International Journal of Entomology Research
ISSN: 2455-4758; Impact Factor: RJIF 5.24
Received: 16-12-2018; Accepted: 19-01-2019
www.entomologyjournals.com
Volume 4; Issue 1; January 2019; Page No. 92-96

Biochemical analysis of phenoloxidase in the haemolymph of *Galleria mellonella* (Lepidoptera, Pyralidae) under natural and artificial envenomation of ecto-parasitoid *Bracon hebetor* (say.) (Hymenoptera, Braconidae)

Muhammad Shehzad[1], Asad Aslam[2*], Zain-ul-Abdin[3], Muhammad Jafir[4], Muhammad Kamil Malik[5], Muhammad Zubair[6], Muhammad Wajid Javed[7], Muhammad Zubair Chaudhary[8]
[1, 3, 4, 7] Department of Entomology, University of Agriculture, Faisalabad, Pakistan
[2, 5] Beekeeping and Hill Fruit Pests Research Station Rawalpindi, Pakistan
[6] Oil Seed Research Institute, AARI, Faisalabad, Pakistan
[8] Pest Warning and Quality Control of Pesticides Shujabad, Pakistan

Abstract
Parasitoids can overpower the phenoloxidase (PO) releasing system through several methods such as inhibition of protease activity or by blocking of pattern-recognizing proteins as well as destruction of immunocompetent cells. The depression of PO activity can be the result of the impact of venom components injected by the female of the parasitoid into the haemocoel of the host insect. The wounding and penetration by parasitic wasp lead to the activation of defense reactions in parasitized host insects. An effective innate immune response against parasites in insects is the encapsulation of the invader followed by melanization. The phenoloxidase (PO) cascade takes part in the process of melanization, is an inactive proenzyme proPhenoloxidase (proPO), present in the cuticle and haemolymph of insects. To understand the impact of venom of the ectoparasitic wasp *Bracon hebetor* (Say.) (Hymenoptera, Braconidae) on the immunity of host *Galleria mellonella* (Lepidoptera, Pyralidae) under natural and artificial envenomation we tested the activity of PO in haemolymph and number of haemocytes with PO activity in haemolymph of the parasitized host larvae. The obtained information was helpful in understanding the insect immune response in host parasitoid relationship, which will ultimately facilitate the development of sustainable plant protection strategies for the control pest insects of Pyralidae and data was analysed statistically.

Keywords: insect immunity, phenoloxidase, *Bracon hebetor*, melanization

1. Introduction

Biocontrol agents can be considered as a unique substitute in place of pesticides for regulating insect pests of valuable crops [28]. Insect parasitoids are a vital components in this regard. They have engrossed a significant consideration from biologists. This interest stems primarily from their importance in biological control where for many years they have been used in attempts to control pest insects on food crops [30]. Parasitoids of insects have developed different multiplicative approaches such as they may be Idiobiont as well as Koinobiont in their lifestyle [4]. Talking about Idiobiont these check the host's growth and immature of these natural enemies utilizes this static and arrested host. While in case of koinobiont parasitoids, contrary to Idiobiont host continue to feed as well as growth also continue after parasitization [20]. During parasitization of their host parasitoids inject a blend of secretions into host body. These secretions inhibits the host cellular and humeral immune responses [1]. In contrary to vertebrates the defence system of invertebrates especially insects does not involve antigen-antibody complex formation yet they respond efficiently to an invader attacking to them via humeral or cellular immune response [12]. One of the most important immune response in insect hosts is the encapsulation and melanization of foreign particles via the phenoloxidase cascade. Phenoloxidase is generally found as its precursor pro-enzyme in the haemolymph [13]. Parasitoides on contrary release substances that are mainly pattern-recognizing proteins and immunocompetent cells to suppress immune phenoloxidase cascade. These immune suppressive components are associated with parasitoide venom [29].

Bracon hebetor is an ecto-parasitoid of Pyralid insect pest and they have been used in many biological control programs in different parts of the world [13, 16, 34]. It parasitizes a number of important lepidopteran pests of stored products and of field crops [26]. One of its most likeable host is *Galleria mellonella* which is a destructive pest of honey bee products [33]. The female of *B. hebetor* inject its venom in host body that have neurotoxic effect. Although a lot of work is going on the parasitoide venom and its components yet little is known about the hemolymph and hemocyte changes in host after parasitization due to venom components. The current project was designed to access the immune response of *Galleria mellonella* after parasitism of *B.hebetor*.

