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# PARASITIZATION AND ENVENOMATION BY THE ECTOPARASITOID, *Bracon hebetor* AFFECT CELLULAR IMMUNE RESPONSE OF *Galleria mellonella*

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The insect immune reactions involve two types of responses i.e., humoral and cellular ones, whereby the overall immunity results from a complex interaction of the two systems. In view of the cellular immune response, parasitoid venom has various effects on host hemocytes depending on the host-parasitoid system. These include alterations in total (THC) and differential hemocyte counts (DHCs), modifications in hemocyte morphology and ultrastructure, induction of hemocytic death and inhibition of hemocyte spreading and encapsulation. The effects of parasitization and envenomation by ectoparasitoid, *Bracon hebetor* Say (Hymenoptera, Braconidae) on total and differential hemocyte counts and hemocyte morphology of its host *Galleria mellonella* Linnaeus (Lepidoptera, Pyralidae) 5$^{th}$ instar larvae were investigated. Five types of hemocytes i.e. prohemocytes, oenocytoids, plasmatocytes, granulocytes and spherulocytes were observed in host hemolymph based on their morphology. A significant decline was observed in total and differential hemocyte counts of the host exposed to parasitization and artificial envenomation. In addition to that, parasitization has induced different morphological alterations in all hemocyte types. These changes mainly include vacuolization in cytoplasm, loss of pseudopods, cell damage, loss of cell boundary and cell membrane deformities. Our data reveals that parasitization has significant impact on cellular immune response of the host which ultimately leads toward the death of the host insect. Therefore, venom of the *B. hebetor* has a strong potential to be considered for the development of novel insect control tools for pest insects of diverse range of Pyralidae (Lepidoptera).
**Keywords**: *Bracon hebetor*, *Galleria mellonella*, venom, parasitization, immunity, hemocytes count

## INTRODUCTION

Insects possess effective immune systems consisting of cellular and humoral immune responses that they collectively defend themselves against invading pathogens and parasites. For instance, through *de novo* synthesis of a group of anti-bacterial and anti-fungal proteins involving mounted humoral response, insects may protect themselves from bacterial and fungal infections (Otvos, 2000; Bulet and Stocklin, 2005). In contrast, larger parasites and pathogens including the eggs of parasitic wasps could be encapsulated by means of host hemocytes, which spread to form manifold layers around the foreign substance (Salt, 1963). Consequently, endoparasitoids that oviposit within the hemocoel of their hosts need to someway avoid these defense responses (mainly encapsulation) so as to make sure the survival of their offspring. Most of them accomplish this by injecting secretions at the time of egg laying that contain antihemocytic and/or immunosuppressive components (see reviews by Vinson, 1990; Strand and Pech, 1995). Some of these components may be derived from poly DNA viruses, present in the parasitoid calyx fluid (Beckage, 1998). Moreover, in some parasitic wasps, it has been defined that venom produced by the adult female contains proteins which play a key role in subduing immune responses of the host (Guzo and Stoltz, 1987; Stoltz *et al.*, 1988).

The most frequent types of hemocytes found in different insect species are prohemocytes (PRs), oenocytoids (OEs), granulocytes (GRs), plasmatocytes (PLs), adipohemocytes (ADs) and spherulocytes (SPs) (Ashhurst and Richards, 1964; Lavine and Strand, 2002). Nevertheless, across different classes of insects, the kind, role and density of hemocytes varies (Lavine and Strand, 2002; Strand, 2008) and among the similar species, hemocyte population can also differ with growth stage or in reaction to injury, strain or disease (Ashhurst and Richards, 1964; Brayner *et al.*, 2007). Extensive studies have been conducted to reveal the impact of parasitization and artificial envenomation by *Pimpla turionellae* on DHC and THCs in two growth phases of the host, *Galleria mellonella* (Er *et al.*, 2010). THC showed a major decrease in number of floating hemocytes in *G. mellonella* larvae and pupae which were exposed to *P. turionellae* (Ergin *et al*., 2006; Er *et al.*, 2010). Similarly, *in vivo* significant changes in the count of different hemocyte of *G. mellonella* have been recorded between venom applications and parasitization and a decline in the percentage of granulocytes and increase in percentages of plasmatocytes

were noticed at various time intervals for larval and pupal life stage of the host, *G. mellonella* (Er *et al.,* 2010).

