Effect of a Microsporidia, *Nosema* on total hemocyte count of 5th instar *Samia ricini* larva

Alika Borphukan* & Dr R. K. Bhola**
*Assistant Professor, University of Science & Technology, Meghalaya Technocity, Killing Road Baridua, Meghalaya-793101
**Professor, Gauhati University Department of Zoology, Gopinath Bardoloi Nagar Guwahati-781014, Assam, India.

Received: May 18, 2018 Accepted: July 04, 2018

ABSTRACT *Microsporidia Nosema is the most common causative agent of Pebrine in silkworm. Eri silkworm, Samia ricini reported to be strong and disease resistant compared to other silkworms is also susceptible to pebrine. Fight against the disease has been going on since a long time but still pebrine is not eradicated. Frequent occurrences of pebrine in S. ricini indicate possibilities of cross infection. Purified Nosema spores from muga silkworm, Antheraea assama smeared on castor leaves inoculated to Samia ricini were found to be pathogenic. Presence of pathogen induced significant changes in total hemocyte count. Number of hemocyte cells/mm³ also progressively increased with larval age during three different seasons in normal as well as infected groups. However, at the corresponding age the hemocyte count was significantly higher in infected group. Increase total hemocyte count may involve the immunological challenge in response to pathogen.*

Keywords: *Microsporidia, pebrine, cross infection, pathogenic, hemocyte*

Introduction

Silkworm larva generally suffers from various diseases causing heavy loss to silk industry. The most destructive parasitic disease is the Pebrine caused by *Nosema*. Changes in total hemocyte counts of a particular insect directly or indirectly affect a number of physiological functions in insect. Hemocytes perform phagocytosis, encapsulation of foreign bodies in the insect body cavity; wound healing, coagulation to prevent blood loss, nodule formation, transport of food material and storage, hormones and detoxification of metabolites and biological active materials. Pathogen in body can alter physiology which can also affect total hemocyte count. So, total hemocyte count was carried on to get a better idea about the physiological change in the organism due to presence *Nosema*.

Any change in the total hemocyte counts of particular insect directly or indirectly affect the insect negatively which can be used as an indicator for change (Pandey and Tiwari, 2012). According to Wigglesworth (1972) the number of circulating cells varies enormously from insect to insect and time to time. Highest number was observed after injury, hemorrhage, parasitic attack and during ecdysis. Increase in the number of hemocytes was recorded in *Antheraea mylitta* infected with *Nosema* (Mudhusudhan *et al*., 2011). To date, reports on parasitic infection in *Samia ricini* are scanty. So far, no systematic investigation has been made on *Samia ricini* infected with *Nosema*. So, present study was carried out to test the change of total hemocyte count caused by presence of a pathogen, *Nosema* collected from *Antheraea assama* (Muga silkworm)

Materials and Methods:

Disinfected eggs of eri silkworm, *Samia ricini* were collected from Central Silk Production centre, Azara. Rearing was done according to sericulture manual, Directorate of Sericulture, Government of Assam. *Nosema* spores were collected from infected larvae of *Antheraea assama*, from the office of the Joint Director, Sericulture, Khanapara. The infected larvae were crushed in the glass mortar with pestle. The crushed suspension obtained from larvae was filtered through muslin cloth and stored in freezer. Spores were purified by 60% sucrose density gradient method.

Inoculation of larva with *Nosema* spores:

Newly hatched healthy V instar larvae were randomly collected from stock room, starved for 6 h and divided into 4 batches. 3 µl spore suspension containing $8x10^5$ spores/ml distilled water was smeared on each castor leaf. Leaves were dried in the air and used to inoculate larvae of three batches. Experimental batches were reared in a separate room. Control batch was provided with leaves smeared with distilled water. The inoculation was done for three different seasons summer, autumn and winter and three consecutive years from 2012 to 2014. For total hemocyte count average was taken for three years.