2. Materials and Methods

2.1 Experimental Insects

The ectophagous larval parasitic wasp *Bracon hebetor* (Say) (Hymenoptera: Braconidae) was reared in the laboratory on the late stage (5th instar) larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) by following a slightly modified approach as described by [14]. The larvae, pupae and adults of the host, greater wax moth, *G. mellonella* was collected from the infested bee hives located at the campus of University of Agriculture, Faisalabad, Pakistan. The host and parasitoid cultures was maintained in two separate glass jars, both

placed at 27-30 ±1ºC, 65 ± 5% relative humidity (R.H) and 18/6h (L:D) photoperiod.

The adults of the parasitoid were reared in the laboratory, on the larvae of *G. mellonella* as a host by using glass vials of (2 cm × 10 cm). Each vial contained 2 to 3, 4th to 5th instar larvae of the host and one female of the parasitoid, provided with cotton swabs/pads soaked in 50% honey and water as food source for *B. hebetor* adult. The females of the parasitoid started to parasitizing the larvae of the host by first injecting a small quantity of paralyzing venom in to mature (5th instar) larvae of the host before egg laying on them which induces partial or complete paralysis and then deposited between three to twenty eggs on the outside of the host. After parasitization, the larvae of the host were become sluggish and later on, the females started egg laying on these larvae. After 24 hours, the parasitized larvae of the host was shifted from the vials to new sterilized glass jars (9 x 5 cm) by using brush, provided with wax, pollen and honey as food for developing host larvae by keeping at the same environmental conditions stated as above and the females of the parasitoid were shifted to new vials containing 2-3 larvae of (4th - 5th instar) the host for further parasitization and this process remained continue until the last moment of the female life. The eggs were hatched within 2-3 days into transparent larvae, which were directly feed on the body of the host larvae. After completing their feeding/development period, larvae started to pupate outside the body of the host.

For natural envenomation, adult parasitoids were released into glass jars containing *G. mellonella* larvae for 3 h. After natural envenomation the larvae of *G. mellonella* were quickly removed from jars, wiped with 70% ethanol and kept in Petri dishes with paper discs until testing. The PO activity in haemolymph and haemocytes of the *G. mellonella* larvae were studied during the first two days after natural envenomation.

2.2 Chemicals

DOPA-3,4-dihydroxy-L-phenilalanine, DTPA diethylene triamine penta acetic acid, CP-3-carboxy-proxyl, sodium chloride, Glucose, EDTA, trisodium citrate, citric acid, HEPES, potassium chloride, Bradford Reagent.

3.3 Haemocyte collection

10 µl of haemolymph was collected in cooled (+4 °C) anticoagulant "N Phenyl thiourea" (Sigma) or (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM Trisodium citrate, 26 mM citric acid, pH 4.6) (AC) by cutting the third proleg with a micro scissors and drawing haemolymph into the tip of a pipette[13]. The sample was centrifuged at 500 g for 5 min for collecting pellets of haemocytes. Precipitate was resuspended and washed three times in cool AC and once in HEPES buffer, pH 7.2 (140 mM NaCl, 5 mM KCl, 6 mM glucose, 10 mM HEPES).

3.4 Analysis of phenoloxidase activity in haemocytes

The phenoloxidase enzyme catalyzes the synthesis of melanin from any of several precursor molecules, including L-3, 4-dihydroxyphenylalanine (LDOPA) [13, 18]. It has been demonstrated that the blood (hemolymph) of many insects contains phenol oxidase, either carried within specialized blood cells (hemocytes) and/or dissolved in solution [19].

A 10 µl suspension of haemocytes (10^4) was placed on a glass slide for 15 min in a moist chamber and then fixed with cold acetone (10 min). Thereafter, cells were washed three times in phosphate buffer (PBS) (10 mM, pH 7.2) and incubated with 40 µl of L-DOPA (4 mg/ml) for 20 min at 28 °C. The monolayer was washed three times by distilled water and then observed by light microscope. Cells with a dark brown color were counted as PO positive haemocytes.

