of proteins and organs have been reported in pupae of *Oryctes rhinoceros and Oecophylla smaragdina (*Adhira and Evans, 2014; Vidhu and Evans, 2014)The observations made by Pant and Kumar (1980) in dipteran flesh fly *Sarcophaga ruficornis* and *Trabala vishnou* (Gakhar et al.,1997) during metamorphosis of final larvae into pupa very well agreed with wide spread histolysis and internal reorganization during pupal life.Increase in total proteins in non-blood fed and newly emerged *Culex quinquefasciatus* may be due to the process of histogenesis of adult tissue and the observation very much agreed with the investigations in the malarial vector *Anopheles stephensi*(Gakhar et al., 1997).

Elevation in total protein content during course of development in *C. quinquefasciatus* was substantiated in 1D SDS-PAGE electropherogram. The intensity of protein bands of different stages gradually increased from first larval instar to fourth relating to increase in protein content obtained in quantitative estimation. The protein profile of pupae was found to be entirely different from earlier instars with the appearance of a high intensity bandbetween 97.4 and 66kDa molecular weight range.Similar observations were reported by previous investigators in*Megachile rotundat*and suggested that these bands may be regulating pupation (Rank *et al*., 1982). Identical observations were obtained in the electropherogram of developing *Anopheles stephensi* by Gakhar *et al* (1997). Adult male and females had a distinct protein banding pattern with well- defined bands just like in pupa. The protein profile of male and female *Culex quinquefasciatus* showed no distinct dissimilarity in number and intensity of protein bands.

In the present study, there is a slight, but gradual elevation in the amount of total free amino acids from first instar larvae to fourth.In the pupae, the total free amino acids decreased sharply and elevated further in adult males and females.The maximum amount of total free amino acids is shown by 4th instar larva. Similar results were obtained by Chen (1958) in various larval and pupal stages of *Culex pipiens* and by Pant and Kumar (1980) in dipteran flesh fly, *Sarcophaga ruficornis*.Increase in free amino acids during larval development suggests the degradation of the ingested dietary proteins and their further utlilization for the formation of larval structures. At the onset of pupation, the significant depletion in free amino acids indicates their involvement in the synthesis of cuticular proteins for the formation of puparium. In adults, on the other hand, there occurs a gradual degradation of stored proteins into amino acids which eventually get involved in the formation of adult tissues. Besides, these amino acids have been reported to participate in energy metabolism in some insects. At metamorphosis, the level of total free amino acids stays either rather constant in some insects or exhibits an initial rise followed by a fall during later pupal development. In the latter case, the variation is interpreted as reflecting the break-down of larval tissues and formation of adult tissues. A part of the amino acids in the developing pupa can be oxidized and converted to fatty acids and carbohydrates in *Lucilia cuprina* (Crompton and Birt, 1967).

In the present investigation, the activity of alanine Aminotransferase (AlAT) and aspartate Aminotransferase (AsAT) differed during the course of development in *C. quinquefasciatus*. AlAT showed a significant elevation from 1st instar larvae to fourth, declined in pupa and rose again in adult males and females. Maximum AlAT activity was shown by adult females among the different stages of life cycle. Our results are consistent with the results obtained in bruchid, *Zabrotes subfasciatus* (Kaur, 1985) and in *Drosophila* (Schneider and Chen, 1981).To the contrary, AsAT activity was maximum in the first instar of *Culex quinquefasciatus,* declined significantly up to pupal stage and elevated again in adult males and females. These results agreed with the observation made by Evans and Kaleysaraj (1992) in *C. quinquefasciatus*.The ratio of AlAT/AsAT, which is the index of transamination and nitrogen balance in lower group of organisms (Adhira and Evans, 2011) and an index of liver function in mammals (Subramoniam*et al*., 1998) and that in *C. quinquefasciatus* first instar to pupa had a ratio d" 0.3. Elevation inAlAT/AsAT ratio shown by adult males and females. (i.e. 0.6) can be considered as an adaptation of aerial life. Increase in the activity of transaminases in adults compared to larval instars and elevated AlAT/AsAT ratio also have been observed in *Oryctes rhinoceros* (Adhira and Evans, 2011) and in *Oecophylla smaragdina* (Vidhu and Evans, 2011). During early adult life AlAT and AsAT are still active, as the early part of adult life of insect is physiologically quite vigorous and energy requirements of the body at this stage are intense. The most important physiological functions of transaminases are the maintenance of an amino acid pool for protein synthesis (Meister, 1965), the supply of metabolites for energy metabolism (Sacktor, 1974) and the catalysis of interactions between protein and carbohydrate metabolism (Katanuma *et al*., 1968).