Hemolymph collection and counting:

The larvae were kept in a jar with ethyl ether mixed cotton for 2 minutes for anesthesia. Then larvae were immersed in hot water at 55-60^{0}C for 2-3 minutes for fixing hemocytes (Rosenberger and Jones, 1960). Insects were removed after heat fixation and thoroughly dried on a filter paper. Hemolymph was obtained by puncturing the abdominal legs with a needle. The hemolymph was collected in a prechilled ependorf tube containing few crystal of thiourea. Thiourea was used to avoid the prophenol oxidase activity followed by melanization of the hemolymph samples. Heat-fixed hemolymph was drawn into a Thoma white blood cell pipette up to the 11 mark with Tauber-Yeager fluid (Tauber and Yeager, 1935). The pipette was shaked for several minutes and first three drops were discarded. The double line with improved Neubauer ruling Hemocytometer was filled with diluted hemolymph and hemocytes were counted in its four corners and one central (1mm^2) square under a microscope. If distribution of cells in the squares were not even, the sample was discarded. The number of circulating haemocytes per cubic millimetre (mm^3) was calculated using the following formula (Jones, 1962)

$$\frac{\text{Hemocytes in five } 1\text{mm}^2 \textbf{ x } \text{Dilution } \textbf{x } \text{Depth factor of chamber}}{\text{No of squares counted}}$$

Depth factor of the chamber=10 (Constant) and No. Of squares counted=5

Everyday 5 larvae were collected into eppendorf tubes from each replication and stored at 4^o C. Results were expressed in total Hemocyte count/mm^3±SD (n=5)

Results:

Total hemocyte count was observed in the control and infected 5th instar larva from day 1-6 during 3 seasons: summer, autumn and winter. In the summer total hemocyte count was found to be 17190 and 21850 on day 1 in control and infected group respectively (Table 1). The total haemocyte count was found to be significantly higher in the infected group compared to control from day 1-6. In control group total hemocyte count increased progressively and highest count was observed on day 6. In the infected group also, the total hemocyte count increased from day 1-6. However, in the infected group total hemocyte count was found to be significantly higher from day1-6 compared to control group.

Table 1. Total hemocyte count in 5th instar larvae of *Samia ricini* infected with *Nosema* during Summer

Days after inoculation	Total hemocyte count (cells/mm^3) ± SD (n=3)		
	Control	Infected	P-value
1	17190±1508	21850±2911*	< 0.001
2	18015±383	23034±1440*	< 0.001
3	20528±316	25478±2606*	< 0.001
4	22091±951	24898±986*	< 0.001
5	21935±1042	25453±1452*	< 0.001
6	23599±1701	26889±1012*	< 0.001

NS- not significant * significantly different from control

In the autumn also the hemocyte were counted in both control and infected groups (Table 2). In the control group, total hemocyte count increased progressively from 21665 to 31332 from day 1-6. Similarly, in the infected group also total hemocyte count increased from 29634 to 36581 from day1-6. It was observed that total hemocyte count in the infected larvae was significantly higher compared to control group from day 1-6.

Table 2. Total hemocyte count in 5th instar larvae of *Samia ricini* infected with *Nosema* in Autumn

Days after inoculation	Total hemocyte count (cells/mm^3) ± SD (n=3)		
	Control	Infected	P-value
1	21665±4244	29634±5719*	< 0.001
2	22749±4680	29806±1477*	< 0.001
3	23969±2720	31414±2080*	< 0.001
4	24848±5994	31203±2223*	< 0.001
5	25008±5028	34893±3659*	< 0.001
6	31322±2069	36581±1528*	< 0.001

NS- not significant *significantly different from control

In the winter, total hemocyte count was found to be 30180 which increased continuously to 38645 on day 6 (Table 3). Similarly in the infected larvae also, the total hemocyte count increased progressively from 38826 on day1 to 45726 on day 6. Total hemocyte count was recorded significantly higher in infected group from day1-6 compared to corresponding control.

