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Research Article

A COMPARATIVE STUDY ON THE HAEMOCYTES OF THE FIFTH INSTAR LARVAE OF A BIVOLTINE BREED AND MULTIVOLTINE BREED OF THE MULBERRY SILKWORM, *BOMBYX MORI* L.

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ABSTRACT

Haemocytes perform various physiological functions in the body of an insect. Total and specific haemocyte populations indicate the immunological status of an insect. The total number of haemocytes (THC) and the different haemocyte count (DHC) vary during the growth and metamorphosis of different breeds of the silkworm. The THC and DHC of a multivoltine breed were compared with that of a bivoltine breed in this work. THC of multivoltine breed ranged from 6.4 x 10^3/ mm^3 on the 1st day to 10x10^3/mm^3 on the 5th day. THC of bivoltine breed ranged from 3.2x 10^3/ mm^3 on the 1st day to 6.4 x 10^3/mm^3 on the 5th day. Only four different types of haemocytes were observed in the haemolymph of both breeds. They were prohaemocyte, granulocytes, spherule cells and oenocytoids. 't' test values indicate that significant differences were observed between the two breeds in terms of THC and DHC.

Keywords: Prohaemocyte, Granulocytes, Spherule cells, Oenocytoids.

INTRODUCTION

Insects have an open system with the blood occupying the general body cavity known as the haemocoel. The haemolymph of insects consists of liquid plasma and numerous haemocytes (Pandey and Tiwari, 2012). The haemocytes of are several types of mesodermal cells which circulate within the haemolymph and they sometimes attach loosely to other tissues (Kerenhap *et al.*, 2005).

Haemocytes perform various physiological functions in the body of an insect. They direct nutrients to various tissues and store them. They perform phagocytosis, encapsulation of foreign bodies in the insect body cavity, coagulation to prevent loss of blood, nodule formation, transport of food materials, hormones and detoxification of metabolites (Patton 1983). Five haemocyte types were identified in the haemolymph of lepidopterous larvae: prohemocytes, plasmatocytes, granulocytes (Coagulocytes), spherulocytes and oenocytoides (Jones 1962; Akai and Sato, 1973; Essawy 1999; Saad, 2005).

The blood cells of the silkworm, *Bombyx mori* L are classified into six types *viz.*, prohaemocytes, plasmatocytes, granulocytes, spherulocytes, imaginal spherulocytes (observed only at adult stage, but occasionally in pupa on the day before emergence) and oenocytes (Nittono, 1960). Literature survey reveals that only a very little information is available on the comparison haemocytes of a bivoltine breed and multivoltine breed of the silkworm *B. mori.*

Therefore, in the present work, an attempt has been made to determine total haemocyte count and the changes in the differential haemocyte count in the haemolymph of the silkworm *B. mori*.

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MATERIALS AND METHODS

Insects

In the present study the commercially exploited multivoltine cross breed (L x CSR2) race and bivoltine breed (CSR6 x CSR12) of *B. mori* were selected. The eggs of these races were procured from Silkworm Rearing Department, Rayanur, Karur district, Tamilnadu, India. They were kept under lab conditions and allowed to hatch. The emerged first instar larvae were fed with young leaves of mulberry variety MR2. The larvae were acclimatized to the lab conditions by rearing them till fifth instar with the relative humidity of 80 - 90 % and the temperature of 27 - 30 ˚C according to the conventional method in trays and provided with suitable amounts of fresh mulberry leaves. All larvae which molted to the last instar at the same time were grouped and used in the experiments.

Haemolymph collection

Haemolymph was obtained by puncturing the abdominal legs with sterilized needle. The haemolymph was collected in pre-cooled tubes containing a few crystals of phenyl thiourea @ 1mg/sample. Phenyl thiourea was used to avoid the activity of prophenol oxidase followed by melanization of the haemolymph samples (Takeda *et al*., 1996). The samples were stored at -20˚C for further use.

Total haemocyte count and differential haemocyte count

Total haemocyte count (THC) was enumerated in the haemolymph using haemocytometer (Cantwell, 1973) using BLX microscope (Weswox). The haemolymph was taken on five clean glass slides every day and a smear was made. The smear was allowed to dry and was stained by using crystal violet, Methylene blue, Giemsa stain and Acetocarmine suggested by Shapiro, (1968); Aronld, (1976) and Essawy, (1990). The smear was examined under microscope and the haemocytes were differentiated according to the classification of Jones (1962), Akai and Sato (1973) and Essawy (1997). For differential haemocyte count (DHC), cell categories were counted in 100 cells in each smear using a Photo plan microscope. The relative numbers of different haemocytes of larvae were determined on 1st day, 2nd day, 3rd day, 4th day and 5th day of the fifth instar.

