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Haemolymph response in silkworm *Bombyx mori*, during infection with Grasserie

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ABSTRACT
Haemolymph is a multifunctional circulatory fluid in the body of silkworms, consisting of liquid plasma and many types of nucleated haemocytes, which are classified into Prohaemocytes (PR), plasmatocytes (PL), granulocytes (GR), spherule cell (SP), and Oenocytes (OE). During infection, pathogens utilize haemolymph as a medium for maintenance and proliferations, affecting not only total haemocyte count (THC), and differential count of haemocyte, but also the haemolymph volume. The present paper deals with these responses of haemolymph in fifth instar silkworm *Bombyx mori* larvae during infection with Grasserie disease. For the present study haemolymph was collected on day two and day five of infected 5th instar larvae, with a cut through one of the prolegs and using prescribed methods, proceeded for hematological examination. In the results we reported, significant decrease in total haemocyte count in the infected worms when compared to healthy worms. With progress of Grasserie, we observed a decrease in differential count of Prohaemocytes (PR) and Oenocytes (OE), while there was an increase in the differential count of plasmatocytes (PL), granulocytes (GR), and spherule cell (SP). Grasserie infection also found to influence the total haemolymph volume and causes reduction in haemolymph volume of infected silkworms over the control. The results obtained are discussed in the light of patho-physiology of larval silkworms during infection.
Key words: *Silkworm, infection, haemocytes, Haemolymph, Grasserie*

Introduction:

The silkworm, Bombyx *mori* is a purely domesticated insect since 4,500 years but like other domesticated animals it is a quite delicate venture and might be easily susceptible to a number of diseases, most of which develops seasonally (Govindan and Devaiah, 1998 and Prasad, 1999). Grasserie is one of the most serious diseases of silkworms, though occurs throughout the year, its intensity varied with seasons. It is also known as the 'hanging disease'. Caused by *Borrelina Bombycis* virus, of the family Baculoviridae causes this disease. The seasonal infection of Grasserie causes patho-physiological changes, at both early and late stages of disease attack. In this infection the virus multiplies and forms polyhedra in the nucleus of infected cells. Haemolymph is a circulatory fluid in the body of insects and performs many diverse functions (Mullins, 1985), and consists of liquid plasma and many types of nucleated haemocytes, which are classified into granular haemocytes, spherule cells, oenocytoids, prohaemocytes and plasmocytes (Arnold and Hinks, 1976). Being the major circulating body fluid haemolymph fills the body cavity or haemocoel (Chapman, 1969) bathing the tissues directly. Investigations on haemolymph give idea about pathophysiological changes associated with the different processes involved in resistance to disease. Hemocytes are the major constituents in haemolymph, move and perform various physiological functions in the body of insect including defense against the pathogen and toxins in the body. Their variety and population index is very important. The most abundant haemocyte types typically described in Lepidopteron larvae are granular cells and plasmatocytes, which are capable of adhesion and phagocytosis of pathogenic agents (Levine and Strand, 2002). Monoclonal antibodies are very useful reagents for distinguishing lepidopteron haemocyte populations based on antigenicity rather than morphology, (Willott *et al.*,1994) which can vary considerably, especially for plasmatocytes. On incidence of infection the haemolymph as well as the tissues gets affected and shows alterations (Watanabe, 1971). The investigation of changes in haemolymph is an appropriate system for studying effects of infectious disease. Hence we carried out the present study to understand the specific responses of haemolymph during attack of Grasserie disease.

Material and Methods:

Collection:

With due consent of the owners of the local sericulture units, healthy and infected silkworms with Grasserie were collected on the basis of gross pathology in their early infection (first 2nd day of fifth instar) and late infection (6th day of fifth instar) states. Fresh haemolymph was collected from all the diseased larvae and from the healthy non infected larvae at same developmental stages, following the methods of Jalal and Rasoul (2010).

Methodology:

Total haemocyte count (THC): Haemolymph was drawn into a the pipette up to 0.5 mark and diluted up to the 11 mark with Toisson's solution (NaCl- 1.0gm, Na2SO4 - 8.0gm, Neutral glycerin - 20ml, Methyl violet - 0.025 gm, Distilled water - 160ml).

Neubauer ruling of Haemocytometer was flooded with diluted haemolymph and the hemocytes counted in its four corner and one central squares under a microscope.

The number of haemocytes per cubic millimeter (mm3) was calculated using the following formula of Jones (1967).

$$\frac{\text{Total haemocyte counted X Dilution X Depth factor of Chamber}}{\text{Number of Square counted}}$$

Where,
Dilution =20 times, Depth factor of the chamber =10 (constant), No. of squares counted = 5.