Table 3. Total hemocyte count in 5th instar larvae of *Samia ricini* infected with *Nosema* in Winter

Days after inoculation	Total hemocyte count (cells/mm^3) ± SD (n=3)		
	Control	Infected	P-value
1	30180±4180	38826±4100 *	< 0.001
2	32954±2214	39850±4415 *	< 0.001
3	35535±5018	43100±2752 *	< 0.001
4	36416±5974	44953±2592 *	< 0.001
5	37155±4428	43827±3563 *	<0.001
6	38645±4748	45726±3437 *	<0.001

NS- not significant *significantly different from control

The Total hemocyte count was recorded lowest in the summer season both in control (17190-23599) and infected group (21850-26889). In the autumn higher number hemocytes were observed both in control (21665-31322) and infected group (29636-36581). However, highest number of hemocytes was recorded in winter both in control (30180-38645) and infected group (38826-45726)

Discussion:

In the present study it was observed that total Hemocyte count (THC) progressively increased from day 1-6 in normal 5th instar larvae. According to Chapman (1982) density of hemocytes in insects generally depends upon blood volume of the insect. Essawy and Saad (2013) found a gradual increase of blood volume starting from the first day which attains its peak on 9th day (the end of feeding period) in *Bombyx mori* (L.) Increase in total hemocyte count was also reported with age in three ecoraces of Tasar silkworm, *Antheraea mylitta* (Singh *et al.* 2008). Similar observations were reported by Kumar and Singh (2015) in a study on cytoplasmic polyhedrosis in *Antheraea mylitta*. Nayaka and Sharma (2014) also found that as the larval age progressed the total hemocyte count increased. Increase in Total hemocyte count in 5th instar larva from 1st day onward has been correlated to increased feeding efficiency (Paul *et al*., 1992). Similar results were also reported by Ling *et al*. (2005) in different life stages of Mediterranean flour moth, *Ephestia kukniella*. Total hemocyte count increases gradually in *Antheraea assama* with developing instars (Bardoloi and Hazarika, 1995). Same was found true in *Rynocoris marginatus* (Ambrose *et al*., 1999). In *Blatta* infected with bacteria the mitotic division of blood cell increased from 0.5% to3.1% and was correlated to increase in number of hemocytes (Tauber *et al*., 1940). Richards and Edwards (1999) and Russo *et al.* (2001) also reported that the presence of pathogens in the hemocoel stimulated the hemopoiesis, increasing the number of hemocytes. Same was found by Andrade *et al*. (2010) in *Anticarsia gemmatalis*, Noctuidae larvae infected by AgMNPV, a multi capsid nucleopolyhedro virus. In the present study it was observed that total hemocyte count varied in different seasons. Kumar *et al*. (2011) reported that number of circulating hemocyte may be temperature dependent and responsible for susceptibility of silkworm races. The total and differential hemocyte count may indicate the susceptibility status of the insect (Nisar *et al*., 2015). During winter highest number of total hemocyte count were observed compared to summer and autumn. Total hemocyte count may also indicate susceptibility status of silkworm *Samia ricini* as mentioned by Singh *et al*. (2008). Similar observations have been made in silkworm *Bombyx mori* against antifungal infection (Kawakami, 1965), *Periplanata americana* (Ennesser and Nappis, 1984) and arthropods (Gupta and Han, 1988). According to Rosenberger and Jones (1960), the presence of pathogens in the hemocoel of the insects can activate their defence system causing alteration in the total number of hemocytes. However, there is no consensus in associating the defence response to decrease or increase in the total number of hemocytes. It has been reported that the presence of pathogen would cause a decrease in the number of circulating hemocytes so as to make the infection successful (Ratcliffe *et al.,* 1985, Morton *et al*., 1987, Rivers *et al*., 2002). This decrease was related to the nodule formation and encapsulation around the invaders as well as the degranulation of some cell types. Velide and Bhagavanulu (2012) also found that reduced numbers of hemocytes are tolerant to pathogen when they worked on BmNPV infection on *A. mylitta*. Singh *et al*. (2008) observed that in tropical Tasar silkworm the total hemocyte count was higher in tolerant eco races compared to susceptible eco race when infected with AmCPV. Anandakumar and Michael (2011) also found that total hemocyte count decreased significantly in *Bombyx mori* when treated with *Bacillus thurungeneses.*

They concluded that a decrease of 15.3% of hemocyte count was due to depletion of prohemocytes. An opposite trend was found in silkworm, *Bombyx mori* against fungal infection (Kawakami, 1965), *Periplanata americana* (Ennesser and Nappi, 1984) and arthropods (Gupta and Han, 1988). Hemocyte count in *Apis mellifera* and *Apis cerana indica* were significantly higher than *Nosema* infected bees (Abrol, 1996). Balavenkatusubbaiah *et al*. (2001) reported changes of total hemocyte count during progressive infection in different breed of *Bombyx mori* L.