RESULTS AND DISCUSSION

Haemocytes are circulating cells found in the haemolymph of insect responsible for the defense mechanism against bacteria. The results regarding the total haemocyte count and differential count are presented in Table 1 and 2.

Total Haemocyte Count

In both breeds, THC was found to be gradually increasing from the first day to the last day of the fifth instar (Table 1). Total haemolymph count (Figure 1) and specific haemocyte count indicate the tolerance status of the insect to diseases (Balavenkatasubbaiah *et al*., 2001). The number of haemocytes is greatly affected by age, stage and physiological status of the insect (Kerenhap *et al*., 2005). In both the races, the increase in THC as the day advanced could be related to the preparation of the silkworm for pupal stage and cocoon formation. This increasing trend ensured a greater immune resistance to any kind of infection. This could be confirmed by the zero mortality observed in both races. Higher THC might be due to higher production of cells which give protection to the later quiescent stage of pupa, antimicrobial proteins, proteins required for the silk synthesis.

The present study showed that multivoltine breed was found to have greater THC than the bivoltine breed on all days of the fifth instar larvae. Statistically significant differences in the THC were observed between the fifth instar larvae of the Multivoltine and Bivoltine silkworm, *B. mori*. The differences observed between the two breeds might be attributed to the racial specificity or ingrained genetic character of the particular race. The higher THC of a multivoltine breed and lower THC of the bivoltine breed must be their specific characters. These breed specific characters might have been the adaptations to the environmental conditions or seasonal variations as the earlier works reported.

The higher THC value of multivoltine breed ensured the higher survival rate. Higher feeding efficiency also could be the reason for the higher THC as reported by (Paul *et al*., 1992). Another reason for the higher THC in multivoltine breed is might be due to the release of more number of pluripotent haemocytes, the prohaemocytes from the haemopoietic organs. Significantly higher THC has been recorded in non-diapausing *Antheraea mylitta*. This was in support of our finding since multivoltine breed was a non-diapausing breed and bivoltine breed was a diapausing breed. The recorded data of the investigation was in conformity with the earlier studies of Chain and Anderson (1982), Dunn and Drake (1983) and Horohov and Dunn (1983). Nappi (1981) and Eslin and Prevost (1998) reported that an increase in the haemocyte number indicates the activation of host defence mechanism. The population of circulating haemocytes may indicate whether the host defence system was activated or not (Brehelin, 1982). Since no mortality was recorded in both breeds both breeds were considered to have good host defence mechanism.

Differential Haemocyte Count

Silkworm, *B. mori* was reported to contain five types of haemocytes based on their morphology and function viz., granulocytes, plasmatocytes, oenocytoids, prohaemocytes and spherulocytes (Akai and Sato, 1973; Balavenkatasubbaiah *et al*., 2001; Ling *et al*., 2003; Kerenhap *et al.*, 2005; Nakahara *et al.*, 2009). However, in the present study only four types of haemocytes viz., prohaemocytes, granulocytes, oenocytoides and spherulocytes (Figure 2-5) were observed in the haemolymph of fifth instar larvae of both breeds. Though similar finding has been reported by Han *et al*. (1998), they have not reported the presence of spherulocytes. Moreover, Nittono (1960) has classified the blood cells in the silkworm, *B. mori* into six types viz. prohaemocytes, plasmatocytes, granulocytes, spherulocytes, imaginal spherulocytes and oenocytes.

In multivoltine breed, of all the four haemocytes, the number of prohaemocytes was found to be the highest on 1st, 2nd and 5th day of the fifth instar. The number of granulocytes was higher than that of the oenocytoids and Spherule cells on all days of the fifth instar. Very less number of Spherule cells was observed on all days. The number of all the cells was found to be the highest on the last day of the fifth instar.

In bivoltine breed, the number of granulocytes was found to be the highest of all haemocytes on all days of the fifth instar. Number of prohaemocytes was found to be higher than that of the oenocytoids and spherule cells. In this breed also, only very less number of spherule cells was observed on all days as recorded in the multivoltine breed.

The number of prohaemocytes was found to be increasing day by day (Table 2). Very significant increase in prohaemocyte was observed on the fourth day and fifth day in both breeds. The number of prohaemocytes in multivoltine breed was greater than that of the bivoltine breed on all days. Differences between the number of prohaemocytes and granulocyte of multivoltine and bivoltine were found to be significant whereas the differences in the number of spherule cells and oenocytoids between these two races were found to be only insignificant.

