Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2582-2845.8747

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2021) *9*(4), 43-49

Indian Journal of Pure & Applied Biosciences

Peer-Reviewed, Refereed, Open Access Journal

Research Article

Haemocytes during Different Stages of Lifecycle in *Bombyx mori* (L.)

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Received: 4.06.2021 | Revised: 12.07.2021 | Accepted: 17.07.2021

ABSTRACT

Haemocytes in insects mediates the cellular responses like phagocytosis, encapsulation and clotting which signifies the immunological functions of any insect. In the domesticated variety of silkworm B.mori five types of haemocytes have been identified in all the stages. The most abundant cell was found to be plasmatocytes followed by Granular cells. Spherule cells and Oenocytoids were found to less during IV and V instars and least or absent in all other stages. Haemocytes found be to fluctuating before and after spinning. Plasmatocytes and granular cells decreased gradually with respect to number of days in pupa. The total haemocytic count increases gradually in the silkworm larval stages and found to be maximum at the last instar and least during adult stages where the role of haemocytes is not required because they die after laying eggs.

Keywords: *Haemocytes, Mulberry, Silkworm, Bombyx mori, Plasmatocytes, Granular cells.*

INTRODUCTION

Haemocytes are several types of cells which circulates within the haemolymph (Kerenap et al., 2005): in most of the insects they are well defined as Prohaemocytes, Plasmatocytes and Granulocytes and one more other types present in some other insects as coagulocytes Spherulocytes, Adipocytes and Oenocytoids (Nittono, 1960). Haemocytes are responsible for the cellular defense mechanism in the insect's immune system (Gupta & Sutherland, & Ribeiro & Brehlin, 2006) as a role to fight against the pathogens involving various physical chemical means were studied in arthropods (Ratcliffe et al., 1976 & Mead, 1986). Most of the haemocytes rest on the surface of various organs of the haemocoel and some cells circulate freely in the haemolymph. The number of cells varies greatly in the developmental as well as during different physiological stages (Wigglesworth, 1973) in the same species and total haemocyte count found to be more in larval stages than nymphal and adult (Webley, 1951).

Cite this article: Arumugavel, S., Selvarani, J., Babila Jasmine, J., Annalakshmi, T., & Xavier Innocent, B. (2021). Haemocytes during Different Stages of Lifecycle in *Bombyx mori* (L.), *Ind. J. Pure App. Biosci.* *9*(4), 43-49. doi: http://dx.doi.org/10.18782/2582-2845.8747

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Tauber and Yeager (1936) studied the haemocytes of Orthoptera, Odonata, Hemiptera, Neuropetra, Coeloptera, Lepidotera and Hymenoptera. It was highly variable among holometabolous and hemimetabolous insects. In hemimetabolous insects nymphs showed lower haemocytic count than adult (Webley, 1951), whereas in holometabolous insects larval forms showed more total haemocyte counts than adults (Han & Gupta, 1951). Similarly physiological conditions like oviposition and ecdysis also associated with excessive total count (Bahadur & Pathak, 1971). Jones, (1982) also showed data on varying differential haemocytic count during different developmental stages. Silkworm *Bombyx mori* is a very sensitive lepidopteran and are susceptible to various diseases. To explore their immune mechanism, it is necessary to learn the total haemocytic count and differential haemocytes related to their different developmental stages.

MATERIALS AND METHODS

Experimental Animal collection area and Rearing of Silkworm:

The model organisms selected for this study is the mulberry silkworm, *Bombyx mori* belonging to the order Lepidoptera, Class Insecta and Family Bombicidae .The race Chosen was a hybrid of LXNB4D2 (Local Multivoltine variety, NB4D2) new bivoltine with oval white cocoon which feeds, mainly on mulberry leaves. It is a holometabolous insect, whose life cycle has four distinct stages namely the egg, larva (5 instars), pupa and adult.