References

- Abrol, D. P. (1996): Effect of mite parasitosis on blood counts of honey bees Apis mellifera L. and Apis cerana indica F. (Hymenoptera: Apidae). Insect Environment. 2(1) : 18-19
- Ambrose, D. P. (1999): Assassin Bugs. Science Publishers, New Hampshire, USA and Oxford & IBH Publishing, New Delhi, India, 337 pp
- Anandakumar, M. D., Michael, A. S. (2011): Haematology and haemochemistry of silkworm, Bombyx mori L. Infected with Bacillus thurungeneses. Int. J. Environ. Sc., 2 (2): 451-457.
- Andrade, F. G., Negreiro, M. C. C., Levy, S. M., Fonseca, B. I. C., Moscardi, F. and Falleiros, A. M. F (2010): Hemocyte quantitative changes in Anticarsia gemmatalis (Lepidoptera: Noctuidae) larvae infected by AgMNPV. Braz Arch. Biol. Technol. 53b (2): 279-284.
- Balavenkatasubbaiah, M., Nataraju, B., Selvakumar, T., Thiagarajan, V. and Datta, R. K. (2001): Haemocyte counts in different breeds of silkworm, Bombyx mori L. and their changes during progressive infection of BmNPV. Ind. J .Seric., 40 (2) : 158-162
- Bardoloi, S., Hazarika, L. K. (1995): Seasonal variation of body weight, lipid reserves, blood volumes and haemocyte population of Antheraea assama. Environ. Entomol, 21: 1398-1409.
- Chapman (1982): The hemolymph. In The Insect Structure and Function. E.L.B.S. Hodder & Stoughton. 92-94.
- Ennesser, A. C. and Nappi, A. J. (1984): Ultrastructural study of the encapsulation response of the American cockroach Periplanata americana. J. Ultrastruct. Res., 87: 31-45.
- Essawy, M. M. and Saad, I. A. I. (2013): Effect of potassium, sodium and its mixture supplementation on –blood volume, total and absolute hemocyte count in mulberry silkworm Bombyx mori(L.) J. Entomol. and Zoo. Stud., 1 (6) : 92-96
- Gupta, A. P. and Han, S. S. (1988) : Arthropod immune system III septate junction in the haemocyte capsule of the German cockroach, Blettella germanica. Tissue & Cell, 20 (4) : 629-634.
- Jones, J. C. (1962): Current concepts concerning insect hemocyte. Am. J. Zool. 2 : 209-246
- Kawakami, K. (1965): Phagocytosis in muscardine diseased larvae of the silkworm, Bombyx mori (Linnaeus). J.Invertebr. Pathol. 7: 203-208.
- Kumar, D., Pandey, J. P., Jain, J., Mishra, P. K., and Prasad, B. C. (2011): Qualitative and quantitative changes in protein profile of various tissues of tropical tasar silkworm Antheraea mylitta Drury. Int. J. Zool. Res., 7: 147-155.
- Kumar, K. K. P., and Singh, G. P. (2015): Hemocyte and biochemical changes of Antheraea mylitta D. infected with Antheraea mylitta Cytoplasmic Polyhedrosis Virus (Am CPV). Int. J. Sci and Res., 4 (12) : 276-279
- Ling, E., Shirai, K., Kanekatsu, R. and Kiguchi, K. (2005): Hemocyte differentiation in the hematopoietic organ of the silkworm, Bombyx mori: prohemocyte have the function of phagocytosis. Cell Tissue Research, 320: 535-543.
- Kawakami, K. (1965) : Phagocytosis in muscardine diseased larvae of the silkworm, Bombyx mori (Linnaeus). J.Invertebr. Pathol. 7: 203-208.
- Madhusudhan, K. N., Devi, N., Lokesh, C. G., Singh, G. P., Sinha, A. K., Kumar, K. K. P., and Prasad, B. C. (2011): Impact of Nosema mylitta (pebrine) infection on the Larval Parameters, Protein Concentration and total Hemocyte Level in Daba Ecorace of Antheraea mylitta D. (Tropical Tasar silkworm). Microb. J., 1: 97-104.
- Morton, D. B., Dunphy, G. B. and Chadwich, J. S. (1987): Reactions of hemocytes of immune and non-immune Galleria mellonela larvae to Proteus mirabilis Dev. Comp. Immunol. 11: 47-55.
- Nayaka, A. R.N. and Sharma, S.D. (2014) : Dynamics of total hemocyte count (THC) in popular bivoltine and multivoltine breeds of mulberry silkworm Bombyx mori L. during the progressive infection of newly isolated microsporidian NIK-5hm. J. Entomol and Zoo. Stud. 2 (1) : 70-72

