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Studies on some Haematological parameters including Cellular Phagocytosis interaction occurring in the larval stages as well as in imago of *Bombyx mori* L. (Lepidoptera: Bombycidae) Breeds

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Abstract

The motive of the present work is to confer different haematological parameters in healthy larval and adult mulberry silkworm *Bombyx mori* L. on the three commercial breeds viz. hybrid (Nistari X M12W), bivoltine (NB4D2) and multivoltine (Nistari) along with cellular defensive role in diseased state. Five types of haemocytes, viz. prohaemocytes, plasmatocytes, granulocytes, Spherulocytes, and Oenocytoids are recorded in both healthy adults and 5th instar larvae in contrast to six types of haemocytes including imaginal Spherulocytes recorded prior this study. Discrete Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC) values in the 5th instar larvae among these breeds showed significant differences with the values decreased in the order multivoltine breed, Nistari >bivoltine breed NB4D2. Under infected conditions, THC value in the 5th instar larvae increased in the order of multivoltine breed > bivoltine breed NB4D2 when counted from day 1 to day 4 of developing 5th instar larvae. In both cases declining haemocyte population turn down sharply on the day 5 of 5th instar larval development just prior spinning. Cellular defense response of Nistari breed, against bacterial infection was also studied in their functional aspects, and sequential steps followed in phagocytosis interaction to defend itself against infection from the beginning were also investigated. This comprehensive study may provide an additional reference for the future researches of insect immunity.

Haemocytes in insect's life play significant roles, either functioning alone or in alliance with the haemolymph. The mulberry Silkworm *B. mori* L. lacks an adaptive immune system but depends solely on innate immunity comprising of humoral and cellular immunity to fight against disease causing pathogens[9]. The "blood cells" in the Silkworm, *Bombyx mori* L. are classified into six types in adult viz. Prohaemocytes, Plasmatocytes, Granulocytes, Spherulocytes, Imaginal Spherulocytes (occasionally observed in pupa or the day before emergence) and Oenocytoids[12]. It was also established long ago that five types of haemocytes in *B. mori* L. larvae functioning in various ways including prevention of pathogenic microorganisms which are known as Prohaemocytes, Plasmatocytes, Granulocytes, Spherulocytes and Oenocytoids[2,3]. It has also been established that the number of haemocytes tend to increase during the larval instars peak in 5th instar and reducing the numbers as observed in the pupa and adult stages[12]. THC and DHC analyses indicate the susceptibility status of the insect which signifies the importance of haematological studies in the field of silkworm physiology. Keeping these in mind, we carried out THC and DHC determination of commercial silkworm breeds along with related haematological parameters to corroborate their susceptible tendencies in healthy and diseased conditions as haemocytes are basically influenced by environmental conditions and disease stresses[13]. Circulating haemocytes carry out cellular defence via phagocytosis, nodulation or encapsulation as reported earlier by Wood and Jasinto[16] and present study also emphasized on phagocytosis interaction against flacherie caused by *Bacillus thuringiensis*. The present study is restricted to the commercial Nistari (multivoltine), NB4D2 (bivoltine) and hybrid (Nistari X M12W) to give a comparable view of the distinct varieties of haemocytes present in both larval and adult stages and to recognize them instantly along with their functional aspects.

Healthy silkworm breeds viz. Nistari (multivoltine), NB4D2(bivoltine) and N X M12W (hybrid) were collected and reared at Krishnath College, Berhampore, Murshidabad presently Murshidabad University, West Bengal during both spring as well as summer season (2018-2020) in the laboratory as per standard rearing protocol with room temperature of 32^{o}C, humidity of 80%-95% and natural photoperiod. Larvae were fed on mulberry leaves S1 variety as per recommendation. The 3rd instar (Nistari, NB4D2), 4th instar (Nistari, NXM12W), 5th instar (NB4D2) larvae, pupae and adults (Nistari) were used for our experiments. Some flacherie infected 4th and 5th instar larvae were also procured from C.S.R & T.I, Government of India, Berhampore, West Bengal for our studies and these larval stocks were separately maintained in the bio-safety laboratory, Division of Entomology, Maulana Azad College, Kolkata with utmost care. Haemolymph collection, staining, THC and DHC determination were done following Jones[8] and Jalali & Salehi[7]. The smear was examined and visualized with a compound microscope (Magnus-MLXDX 11E634) at 40X magnification and after that images were acquired. Morphometric analyses were executed with the help of ocular and stage micrometer and the average size of the haemocyte types was

estimated by measuring the length and width of five cells of each type by calibration and standardization of the microscope. Close observations were made under Phase Contrast Microscope to study phagocytosis interactions of the haemocytes.

