ISSN 0974 - 9373

Vol. 14 No.4 (2010) **Journal of International Academy of Physical Sciences** pp. 521 - 530

Effect of Cholesterol Supplementation on Lipids: Role of Carboxylesterase in *Philosamia ricini* during 5th Instar Larval Development

Deepti Pandey and Dwijendra K. Gupta
Department of Biochemistry,
University of Allahabad, Allahabad-211002
E-mail: dwijenkumar@gmail.com

(Received July 12, 2010)

Abstract: Effect of cholesterol on lipid content of Eri silkworm *Philosamia ricini* was investigated by spray supplementing cholesterol at 1mg/ml in their diet. The biochemical changes in the hemolymph and fat body of Eri silkworm *Philosamia ricini* larvae, as a result of cholesterol supplementation showed initially sudden 10-fold increase in hemolymph lipid and decrease in fat body lipid in 5th instar first day larvae. Lipid content in fat body found to be increased 2-3 times in 5th instar third and fifth day larvae. A significant change was also observed in neutral, polar and non-polar lipids. Cholesterol is accumulated in fat body as a neutral lipid due to dietary cholesterol. Results done on TLC of neutral lipid component showed that cholesterol was present in low level on the 3rd and 5th day of larval hemolymph as compared to fat body cholesterol. Carboxylesterase activity decreased gradually in early 5th instar to late 5th instar larvae due to cholesterol supplementation. The enzyme activity appears to be associated with reducing juvenile hormone titer during metamorphosis and mobilization and energetic catabolism of fat substance.

Keywords: Eri silkworm, Hemolymph, Fat body, Juvenile hormone, Metamorphosis.

1. Introduction

Insects like other arthropods are unable to synthesize cholesterol or its precursor squalene de novo and show requirement of sterols in other insects order as nutrients[1-4]. The importance of sterols in insects is essential structural components of cellular membranes as well as precursors of molting hormone, ecdysteroid has been well documented[5]. In insects, certain sterols also have brain hormone activity[6] and juvenile hormone activity[7]. Interestingly however, most phytophagous insects apparently lack the capacity for the biosynthesis of the steroid nucleus and therefore show a dietary requirement for sterols[8]. In contrast, some that feed on plants or

fungi consume 24-alkylsterols and so must absorb and either use these sterols directly in their tissues or metabolize them to more utilizable forms. They are thus capable of carrying out limited modification of the ingested cholesterol and some of them even are able to synthesize sterol from the precursor acetate.

Cholesterol biosynthesis in insects involves the Isoprenoid–sterol pathway with the operation of classical acetate-mevalonate isoprenoid sequences resulting in synthesis of 30-carbon olefin, squalene oxide called cycloartenol which is finally converted into sitosterol[9-11]. Next, four steps occur in the 24-dealkylation pathway that converts sitosterol to cholesterol.

Apart from the role of sterols as structural components of the cell membrane and cuticle, other lipids provide an important source of metabolic fuel for insects growth and development[12]. As a complex array of physiological and biochemical events and processes occur following massive dietary intake during the 5th Instar larval stage, it was considered worthwhile to study the changes in biochemical parameters concerning lipid following dietary supplementation of cholesterol. In insects, Carboxyl esterases (CE) also are known to play important role in the mobilization and energetic catabolism of fat substances [13, 14], control juvenile hormone titers[15] and in reproduction and vitellogenesis processes[16,17]. Therefore, the biochemical status of CE as a corollary to cholesterol's role was also undertaken during 5th insect larval development. Carboxylesterases (CE 3.1.1.1) distribute broadly in insects, and play an important role in the metabolism with various functions.

2. Experimental

The larvae of silkworm *P. ricini* were obtained from Resham Nideshalaya Uttaranchal, Dehradun and were reared in the laboratory at 25±5 ºC with room humidity maintained at 80±5%. The larvae were fed fresh, tender castor leaves (*Ricinius communis*) twice a day. The newly ecdysed worms from 4th instar stage were taken as 'Day Zero' of 5th Instar larval period.