The number of granulocytes was found to be increasing day by day in both breeds (Table 3). The increase was highly significant on the fourth day. Plasmatocytes and granulocytes are the main haemocytes involved in phagocytosis of foreign microorganisms and thus play a role in defense mechanism against entry of foreign body in *B. mori* (Sato *et al.*, 1976; Akai and Sato, 1973; Rowely 1977; Ratcliffe and Rowely, 1975; Strand and Pech, 1995). Granulocytes and plasmatocytes have been reported to be deployed in combating invading bacteria in *B. mori,* at 1 hr and 6 hr post infection with bacteria (Krishnan *et al.*, 2000). Though plamatocytes were not found, increased population of granulocytes can be interpreted as both the breeds were having a good defense mechanism. The changes in the number of haemocytes especially THC and DHC with regard to granulocytes, plasmotocytes and spherulocytes in silkworm reflected the greater resistance against diseases (Salt 1970; Ratcliffee and Rowley, 1975).

The number of oenocytoids was found to be increasing day by day in the multivoltine breed whereas it was found be fluctuating in the bivoltine breed (Table 4). On the second and fifth day the number of oenocytoids in bivoltine breed was very significantly lower than that of the multivoltine breed whereas only insignificant differences were observed on the other days. These cells are responsible for lipid processing and detoxification (Makki *et al*., 2014).

In the present investigation, initially the number of spherulocytes enhanced slowly and then increased abruptly from the 3rd day in both breeds (Table 5). Similarly, Bora and Handique (2008) observed an initial increase but it decreased on later days of the fifth instar. Ribeiro *et al*. (1996) reported that the functions of spherule cells are unknown. Spherulocytes have been suggested to transport cuticular components (Sass *et al*., 1994). Nittono (1960) reported that spherule cells of *B. mori* could be related to silk synthesis. Maximum number of spherule cells was observed on the 5th day of breeds. Number of spherule cells was found to be increasing day by day in both breeds. A sudden highly significant increase was observed on the third day in both breeds. The number of spherule cells in bivoltine breed was greater on all days than that of the multivoltine breed. Effective utilization of fat reserves, less respiratory metabolism and also extra energy production in the bivoltine breed might be the reasons for such greater number as suggested by Begum *et al*. (1998). But ststistically, the difference was only insignificant on the first two days and significant on the later days. With reference to the size of different haemocytes, no significant difference was observed between two breeds (Table 6).

The differences in the THC and DHC of bivoltine breed and multivoltine breed might be due to racial specificity or genetic characteristics inherited for their parental stocks. Hence THC and DHC values can be used as indices used for screening germplasm stocks by breeders to develop a breed with higher survival rate.

Table 1. Total haemocyte count (THC) in the fifth instar larvae of a multivoltine breed (LxCSR2) and a bivoltine (CSR6 x CSR12) breed of the mulberry silkworm, *Bombyx mori*.

S. No.	Days	THC Multivoltine breed (L x CSR2) (10^3/mm^3)	THC Bivoltine breed (CSR6 x CSR12) (10^3/mm^3)	‘t’ test value ($p < 0.05$)
1.	1 day	6.4±1.55	3.2±1.61	3.17 Significant
2.	2 day	6.2±1.62 (3.12%)	4.2±1.71 (31.2%)	1.93 Significant
3.	3 day	8.2±1.58 (32.2%)	4.4±1.77 (4.7%)	3.63 Significant
4.	4 day	9.4±1.76 (14.6%)	5.2±1.58 (18.8%)	3.93 Significant
5.	5 day	10±1.64 (6.3%)	6.4±1.36 (23.07%)	3.78 Significant

Values inside the parentheses indicate the percentage of increase in total haemocyte count over the previous day.