Our test insects include *G. mellonella*, a host organism and its parasitoid, *Bracon hebetor* (Hymenoptera: Braconidae). Larvae of the honey comb moth or greater wax moth, *G. mellonella* are considered as an infection model particularly used to evaluate the efficacy of novel antimicrobial medicines and to explicate biochemical characteristics of innate and humoral immune responses and phagocytic cell functions by insects counter to several important human and veterinary infectious agents (e.g. nematodes, fungi and gram-negative or gram-positive bacteria) (Christen *et al.,* 2007; Ciesielczuk *et al.,* 2015). The reason behind this is that *G. mellonella* larvae are comparatively low-cost and less labor-intensive to maintain and have a short lifetime, making them ideal for high-throughput research (Ramarao *et al.* 2012). The *B. hebetor* is a larval ectoparasitic wasp that parasitizes numerous Lepidopteran pests such as host insects related to the family, Pyralidae (Dweck *et al.,* 2010). It also utilizes larvae of many Lepidoptera associated with stored commodities (Richards and Thomson, 1932) and other host insects in the meadow crops (Harakly, 1968; Dweck *et al.,* 2010).

In this project we have focused on the study of the effects of parasitization by the wasp *B. hebetor* or artificial venom injections in its host, *G. mellonella* on the cellular immune reactions of its host. Total and differential hemocyte counts and modifications in their morphology were observed, suggesting that venom of the wasp has significant impact in modulating host immunity.

## MATERIALS AND METHODS

***Insects*:** The ectophagous larval parasitic wasp *B. hebetor* was reared in the laboratory (Insect Molecular Biology Lab) Department of Entomology, UAF., on the final stage (5th instar) larvae of the host greater wax moth *G. mellonella* by following an approach as described by Manzoor *et al.* (2011). For parasitization purpose the vials (2 cm × 10 cm) containing a host larvae with pair of parasitoids (male & female)/each vial were kept under controlled conditions (27-30±1°C, 65 ± 5% relative humidity (RH) and 18 h light /6 h dark photoperiod). Cotton swabs/pads soaked in 50% honey solution were provided as food source for *B. hebetor* adults whereas the raw wax was replaced occasionally for host larvae feeding i.e., the honey combs containing host larvae were collected from the fruit and ornamental trees located in the main campus of the University of Agriculture, Faisalabad, Pakistan.

***Blood smear preparation*:** Ten unparasitized host larvae acted as control and twenty parasitized larvae i.e., ten after one day of parasitization and ten after two days of parasitization were collected from vials. For smear slide preparation of each larvae, a small drop of hemolymph was taken by cutting the 7$^{th}$ abdominal proleg of the 5$^{th}$ instar host larvae. The drop was spread to form a thin film by using edge of another slide and it was dehydrated in air. A diluted solution (1:10) of "Giemsa stain" was prepared for staining of the slides. The diluted stain was used for staining of the air dried smear for 20 minutes. The slide was rinsed in distilled water and observed under microscope.

***Differential and total hemocyte counts*:** To determine the DHC, nearly 200 cells were selected from different spots of the stained hemolymph of each larva. For the THCs, the control and treated larvae were collected from the vials and for each larvae the protocol was followed as described by Jalali and Salehi (2008). Briefly, Thoma white blood cell pipette was used to suck the hemolymph up to the 0.5 mark and filled up to the 11 mark with Tauber-Yeager fluid to perform hemolymph dilution. The first three drops were released following several minutes shaking of the pipette. The diluted hemolymph was filled into double line hemocytometer with improved Neubaur ruling and four corner squares plus one central square were considered for counting hemocytes. Formula of Jones (1962) was practiced for calculating the number of circulating or floating hemocytes/mm$^3$.

$$\frac{\text{Hemocytes in five 1 mm2} \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{No. of squares counted}}$$

The experiment was replicated thrice both for differential and total hemocyte counts and the data was analyzed by ANOVA and Tukey's test ($P<0.05$).

***Venom extraction and microinjections*:** The extraction of crude venom was performed and briefly described

the whole reproductive tracts of adult females were pulled out by grasping the ovipositor tip with fine forcep, while holding and keeping the abdomen of the female with another forcep in a drop of Pringle's saline solution (1938) placed on a petri dish under microscope. The venom glands with their reservoirs obtained from each female were separated from the ovaries and other unnecessary tissues. Subsequently the venom reservoirs were ruptured in a 20-µl drop of ice-cold Pringle's saline solution and transferred in to 1.5 mL Eppendorf tube, centrifuged at 5000 rpm for 5 min. and supernatant was transferred in to another clean 1.5 mL Eppendorf tube and stored at -20°C.