Differential count of haemocyte, (DHC): Differential hemocyte counts (DHC) were realized on haemolymph air dried smear slides stained with Giemsa-Rosenfeld. The slide was rinsed in distilled water and mounted in DPX. To determine the DHC, cell categories were counted in 200 cells chosen from random areas of the stained haemolymph smear.

Total haemolymph volume: It was measured according to the method described by Liji (2008). By this method the volume of haemolymph was determine directly by filing the fine calibrated capillary tube in which oozed out haemolymph was immediately drawn.

Results & Discussion:

Table 1, depicted that Infection with Grasserie caused reduction of haemolymph volume over the control during early and late Grasserie infection. During early infection the volume was 0.31µl, as compare in control 0.36 µl. At late infection more reduction in haemolymph volume was observed as compared to controls, which were 0.55 µl and 0.68 µl respectively. The reported reduction in haemolymph volume, according to, Roan and Hopkins 1961, Mehrotra and Sethi (1966) and Samaranayaka (1977) was due to any stress. In response to stress insects lose water which affect the haemolymph volume. Such reduction in haemolymph volume during stress, as said earlier could be due to loss of water, either through excretion or through spiracles. Samaranayaka (1977) documented that it is due changed in distribution of water in haemocoel, tissues and lumen of gut. He reported that the moisture content of the body tissues remained high at a time when haemolymph volume was low.

The observation on Quantitative analysis of THC of silkworms infected with Grasserie is mentioned in the Table 2. As depicted in the table the infection with Grasserie envisaged significant change in total haemocyte count on the second day of infection with in the 5th instar silkworm larvae. Chiang, (1988) and Abd- El-Aziz and Awad (2010) too reported similar results on account of infection with pathogenic infections in insects. we reported reduction in THC, as on second day with Grasserie, which was (335.0 THCx103/mm^3) as compared to control healthy non infected worm of the same developmental stage, which had THC (389.8 THCx103/mm3) With the progress of the infection, the reduction in THC was more prominent as on day 5 it had THC (288.0THCx103/mm3) as compared to control healthy non infected worm (465.0 THCx103/mm3) of the same developmental stage. Ericsson *et al.,* (2009) also reported significant reduction in the and are in accordance with haemocyte count in Trichoplusiani after bacterial infection of E. coli. Abir *et al.,* (2013) too reported reduction in the silkworm, Bombyx mori larvae, THC due to infection with (G+) *Bacillus thuringeinsis* on after 48 hrs post-infection. Study by Morton *et al.,*(1987) and Rivers *et al.,* (2002), suggested that this decrease is associated with the nodule formation and encapsulation around the invading pathogen, as well as the degranulation of some cell types.

In accordance with Wittig, (1968) and Gillepsie, (1997) our results, distinguished the five well-defined haemocyte types found in the haemolymph of healthy and infected silkwrom larvae (micrograph 1): Prohaemocytes, plasmatocytes, granulocytes, oenocytoids and spherulocytes. They suggested that, haemocytes, altered during occurrence of disease as changes in the population of different haemocytes during the incidence of Grasserie is reported in the present study. Ayesha and Khowaja (2009) said that information of normal haemocytes of an insect is necessary to physiologists, toxicologists and biochemists, as alterations in structure, types and number of cells reflects changes in physiological and biomolecular processes. Insect haemocytes respond to internal changes during development (at ecdysis) and to conditions such as starvation, wounding, parasitism, diseases, chemicals including insecticides. That is why, (table, 3) the count of Prohaemocytes (PR) remain unchanged in response to Grasserie infection at early infection was 24.29 DHCx103/mm^3 as compared to healthy control 24.5 DHCx10^3/mm^3 but with progress of infection, at later stage, it becomes (18.66 DHCx10^3/mm^3) as compared to control 32DHCx10^3/mm^3. This reduction is more significant at the late infection.

The number of Plasmatocytes (PL) increased in response to Grasserie pathogens at early stage, 37.42 DHCx10^3/mm^3), in comparison to control 14.25 DHCx10^3/mm^{3}and at the late infection increased to 29.84 DHCx10^3/mm^3 as compare to the control 20.23 DHCx10^3/mm^3.