Rearing of silkworm

Eggs were collected form the Sericulture Centre of V.M. Chathram, Tirunelveli. The larvae were reared by tray rearing method and maintained at required temperature (27±10ºC) and (80±20) humidity. MR_2 variety of mulberry was used to feed the worms, fresh healthy leaves were collected during the morning fresh healthy leaves were collected during cool hours of the day and stored in wet gunny bags. The leaves were chopped and fed to the early larval instars. The larvae were fed 5 times a day. The beds were cleaned every day and sufficient spacing was adapted during rearing (Krishnaswami et al., 1970).

THC (Total Haemocytes Count)

The *B. mori* prolegs were cut to collect the haemolymph in larva and adults and in pupa a small incision made at the abdominal region. The haemolymph was immediately diluted with 0.8% stained saline solution upto mark of 1 ml and shaken well. The counting of the free haemocytes, was done by using haemocytometer with improved double Naubauer ruling. The mixed fluid was drawn into the chamber and the cells were counted under the light microscope. This was repeated for atleast 5 times/worm. SPSS 16 version used for calculation of ANOVA.

DHC (Differential Haemocytic Count)

The haemolymph was drawn on a glass slide which was already washed with alcohol chocked cotton to remove impurities and a drop of 20% formalin was placed on the slide already. A rectangular thin cover glass was taken and a thin blood smear was prepared. Then 25% Giema stain (prepared in methanol or 70% alcohol) was allowed to spread on the smear and air dried for 2 minutes. Then it was gently washed by adding distilled water and dried. Thus the prepared slide was then observed under high power (45X) light microscope. Totally 100 cells were counted from each and five insects as prohaemocytes, plasmatocytes, oenocytoids, adipohaemocytes, spherical cells and granular cells.

RESULTS

Table.1 shows the total haemocytic count (THC) during the larval stages in *B.mori*. The One way ANOVA on THC shows the difference occurring among the different stages and the statistical difference (different groups were represented in different alphabets) was noted on the last days of 3^{rd}, 4^{th} and 5^{th} instar larval stages. Where II and III larval instars total haemocytic count was between 3612 ± 465 to 4680 ± 352 cells/mm^3. During the IV instar and drastic increase was observed as 7080±356 (day 1) to 13272 ± 418 cells/mm^3 (day 5) and after moulting THC decreased to

9440± 180 cells/mm^3 during the first day of V instar, but a rapid increase was observed during the 2nd day of V instar larva. A peak of 349376± 483 and 352504 ± 424 cells/mm^3 were observed during 6th and 7th day of V instar and on 8th day reduction in the THC were observed (226728±532 cells/mm^3). The ripen larva before spinning larva which stopped eating showed a decreased count (19448±800 cells/mm^3) compared to the feeding larva. And after spinning THC was reduced to 7016±545 cells/mm^3. Similarly, pupa showed a lesser amount of haemocytic count compared to the larval stages. The adult stage showed least haemocytic count as 1152±113 cells/mm^3 before mating in females and after mating it was 521±30 cells/mm^3.

Table 2 shows the percentage of differential haemocytic counts of *B.mori* from II instar to V instar larva. The haemocytes observed are Prohaemocytes, Plasmatocytes, Granular cells, Spherule cells and Oenocytoids. Count of prohaemocytes fluctuated from 1st instar to 5th from 39.2±23.28 to 77.8 ± 5.62) and it was followed by plasmatocytes, granular cells and others. Prohaemocytes dominated the count over the other cells present in the haemolymph of *B.mori* and it was followed by plasmatocytes and granular cells. Spherical cells and Oenocytes were found to be the least. ANOVA analysis also shows a statistical difference among the prohaemocytes, plasmatocytes, granular cells followed by spherule cells and oenocytoids. The prohaemocytes are more in IV instars compared to the other cells. The granular cells decreased with reference to the number of days in IV instar. The plasmatocyte count was found to remain constant throughout this stage. In contrast the V instar larva showed a decrease in prohaemocytes from day 1 to day 8. Whereas, the plasmatocytes and granulocytes increased throughout the V instar. The granulocytes were more during this stage as well as the other stages of *B.mori* and these cells were followed by spherical cells and oenocytoids.