The bioassay experiment was conducted to check the biological activity of crude venom on 5$^{th}$ instar larval stage of *G. mellonella* by using microinjection technique. Twenty five larvae were placed in each petri dish (total 3 petri dishes, one for control, one for 24 hours treated larvae and one for 48 hours treated larvae) after performing microinjections. Out of 25, 10 larvae were utilized for hemocytic analysis and remaining 15 were used to check the mortality data. 1µL microinjection / each host larvae of 4 concentrations of crude venom (0.1, 0.05, 0.025 and 0.012 wasp equivalent/host) was performed. Firstly, 0.1 wasp equivalent/ host concentration

was prepared by taking 10 wasps venoms in 100µL of Pringle saline solution and were diluted further to make other concentrations. The host larvae injected with saline solution were acted as control. The experiment was replicated thrice and the data was analyzed statistically by ANOVA and Tukey's test ($P<0.05$).

## RESULTS

Hemocytic cell counts and modifications in the morphology of the different hemocyte types were observed under microscope. Types of the hemocytes commonly found were PRs, PLs, GRs, SPs and OEs (Fig. 1).

***Effect of parasitization on hemocyte count*:** THC decreased under the effect of parasitization. Parasitized larvae (after 24 hours) showed declining number of hemocytes (6423.3 ± 12.02) as compared to the control or unparasitized larvae (8516.7 ± 26.03) and same was observed for the parasitized larvae after 48 hours of parasitization which showed more decline in hemocyte count as compared to the parasitized host larvae (after 24 hours) (Table 1). Similar declining trend of hemocytes number were also observed in case of DHCs of cells. For most of the cell types 15 to 20% decline in hemocytes number was observed except prohemocytes (36.2%) in 24 hours parasitized larvae as compared to the control (unparasitized larvae) whereas for 48 hours parasitized larvae the decline was 40 to 50% except granulocytes (38%) as compared to the control larvae.

**Table 1. Changes in THC and DHC of the *G. mellonella* 5th instar larvae under parasitization by *B. hebetor*.**

| Host larvae | THC (No. of cells/mm$^3$ of hemolymph) (Mean±SE) | Percentage of different hemocyte types (Mean ± SE) | | | | |
|---|---|---|---|---|---|---|
| | | PRs | PLs | GRs | SPs | OEs |
| Unparasitized (control) | 8516.7±26.03a | 7.33±0.33a | 31.67±0.88a | 26.33±0.88a | 13.33±0.88a | 5.0±0.58a |
| Parasitized (24 hours) | 6423.3±12.02b | 4.67±0.33b | 25.33±0.88b | 22.67±0.88a | 11.00±0.58a | 4.0±0.33a |
| Parasitized (48 hours) | 5163.7±17.64c | 3.67±0.33b | 17.67±1.20c | 16.33±0.88b | 7.00±0.58b | 3.0±0.58a |

Means followed by different letter(s) within each column (denoted by lower-case letters) are significantly different by Tukey's HSD test at $P<0.05$.

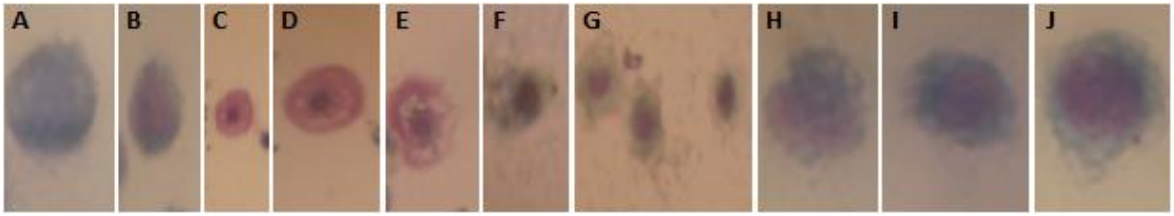


**Figure 1. Different hemocyte types in *Galleria mellonella* (5th instar control larvae) observed under microscope:** Prohemocytes (A, B), Oenocytoids (C, D), Plasmatocytes (E, F, G), Granulocytes (H) and Spherulocytes (I, J). Cells were stained with Geimsa stain, observations were taken by microscope (40X and 100X).

