RESEARCH

Investigation into the Effect of Altitude on the Differential Hemocyte Count of Circulating Plasmatocytes and Granulocytes of Larval Stage of *Antheraea assama* (Lepidoptera: Saturniidae)

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ABSTRACT. Differential hemocyte count of circulating plasmatocytes (PL) and granulocytes (GR) of fifth-instar larvae of Muga Silkworm *Antheraea assama* Westwood (Lepidoptera: Saturniidae) reared at four different sericulture farms situated at different altitudes, viz, Khanapara State Sericulture Farm, Assam, altitude 55.5 m above sea level (ASL); Nongpoh (Central Silk Board farm), Meghalaya, altitude 464 m ASL; Tura (Central Silk Board farm), West Garo Hills, Meghalaya, 657 m ASL; and Kalimpong (Central Silk Board farm), West Bengal, altitude 1,247 m ASL, were calculated and compared to investigate the effect of altitude on the number of PL and GR per mm^3 of larval hemolymph. The investigation showed that the mean circulating PL and GR were highest at Khanapara (55.5 m ASL) located at the lowest altitude, whereas their numbers gradually decreased with increase in altitude at Nongpoh (464 m ASL), Tura (657 m ASL), and Kalimpong (1,247 m ASL). This may be attributed to the average environmental temperatures observed at different altitudes, which may affect the overall hemocyte load of larval stages reared at those altitudes.

Key Words: *Antheraea assama*, hemolymph, hemocyte, plasmatocyte, granulocyte

The hemolymph of insects and other invertebrate groups have cellular inclusions called hemocytes. These hemocytes play important role in the physiology of the organism to which they belong, being responsible for coagulation of hemolymph (Gregoire 1955, 1957), connective tissue synthesis (Wigglesworth 1955, 1956, 1973), wound healing, self recognition, general and specific immune response and opsonization (Gupta 1986; Millar and Ratcliffe 1989; Xylander 1992, 1994, 2009), cellular immune reactions like phagocytosis and encapsulation (Salt 1970), melanization and discharging elements of the phenoloxidase system (Xylander 2009), and production and storage of the respiratory pigments in some arthropods (Xylander 2009). As such, it is evident that hemocytes and their numbers in the hemolymph (i.e., hemocytes per mm^3 of hemolymph) play a significant role in indicating the overall physiological condition of the insect.

However, hemocyte numbers in the hemolymph of any particular insect may vary depending on various factors, such as disease and meteorological factors, including altitudes and accompanying temperature variations. This study involves the fifth-instar larval stage of Muga Silkworm *Antheraea assama* Ww, a sericigenous insect native to the state of Assam, India, and is world famous for producing the golden-hued muga silk (Bardoloi and Hazarika 1992). Our investigation aims to ascertain whether the different altitudes with varying temp conditions have any effect on the numbers of circulating plasmatocytes (PL) and granulocytes (GR) of the larvae and, in turn on their immunity, as PLs and GRs are known to play important role in various cellular immune reactions. Further, changes in PLs and GRs would also affect the overall physiological conditions of the insect, which would ultimately have its impact on the yield of Muga silk production and quality.

Materials and Methods

Field investigations were carried out at different sericulture farms located at different altitudes during the months of April–May to realize the objective of the present investigation. These farms were randomly selected, which include the following:

- Khanapara State Sericulture Farm, Assam, situated at an altitude of 55.5 m above sea level (ASL); average temperature of the farm during the collection period was 32–34°C.
- Nongpoh (Central Silk Board farm), Meghalaya, altitude 464 m ASL; average temperature of the farm 27–29°C.
- Tura (Central Silk Board farm), West Garo Hills, Meghalaya, altitude 657 m ASL; average temperature of the farm 23–25°C.
- Kalimpong (Central Silk Board farm), West Bengal, altitude 1,247 m ASL; average temperature of the farm 18–21°C.

Insects. Fifth-instar larvae were directly collected from the four different sericulture farms as mentioned above, situated at different altitudes, and transported to the laboratory for conduction of the experiments.

Host Plant. Larvae of *A. assama* which were reared on Som plants (*Machilus bombycina* King) were considered for the experiments, as it is the most preferred primary host plant of the insect.

Measurement of Differential Hemocyte Count of Circulating PL and GR. For this experiment, total hemocyte count (THC) and differential hemocyte count (DHC) were performed on the same insect.