In Granular cells (GR), no significant difference was found between healthy control, however the count at late infection with the Grasserie reported to be 41 DHCx10^3/mm^3 as compared to control 31 DHCx10^3/mm^3. The count of the Spherule cells (SP), increase on day two after infection with Grasserie but on day five significant increase recorded as 35 DHCx10^3/mm^3 as compared to control healthy which was 22 DHCx10^3/mm^3. No significant change in Oenocytoids (OE): was observed in the beginning of infections, But with the progress of the disease on day 5, a decrease in OE count 4.17 DHCx10^3/mm^3, of was reported, as compared to control 9.35 DHCx10^3/mm^3. Variation reported in present study in haemocyte during Grasserie infection also have been extensively studied as part of research on insect pathogenecity by Wittig, (1968) and Gillepsie, (1997). Balavenkatasubbaiah and Nataraju (2005) and Krenhap *et al.,* (2005) reported period of survival of haemocytes with regard to granulocytes, plasmatocyte and spherulocytes under normal condition and during infective BmNPV infection that causes Grasserie.

In the present study Prohaemocytes (PR) and oenocytoides (OE) decreased in number whereas the number of plasmotocytes (PL), granulocytes (GR), and spherule cell (SP) increased as the experimental diseases progresses. The overall values obtained for the DHC of the 5 cellular types in Grasserie infected silkworm was (PR> GR> SP> PL> OE). Arnold (1979) and (1982) suggested that alteration in variety of haemocytes indicated the specific physiology in insect which might be involved to defend the invading pathogens causing disease like Grasserie.

Finally it can be concluded that the results obtained, here are indicative of the pathogenic attack on the defense mechanisms of the silkworm against the infecting agent of Grasserie, which may help in the understanding of the role of the haemolymph during infection in Silkworm *Bombyx mori* at small sericulture units in the study area.

References:

Abd El-Aziz NM and H. Awad (2010). Changes in the haemocytes of *Agrotis ipsilon larvae* (Lepidoptera: Noctuidae) in relation to dimilin and *Bacillus thuringiensis* infections. *Micron*, **41** : 203–209.

Abir A. Gad and Ahmed A. Abdel-Megeed. (2013). Physiological and mutagenic effects of pathogenic and non-pathogenic bacteria on the last larval instar of *Bombyx mori* (Lepidoptera: Bombycidae) *African Journal of Microbiology Research*., **7**(48) : 5433-5439.

Arnold J. W. and Hinks, C. F (1976). "Haemopoiesis in Lepidoptera I. The multiplication of calculating haemocytes". *Cannadian Journal of Zoology*, **54** : 1003 - 1012.

Ayesha Qamar and Khowaja Jamal (2009). Differential haemocyte counts of 5th instar nymphs and adults of Dysdercus cingulatus Fabr (Hemiptera: Pyrrhocoridae) treated with acephate, an organophosphorus insecticide. *Biology and Medicine,***1** (2) : 116-121.

Arnold J.W. (1979). Controversies about haemocyte types in insects, Insect hemocytes, A.Gupta (Ed)., Cambridge University Press, Cambridge, pp.231-258.

Arnold J. W. (1982). Larval haemocytes in *Noctuidae* (Insecta:Lepidoptera). *Int. J. Insect. Morph. Embrol.,* **11**: 173-188.

Balavenkatasubbaiah M. and Nataraju B. (2005). Haemocyte changes during the progressive infection of *Beauveria bassiana* in different breeds of silkworm (*Bombyx mori* L.). *Madras Agric. J.,* **92** (7-9) : 431 - 437.

Chapman R. F. (1969). The Insects Structure and Function, 659-691. New York: American Elsevier.

Chiang A. S., Gupta A. P. and Han S. S. (1988). Arthropod immune system, 1 Comparative light and electron microscopic account of immunocytes and other haemocytes of *Blattella germanica* (Dictyopters: Blattellidae). *J. Morphol.,***198** : 257.

Govindan R, Narayanaswamy T. K. and Devaiah M. C. (1998, 2001, 2003, 2006). "Text book of Principles of Silkworm Pathology" Seri Scientific Publishers, Bangalore. pp 239.

Gillespie J. P., Kanost M. R, and Trenczek T. (1997). Biological mediators of insect immunity. *Annu Rev Entomol*, **42** : 611-643.

Jalal Jalali Sendi and Rasoul Salehi (2010). The Effect Of Methoprene On Total hemocyte counts And Histopathology Of Hemocytes In *papilio Demoleus L.* (Lepidoptera) *Mun. Ent. Zool.,*. **5**(1).

Krenhap W., Balasingh J., Thiagarajan V. and Kumar V. (2005). Studies on the influence of feeding frequency on the total and differential haemocyte count in *Bombyx mori* Linn. *Indian. J. Seric.,* **44**(1) : 113-117.

Lavine M. D., Strand M. R. (2002). Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol.*, **32** : 1295 - 1309.

Liji, C. (2008). Effects of Antioxidants On *Bombyx Mori* Ageing. Thesis submitted to University Of Calicut, Kerala, India.

Mehrotra K. N. and Sethi G. R. (1966) Water loss in *Schistocerca gregaria* (*Forskal)* treated with mala-thion, *Indian J. Km.*, **28** : 114-116.