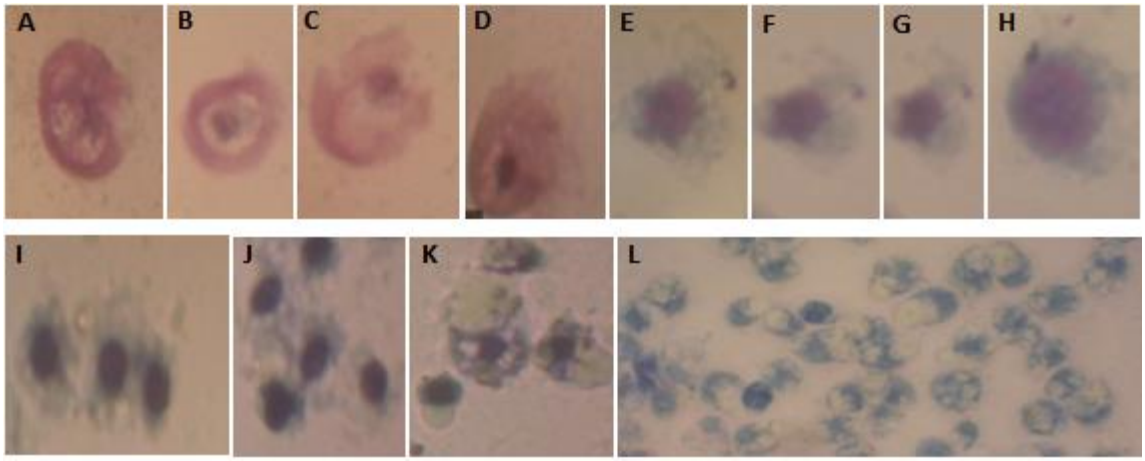


**Figure 2. Morphological alterations in hemocytes of *Galleria mellonella* (5th instar parasitized larvae) observed under microscope:** Vacuoliztion in cytoplasm (A, B, K, L), Cell damage (C, D), Loss of pseudopods and cell boundary (E, F, G, H), Loss of cytoplasm and cell membrane irregularity (I, J, K). Cells were stained with Geimsa stain.

**Table 2. Means for total hemocyte count and mortality percentage of the *G. mellonella* larvae under effect of differentially diluted venom microinjections by *B. hebetor*.**

| Venom concentrations injected (wasp equivalent/host/µL) | Total hemocyte count (24 hours later) (Mean±SE) | Total hemocyte count (48 hours later) (Mean±SE) | Mortality Percentage (Mean±SE) |
|---|---|---|---|
| 0.1 | 6403.0 ± 49.78e | 5144.3 ± 55.64e | 100.00 ± 0.00 a |
| 0.05 | 6845.0 ± 114.05d | 5633.4 ± 69.36d | 86.66 ± 3.83 a |
| 0.025 | 7296.6 ± 89.50c | 6206.7 ± 75.13c | 64.43 ± 4.43 b |
| 0.012 | 8000.0 ± 115.47b | 7153.3 ± 109.7b | 46.66 ± 3.83 c |
| Control (saline injection) | 8510.7± 87.34a | 8511.0 ± 62.69a | 2.23 ± 2.23 d |

Means followed by different letter(s) within each column (denoted by lower-case letters) are significantly different by Tukey's HSD test at P <0.05.

***Effect of parasitization on hemocyte morphology*:** Several morphological changes were observed in hemocytes of parasitized larvae i.e., vacuolization in various types of cells (Fig. 2 A, B, K, L), cell damage (Fig. 2 C, D), loss of pseudopods in PLs and loss of cell boundary in SPs and GRs (Fig. 2 E, F, G, H) are obvious. Some other alterations like loss of cytoplasm and cell membrane irregularity in PLs and other cell types (Fig. 2 I, J, K) were also observed.

***Effect of artificial envenomation on hemocytes count and larval mortality*:** Similarly, the artificial envenomation by venom injections in the host larvae have also significant effects on the hemocytes population. Venom injected larvae showed declining number of total hemocytes (6403.3 ± 49.78) as compared to the control larvae (8510.7 ± 87.34) in case of 24 hours parasitized larvae and similar declining trend was also observed in case of 48 hours parasitized larvae (Table 2). Moreover, the hemocyte population was increased gradually with decreasing venom concentration i.e., 0.012 wasp equivalent/host it was 8000 ± 115.47 as compared to the 6845 ± 114.05 at 0.05 wasp equivalent/host (Table 2). Similar increasing trend was also observed in case of 48 hours parasitized larvae but the number of total hemocytes were less as compared to the 24 hours parasitized larvae (Table 2). Mortality data shows that by decreasing the concentration of crude venom the mortality percentage was decreased gradually i.e., 100% mortality was observed with 0.1 wasp equivalent/host and it was decreased to 86.66%, 64.43% and 46.66% at 0.05, 0.025 and 0.012 wasp equivalent/host concentrations, respectively.

