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Haemocytes and their total count in 5th instar larvae of Eri silkworm, *Samia cynthia ricini* Boisduval (Lepidoptera: Saturniidae) reared on two host plants (castor and tapioca)

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Abstract
Haemolymph is the circulatory fluid of various invertebrate animals that is functionally comparable to the blood of vertebrates that contain organic and inorganic components in addition to cellular element termed haemocytes. Present study revealed that five types of haemocytes in the haemolymph of eri silkworm *Samia cynthia ricini* were found *viz.,* prohaemocytes, plasmatocytes, granulocytes, oenocytes and spherulocytes. Morphological features say that prohaemocyte are small cells with large nucleus. Spherulocyte includes cytoplasm with vacuoles and are observed least in the haemolymph of eri silkworm. Plasmatocyte show polymorphism and have large nucleus and are among the most abundant types. Whereas granulocytes are seen to be compact cell with small nucleus. Oenocytes are small cell with elongated nucleus. In term of total haemocyte count, maximum haemocytes count was found to be (2156 cells mm^3) in 5th instar castor (*Ricinus communis*) fed larvae compared to tapioca (*Manihot esculenta*) fed larvae (1470 cells mm^3).

Keywords: haemocytes, granulocytes, oenocytes, plasmotocytes, prohaemocytes, *Samia cynthia ricini* boisduval, spherulocytes

Introduction

North-East region of India is one of the mega biodiversity centres with flourishing green forests and harbouring varied flora and fauna. Silk producing insects along with their host plants are endemic to this region (Unni *et al*., 2009) [38]. Their rearing is a traditional culture that is inherited from generation to generation among different tribal communities. The tribal women of this region are keeping this practised in existence as a source of extra income as well as to supplement in their diet. The silkworms are mainly reared for their silk, the quality of silk mainly depends on the quality of host plants for their growth, immunity and development. In certain area of Assam, the production of silk varies due to the mortality of the worm by different diseases causing. Therefore, it necessary to feed good quality plants to enhances the quality of silk as well as immunity of the silkworms (Hirayama *et al*., 2007) [11]. Among all the silkworms, Eri silkworm, *Samia cynthia ricini* (Boisduval) is a sericigenous insect and largely reared in North-eastern part of India, particularly in Assam (Sahu *et al*., 2006) [28]. They are polyphagous in nature as they feed on a variety of plants species. Castor and kesseru are considered as the major primary host plant whereas tapioca and gomari are secondary host plants for the silkworm. In the rural areas eri silkworm culture are mostly carried out in castor plants because of the abundant availability (Rao *et al*., 2005) [25]. In present study, castor and tapioca are selected as host plant for the rearing of the silkworm. Both of the host plants are belonged to the Euphorbiaceae family, where castor is a heat loving intermediate annual plant and tapioca is a biennial plant.

Insects have an efficient innate immune system to discriminate an invading pathogenic microorganism (Sheehan *et al*., 2020) [29]. These are mainly performed by special cell haemocytes in the hemolymph of silkworm (Strand, 2008) [34]. Based on the morphology and function silkworm haemocytes are divided into five types namely Granulocytes, Plasmatocytes, Oenocytoids, Prohaemocytes and Spherulocyte (Akai & Sato, 1973) [2]. They can be easily identified using light and fluorescent microscope following different staining methods (Gillespie *et al*., 1997) [9]. Their important role in silkworm is coagulation and phagocytosis as defence mechanism against foreign body (Tungjitwitwitayakul & Tatun, 2019) [37]. Apart from the silkworms, other insects' immune response is also depended on the number and types of haemocytes available (Russo *et al*., 2001) [27]. Insect doesn't have special adaptive immune system, they have to defend against foreign bodies via this small innate immune cell (Jiang *et al*., 2010; Tanaka & Yamakawa, 2011) [14, 36]. Their innate immunity is composed of humoral response which include the production of protein like PPO (Kanost *et al*., 2004; Jiang *et al*., 2010; Tanaka & Yamakawa, 2011) [15, 14, 36] and cellular response were performed by haemocytes like GR and PL (Lavine & Strand, 2002) [17]. Therefore, present study was done to characterised the types of haemocytes in *Samia cynthia ricini* Boisduval and determine the total haemocyte count in 5th instar larvae reared on two host plant *viz*., castor and tapioca.