- Nisar, A., Ganie, Afifa, S., Baqual, K. M. F., Sharma, R. K., Dar, K. A. and Bashir, M. (2015): Studies on the total and differential hemocyte count in some breeds of silkworm, (Bombyx mori L.) I. J. A. B. R, 5 (1) : 58-61
- Pandey, J. P. and Tiwari, R. K. (2012) : An Overview of Insect Hemocyte Science and its Future Application in Applied and Biomedical Fields. Amer. J. Biochem. and Mol. Biol., 2 : 82-105.
- Paul, D. C., Rao, S. G. and Deb, D. C. (1992) : Impact of dietary moisture on nutritional indices and growth of Bombyx mori and concomitant larval duration. J. Insect Physiol., 38: 229-230.
- Ratcliffe, N. A., Rowley, A. F., Fitzgeald, S. W. and Rhodes, C. P. (1985): Invertebrate immunity: basic concepts and recent advances. Int. CRev. Cytol. 97: 183-279.
- Richarda, E. H. And Edwards, J. P. (1999): Parasitization of Lacanobia oleracea (Lepidoptera: Noctuidade) by the ectoparasitic wasp Eulophus pennicornis: effects of parasitisation, venom and starvation on host haemocytes. J. Insect Physiol. 45 : 1073-1083.
- Rivers, D. B., Ruggiero, L. and Hayes, M. (2002): The ectoparasitic 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.
- Rosenberger, C. R. and Jones, J. C. (1960): Studies on total blood cell counts of the southern armyworm larvae Prodenia eridania (Lepidoptera) Ann. Entomol. Soc. Am. 53: 351-355.
- Russo, J., Brehelin, M. and Carton, Y. (2001): Haemocyte changes in resistant and susceptible strains of D. melanogaster caused by virulent and a virulent strains of the parasitic wasp Leptopilina boulardi. J. Insect Physiol. 47 : 167-172
- Singh, G. P, Zeya, S. B., Srivastava, A. K., Prakash, B., Ojha, N. G. and Suryanarayana, N. (2008): Susceptibility of three eco-races of tropical Tasar silkworm to Antheraea mylitta cytoplasmic polyhedrosis virus (AmCPV). Caspian J. Env. Sci., 6 (2) : 161-165
- Tauber, O. E. and Yeager, J. F. (1935): Total blood cell count in various insects. Ann. Entomol.. Soc. Am, 28 : 229-240
- Tauber, O. E. (1940): Mitotic response of roach hemocytes to certain pathogen in the hemolymph. Ann. Ent. Associ. Ameri. 33 : 113-119
- Velide, L. and Bhagavanulu, M. V. K. (2012): Estimation of efficient concentration of bavistin in controlling microsporidiasis and improving cocoon characters in Anthereae mylitta Drury (Andhra local ecorace). I. J. P. A. E. S., 2 (1): 117-182.
- Wigglesworth, V. B. F. R. S. (1972): Circulatory system and associated tissues. In The principles of insect physiology. ELBS and Chapman and Hall. pp 434