Morton D. B., Dunphy G. B. and Chadwich J. S. (1987). Reactions of hemocytes of immune and nonimmune *Galleria mellonela* larvae to *Proteus mirabillis.Dev. Comp. Immunol.*, **11** : 47-55.

Mullins D. E. (1985). "Chemistry and physiology of the haemolymph". In Comp.Insect. Physiol.Biochem. Pharm. Vol. 3.(Ed.,by Kerkut GA, Gilbert LF) Pergamon Press, Newyork, pp 355400.

Prasad, N. R. (1999). Silkworm disease management and its limitations. *Indian Silk*, **39**(4): 7 – 9.

Rivers D. B., Ruggiero L. and Hayes M. (2002). The ectoparatisitic wasp *Nosonia vitripennis (Walker)* (Hymenoptera: Pteromalydae) differentially affects cells mediating the immune response of its flesh fly host, *Sacophaga bullata Parker* (Diptera :Sarcophagidae). *J. Insect Physiol*, **48** : 1053-1064.

Roan C .C and Hopkins T. L (1961**).** Mode of action of insecticide., *Rev. Ent.*, **6** : 333 – 345.

Samaranayaka M. (1977).The efi'ect of insecticide on osmotic and ionic balance in the locust S. gregariak. *Pesticide Biochem. Physiol.*, **7** : 510 – 516

Watanabe H. (1971). DISC electrophoretic patterns of hemolymph protems of larvae of the armyworm, *Pseudaletia unipuncta,* Infected with a nuclearpolyhedrosiss and granulosis virus. *Journal of Invertebrate Pathology*, **17** : 127 – 129.

Willott E., Trenczek T., Thrower L. W. (1994). Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte

types in Manduca sexta. *Eur. J. Cell. Biol.*,**65** : 417 - 23.

Witting G. (1962). The pathology of insect blood cells: a review. *Amer. Zool.,* **2 :** 257.

Witting G. (1968). Phagocytosis by blood cells in healthy and diseased caterpillars, III Some observations concerning virus inclusion bodies. *J. Invertebr. Patho.,* **10** : 211.

Yeager J. F. (1945). The blood picture of the southern armyworm (*Prodenia eridania*). *J. Agric. Res.,***71** : 1 – 4.

Micrograph-I: Normal Haemocytes in silkworm

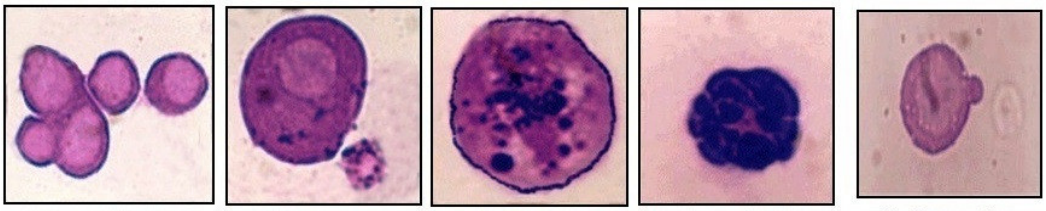

Table1: Changes in the volume of haemolymph of Silkworm during seasonal diseases.

Duration of infection	**Control Larva**	**Grasserie Infected**
2nd day(Early)	**0.36 ± 0.03**	0.31
5th day(Late)	0.68 ± 0.05	0.55 ± 0.05*

*Values are the means of 5 determinations; *Values are significant at P<0.05 against the normal for n=5,*

Table 2: Total Haemocyte Count in Haemolymph of Silkworm during Grasserie diseases.

Duration of infection	**Control Larva**	**Grasserie Infected**
Early infection (2nd day of 5th instar)	389.8 ±18.44	335.0 ±18.07
Late infection (5th day of 5th instar	465.0 ± 16.26	288.0 ±21.16*

Table3: Differential Haemocyte Count in Haemolymph of Silkworm during seasonal diseases.

	Healthy Control		**Grasserie Infection**	
Haemocytes ↓	**Early**	**Late**	**Early**	**Late**
Prohaemocytes	24.5 ±1.29	32 ±1.39	24.29 ±1.50	18.66 ±2.70
Plasmatocytes	14.25 ±1.71	20.23 ±1.41	37.42±3.6	29.84±1.61
Granulocytes	26 ±1.83	31 ±1.88	27 ±1.33	41 ±1.53
Spherulocytes	19 ±0.82	22 ±0.62	19 ±1.23	35 ±1.79
Oenocytes	4.75 ±3.22	9.35 ±2.92	5.67 ±0.75	4.17 ±1.92

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