Materials & Methods

Insect Rearing

Surface rearing technique and bunch hanging technique were used for eri silkworm rearing. After hatching from the egg, the larvae were reared separately on two host plants via castor and tapioca using surface rearing technique up to third instar larvae. In the later stages, larvae were fed with mature leaves using bunch rearing technique where 6-7 host plant leaves were tied together as a bunch and hanged pointing downward on a rope or stick. Proper hygiene and cleanliness were also maintained during its rearing to prevent the occurrence of any disease in larvae.

Length and weight measurement

Morphological characteristics (length and weight) of 5th instar eri silkworm larvae reared on two host plants were observed during the rearing period by using scale mark on a paper and weight with the help of digital electronic balance.

Haemocytes Observation

Healthy 5th instar larvae of eri silkworm feeding castor and tapioca were selected in the present study. For microscopic observation, haemolymphs were collected by puncturing the proleg of larvae with a sterilized blade; a few drops of haemolymph were placed quickly on one end of a clean slide. Haemolymph smears were immediately made, air dried, and stained using eosin and methylene blue and kept for about 15 minutes. After staining, the permanent slide is observed under light microscope to determine the haemocyte types of Eri silkworm, *Samia cynthia ricini* Boisduval based on their morphological features.

Determination of THC

Total haemocytes count was estimated by Neubauer haemocytometer using standard formula. First, the larvae were kept in hot water of 50^0 C for two minutes to fix the haemolymph. After fixing, the larvae are removed and dried using filter paper. Then by puncturing the proleg haemolymph were collected in a clean Eppendorf pipette. The haemolymph is sucked up to mark 0.5 of a white blood cell diluting pipette, then the tip was carefully wiped clean and immediately diluted in WBC diluting fluid up to 11 mark and shaken vigorously for several minutes. Then the sample was poured in clear counting slide and covered with special cover slip and observe under microscope. The picture shows that haemocytes are uniformly dispersed on square of the counting slide. Then the number of cells per mm^3 of haemolymph was calculated using formula suggested by (Jalani *et al*., 2008) [13].

Haemocytes in five 1mm2 squares x Dilution x depth factor of the chamber

$$\frac{\text{Haemocytes in five 1mm}^2\text{ squares x Dilution x depth factor of the chamber}}{\text{Number of squares counted}}$$

Where,

Dilution = 20 times

Depth factor of the chamber = 10 (constant)

Number of squares counted = 05

Result

Eri silkworm

Eri silkworm, *Samia cynthia ricini* Boisduval is a non-mulberry fully domesticated, multi volatile, polyphagous species and are mainly reared by the tribal communities of North-east India. The silk produced by the silkworm referred to as endi or errandi is highly attractive and lustrous. Their rearing in turn provides employment to thousands of people in rural areas. Proper rearing of the worms is an important factor for the production of good quality silk in the region as they are more susceptible to diseases caused by bacteria and fungi. Otherwise, the farmers face challenges in the harvest which in turn create hurdles in their economic sectors. The present study reported that silkworms have developed their own self defensive cells called haemocytes to fight against such challenges. Some morphological parameters of silkworm that are affected by the two different host plant species namely *Ricinus communis*, Linnaeus (Castor); *Manihot esculenta*, Crantz (Tapioca) have also been focussed during the study. Morphological parameters namely body length and weight are taken into consideration and it has been observed that larvae fed with castor leaves shows comparatively high length and weight parameter (7.4 cm & 11.2 g) compared to tapioca leaves (6.5cm & 9.3 g) (Table 1). Thus, we can say that appropriate food influences the quality of silkworm growth and health.

Table 1: Showing (mean ± SD) body length and weight in 5th instar larvae of *Samia cynthia ricini* fed on castor and tapioca host plant.

Food Plants	Body Length (CM) Mean±SD	Body Weight (GM) Mean±SD
CASTOR	7.4±0.23	11.2±0.71
TAPIOCA	6.5±0.31	9.3±0.66
Values are mean ± SD (n=10 samples in each group).		

Haemocyte types

The haemolymph of silkworm feeding on two host plants castor and tapioca contained five types of haemocytes namely, PL, PR, GR, OE and SP (Table 2). The primary method to identify and classify insect haemocytes is according to haemocytes morphological features. Present study reveals that PR are small, round, oval, spherical cells having small cytoplasmic area with large nucleus (Figure 1a). SP are oval in shaped having small nucleus, vacuoles within cytoplasm (Figure 1d) and are observed least in the haemolymph of silkworm. PL are variable in shape (polymorphic) and are among the most abundant type of haemocyte in eri silkworm. PL are ovoid or fusiform or pear shaped with large nucleus and having cytoplasmic expansions (pseudopodia) (Figure 1b), whereas GR are round, ovoid, compact cells containing small nucleus (Figure 1c). OE are spherical or oval in shape with granulated cytoplasm and small elongated nucleus (Figure 1e).