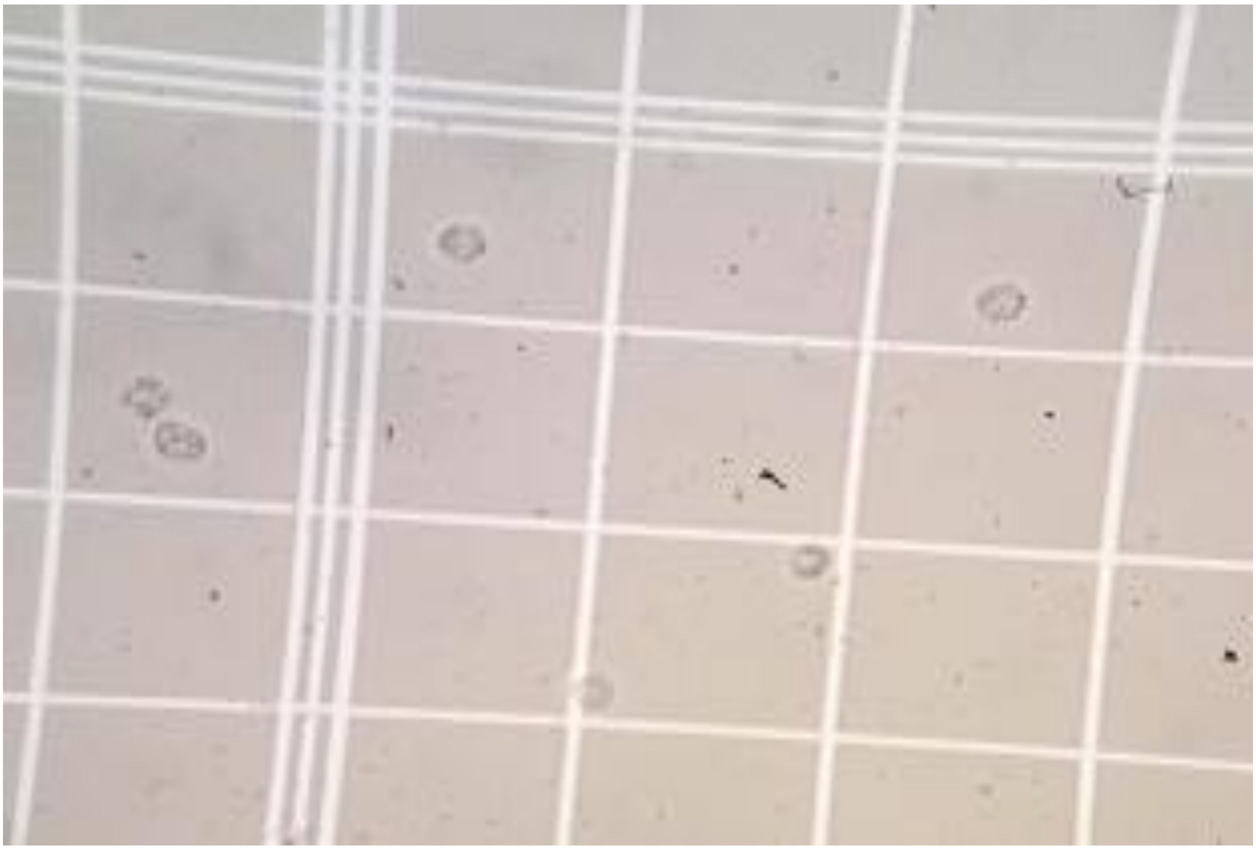

Figure 1. Microscopic view (Photoplan Weswox Optik) of the haemocytes in the squares of Haemocytometer used for Total Haemocyte Count (THC) in the Haemolymph of the mulberry silkworm *Bombyx mori.*

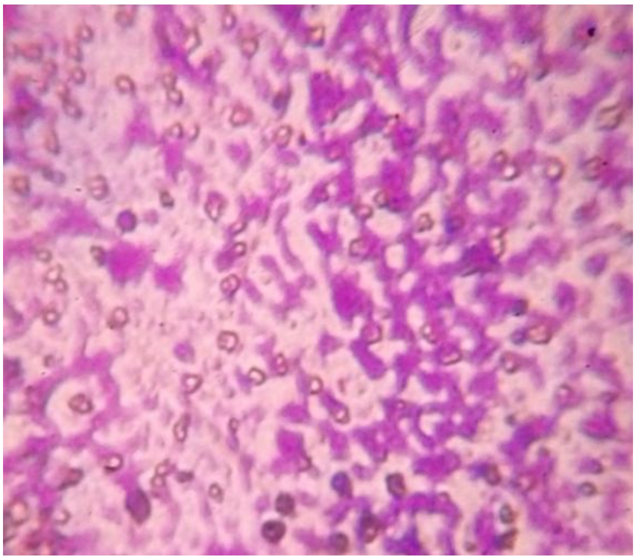

Figure 2. Prohaemocyte of *B. mori*.