THC and DHC analysis of haemocytes showed significant differences with the values in observing seasonal and day to day occurrences among studied breeds under both healthy and infected conditions. Haemocytes of healthy mulberry silkworm larvae *Bombyx mori* L at their 4th and 5th instars were observed for THC (Table-1).

Table-1. THC values of silkworm breeds (Number/ mm^3) in two seasons.

Silkworm Breeds	Spring, 2019	Summer, 2019
Pure Mysore	9300	9800
Nistari Silkworm	9900	10600
NB4D2	8300	8200

Higher THC values in the healthy 5th instar larvae of multivoltine breeds occurring than the bivoltine breeds is due to the development of primary haemocytes.

Above findings of two commercial breeds of West Bengal in comparison to that of Pure Mysore race as reported earlier showed deviations with the results of Paul *et al.*,[14] and they explained that feeding efficiencies in the larval stages are responsible to increase the number of haemocytes in both the seasons. Our study is also adding another important factor that influencing increased haemocytes in both the breeds of *Bombyx mori* L. Higher THC value in multivoltine breeds is probably due to a large number of haemocyte populations producing from the haematopoietic organs as hematopoietic tissue (Fig-1). These primary haemocytes are prohaemocytes and plasmatocytes. These are pluripotent and the main sources for other cell types. Further, it can be explained as higher survival chances for multivoltine breed during summer due to increased number of haemocyte populations. In healthy 5th instar larvae THC values in both multivoltine and bivoltine breeds recorded significant differences during 1st day to 5th day of 5th instar. The present study showed that multivoltine breed was found to have greater THC value than the bivoltine breed on all the days of the 5th instar larvae. In both breeds THC was found to be gradually increasing from the first day to the last day of the 5thinstar (Table-2). In healthy 5thinstar larvae THC value of multivoltine breed, Nistari ranged from 6.4 x $10^3/mm^3$ on the 1stday to 10 x $10^3/mm^3$ on the 5thday and in bivoltine breed NB4D2 value recorded from 3.2 x $10^3/mm^3$ on the 1stday to 6.4 x $10^3/mm^3$on the 5thday (Fig- 2).

Table-2. Day wise THC records in the 5th instar larvae of a multivoltine (Nistari) and a bivoltine (NB4D2) breed of the mulberry silkworm, *Bombyx mori* L. always pointed out greater population

Days	Multivoltine breed (Nistari) ($10^3/mm^3$)	Bivoltine breed (NB4D2) ($10^3/mm^3$)
Day 1	6.4	3.2
Day 2	7.2	4.2
Day 3	8.2	4.4
Day 4	9.4	5.2
Day 5	10.0	6.4

So, all these records can be explained for normal growth of larvae during their developmental period to attain maturity. Differences of THC values also noted in both these breeds.

Table-3. Types of haemocytes form and state of larval haemocytes

Haemocytes	Appearance	Position of nucleus	Nature of cytoplasm
Prohaemocytes (PR)	Round or spherical	Central	Basophilic
Plasmatocytes (PL)	Elliptical, fusiform	Largely central	Basophilic
Granulocyte (GR)	Spherical or oval	Central or eccentric	Slightly acidophilic
Spherulocytes (SP)	Round or oval	Generally eccentric	Basophilic
Oenocytoids (OE)	Rounded	Eccentric	Slightly acidophilic

Table-4. Haemocyte types in Nistari, NB4D2 and NXM12W breeds

	Instars	Haemocyte types				
Nistari	3rd instar	PR	PL	GR	OE	-
	4th instar	PR	PL	GR	OE	SP
	5th instar	PR	PL	GR	OE	SP
NB4D2	3rd instar	PR	PL	GR	-	-
	4th instar	PR	PL	GR	OE	SP
NXM12W	4th instar	PR	PL	GR	-	-

Characterization of larval haemocytes, morphometric analysis and their immune functions:

Adult *Bombyx mori* L. was reported to contain six types of haemocytes viz. Prohaemocytes (PRs), Plasmatocytes (PLs), Granulocytes (GRs), Spherulocytes (SPs), Imaginal Spherulocytes and Oenocytoids (OEs). However, in the mature 5th instar larvae five types of haemocytes were recognized earlier (Table-3) based on their morphology and functions as known from the works of Akai and Sato[1]. Further Balavenkatasubbaiah *et al*.,[2]; Ling *et al*.,[10], and Nakahar *et al*.,[11] described in detail for better understanding of all these haemocytes though Jones[8]; Gupta[6] and recently Tan *et al*.,[15] explained lesser numbers of larval haemocytes observed in Silkworm. In the present study following observations have been noted in 3rd to 5th instar larvae of those commercial breeds (Table-4).