Table 2: Morphological properties of haemocytes in 5th instar larvae of *Samia cynthia ricini viz*., PR, PL, GR, SP and OE.

Parameter	Types of haemocytes				
	PR	PL	GR	SP	OE
Cellular Shape	Small round, Oval	Polymorphic, round, fusiform, irregular	Round, ovoid, compact cell	Oval or elliptica granules are present	Oval or spherical cell
Nucleus Shape	Large nuclei	Large nuclei	Smaller compared to PL	Small nucleus	Small nucleus, round and elongated
Nucleus position	Centrally located	Centrally located	Centrally, sometimes eccentrically located	Eccentrically located	Eccentrically located

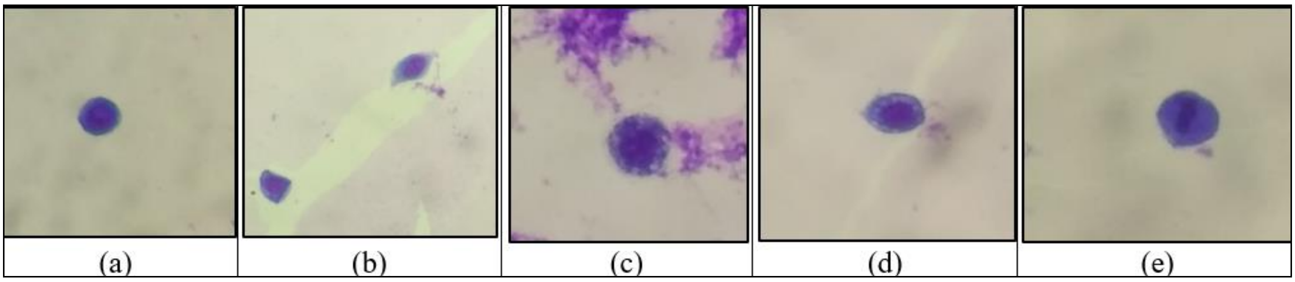

Fig 1: a) Prohaemocyte: small cytoplasmic area with large nucleus. b) Oval and triangular plasmatocyte. c) Granulocyte: compact cell. d) Spherulocyte: cell with granules. e) Oenocyte: with elongated nucleus.

THC

The overall THC in 5th instar larvae of eri silkworm, *Samia cynthia ricini* Boisduval is found to be higher in castor fed larvae (Figure 2). Present study also reported that the THC gradually decreases in the later stages of 5th instar. The observation made in the 1st day, 2nd day, 3rd day, 4th day and 5th day larvae of eri silkworm fed on castor leaves showed a higher amount of THC compared to larvae fed on Tapioca leaves (Table 3). The results of THC in castor fed 5th instar larvae are as follows: 1st day (2233 cells mm^3), 2nd day (2224 cells mm^3), 3rd day (2217 cells mm^3), 4th day (2098 cells mm^3), 5th day (2010 cells mm^3) whereas tapioca fed 5th instar larvae reveals results as such: 1st day larva (1534 cells mm^3), 2nd day (1495 cells mm^3), 3rd day (1471 cells mm^3), 4rd day (1438 cells mm^3), 5th day (1412 cells mm^3). The overall haemocyte counts are found to be 2156 cells mm in 5th instar castor fed larvae and 1470 cells mm^3 in 5th instar tapioca fed larvae.

Table 3: Showing THC in 5th instar larva of *Samia cynthiaricini* fed with two host plants Castor and Tapioca.

Host Plant	Haemocyte's count (no. per mm³)					
	1st day	2nd day	3rd day	4th day	5th day	Mean±SD (Overall)
Castor	2233±1.66	2224±1.37	2217±1.39	2098±0.94	2010±1.17	2156±98.68
Tapioca	1534±2.44	1495±1.42	1471±1.33	1438±1.59	1412±1.26	1470±47.72
Values are mean ± SD (n=10 samples in each group).						