During larval growth and pharate- adult development,the high AsAT activity involves the synthesis of proteins which subsequent to their participation in the formation and differentiation of larval and adult structures get transformed in to insoluble structural proteins (Pant and Kumar, 1980). This leads one to conclude that during growth and differentiation (histogenesis) the aminotransferases are highly active, while during histolysis, it is the reverse. Elevation of transaminase activity (AlAT/AsAT ratio) in adult *C. quinquefasciatus* may be related tothe aerial life of adults which necessitated the increased turnover of metabolites than the larvae.

Gradual decrease in the activity of glutamate dehydrogenase (GDH) from 1st instar up to pupa, together with a steep elevation in GDH activity in non- blood fed newly hatched female and male mosquitoes, can be correlated with their physiological states. Through the decrease in GDH activity, the biological availability of alpha-ketoglutarate formed from glutamate is restricted so that shuttling of all glucogenic amino acids into Krebs cycle is also restricted. This will help the fourth instar larvae to conserve amino acids for future use to synthesize new proteins. Adult females showed more activity than adult males.In all larval stages, pupae and adults there exhibited a close relation between AsAT and GDH activity. It has been reported that the AsAT and GDH activity are closely related and transaminase – dehydrogenase complex is necessary in insects for the continuous supply of glutamate (Bursell,1970). Glutamate is the most abundant amino acid of free amino acid pool, playing significant roles as neurotransmitter, energy releaser and also as an amino acid responsible for acid- base balance of the haemolymph (Chen, 1966).

In the present investigation, activity of phenol oxidase (PO) showed a sharp significant elevation from 1st instar larvae to emerging adults. Adult females showed the maximum PO activity. However, the activity of PO declined significantly after 48 hours of emergence. Our findings agree very well with the findings in red turpentine beetle, *Dendroctonus valens* (Shi *et al*., 2010). PO is an oxidoreductase produced in inactive prophenol oxidase form, and catalyzes the oxidation of phenols to quinines, which then polymerize non-enzymatically to produce melanization (Sugumaran, 1996). Melanization involves a series of diet-dependent chemical reactions involved in cuticle pigmentation, moulting, tissue repair and defense against pathogens (Gillespie *et al*., 1997, Rolf and Siva- Jothy, 2003; Schmid- Hempel, 2005; Siva-Jothy *et al*., 2005) In many species, the degree of cuticle melanization is a strong indicator of resistance to pathogens and is correlated with PO activity in the cuticle, haemolymph and midgut (Barnes and Siva- jothy, 2000; Wilson *et al*., 2001). The level of active PO seems to be correlated with the degree of pigmentation of the cuticle during stages of development (Giglio and Giulianini, 2013).

The first instar *Culex quinquefasciatus* larvae are almost transparent and the activityof PO measured is very low. From 2nd instar larvae to pupa, gradual darkening of cuticle was observed and this increase in pigmentation and sclerotisation may have an adaptive advantage in decreasing the ability of fungal and bacterial proteases of polluted aquatic ecosystems. Newly hatched pharate adults are with fragile flimsy body, which gradually hardens and darkens within 2 days. Elevated ability of PO may facilitate the newly hatched mosquitoes to lead a successful winged life by providing a hard structural frame to the body. However, after 48 hours of adult emergence, cuticular melanization and sclerotization seems to be completed and hence there is a decline in activity of Phenol oxidase.

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