3.5 Analysis of phenoloxidase activity in the haemolymph

Phenoloxidase activity was assayed by using a method modified from that described by [2]. 15 µl of haemolymph will be added to 20 µl 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl with 1 mM pheny 1 methane sulfonyl fluoride (PMSF). Each sample will then be centrifuged at 500 g for 5 min to remove the hemocytes. Aliquots of samples (15 µl) were added to microplate walls containing 200 µl of 10 mM DOPA. After 20 min phenoloxidase activity was determined by measuring absorbance at 490 nm at 28 C with a plate reader. The concentration of protein in the haemolymph was determined by Bradford method (1976). For the calibration curve bovine serum albumin (BSA) was used. Phenoloxidase activity was measured in units of transmission density (**ΔA**) of the incubation mixture during the reaction per 1 min and 1 mg of protein.

3.6 Statistics

The obtained data was presented as the average ± its standard error. To check the normality of the sampling, the Wilk. Shapiro W criterion was used. Statistical significance was determined by Student's t-criterion.

4. Results

4.1 Effect of *B. hebetor* venom on PO-activity in the hemolymph of *G. mellonella* after natural parasitism and artificial envenomation

Phenoloxidase activity was assayed by using a method modified from that described by[2] post parasitization by *B. hebetor* and artificial envenomation after 12, 24, 36 and 48 hour time interval and the results was compared with the control i.e. which was not envenomated by neither natural parasitism nor artificial envenomation. The concentration of protein in the haemolymph was determined by [6]. For the calibration curve bovine serum albumin (BSA) was used. Phenoloxidase activity was measured in units of transmission density (**ΔA**) of the incubation mixture during the reaction per 1 min and 1 mg of protein as shown given below (Fig. 4.1).

This experiment was repeated five times and the given (Fig.1) graph was drawn by calculating the average value of recorded concentration of the protein from all the treatments i.e. post parasitization by *B. hebetor*, artificial envenomation and control after 12, 24, 36 and 48 hour time interval.

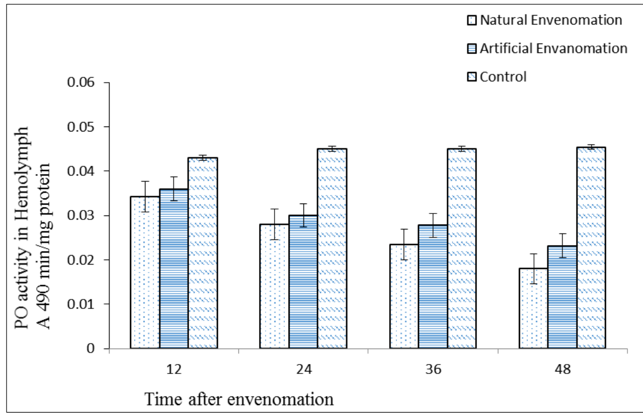

Fig 1: Effect of *Bracon hebetor* venom on PO-activity after artificial and natural envenomation with the passage of time

The above (Fig.1) proves that there was a strong decline in the PO activity in the haemolymph of naturally as well as artificially envenomated larvae of the *G. mellonella* due to immune-suppressive effect of the venom of parasitic wasp *B. hebetor* as compared to control. The graphical data shows that maximum phenoloxidase activity reduction (0.018± 0.001) was occurred in the hemolymph of naturally envenomated larvae after 48 hour that was more than half of the control (0.0454± 0.000245). However the least change in PO activity of the natural envenomated host larvae was observed after 12 hours of post-parasitism (0.0342± 0.00086) as compared to that of control (0.043± 0.000316). After the 24 hours of natural parasitism the activity of phenoloxidase was reduced further then the 12 hours (0.028± 0.001068) as compared to control in which the activity of the protein changes a little (0.0452± 0.0002). The concentration of protein in the haemolymph of parasitized larvae was reduced further (0.0234± 0.000927) while in control shows a little variation (0.045± 0.000316).
The activity of phenoloxidase in artificial envenomated larvae also decreased(0.036±0.001068, 0.03±0.001049, 0.0278±0.001158, 0.0232±0.001068) after 12, 24,36, 48 hours but to a lower levels as compared to natural parasitized host larvae but the decrease in activity was higher than that of control larvae that remain almost same (0.045±0.000316)