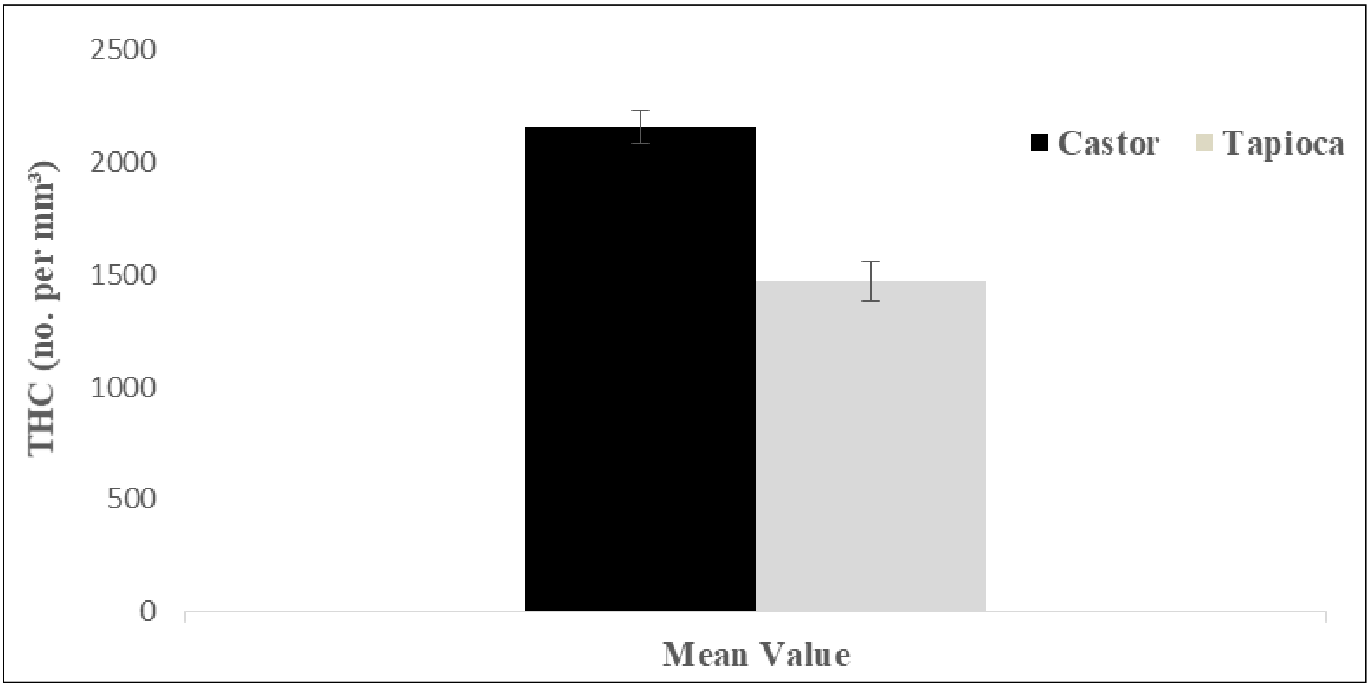

Fig 2: THC (Mean±SD) in 5th instar larva of *Samia cynthia ricini* feeding with two host plants Castor and Tapioca.

Discussion

Eri silkworm rearing with castor and tapioca host plants shows a marked difference in length-weight parameters and total haemocyte count. These may be due to the different nutritional content of host plants that directly influence the growth and development of the larvae. Similar result was reported by Murugan *et al*., (1998) [21] & Alipanah *et al*., (2020) [3] that dietary nutritional elements of host plant have a direct influence on the health of silkworm. Slansky & Scriber, (1985) [32] also demonstrated that suitable diet promoting growth effect on silkworm which directly enhance the qualities of silk. Castor is considered as the best primary host plant species for eri silkworm compared to tapioca. This result agrees with Nangia *et al*., (2000) [22] who reported that castor (*Ricinus communis* Linnaeus.) is main host plant followed by tapioca (*Manihot esculenta* Crantz.), kesseru (*Heteropanax fragrans* Seem.) and papaya (*Carica papaya* Linnaeus).