Haemocyte types and their characteristics in silkworm (***Bombyx mori* L**.) have been studied extensively and are known from a large number of literatures. Here for instant recognition of those haemocytes, typical

morphology of its type and morphometric analysis along with a particular role in immunological studies have been focused for further works in this area of study. Prohaemocytes (PRs) were the smallest among all haemocytes and the nucleus occupied most of the cytoplasm which forms a very thin layer around the nucleus. Plasmatocytes (PLs) were highly polymorphic cells and significantly larger than PRs and their irregular shapes were due to cytoplasmic projections. Granulocytes (GRs) were the most common in all larval instars having variable in shape and size and contained large amount of different sized granules in the cytoplasm. Oenocytoids (OEs) were opaque in appearance and the cytoplasm contained fine and weak granulations. Spherulocytes (SPs) were irregular in shape and the cytoplasm was characterized by large vesicles with membrane-bound vacuoles containing spherules and appeared as bulbous swellings on the cell surface.

Morphometric Analysis :

The average size of each haemocyte type for healthy silkworms were evaluated by measuring the length and width of five cells of each type with the help of ocular and stage micrometer. The morphometric analyses of distinct haemocytes were measured and are depicted in the table-5.

Table-5. Micrometric measurements (μm) of haemocyte types in studied multivoltine and bivoltine breeds

	Instars	PRs	PLs	GRs	OEs	SPs
Nistari	3rd instar larvae	L=13.55 W=12.3	L=12.4 W=8.1	L=15.5 W=13	L=18.2 W=16.6	-
	4th instar larvae	L=10.56 W=8.4	L=13.55 W=10.58	L=16.38 W=13.8	L=23.47 W=19.46	L=16.25 W=14.8
	5th instar larvae	L=13 W=12	L=22 W=17	L=24 W=21	L=24 W=22	L=27 W=12
	Pupae	L=10.82 W=9.32	L=13.15 W=10.32	L=12.98 W=10.49	L=19.49 W=14.82	L=25.82 W=20.41
	Adult	L=10.32 W=9.49	L=14.32 W=9.98	L=12.82 W=10.48	L=19.15 W=17.49	L=16.65 W=13.73
NB4D2	3rd instar larvae	L=13.99 W=12.66	L=11.86 W=9.15	L=17.69 W=15.41	-	-
	5th instar larvae	L=10.61 W=8.73	L=14.15 W=11.65	L=16.48 W=14.49	L=21.99 W=18.65	L=12.78 W=11.94
NXM12W	4th instar larvae	L=0.99 W=7.99	L=10.66 W=8.49	L=14.15 W=12.8		

Micrometric observations showed instar wise little deviations and noted Oenocytoids are the largest of Bombyx haemocytes.

Table-6. Measurements of Standard Error (SE) of haemocyte types in studied multivoltine and bivoltine breeds

	Instars	Mean ± SE	
		Length	Width
Nistari	3rd instar larvae	14.91 ± 1.09	12.5 ± 1.50
	4th instar larvae	16.04 ± 1.91	13.40 ± 1.69
	5th instar larvae	22 ± 2.13	16.8 ± 1.91
	Pupae	16.45 ± 2.46	13.07 ± 1.85
	Adult	14.65 ± 1.36	12.23 ± 1.35
NB4D2	3rd instar larvae	14.51 ± 1.38	12.40 ± 1.47
	5th instar larvae	15.20 ± 1.74	13.09 ± 1.48
NXM12W	4th instar larvae	8.6 ± 3.21	9.76 ± 1.24

Table-7. THC (*10^3/mm^3) of Bivoltine & Multivoltine Breed under infected condition

Breeds	Day 1	Day 2	Day 3	Day 4	Day 5
NB4D2 Bivoltine	1.68	1.98	3.03	2.48	1.74
Nistari Multivoltine	1.96	2.25	3.21	3.64	2.23