Cholesterol Supplementation

Fresh castor leaves washed copiously with water and air dried were sprayed uniformly with alcoholic solution of cholesterol at 1 mg/ml and air dried for 15-20 min prior to feeding. The 5th instar larvae were divided into four groups, one group as control and rest three groups as experimental ones. Following feeding on cholesterol sprayed castor leaves once every day, the larvae belonging to three experimental groups were processed for

samples of fat body and hemolymph on day 1, 3 and 5 .The control group was fed castor leaves sprayed with alcohol and air-dried.

Collection of hemolymph

Larvae were picked randomly from the groups of both control and experimental. A small incision was made on the dorsal side of the insects with a sharp sterilized stainless steel blade and pale yellow colored fluid was drained in ice-chilled centrifuge tubes containing approximately 0.05% (w/v) phenylthiourea crystals for preventing melanization of hemolymph. Samples were stored at -20ºC for further studies.

Preparation of tissue homogenate

Randomly picked larvae from control and experimental were dissected dorsally in chilled Bodenstein's Ringer solution[18,19]with clean sharp pair of scissors and homogenized in Potter Elvehjem homogenizer. The homogenate (20 % w/v) of fat body was prepared in ice-cold Tris-HCl buffer (50 mM, pH 6.8) and next centrifuged at 10,000 rpm at 4 ^{0}C for 30 minute. The supernatant was collected and kept at -20ºC for further studies.

Biochemical Parameters

Total lipid was extracted as per procedure of Folch etal[20]. Briefly, the organic layer containing total lipids was next twice washed with 0.2 volume of NaCl solution (0.9%, w/v) to remove protein and non-lipoidal substances. The total extract was taken in pre-weighted beaker (25 ml) and the solvent was evaporated to dryness at 60°C in an oven for 3 h. Total lipids were quantitated next and stored overnight in a desiccator. Lipid fractionation was performed by column chromatography on the basis of different polarity[21]. Briefly, silicic acid was washed thrice with double distilled water to remove fine particles and activated at 125°C for 1 h. Slurry of activated Silicic acid in chloroform was carefully poured into the glass column (2.5 cm diameter × 15 cm length) with a bed height of 9 cm. Total lipids were fractionated into neutral, polar and non-polar lipids eluted using chloroform acetone and methanol. Total cholesterol was estimated by Babson Shapiro and Phillips method[22].Thin layer chromatography (TLC) of neutral lipid was performed on a 2 mm thin adsorbent of Silica gel G. TLC layer using glass plates (2.5 cm ×7.5 cm) and developed for 2 h in a chloroform-methanol (2:1,v/v) solvent system to remove organic contaminants. The plates were air dried at room temperature for 20-25 min and then activated overnight in an oven at 110°C.A small sample aliquot (50µl) was loaded onto glass plate and chromatogram developed using solvent system- petroleum ether-diethyl ether-acetic acid (80:15:2, v/v/v) and spraying the air-dried plates with Sulfuric acid (70%, v/v). The plates were next charred by heating at 180°C

for 15-30 min and the resultant dark spots were visualized in a UV transilluminator. Identification of cholesterol and triglycerides on TLC plates were done by comparing with the R_f (Retardation factor) of the authentic standard of cholesterol (0.38) and triolein (0.70).

Carboxylesterase (CE) activity was determined by the method of Grant et al[23] using ρ-nitrophenol acetate (p-NPA; 75mM) substrate. All assays were performed on triplicate sets and standard deviation has been worked out in the data sets.

2. Results

Investigations on various lipid classes (total, neutral, polar and non-polar etc) were carried out on fat body and hemolymh of both control and experimental sets of 5th Instar larvae on days 1, 3 and 5. When compared to controls, cholesterol supplementation brought about a 10-fold dramatic increase in total lipid levels in larval hemolymph on day 1, which then progressively decreased to 28% on day 3 and 19% day 5(Fig 1 A). Conversely, fat body showed a substantial 46% decline in control but later increased on day 3 (58%) and day 5 (90%) , respectively (Fig 1 B). Among the fractionated lipid classes of hemolymph, neutral lipids revealed a significant 36% increase on day 1 and 43% on day 3 and 5 while polar lipids and non-polar lipids underwent over 2 fold decline (Fig 2 A). In contrast, fat body neutral lipid showed a modest decrease (~ 10%) on day 1 and a further slight increase upto 22% on days 3 and 5 (Fig 2 B). Polar lipid fraction in fat body was found to be decreased (49%) on day 3 with insignificant increase on day 5 whilst non-polar lipid level increased by 31% (Fig 2 A, B).