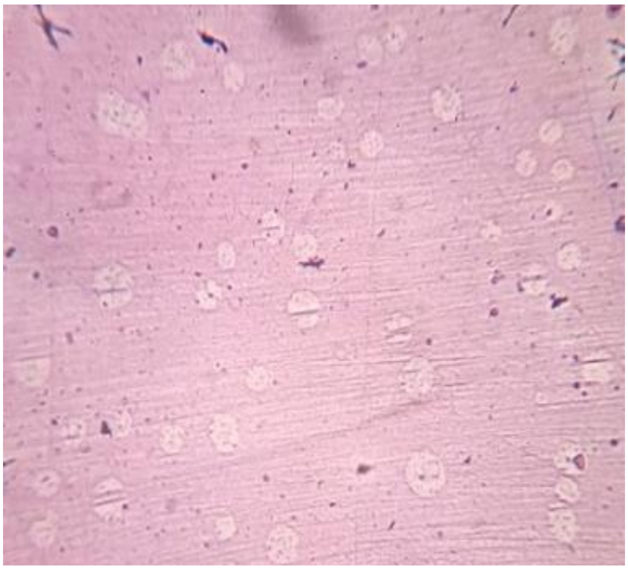

Figure 3. Granulocyte of *B. mori.*

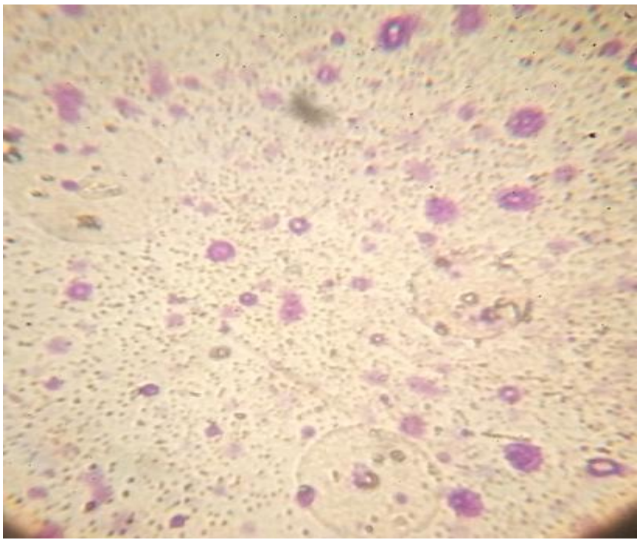

Figure 4. Oenocytoids of *B. mori.*

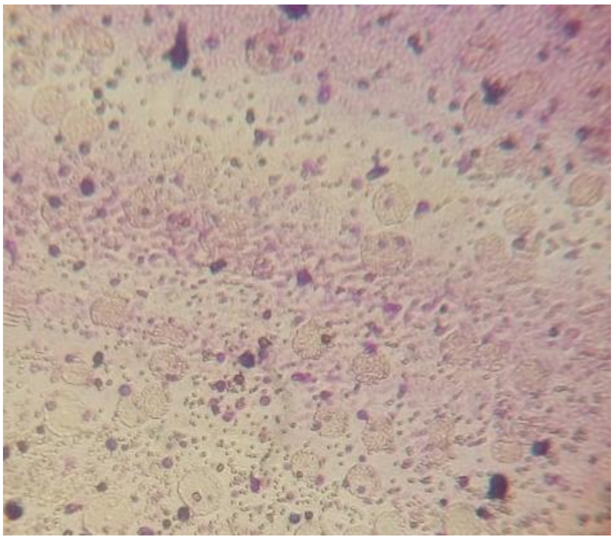

Figure 5. Spherule cells of *B. mori.*

Table 2. Number of prohaemocyte of the fifth instar larvae of a Multivoltine (L x CSR2) breed and a Bivoltine breed (CSR6 x CSR12) of the mulberry silkworm *Bombyx mori*.

S. No.	Number of Days	Number of Prohaemocytes (Mean%±S.D)		't' test value ($p < 0.05$)
		Multivoltine breed (L x CSR2)	Bivoltine breed (CSR6 x CSR12)	
1.	Day 1	14.2±1.60	11.2±1.42	3.12 Significant
2.	Day 2	18.4±1.75 (29.5%)	13±1.47 (16.07%)	5.17 Significant
3.	Day 3	23.4±1.50 (27.1%)	18.8±1.52 (44.6%)	-4.76 Significant
4.	Day 4	40.2±1.52 (71.7%)	31±1.54 (64.8%)	9.26 Significant
5.	Day 5	64.8±1.58 (61.1%)	53.8±1.58(73.5%)	11.01 Significant

Values inside the parentheses indicate the percentage of increase in prohaemocyte over the previous day.