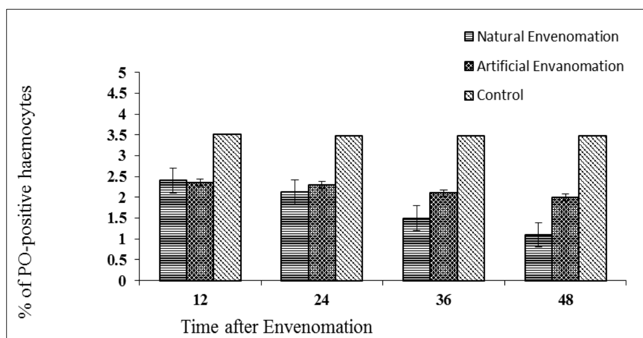

Fig 2: Percentage PO positive haemocytes of *G. mellonella* after natural parasitism and artificial envenomation with different time intervals.

Phenoloxidase activity was assayed in the haemocytes of the host larvae by using a method modified from that described by [2] post parasitization by *B. hebetor* and artificial envenomation after 12, 24, 36 and 48 hour time interval and the results was compared with the control i.e. which was not envenomated by neither natural parasitism nor artificial envenomation. The percentage of protein in the haemocytes was determined by light microscopy.
This experiment was repeated five times and the given (Fig.2) graph was drawn by calculating the average value of recorded concentration of the protein from all the treatments i.e. post parasitization by *B. hebetor*, artificial envenomation and control after 12, 24, 36 and 48 hour time interval.
The above (Fig.2) proves that there was a strong decline in the PO positive haemocytes of greater wax moth host parasitized naturally as well as artificially due to immune-suppressive effect of the venom of parasitic wasp *B. hebetor* as compared to control. Percentage of PO positive haemocytes was half in natural envenomated host larvae then that of the control.
The graphical data shows that maximum percentage reduction of PO positive haemocytes was observed after the 48 hours of natural parasitization (1.01± 0.06738) as compared to control (3.472± 0.003742) yet the maximum percentage of PO-positive haemocytes was observed after 12 hours of parasitization (2.4± 0.082244). After the 24(2.12±0.12806)hours of natural envenomation the percentage of PO-positive haemocytes was observed to be lower than the 12 hours however the percentage positive haemocytes in control remains almost the same (3.472± 0.003742). Further the percentage of positive cells reduced further after 36 hour of natural envenomation (1.5±0.1) as compared to control which shows a little variation (3.46±0.003742).
In contrast to natural envenomation the percentage of PO-positive haemocytes in artificial envenomated larvae changes to a lesser extent after 12,24,36,48 hours of data collection(2.35±2.3±0.008602, 2.1±0.005831, 2±0.009274) but more than the control conditions that remains almost the same (3.47±0.003742)

5. Discussion

Our data indicate the suppression of the main immune reaction in *G. mellonella* larvae during natural parasitization and artificial envenomation by *B. hebetor*. It is well known that although parasitoids can reduce host immune responses through a number of mechanisms [5,29], host immunity is generally not totally suppressed [1,10], presumably in order to allow the parasitized host to protect itself against opportunistic infections and further parasitization. Such opportunistic infections might be disadvantageous to the original parasite. Our results support the studies which show that the physiological effects of parasitization and artificial envenomation by the venom of ectoparasites may include changes in host hemocytes morphology and viability, as well as suppression of immune reactions, such as phagocytosis and encapsulation [20, 21, 22, 23, 24]. In parasitoid-host systems suppression of immunity of the host frequently can be caused by venom components, which the female of the parasitoid injects to induce the paralysis of the host while egg laying [25]. However this has not been detected on *B. hebetor*. It is known that the ability of *B. hebetor* venom to cause host paralysis is mediated by three partially characterized proteins that presynaptically block glutaminergic transmission [11]. It is not known, however, whether the suppression of immunity has anything to do with these three proteins, or the other venom components that affect host endocrine and metabolic activity. The suppression of phenoloxidase activity noted in our study can be beneficial for the parasitoid because products of the proPO cascade take part in the immune reactions such as

haemolymph coagulation and hemocytes aggregation that result in wound thrombosis and, accordingly, result in difficulty of parasite feeding [7, 10, 13, 27]. Probably, inhibition of the PO cascade following coagulation facilitates feeding by the larvae of the parasitoid since these parasitoids use their mouthparts to penetrate the host body wall and imbibe the host haemolymph. Obviously, a rapid host coagulation reaction would prevent haemolymph flow into the larvae and also block the mouthparts of the larvae.