Cholesterol as determined by TLC showed spots in chromatograms for hemolymph and fat body typically with R_f values ranging between 0.33-0.40 in agreement with authentic cholesterol sample (R_f 0.38). Hemolymph collected from experimental larvae registered low levels on the 3rd and 5th day during 5th instar larval development though cholesterol accumulation was seen in fat body on day 5 (Fig 5 A, B) as seen in chromatograms. This was also in agreement with colorimetrically determined cholesterol values for hemolymph (2.7 fold decline over controls) and for fat body (34% increase over controls) on the 5th day. (Fig 3 A, B).

Triglycerides as revealed by TLC did not show any significant in hemolymph and fat body levels. This could be due to low resolution of the data as well as the unsatisfactory limit of detection in TLC experiments in as much as no further experiments could be undertaken to quantitate the spots.

Carboxyl esterases (CE) are also known to regulate juvenile hormone titers and as well as to affect reproduction and vitellogenesis processes**.** Remarkably, cholesterol supplementation in *P. ricini* significantly brought

about a modest decrease in the levels of CE in both hemolymph (about 2-3 fold) and fat body from 27%-45 % (Fig 4 A, B).

3. Discussion

Insects cannot de novo synthesize cholesterol or squalene but require dietary sterol for their normal growth, development and reproduction.[8] Aside from cholesterol's importance as an essential structural component of cellular membranes, it acts as a precursor of molting hormone[5], ecdysteroid. Due to complex physiological and biochemical changes occurring after massive dietary intake during the 5th Instar larval stage, the role of supplementation of cholesterol on lipid and general carboxylesterase was investigated.

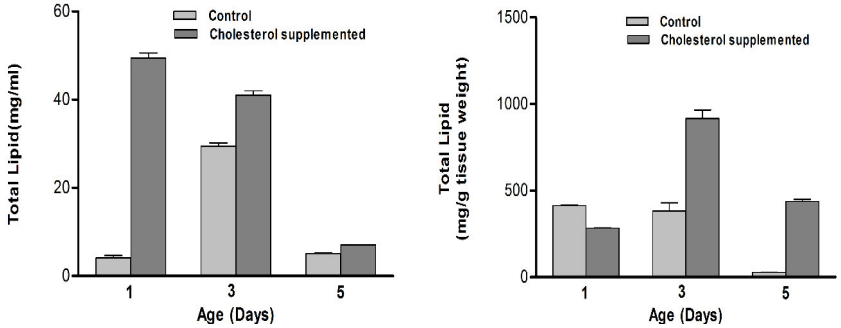
There is a fine-tuning of two hormones: juvenile hormone that is broken down in the hemolymph, controls juvenoid state of larvae whereas ecdysone derived from cholesterol sets the molting stage of larvae[24] Hemolymph of the larval tobacco hornworm has been shown to contain one or more enzymes (carboxyl-esterases, CE; EC 3.1.1.1) that cleave the methyl group from the ester linkage of juvenile hormone (methyl trans, trans, cis 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate) and generate free carboxylate. This enzyme action is the probable first step in juvenile hormone catabolism[25,26].

Esterases have been also shown to occur in *Hyalophora gloveri* pupae. Whitmore et al[24] have also shown that juvenile hormone can induce the appearance of specific multiple molecular forms of CE that degrade the juvenile hormone, suggesting that this is one mechanism an insect uses in regulating the titer[27] of circulating juvenile hormone to ensure normal development. Therefore, the biochemical status of CE as a corollary to cholesterol's role was also undertaken during 5th insect larval development.