Total and Differential haemocyte count of flacherie infected larvae in Multivoltine and Bivoltine breeds :

Flacherie is the most common bacterial disease that inflicts the maximum damage to sericulture practices. Due to varied symptoms the disease is also named as Sotton disease, shrinking disease, softening disease, faecal disease etc. The changing of reactions of 5th larval instars against *Bacillus thuringiensis* in both the breeds revealed highly prominent changes in the THC (Table-7). It was noted that initially there is a sharp fall in the circulating haemocytes than normal THC levels in the 5th instar larvae. So, it is indicating the deployment of defence cells chiefly plasmatocytes and granulocytes to fight against bacteria. The initial decrease in the THC's is indicative of the quick deployment of cells to the infection site to combat the invading pathogens.

Day wise THC values of infected 5th instar larvae in two breeds showing multivoltine breed> bivoltine breed when analysed for comparison.

Under infected conditions, THC value showed in multivoltine breed from 1.96 x 10^3/mm^3 on day 1 and increased to 3.64 x 10^3/mm^3on the 4th day of 5th instar larvae. In contrast to above breed, THC value showing less in bivoltine breed from 1.68 x 10^3/mm^3on the 1stday and increased to 2.48 x 10^3/mm^3 on the 4th day of 5th instar larvae (Fig-3). With the commencement of spinning there was a sharp fall in THC which drastically reduced in

the pupal stage (0.340×10^3+-0.303/mm^3) and it was higher in adults. These results indicated capacity to endure against diseases and the differences in between the two breeds due to their acquired characters.

In our observations THC value in both larval groups showed increased level first and immediately declining phases started under flacherie infected conditions and sharply declined just before spinning.

Bacterial infection decreased the number of prohaemocytes, granulocytes, plasmatocytes and Oenocytoids as observed in differential haemocyte count (DHC). On the other hand, the infection of Bombyx mori 5^{th} larval instar with Bacillus thuringiensis gradually increased the granulocyte count but still less than healthy ones.

Cellular defence and Phagocytic Interaction :

The cellular immune response includes the identification of pathogens, phagocytosis of invasive bacteria and viruses, nodulation of large microbial pathogens such as fungi and bacterial clusters and encapsulation of multicellular (parasitic) organisms. Zafar *et al.*,[17] clearly mentioned immunological responses in silkworm are accomplished by circulating haemocytes which play a significant role in innate immune mechanism. Present study reveals that pathogenic bacteria invade into the haemocoel of *Bombyx mori* L. at larval stage led to humoral and cellular immune response. Due to bacterial infection haemocytes underwent considerable structural changes. The contents of the granulocytes seem to swell giving the cell an extremely vacuolated appearance. Haemocytes (plasmatocytes and granulocytes) that have phagocytic response to the bacteria tend to form aggregations. These unstructured aggregations may later be encapsulated by other haemocytes or by cells may be released from the aggregations. The aggregated haemocytes appeared in haemolymph due to *B. thuringiensis* infection. A number of phagocytosing plasmatocytes, granulocytes and attached bacteria were also observed. Oenocytoids showed to have numerous patches of crystal like inclusions in the cytoplasm. Hyperphagocytic haemocytes are involved in nodule formation. It was earlier demonstrated that granulocytes and plasmatocytes are the major cells that phagocytized pathogenic bacteria in the larval stages of *Bombyx mori* L. According to Carton and Nippi[4] phagocytosis, encapsulation and nodule formation is the main reaction for clearance of pathogen and other foreign particles. As we know that the process of phagocytosis is accomplished in a single cell, involving the identification, phagocytosis, destruction of invasive pathogens and death of cells occur as described by Gray and Botelho[5]. Our findings facilitated phagocytosis where phagocytic cells recognized foreign particles through a series of receptors on their cell membrane for pathogen associated molecules. These receptors in turn initiate a series of signaling pathway that instruct the cells to ingest and eventually destroy the foreign particles. Following steps have been evaluated during the process of phagocytosis- 1) when foreign particles or organisms are too large for either phagocytosis or nodule formation is completely destroyed by encapsulation. 2) At the initial stages foreign