Quantitative estimation of hemocytes per cubic millimeter of hemolymph (THC) from healthy well-fed fifth-instar larvae (48-h post molt) were carried out as per the method of Hazarika and Gupta (1987), after fixation of whole insect in hot water at 56–60°C for 2–3 min (Rosenberger and Jones 1960). After heat fixation, the insects were removed and rapidly dried on a filter paper. A metathoracic proleg was severed at the tip, and first two to three drops of pale greenish blood were allowed to flow into a clean glass slide. A portion of the blood was quickly drawn to a 0.5 mark of a white blood cell diluting pipette, the tip was carefully wiped clean and the blood then diluted to the 11 mark
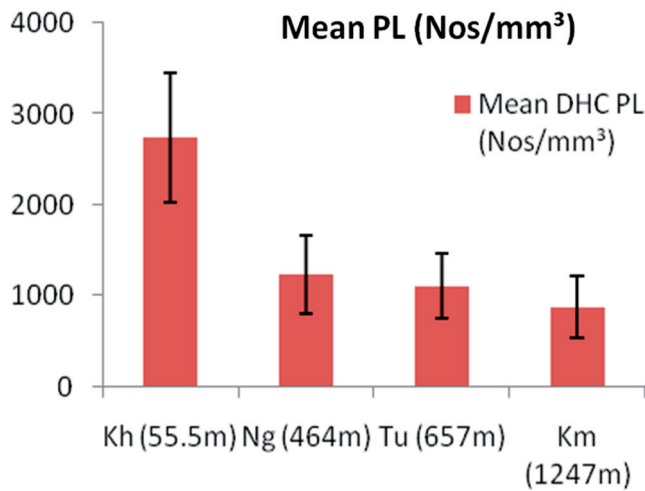
Fig. 1. Bar diagram showing comparison of mean circulating PLS at different altitudes.

Fig. 2. Bar diagram showing comparison of mean circulating GRs at different altitudes.

(i.e., 20 times dilution) with physiological saline (NaCl 0.9 g, KCl 0.041 g, CaCl$_2$ 0.048 g, NaHCO$_3$ 0.002 g, distilled water 100 ml) containing acetic acid (1%). The pipette was then shaken vigorously for several minutes; the first drop was discarded and a hemocytometer was filled. Using a levy double line hemocytometer with improved Neubauer ruling, cells were counted in the four corner squares, and total numbers were counted per cubic millimeter by the following formula:

$$\frac{\text{Hemocytes counted in} \times 1\,\text{mm squares} \times \text{dilution} \times \text{depth of chamber}}{\text{Number of 1 mm squares counted}}$$

For DHC of circulating PLs and GRs, hemolymph samples were taken from fifth-instar larvae, 48 h post-molt, since at this time the blood (hemolymph) usually carries a full complement of all the hemocyte types (Arnold and Hinks 1975). Hemolymph drops were obtained by severing the tip of one of the prolegs of the larvae. Unfixed hemolymph drops were directly collected on clean slides; smear was prepared and then air dried. Air-dried hemolymph smear was fixed in methanol and stained with Giemsa stain. The stained films were mounted in DPX. A minimum of 200 cells were classified per insect (fifth-instar larva) and were replicated in a minimum of three sets. The percentage of both prohemocytes (PR) and GR was calculated on the basis of the total number of all the hemocytes which had been obtained in a number of hemolymph smears (Mall and Gupta 1982). The percentage of PLs and GRs were then converted to circulating PLs and GRs from the THC, i.e., no./mm^3.

Statistical analysis of the data, i.e., one-way analysis of variance was performed using the statistical software OriginPro8.

Results

It is evident from the results plotted in Fig. 1 that the mean circulating PLs is highest at Khanapara (situated at the lowest altitude from the sea level), while its number is observed to be lowest at Kalimpong (situated at the highest altitude). However, another point evident from Fig. 1 is that although the mean circulating PLs obtained from Nongpoh, Tura, and Kalimpong are different in their values, these means are not significantly different from one another except the values between Khanapara and Kalimpong. Thus, it can be asserted that as the altitude increases from Khanapara to Kalimpong, there is a gradual decrease in the number of circulating PLs in the larvae with the highest and lowest altitudes showing significant differences.