In our experiment the cholesterol supplementation results in an initial increase in lipid content of hemolymph which probably is a consequence of release of fat body lipids into hemolymph as initial step towards early histolytic events. The release or transport from fat body into hemolypmh could be in the form of lipoprotein as demonstrated in some insects[28,29,30]. Interestingly, TLC and biochemical results described that the cholesterol content progressively increased in fat body and decreased in hemolymph due to supplementation of cholesterol indicating that the fat body stored excess dietary cholesterol[31]**.** This speculation was supported by the report on *Bombyx mori* by Sridhara and Bhat[32] that cholesterol had no observable effect on growth and development except for an increase in the sterol content. Excess cholesterol content, after supplementation causes

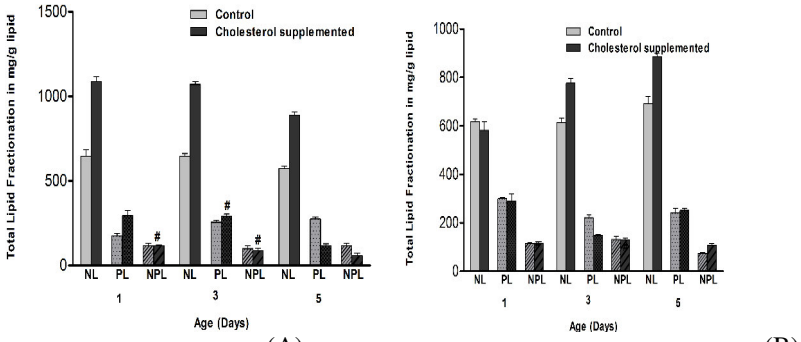
physiological stress could be attributed to the interruption in absorption system[33]. Besides the interplay between fat body and hemolymph, lipids could provide as energy source for growth as well as for hormone synthesis in preparatory to metamorphosis events.

The results obtained with cholesterol supplementation on various lipid classes also suggest that exogenously derived cholesterol can influence various metabolites and hormones involved in bringing major developmental changes. A systematic time-and dose-dependent effect of cholesterol supplementation on the levels of Juvenile hormone and Ecdysteroid as well as different metabolites needs to be done to conclusively prove the positive stimulatory influence on juvenoid activity of *P. ricini* larvae and whether this can be prolonged with delayed pupation. A requirement of a cholesterol transporter protein like the one encoded by a Drosophila gene Start1[34]that regulates ecdysteroidogenesis, remains to be established.



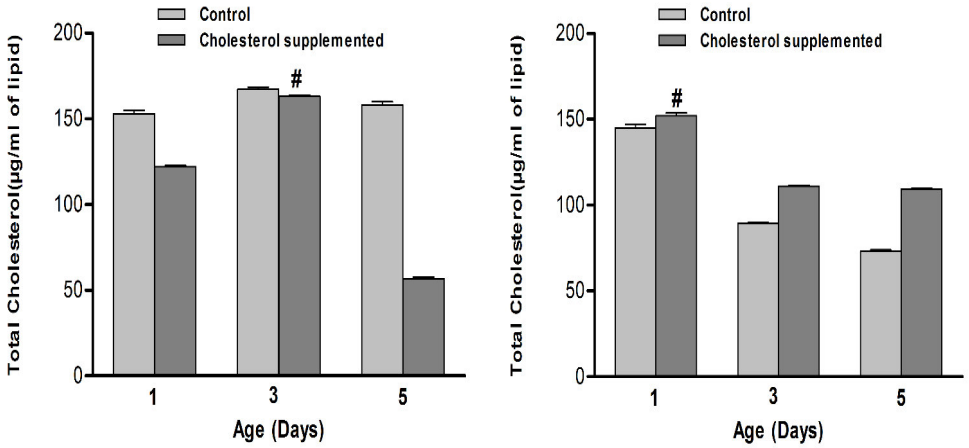
(A) (B)

Figure1. Age-dependent changes in total lipid content of 5th instar larvae of *P. ricini* following cholesterol supplementation in (A) Hemolymph (B) Fat Body compared to their respective controls. Values represent Mean ± SEM of three independent experiments with a significance $P < 0.05$.