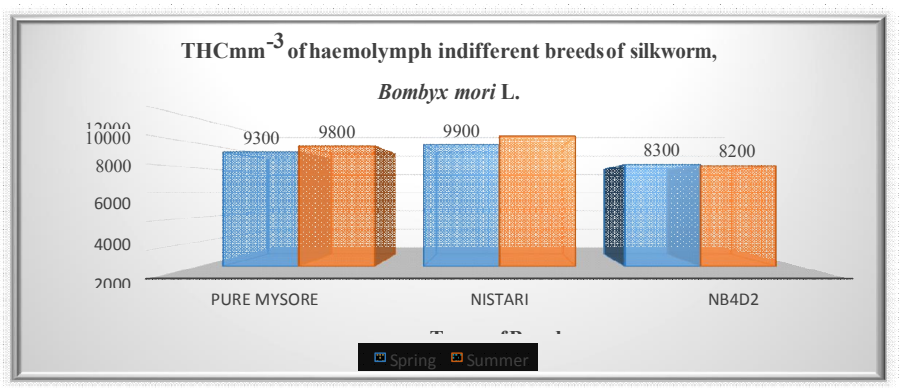


Fig. 1. Seasonal comparisons of THC values

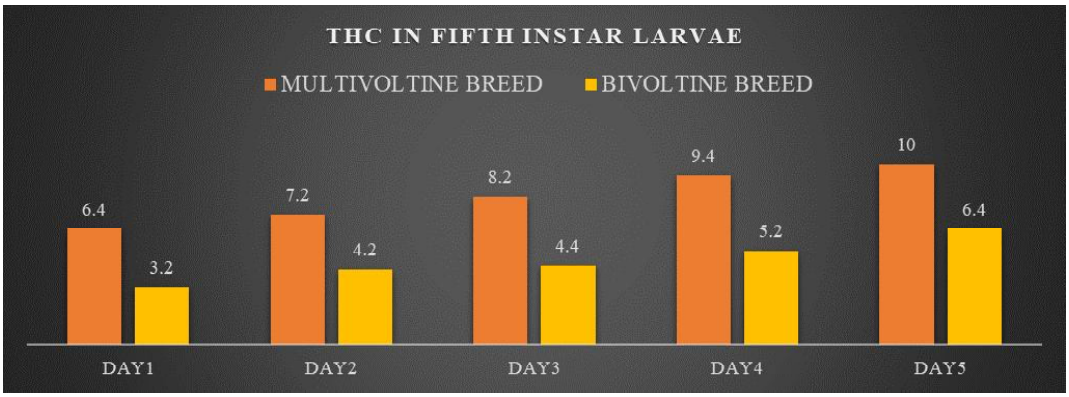


Fig. 2. Day wise THC analysis. Gradual increased THC values in both breeds indicating healthy larval growth