Similarly, Fig. 2 shows that mean circulating GRs are found to be highest at Khanapara, whereas its number is lowest at Kalimpong. Mean circulating GRs at Nongpoh and Tura are not significantly
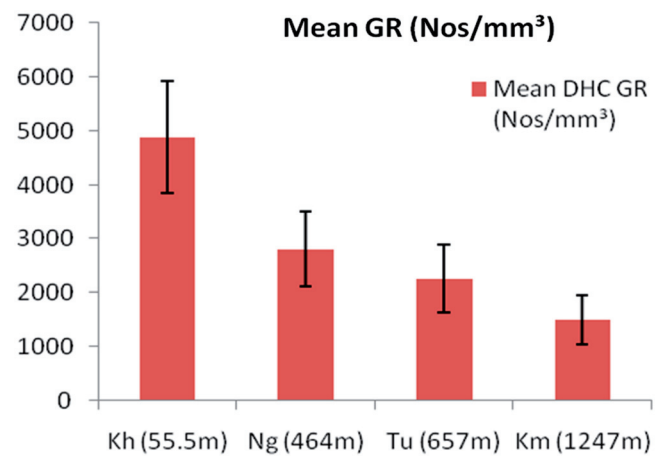
different from one another; however, their values are significantly different between Khanapara and Kalimpong. Thus, in this case also, the results have shown a similar trend; that is, with increase in altitude, circulating GR in the larvae have been found to gradually decrease in their numbers.

Discussion

DHC of circulating PLs and GRs of fifth-instar larvae collected from farms located at different altitudes, viz. Khanapara (55 m ASL), Nongpoh (464 m ASL), Tura (657 m ASL), and Kalimpong (1,247 m ASL) were observed to be significantly different from each other. Both circulating PLs and GRs were found to be highest at Khanapara and lowest at Kalimpong, i.e., with increase in altitude there is a gradual decrease in cell count. This may be attributed to the effect of low environmental temperature at high altitude and vice versa. It has been reported by various workers that temperature does have an effect on hemocyte count; low temperature treatment to insects decreases cell count, whereas at high temperature, increase in hemocyte count was observed (Tauber and Yeager 1935, Rosenberger and Jones 1960, Tiwari and Shukla 2000). The high hemocyte load at higher temperature may be attributed to loss of body fluid due to dessication. Another explanation suggests that at higher temperature, probably as a defense mechanism, hemocytes including PRs and GRs get detached from tissue surfaces and increase their rate of multiplication leading to higher hemocyte production so as to promote cellular defense to the silkworm larvae, which are supposed to be more prone to infections at higher temperatures (since microbial growth increases during hot and humid seasons) (Pandey et al. 2010). Similarly, declining hemocyte counts in lower temperature (higher altitudes) may be attributed to clumping of hemocytes due to chilling stress and thus making the hemocytes unavailable in circulating hemolymph (Pandey et al. 2010). This may be the reason for the observed lower hemocyte counts in higher altitudes with lower temperature regimes. Moreover, it has been reported in *A. mylitta* that PRs increase at high temperature, and since they have been reported to serve as stem cells by many workers (Gupta 1985, etc), these PR cells probably undergo mitotic divisions at higher temperature giving rise to other hemocyte types, including circulating PRs and GRs, as is evident from our study.

The GRs are also reported to carry nutrients under stress conditions as earlier reported by Arnold (1979). At higher altitudes, characteristic lower temperature affects the activity period of larvae significantly. Lower activity period means lesser foraging time leading to nutrient stress. Probably at such situations, GRs breakdown and release additional nutrients to compensate the deficiency. This may be the cause of lower observed number of GRs in higher altitudes.

GR in *A. assama* has previously been suggested to be associated in immunity, as a recognition factor (Bardoloi and Hazarika 1995), releasing substances upon coming in contact with foreign substances which subsequently attract PLs for phagocytosis (Ratcliffe and Rowley 1979). Thus, lower GRs in higher altitudinal broods results in reduced recognition factors, which probably fail to give rise to increased PLs in such broods. This can be supported by another study, which states that temperature influences foraging behavior in honeybee (Rajkhowa and Deka 2013); higher temperature having a positive relationship with foraging. Therefore, it can be assumed that since silkworms are cold blooded, their foraging activity diminishes at lower temperatures, which leads to nutrient deficiency which is in turn compensated by the disintegration of GRs to release nutrients into the hemolymph as discussed earlier, thereby decreasing circulating GR numbers.

The observed increased numbers of immunocytes (PLs and GRs) at lower altitudinal broods, when compared with those observed at higher altitudinal broods probably correspond to the growing demand for cellular immunity (Bardoloi and Hazarika 1995). In fact, as the effectiveness of phagocytosis, encapsulation, and other related defense mechanisms are primarily a result of the available circulating immunocytes (Gupta 1985, 1986), it is reasonable to suggest that larger the PL and GR population, stronger is the cellular defense (Bardoloi and Hazarika 1995). Therefore, from this study, it can be stated that silkworm broods or races reared at the plains are better equipped in terms of cellular immunity in comparison to their counterparts at higher altitudes.

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