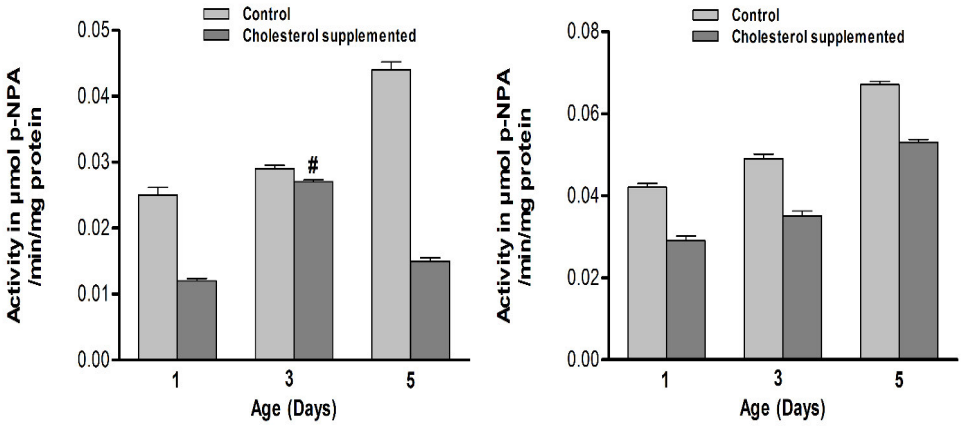
(A) (B)

Figure2. Age-dependent changes in total fractionated lipid content of 5th instar larvae of *P. ricini* following cholesterol supplementation in (A) Hemolymph (B) Fat Body compared to their respective controls. Values represent Mean ± SEM of three independent experiments with a significance $P < 0.05$.



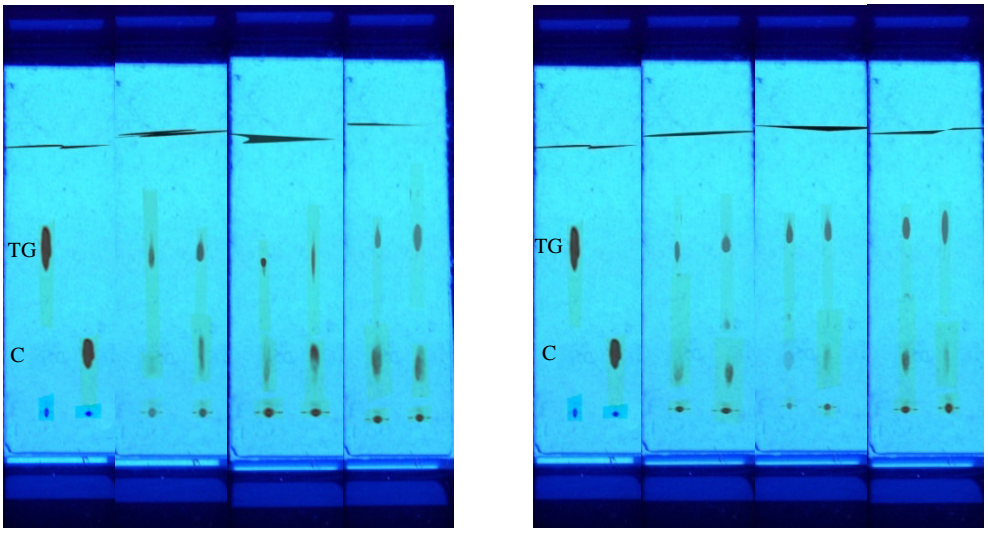
(A) (B)

Figure3. Age-dependent changes in total Cholesterol content of 5th instar larvae of *P. ricini* following cholesterol supplementation in (A) Hemolymph (B) Fat Body compared to their respective controls. Values represent Mean ± SEM of three independent experiments with a significance $P < 0.05$



(A) (B)

Figure4. Age-dependent changes in Carboxylesterase activity of 5th instar larvae of *P. ricini* following cholesterol supplementation in (A) Hemolymph (B) Fat Body compared to their respective controls. Values represent Mean ± SEM of three independent experiments with a significance $P < 0.05$



(A) (B)

Figure5. Lipid Analytics by T.L.C. of 5th instar larvae from (A) Hemolymph, (B) Fat body.
Thin layer chromatograms were developed in a Solvent system: Petroleum ether-Diethyl ether: Glacial acetic acid (80:15:2, v/v/v). Lane 1. Triolein; lane 2: Cholesterol; Lane 3: Control day1; Lane 4: Experimental day 1; Lane 5: Control day 3; Lane 6: Experimental day 3; lane 7: Control day 5; Lane 8: Experimental day 5; TG: Triglycerides and C: Cholesterol