Table 3 shows the haemocytic count before and after spinning which was the last larval stage and beginning of pupa. The prohaemocytic count was less before spinning (7±1.32) but increases after spinning (53±2.4). A vice versa increase on plasmatocytes and decrease of granulocytes and a decrease in plasmatocytes and decrease in granulocytes was noted before and after spinning. The differential haemocytic populations during pupal stages are shown in Table 4. The prohaemocytes and plasmatocytes remained constant (no statistical difference among days were noted) but the granular cells decreased drastically from day 1 to day8 and the difference was statistically significant. Spherule and oenocytoids remained constant throughout this stage

The female adult haemocytic population is given in Table 5. The prohaemocytic and plasmatocytes count decreased after mating, but the granular (34.4±1.36cells/mm^2) and spherule cells (3.6±0.94 cells/mm^2) increased after mating. Here in adult when the prohaemocytes increased the granulocytes were decreased.

DISCUSSION

Silkworm being a holometabolous insect, the rate of THC reaches its peak during the larval developmental stages and it falls to the minimum during the adult stage. During the larval IV and V instars the drastic increase in THC may be related with food intake. Because, it is apparent that THC decreases after the worm went for molting which is the non-feeding period. The decrease in THC at each molt was reported by Wigglesworth (1995), Jones (1962) and Wheeler (1963) they reasoned it that in *Periplanata americana* the decrease in THC was due to increase in the blood volume during molting. Increase in THC on *Oncopelitus fasciatus* after ecdysis was reported by Fier and O'Conner, (1969). In *Anagasta Sp* also Nittono (1960) observed that the THC was higher in larvae than the other developmental stages.

It was reported by Wago and Ichikawa (1979) that gradual increase of THC from I to the III instar and a remarkable increase from IV to the V instar larvae of

B.mori. And in this study also the larva of *B.mori* shows high THC and a least count during the adult stage. May be the animals, most of the developmental process takes place during the larval stages and the adults are involved only in laying eggs. A similar drop in THC during the adult emergence was reported in *Glossina morsitanus* (Kaaya & Otieno, 1981). Major developments like silk gland development and enormous increase in size of the animal takes places during the V instar larval stages, and are most susceptible for the diseases to occur through pathogens via feed. It is the longest stage in this animal lifetime. In order to combat the pathogens larva may synthesis more haemocytes than in other stages, since haemocytes role in the survival of the insect species is well known (Siddiqui & AL-khalifa, 2014).

The THC may be associated with the non-feeding period like pupa, where the THC decline in this stage. The drop in plasmatocytes population after molting was also reported by Hrdy (1959) in *Acheta domesticus L.*, by Jones (1967) in *G. mellonella*. Here in this study the *B. mori* larval plasmatocytes increases before molting and it drops down after molting, whereas the prohaemocytes increased after molting.

Heamocytes plays an important role in the survival of insect species and are of economic and medical importance (Siddiqui & Kalifa, 2014). The plasmatocytes and prohaemocytes found to be predominant in all the larval stages of *B.mori* during active feeding larval period. Granulocytes percentage was more during the final instar. The decrease during pupal stages shows their less important part in larval to pupal metamorphosis. The role of granulocytes is the attraction of plasmatocytes (Riberio & Brehélin, 2006), hence when their population decreases the plasmatocytes also shows a decline phase in *B.mori*. Shapiro et al. (1969) found that spherule cell to be decreased during pupal stage when compared to larval stage and according to Jones (1967) no spherule cells found during pupal stage and during non-feeding period, but in this study during the pupal stage especially at 1st and 7th day spherule cells found to be more compared to all other stages. No spherule cells were observed during the adult stages before mating but they increased after mating may be the spherule cells have roles to be accomplished, but the roles of spherule cells are yet to be known. After mating it was noted that prohaemocytes and plasmatocytes decreasing and increase in granular cell population, may be conversion of the prohaemocytes into granular cells may take place in case of egg laying adults.