## DISCUSSION

Previously, different studies have been conducted regarding the effect of parasitization by different parasitic wasps on the immunity of their host insects. There are a lot of evidences relating the impact of ectoparasitoid, *Eulophus pennicornis* Nees on immunity of the host insect (Richards and Edwards, 1999, 2000a, 2000b). Though, there is less information available regarding the state of the insect's immune system during parasitization by the ectoparasitic wasp, *B. hebetor*. Baker and Fabrick (2000) have observed negligible changes in the formation of hemolymph proteins in case of parasitized or paralyzed hosts previously. Studies relevant to the cellular and humoral immune response of host insects during parasitization by *B. hebetor* have also been scarce (Hartzer *et al.,* 2005).

Kryukova *et al.* (2011) has investigated the immune responses of the *G. mellonella* larvae naturally envenomated by *B. hebetor*. A significant decline in PO activity of parasitized wax moth and the number of hemocytes in the hemolymph were detected. Similarly, in 2015 they have observed that *B. hebetor* venom triggers calcium-dependent degradation of *G. mellonella* larval hemocytes. The decline in hemocyte viability was noticed 1, 2 and 24 h following envenomation. These findings conclude that the natural envenomation by *H. hebetor* suppress immune responses in *G. mellonella* larvae.

We have observed different types of hemocytes under light microscopy and classified them into different categories like prohemocytes, oenocytoids, plasmatocytes, granulocytes and spherulocytes. Our results are in accordance with the study undertaken by Jalali and Salehi (2008) to identify the types of hemocytes of *Papilio demoleus* (Lepidoptera: Papilionidae) by using electron microscopy. They have organized the *P. demoleus* hemocytes in six classes on the base of distinguishing morphological and cytological features exposed by light, phase and scanning electron microscopies called as the PRs, GRs, PLs, ADs, SPs, OEs and two extra subtypes, the POs (Podocytes) and VEs (Vermicytes).

Our findings reveal that following parasitization/envenomation hemocytes count decreased after one day and two days intervals. These results are in contradiction by Dubovskiy *et al.* (2013) in which they have evaluated several features of immune response in insects treated with pirimiphosmethyl. Both cellular (hemocyte count and encapsulation rate) and humoral (phenoloxidase activity) immunity changes were observed in larvae of the insects belonging to two insect orders, the wax moth *G. mellonella* and the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera, Chrysomelidae).

Moreover, the work done by Ignoffo *et al.* (1983) somehow support our results in which following insertion of blastopores of Nomuraea rillevi inside Trichoplusia sp. McDunnough,

1944 they have observed about 6–7 times decline in THC i.e., number of average hemocytes decreased from 8990/µl (2 days after inoculation) to 1350/µl (6 days after inoculation). Similarly our results are supported by the study conducted by Gupta and Sutherland (1968) which reported that after treatment with chlordane, the decline in the number of PLs, SPs and GRs was observed in *Periplanata americana* Linnaeus (Blattidae: Orthoptera).

Our data also show certain morphological changes in addition to total and differential hemocyte count and further, complemented by the study of Sezer and Ozalp (2015) in which effects of juvenile hormone analogue pyriproxyfen on THC and hemocyte morphology of *G. mellonella* pupae were investigated. A major decline in total hemocyte count was recorded when pupae were treated with 1, 5 and 10 µg/ml of pyriproxyfen. Different levels of malformations were also detected nearly in all hemocyte types.

Artificial envenomation results indicated that *B. hebetor* crude venom injections has 100% mortality in 0.1 wasp equivalent/host or above concentrations, the mortality percentage was reduced in more diluted form of the wasp venom i.e., 0.05 wasp equivalent/host or lower concentrations as shown in Table 2 which indicates that it has a strong insecticidal potential. Our findings are in line with Beard (1978); Piek and Spanjer (1986); Moretti and Calvitti (2014), in which concluded that venoms from the ectoparasitic wasps have been studied so far, are involved in paralysis and either instantly kill the host or entirely suppress the host growth.

***Conclusion*:** Venom of the ecto-parasitic wasp *B. hebetor* has a strong potential to interfere with the host immunity and a rich source of immunosuppressive or insecticidal potential which may be exploited through an integrated approach of insect genomics and proteomics for in depth analysis of the venom of the wasp for the development of innovative and sustainable insect control tools which are safer to nontarget organisms and environment. Further, our work has generated a useful scientific information for practical application for insect control in the field of Agriculture.

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