4. Conclusion

The study shows the impact of cholesterol supplementation during 5th instar larvae appears to be time dependent. But we have not evaluated the dose dependence. The results also suggest that continuous exogenously added cholesterol can cause accumulation of cholesterol in the fat body and thus severely affect insect sterol metabolism by causing feedback inhibition on 24, 25-reductase enzymes and thereby inhibiting the dealkylation pathway. The 24, 25-reductase is a key enzyme of sterol metabolism and responsible conversion of phytosterol to ecdysteroid[35].

CEs may play important roles in the adaptation of these insects to their specific biological function[36]. Decreased activity of CE may also cause improper mobilization of lipid from fat body to hemolymph and consequently, disturb the hormonal homoeostasis during metamorphosis. Hence, accumulated cholesterol can act as an inhibitor for the biosynthesis of cholesterol and thereby inhibiting the larval growth and development and thus delayed pupation.

Acknowledgements

DP is a recipient of UGC research fellowship. The facility extended by DBT-Bioinformatics Infrastructure Facility of Bioinformatics Center of University of Allahabad is also gratefully acknowledged.

References

1. J. A. Svoboda, Steroid metabolism as a target for insect control, *Biochem. Soc. Trans*., **22** (1994) 635–641.
2. J. A. Svoboda and W.R. Lusby, Variability of sterol utilization in stored product insects, *Experientia.,* **50** (1994) 72–74.
3. J. A. Svoboda, M.F. Feldhaufer and G.F. Weirich, Isopentenoids and Other Natural Products, *ACS Symposium series,* **562** (1994) 126-139.
4. S. T. Behmer and W.D. Nes, Insect sterol nutrition and physiology: a global overview, *Adv. insect physiol*., **31** (2003)1-72.
5. J.A. Svoboda, Variability of metabolism and function of sterols in insects, *Crit. Rev. Biochem. Mol. Biol*., **34** (1999) 49–57.
6. M. J. Kobayashi and M. Kirimura Saito, The brain hormone in an insect, *Bombyx mori* L. (Lepidoptera), *Mushi,* **36** (1962) 85.
7. L. I. Gilbert, Endocrine control of growth, *Science,* **139** (1963) 928-929.
8. H. Lipke and G. Fraenkel, Insect nutrition, *Annual Reviews Inc. Palo. Alto.,* **1** (1956) 17.
9. H. W. Kircher, *Cholesterol Systems in Insects and Animals,* Boca Raton, FL: CRC Press, (1982) 1–50.
10. B. C. Campbell and W. D. Nes, A reappraisal of sterol biosynthesis and metabolism in aphids, *J. Insect Physiol*., **29** (1983) 149–156.
11. M. Silberkang, C. M. Havel, D. S. Friend, B. J. McCarthy and J. A. Watson, Isoprene synthesis in isolated embryonic Drosophila cells. I. Sterol-deficient eukaryotic cells, *J. Biol. Chem.,* **258** (1983) 8503–8511.
12. R. G. H. Downer, *Functional role of lipids in insects*, Biochemistry of Insects, Academic Press, New York, (1978) 57-92.
13. W. Lands, Lipid metabolism, *Ann. Rev. Biochem.,* **34** (1965) 313-339.
14. S. Ahmad, Larval and adult housefly carboxylesterase: isozymic composition and tissue pattern, *Insect Biochem.*, **6** (1976) 541-547.
15. B. Hammock, Regulation of juvenile hormone titer: degradation, *Comparative Insect Physiology, Biochemistry & Pharmacology,* **7** (1985) 431-72.
16. J. Hooper and T. Wan, Aliesterase activity and reproduction in *Blattella germanica*, *Entomol. exptl. et appl.,* (1969) 211-225.
17. H. Kai and K. Hasegawa, An esterase in relation to yolk cell lysis at diapause termination in the silkworm, *Bombyx mori*, *J. Insect Physiol.,* **19** (1973) 799-810.
18. S. Ringer, Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle, *J. Physiol. Cambridge*, **3** (1980–1982) 380–393.
19. S. Ringer, Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle, *J. Physiol. Cambridge,* **4** (1983–1984) 29–42,222–225.
20. J. Folch, M. Lees and G. H. S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem.,* **226** (1957) 497-509.
21. G. Rouser, G. Kritchevsky and A. Yamamoto, Column chromatographic and associated procedures for separation and determination of phosphatides and

glycolipids, *In Lipid Chromatographic Analysis, Ed. G.V. Marinetti. Marcel Dekker, New York*, **3** (1976) 713.