Table 1: The total Haemocytic count during the different stages in *Bombyx mori*

Instar	days	Cells/mm^3
2th instar	1	4072±604^{b}
	2	4392±505 ^{b}
	3	4020±465 ^{b}
3th instar	1	3612±465 ^{b}
	2	4200±271 ^{b}
	3	4680±352 ^{b}
	4	4392±504 ^{b}
4th instar	1	7080±356^{c}
	2	6888±408 ^{c}
	3	6352±813 ^{c}
	4	9392±214 ^{c*}
	5	13272±418 ^{c*}
5th instar	1	9440±180^{e}
	2	26768±626 ^{e}
	3	32064±504 ^{e}
	4	38744±211 ^{e}
	5	45136±995 ^{e}
	6	349376±483 ^{e*}
	7	352504±424 ^{e*}
	8	226728±532 ^{e*}
Before spinning		19448±800^{d}
After spinning		7016±544^{c*}

Pupa	1	13752±873^{c}
	2	13709±815 ^{c}
	3	13072±779 ^{c}
	4	7088±254 ^{c*}
	5	4040±354^{c*}
	6	1864±124 ^{c*}
	7	1852±672 ^{c*}
	8	1824±164 ^{c*}
Adult Females	before mating	1152±113 ^{b}
	After mating	521±30 ^{a*}

The mean of 5±S.D. letters above each level of the independent variable to show which groups are statistically different from one another according to Duncan's multiple range at p=0.05. *significant difference among the same group.

Table 2: Differential haemocytic counts during the larval stages in *Bombyx mori*

Instar	Days	Types of cells				
		Prohaemocyte	Plasmatocyte	Granular cells	Spherule cells	Oenocytoids
2th instar	1	45.40±2.2^{d}	53.60±2.40^{c}	5.60±1.60^{b*}	1.60±2.07^{a}	0.2±0.20^{a}
	2	63.6±3.41^{d*}	57.80±4.96^{c}	12.6±2.11^{b}	3.2±0.86^{a}	0.4±0.24^{a}
	3	59.4±0.00^{d}	54.8±2.51^{c}	11.0±1.39^{b}	0.8±0.37^{a}	0.4±0.24^{a}
3th instar	1	40.0±2.51^{d}	37.6±3.82 ^{c}	7.4±1.32 ^{b}	0.6±0.40^{a}	0.4±0.24^{a}
	2	40.0±3.82^{d}	31.4±2.76 ^{c}	13.4±2.24^{b}	2.2±0.64^{a}	1.2±0.58^{a}
	3	65.2±2.61^{d*}	31.4±2.76 ^{c}	13.4±2.76^{b}	0.0±0.00 ^{a}	0.00±0.00 ^{a}
	4	62.0±2.94^{d*}	40.0±2.02 ^{c}	2.0±0.63^{b*}	0.0±0.00 ^{a}	0.00±0.00 ^{a}
4th instar	1	64.2±3.67^{d*}	34.0±2.76 ^{c}	14.0±1.87^{b}	0.4±0.24 ^{a}	0.2±0.24 ^{a}
	2	54.8±3.76^{d}	38.2±2.58^{c}	15.2±2.03^{b}	0.4±0.24 ^{a}	0.6±0.24 ^{a}
	3	77.8±5.16^{d*}	30.2±1.24^{c}	7.2±0.48^{b}	0.8±0.20^{a}	1.0±0.24^{a}
	4	61.8±2.65^{d*}	38.0±2.81^{c}	7.8±1.80^{b}	0.8±0.37^{a}	2.0±0.31^{a}
	5	60.0±2.62^{d}	39.0±2.62^{c}	6.80±1.06^{b}	2.0±0.31^{a}	1.30±0.58^{a}
5th instar	1	67.2±4.36^{d}	36.8±2.35^{c}	7.8±1.28^{b}	0.8±0.37	1.6±0.39^{a}
	2	24.4±2.46^{d*}	46.4±2.98^{c}	37.4±3.33^{b*}	0.8±0.37	1.2±0.58^{a}
	3	37.4±1.29^{d*}	33.0±1.87^{c}	6.4±1.03^{b}	1.2±0.37	1.6±0.51^{a}
	4	39.2±3.28^{d*}	37.8±1.88^{c}	33.0±1.52^{b*}	0.6±0.24	0.4±0.39^{a}
	5	44.4±3.95^{d}	42.4±2.48^{c}	28.0±3.02^{b*}	3.8±0.83	0.8±0.37^{a}
	6	50.8±2.51^{d}	48.8±1.91^{c}	6.0±1.13^{b}	0.6±0.24	0.4±0.24^{a}
	7	39.6±4.65^{d}	46.6±3.36^{c}	30.0±2.0^{b*}	1.6±0.51	0.2±0.19 ^{a}
	8	51.0±4.78 ^{d}	45.8±2.81 ^{c}	23.8±2.35^{b*}	0.8±0.37	0.4±0.24