Table 3. Number of Granulocyte of the fifth instar larvae of a Multivoltine (L x CSR2) breed and a Bivoltine breed (CSR6 x CSR12) of the mulberry silkworm *Bombyx mori*.

S. No.	Days	Number of Granulocyte		't' test value ($p < 0.05$)
		Multivoltine breed	Bivoltine breed	
1.	Day 1	7.8±1.52	11.8±1.56	-4.11 Significant
2.	Day 2	15±1.51 (92.3%)	18±1.58 (52.5%)	-2.99 Significant
3.	Day 3	29.6±1.62 (97.3%)	31.8±1.56 (76.6%)	-2.17 Significant
4.	Day 4	47.8±1.55 (61.4%)	54.2±1.57 (70.4%)	-6.48 Significant
5.	Day 5	56.8±1.52 (18.82%)	64±1.59 (18.08%)	-7.31 Significant

Values inside the parentheses indicate the percentage of increase in granulocyte over the previous day.

Table 4. Number of Oenocytoids of the fifth instar larvae of a Multivoltine (L x CSR2) breed and a Bivoltine breed (CSR6 x CSR12) of the mulberry silkworm *Bombyx mori*.

S. No.	Days	Number of Oenocytoids (Mean % ± S.D)		‘t’ test value (p < 0.05)
		Multivoltine breed (LxCSR2)	Bivoltine breed (CSR6 x CSR12)	
1.	Day 1	1.2±0.1	1.4±0.29	-1.45 Not significant
2.	Day 2	2.2±0.56 (83.3%)	1.2±0.1 (-14.2%)	3.98 Significant
3.	Day 3	2.4±0.32 (9.09%)	2.4±0.25 (100%)	0.10 Not significant
4.	Day 4	3±1.58 (25%)	2.8±0.07 (16.6%)	0.28 Not significant
5.	Day 5	3.2±1.92 (6.6%)	1.4±0.54(-50%)	2.11 Significant

Values inside the parentheses indicate the percentage of increase in oenocytoids over the previous day.

Table 5. Number of Spherule cells of the fifth instar larvae of a Multivoltine (L x CSR2) breed and a Bivoltine breed (CSR6 x CSR12) of the mulberry silkworm *Bombyx mori*.

S.No	Days	Number of Spherule cells (Mean%±S.D)		‘t’ test value (p < 0.05)
		Multivoltine breed (LxCSR2)	Bivoltine breed (CSR6 x CSR12)	
1.	Day 1	5±1.58	6±1.58	-1 Not Significant
2.	Day 2	7.8±1.55 (56%)	8.8±1.61 (46.6%)	-1.01 Not Significant
3.	Day 3	20.4±1.69 (161.5%)	27±1.58 (206.8%)	-6.3 Significant
4.	Day 4	24.8±1.59 (21.5%)	39.2±1.73 (45.1%)	-13.6 Significant
5.	Day 5	49.8±1.48 (100.80%)	53.8±1.52 (37.2%)	-4.2 Significant

Values inside the parentheses indicate the percentage of increase in spherule cells over the previous day.

Table 6. Size of the haemocytes of the fifth instar larvae of a Multivoltine (L x CSR2) breed and a Bivoltine breed (CSR6 x CSR12) of the mulberry silkworm *Bombyx mori*.

S.No.	Types of haemocytes	Multivoltine breed (L x CSR2)	Bivoltine breed (CSR6 x CSR12)
		Length (μm)	Length (μm)
1.	Prohaemocyte	4-11	3-9
2.	Granulocytes	7-19	8-15
3.	Oenocytoids	5-12	6-13
4.	Spherule cells	12-22	10-21

CONCLUSION

In both multivoltine and bivoltine breeds, THC was found to be gradually increasing from the first day to the last day of the fifth instar. Multivoltine breed was found to have greater THC than the bivoltine breed on all days of the fifth instar larvae. The higher THC of a multivoltine breed and lower THC of the bivoltine breed must be their specific characters. These breed specific characters might have been the adaptations to the environmental conditions or seasonal variations. Only four types of haemocytes viz, prohaemocytes, granulocytes, spherulocytes and oenocytoides were observed in the haemolymph of fifth instar larvae of both breeds. The differences in the THC and DHC of bivoltine breed and multivoltine breed might be due to genetic characteristics inherited for their parental stocks and so THC and DHC values can be used as an index to develop a breed with higher survival rate.

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