22. A. L. Babson, P. O. Shapiro and G. E. Phillips, A new assay for cholesterol and cholesterol esterase in serum which is not affected by bilirubin, *Clin. Chim. Acta*., **7** (1962) 800.
23. D. F. Grant, D. M. Blender and B. D. Hammock, Quantitative kinetic assays for glutathion S-transferase and general esterase in individual mosquitoes using an EIA reader, *Insect Biochem.,* **19** (1989) 741-751.
24. D. Whitmore, E. Whitmore and L. I. Gilbert, Juvenile Hormone Induction of Esterases: A Mechanism for the Regulation of Juvenile Hormone Titer, *Proc. Nat. Acad. Sci. USA*, **69** (1972) 1592-1595.
25. M. Slade, C. H. Zibitt and J. B. Siddall, Metabolism of exogenous cecropia juvenile hormone in lepidopterans, Proc. *Int. Symp. International Union of Pure and Applied Chemistry*, (1971).
26. J. B. Siddall, R. J. Anderson and C. A. Henrick, Studies on insect hormones, *Proc. 23rd Int. Cong. Pure Appl. Chem.*, **3** (1971) 17-25.
27. L. M. Riddiford, K. Hiruma, X. Zhou and C. A. Nelson, Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem. Mol. Biol*., **33** (2003) 1327-1338.
28. H. Chino, S. Murakami and K. Harashima, Diglyceride-carrying lipoproteins in insect hemolymph isolation, purification and properties. *Biochim. Biophys. Acta*, **176** (1969) 1-26.
29. K. K. Thomas and L. I. Gilbert, Isolation and characterization of the hemolymph lipoproteins of the American silkmoth, *Hyalophora cercropia*, *Arch. Biochem. Biophys*., **127** (1968) 512-521.
30. K. K. Thomas and L. I. Gilbert, The haemolymph lipoproteins of the silkmoth *Hyalophora gloveri:* studies on lipid composition, origin and function, *Physiol. Chem. Physics,* **1** (1969) 293-311.
31. K. Tsuchida and M. A. Wells, Digestion, absorption, transport and storage of fat during the last larval stadium of *Manduca sexta*. Changes in the role of lipophorin in the delivery of dietary lipid to the fat body, *Insect Biochem.,* **18** (1988) 263–268.
32. S. Sridhara and J. V. Bhat, Effect of cholesterol and ergosterol on the silkworm *Bombyx mori L.*, *J. Insect Physiol.,* **9** (1963) 693-701
33. K. Etebari, A. R. Bizhannia, R. Sorati and L. Matindoost, Biochemical changes in haemolymph of silkworm larvae due to pyriproxyfen residue, *Pesticide Biochemistry and Physiology,* **88,** (2007) 14-19.
34. G. E. Roth, M. S. Gierl, L. Voldborn, M. Meise, R. Lintermann and G. Korge, The Drosophila gene Start1: A putative cholesterol transporter and key regulator of ecdysteroid synthesis, *Proc. Nat. Acad. Sci. USA*, **101** (2004) 1601–1606.
35. J. A. Short, D. A. Guo, J. A. Svoboda and W. D. Nes, Mechanistic and metabolic studies of sterol 24,25-double reduction in *Manduca sexta*, *Arch. Insect Biochem. Physiol.,* **31** (1996) 1–22.
36. Q. Y. Yu, C. Lu, W. L. Li, Z. H. Xiang and Z. Zhang, Annotation and expression of Carboxylesterases in the silkworm, *Bombyx mori, BMC Genomics,***10** (2009) 553.