The mean of 5±S.D. letters above each level of the independent variable to show which groups are statistically different from one another according to Duncan's multiple range at p=0.05. *significant difference among the same group.

Table 3: Differential haemocytic counts during prepupal stages of *B. mori.*

Cells	Before spinning	After spinning
Prohaemocytes	7.0±1.32 ^{c}	53.0±2.40 ^{c*}
Plasmatocytes	64.8±3.67^{d}	34.6±2.76 ^{d*}
Granular cells	29.8±3.02 ^{b}	1.4±0.58 ^{b*}
Spherule cells	1.2±0.37 ^{a}	0.8±0.20 ^{a}
Oenocytoids	0.4±0.24 ^{a}	0.1±0.31 ^{a}

The mean of 5±S.D. letters above each level of the independent variable to show which groups are statistically different from one another according to Duncan's multiple range at p=0.05. *significant difference among the same group.

Table 4: Differential haemocytic counts at pupal stages of *B .mori.*

Cells	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Prohaemocyte	43.4±1.63 [d]	45.4±3.48 [d]	48.8±3.12 [d]	56.6±2.44 [d]	49.6±3.42 [d]	46.0±3.33 [d]	43.6±2.37 [d]	51.0±1.37 [d]
Plasmatocyte	39.8±2.47 [c]	40.40±3.78 [c]	37.0±2.70 [c]	22.8±1.65 [c]	32.2±1.98 [c]	38.0±3.09 [c]	44.4±2.03 [c]	6.2±1.56 [c*]
Granular cells	23.4±1.4 [b]	24.8±1.95 [b]	13.4±1.5 [b]	13.2±1.65 [b]	10.2±1.8 [b*]	11.4±1.47 [b*]	8.2±1.65 [b*]	1.60±0.40 [b*]
Spherule cells	1.40±0.4 [a]	1.80±0.58 [a]	2.0±0.70 [a]	0.0±0.00 [a]	2.20±0.58 [a]	4.80±0.37 [a]	0.80±0.83 [a]	0.00±0.00 [a]
Oenocytoids	2.20±0.37 [a]	1.80±0.37 [a]	0.80±0.37 [a]	0.00±0.0 [a]	1.00±0.31 [a]	2.2±0.37 [a]	1.60±0.67 [a]	0.8±0.34 [a]

The mean of 5±S.D. letters above each level of the independent variable to show which groups are statistically different from one another according to Duncan's multiple range at p=0.05. *significant difference among the same group.

Table 5: Differential haemocytic counts of adult female *B.mori.*

Cells	Before Mating (cells/mm^3)	After Mating
Prohaemocytes	59.20±1.85 [d]	32.8±3.10 [d*]
Plasmatocytes	43.8±1.37 [c]	34.8±1.98 [c]
Granular cells	4.6±0.67 [b]	34.4±1.36 [b*]
Spherule cells	0.00±0.00 [a]	3.6±0.94 [a*]
Oenocytoids	0.00±0.00 [a]	0.20±0.20 [a]

The mean of 5±S.D. letters above each level of the independent variable to show which groups are statistically different from one another according to Duncan's multiple range at p=0.05. *significant difference among the same group.

CONCLUSION

In silkworm *B.mori* the THC and DHC number varies with different developmental stages in the single species of *B.mori*. The larval stages shows more haemocytes proves the importance of physiological changes happening in holometabolous insect *Bombyx mori*. Haemocytes studies should be carried out after egg laying in females so that their role of apoptosis can be known.

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