In contrast to our results it was found that the activity of phenoloxidase in the haemolymph of larvae *Plodia interpunctella* is increased after natural envenomation with *B. hebetor* [9]. Possibly, this is connected with different host specific impacts of the venom on the various hosts [31]. Also it may be related to methodological differences in PO testing. Specifically, [9] used dopamine as a substrate and Micrococcus lysodeikticus as an elicitor. In our work we used cell-free haemolymph diluted by PBS and L-DOPA as a substrate according to the classic procedure.

Venom of the parasitoid is remarkably recognized to persuade paralysis, to interfere with its immune system, or to upset the host development unaided or in combination with other factors [17, 32]. Such influence on the host organism can be beneficial for a parasitoid because the products of pro PO cascade takes part in the coagulation of haemolymph and in hemocytes aggregation, mechanisms leading to wound thrombosis and consequent. Difficulties in the feeding of parasitoids.

6. Reference

1. Asgari S, Zhang G, Zareie R, Schmidt O. A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits melanization of the host hemolymph. Insect Biochemical and Molecular Biology. 2003; 33:1017-1024.
2. Ashida M, Soderhall K. The prophenoloxidase activating system in crayfish. Comparative Biochemistry and Physiology Part B. Comparative Biochemistry. 1984; 77:21-26.
3. Ashida Y, Saito Y, Kawato A, Suginami K, Imayasu S. Effects of dietary sake cake on cholesterol metabolism in rats.Journal of the Agricultural Chemical Society of Japan. 1997; 71:137-143.
4. Askew RR, Shaw MR. Parasitoid communities: their size, structure and development. Journal of Parasitology. 1986; 155:225-63.
5. Beckage NE. Game's parasites play: the dynamic roles of proteins and peptides in the relationship between parasite and host. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 1. Academic Press, New York. 1993; pp. 25-57.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976; 72(12):248-254.
7. Brehelin M. Depression of immune reactions in insects. Research in Immunology 1990; 141:935-938.
8. Fagundes GG, Mohamed H, Solis DR. Biological responses of *Anagasta kuehniella* and its parasitoid, *Bracon hebetor*, to microwaves radiation (2450 MHz). Rev. Agriculture Piracicaba. 2005; 80:12-34.
9. Hartzer KL, Zhu KY, Baker JE. Phenoloxidase in Larvae of *Plodia interpunctella* (Lepidoptera: Pyralidae): Molecular Cloning of the Proenzyme cDNA and Enzyme Activity in Larvae Paralyzed and Parasitized by *HabroBracon hebetor* (Hymenoptera: Braconidae). Archives of Insect Biochemistry and Physiology. 2005; 59(2):67-79.
10. Kinuthia W, Li D, Schmidt O, Theopold U. Is the surface of endoparasitic wasp eggs and larvae covered by a limited coagulation reaction? Journal of Insect Physiology. 1999; 45:01-506.
11. Labrosse C, Eslin P, Doury G, Drezen J, Poirie M. Haemocyte changes in D. melanogaster in response to long gland components of the parasitoid wasp *Leptopilina boulardi*: a Rho-GAP protein as an important factor. Journal of insect physiology. 2005; 51(2):161-170.
12. Lackie AM. Transplantation Immunity in Arthropods: Is Immunorecognition Merely Wound-Healing. Immunity in invertebrates. 1986; pp. 125-135.
13. Leonard WJ, Depper JM, Krönke M, Robb RJ, Waldmann TA, Greene WC. The human receptor for T-cell growth factor. Evidence for variable post-translational processing, phosphorylation, sulfation, and the ability of precursor forms of the receptor to bind T-cell growth factor. Journal of Biological Chemistry. 1985; 260(3):1872-80.
14. Manzoor A, Zain-ul-Abdin, Arshad M, Gogi MD, Shaina, Mubarik E, Abbas SK, *et al*. Biological activity of the toxic peptides from venom of *Bracon hebetor* (Say.) (Hymenoptera: Braconidae). Pakistan Entomology. 2011; 33(2):125-130.
15. Mohapatra SD, Duraimurugan P, Saxena H. Natural parasitization of *Maruca vitrata* (Geyer) by *Bracon hebetor* Say. Pulses Newsl. 2008; 19:11.
16. Moreau SJM, Dingremont A, Doury G, Giordanengo P. Effects of parasitism by *Asobara tabida* (Hymenoptera: Braconidae) on the development, survival and activity of *Drosophila melanogaster* larvae. Journal of Insect Physiology. 2002; 48:337-347.
17. Nappi AJ, Ottaviani E. Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays. 2000; 22(5):469-80.
18. Nappi AJ, Seymour J. Hemolymph phenol oxidases in *Drosophila melanogaster, Locusta migratoria,* and *Austropotamobius pallipes*. Biochemical and biophysical research communications. 1991; 180:748-754.
19. Pennacchio F, Strand MR. Evolution of developmental strategies in parasitic Hymenoptera. Annu. Rev. Entomol., 2006; 51:233-258.
20. Resh VH, Cardé RT. Encyclopedia of insects. 2nd Ed, Academic Press Amsterdam. 2009; pp. 51-748.
21. Richards EH, Edwards JP. Parasitism of *Lacanobia oleracea* (Lepidoptera) by the ectoparasitoid, *Eulophus pennicornis*, is associated with a reduction in host haemolymph phenoloxidase activity. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2000; 127(3):289-298.
22. Richards EH, Edwards JP. Parasitism of *Lacanobia oleracea* (Lepidoptera, noctuidae) by the ectoparasitic wasp *Eulophus pennicornis*, results in the appearance of a 27 kDa parasitism-specific protein in host plasma. Insect biochemistry and molecular biology. 1999; 29(6):557-69.
23. Richards EH, Edwards JP. Parasitism of *Lacanobia oleracea* (Lepidoptera, Noctuidae) by the ectoparasitic