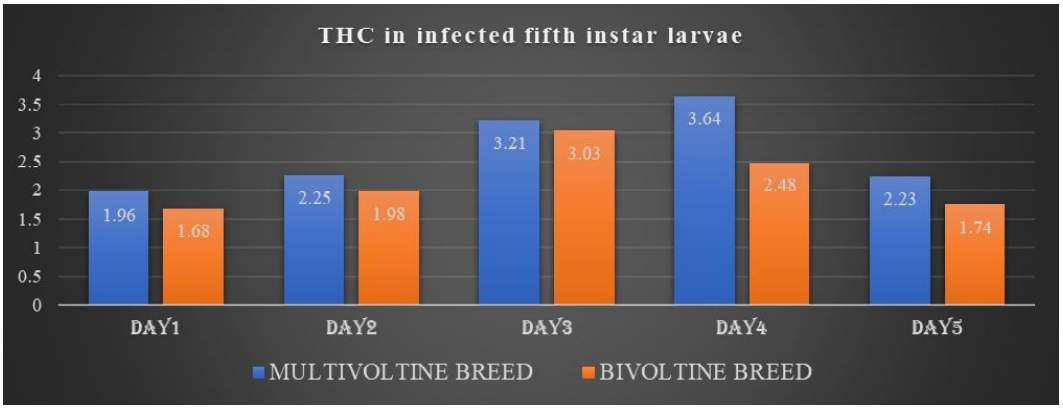
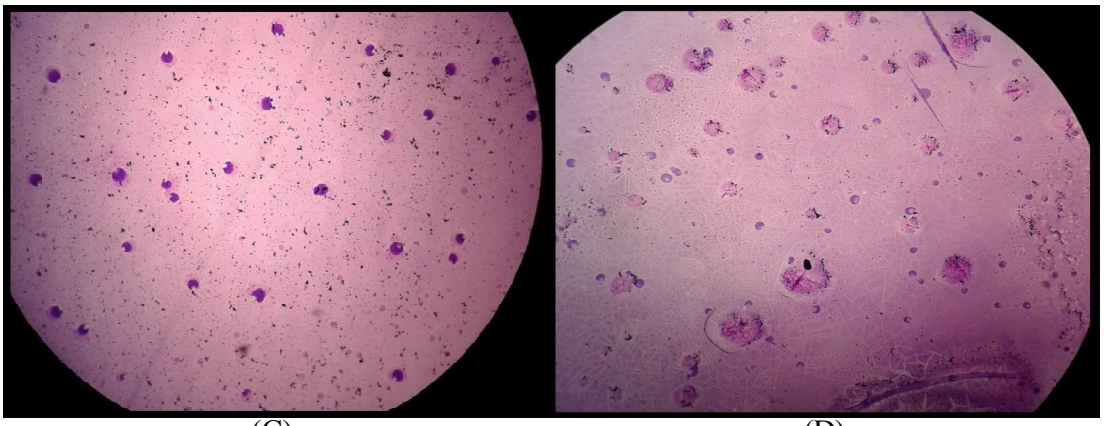
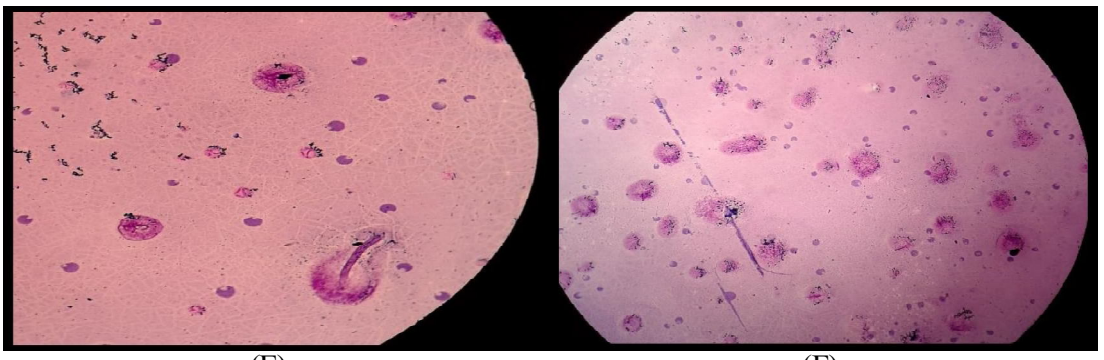


Fig. 3. Day wise THC values of infected 5th instar larvae in two breeds showing multivoltine breed> bivoltine breed when analysed for comparison

(A) (B)



(C) (D)



(E) (F)

ILLUSTRATIONS:

Fig-4

A – Healthy 5th instar larvae of Nistari	**B** – Infected 4th instar larvae of Nistari (First day)
C – Prohaemocytes (Nistari)	**D** – Granular haemocytes (Nistari)
E – Encapsulation	**F** – Lysis of Granulocytes

body is randomly contacted by a granulocyte which recognizes the particular existence. 3) The Granulocyte degranulates and material sticks to foreign body which is responded by additional granulocytes attacking to foreign bodies. 4) Lysis took place and granulocytes release a haemocytic recognition factor that attracts and recruits plasmatocytes to attach foreign body. 5) Plasmatocytes then flatten and spread over the foreign body surface increasing the number of layers around the foreign food so long that it is no longer recognized as foreign particles.

Information on the haemocyte population within an insect is essential for strengthening physiological studies. The haemocytes of *Bombyx mori* L. are also the examples of classic types as found in *Drosophila* (Diptera) which are engaged in different roles in host defence reactions. All our findings in regard to phagocytic response are consistent with the general idea that the nature of infecting bacteria influences interaction. The THC and DHC values in the 5th instar larvae among the studied breeds showed significant differences with the values decreased in the order multivoltine breed, Nistari >bivoltine breed NB4D2. Finally, it is clear from the study that, under infected conditions, THC value in the 5th instar larvae increased in the order of multivoltine breed > bivoltine breed NB4D2 when counted from day 1 to day 4 of developing 5th instar larvae. In both cases declining haemocyte population turn down sharply on the day 5 of 5th instar larval development just prior spinning. So, multivoltine of Silkworm contains more circulating cells and more defence cells against bacterial invasions than the bivoltine breed so far examined. Further investigations will certainly add more information about activation of haemocytes against different pathogens to defend.

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