Morphological evidences show that haemolymph of eri silkworm contained five types of haemocytes *viz*., prohaemocytes, plasmatocytes, granulocytes, oenocytes and spherulocytes. Gupta & Han, (1988) [10] also used haemocyte morphological differences via histochemistry to identify and classify the insect haemocytes. Generally, haemocytes are classified based on morphology and function rather than other markers (Strand, 2008; Lavine & Strand, 2002; Ribeiro & Brehelin, 2006) [34, 17, 26]. Present study show that PL are found to be abundant in eri silkworm thus providing a wide range of immunity to the worm. Immunity like encapsulation and phagocytosis are performed by PR and PL reported by (Yamashita & Iwabuchi, 2001) [41]. Ashida & Brey, (1998) [5] reported that prophenoloxidase (PPO) is an important immunity protein and are present in OE of different insects. The polymorphic nature of PL is due to the different irregular shape. According to Das *et al*., (2017) [7] the irregular shaped of the PL is due to cytoplasmic extensions. Pseudopodia as a cytoplasmic expansion is a distinct characteristic of PL (Wu *et al*., 2016) [40]. Whereas spherical vacuoles in SP make it distinct from other haemocytes (Silva *et al*., 2002) [30]. Compact cell GR have acidophilic cytoplasmic inclusions reported by (Lawrence, 2008) [18]. Morphological features like; polymorphic nature of PL, PR as smallest cell, GR as compact cell, SP with vacuoles and OE with elongated nucleus was also reported by (Ozturk, *et al*., 2018) [23]. The size and other characteristics of each type of haemocyte in *Samia cynthia ricini* Boisduval were similar to the haemocytes of other insects such as (*Bombyx mori* Linnaeus), (*Phutella xylostella* Linnaeus), (*Hyphantria cunea* Drury), (*Ectomoyelois ceratoniae* Zeller), (*Calliptamus barbarus* Costa), (*Phlaeoba infumata* Brunner von Wattenwyl), (*Musca domestica* Linnaeus), (*Gryllus bimaculatus* De Geer) (Ghasemi *et al*., 2014; Tan *et al*., 2013; Huang *et al*., 2010; Khosravi *et al*., 2012; Ajamhassani *et al*., 2013; Zhang *et al*., 2021; Pal & Kumar, 2014; Sokolova *et al*., 2000) [8,35,12,16,1,43,24,33]. Insects do not have an acquired immune system but have a well-developed innate immune system (Lavine & Strand, 2002) [17]. The insect haemocyte is the main component of this innate system that directly clears pathogen from circulation (League & Hillyer, 2016) [19].

In the present study it was observed that THC progressively increased from day 1-5 in normal 5th instar larvae of eri silkworm. Decreasing in the number of haemocytes in the developmental stage is not only found in silkworm but also reported in other insects like (*Ephestia kuehniella* Zeller), (*Antheraea assamensis* Helfer) and (*Rynocoris marginatus* Febricius) (Ling *et al*., 2005; Bardoloi & Hazarika, 1995; Ambrose *et al*., 1999) [20, 6, 4]. The study results also shows that castor fed larvae have maximum THC compared to tapioca fed larvae. Castor leaves enable silkworm to rapidly produce more haemocytes (Vogelweith *et al*., 2016) [39]. Hence, larval diet is considered as one of the important factors that determine the abundance of immune cells which was experimentally proved by (Siva-Jothy & Thompson, 2002; Yang *et al*. 2008) [31, 42]. Silkworm larvae are more susceptible to various diseases caused by bacteria, protozoan, virus and fungus that directly influence the growth and the quality of silk produce by the silkworm. Feeding with proper di*et al*ong with proper care is vital in the cellular fitness of silkworms as it provides a defensive mechanism against diseases.

Conclusion

A total of five types of haemocytes were present in eri silkworm, *Samia cynthia ricini* Boisduval with different morphological features via PR, PL, GR, SP and OE. PL is responsible for cell ingestion (phagocytosis) as a defense mechanism to protect the insect's body from invasion particularly from pathogens. PL also represents the highest number of circulating haemocytes in the haemolymph of eri silkworm. The volume of haemolymph not only determines the immunity but also maintain the body size of silkworm. Also selected plant species play a significant role in enhancing the haemocyte count which is turn aid as a defense mechanism against pathogens. Thereafter in order to enhance the growth and immunity of silkworm and eliminate associate diseases it is important to select proper di*et al*ong with proper maintenance.

Abbreviations

PL: Plasmatocytes
PR: Prohaemocytes
GR: Granulocytes
SP: Spherulocytes
OE: Oenocytes
THC: Total Haemocytes Count
PPO: Prophenoloxidase

Competing Interests

"Not Applicable"

Funding

"Not Applicable"

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