wasp, *Eulophus pennicornis,* results in the appearance of a 27 kDA parasitism-specific protein in host plasma. Insect Biochem. Mol. Biol. 2000b; 29:557-569.

24. Richards EH, Edwards JP. Larvae of the ectoparasitic wasp, *Eulophus pennicornis*, release factors which adversely affect haemocytes of their host, *Lacanobia oleracea.* Journal of Insect Physiology. 2002; 48:1041-1052.
25. Shelby KS, Adeyeye OA, Okot-Kotber BM, Webb BA. Parasitism-linked block of host plasma melanization. Journal of Invertebrate Pathology. 2000; 75(3):218-25.
26. Shojaei S, Safaralizadeh MH, Shayesteh N. Effect of temperature on the functional response of *HabroBracon hebetor* (Hymenoptera: Braconidae) to various densities of the host, *Plodia interpunctella* H.(Lepidoptera: Pyralidae). Pak. Entomol. 2006; 28:51-6.
27. Soderhall K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Current opinion in immunology. 1998; 10(1):23-8.
28. Van Driesche RG, Bellows TS. Biology of arthropod parasitoids and predators. InBiological control. Springer, Boston, MA, 1996, pp. 309-336.
29. Vinson SB. How parasitoids deal with the immune system of their host: an overview. Archives of Insect Biochemistry and Physiology. 1990; 13(1-2):3-27.
30. Waage JK, Mills NJ. Biological control, pp. 412-430. *In* Crawley, M. J. (ed.). Natural Enemies: the Population Biology of Predators, Parasites and Diseases. Blackwell, Oxford, United Kingdom, 1992.
31. Wang X, Yang Z. Ecological mechanisms and prospects for utilization of toxins from parasitic hymenopterans. Frontiers of Forestry in China. 2008; 13(1):1-9.
32. Weaver RJ, Marris GC, Bell J, Edwards JP. Identity and mode of action of host endocrine disrupters from the venom of parasitoid wasps. Comparative Biochemistry and Physiology--Part B: Biochemistry and Molecular Biology. 2000; 126:101.
33. Williams JL. Insects: Lepidoptera (moths). In Honey Bee Pests, Predators, and Diseases; Morse, R., Flottum, K., Eds.; AI Root Company: Medina, OH, USA. 1997; pp. 121-141.
34. Yasodha P, Natarajan N. Diversity of natural enemies of *Leucinodes orbonalis* Guenee (Lepidoptera: Pyraustidae). Entomon-trivandrum. 2006; 31(4):323.