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Haemocyte morphology and differential haemocyte counts of giant ladybird beetle, *Anisolemnia dilatata* (F.) (Coleoptera: Coccinellidae): a unique predator of bamboo woolly aphids

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Changes in haemolymph characteristics such as differential counts of haemocytes have direct bearing on the general performance of insects. The present study was carried out to generate data on the morphology of different haemocytes and their differential counts of giant ladybird predator, *Anisolemnia dilatata* (F.), unique to woolly aphid pests of bamboo habitat. Five types of haemocytes, viz. prohaemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytes were morphologically characterized in the haemolymph of larvae, pupae, virgin females and males. Among these, plasmatocytes were dominant followed by granulocytes, prohaemocytes, spherulocytes and oenocytes. Granulocytes showed consistency in numbers in all life cycle stages from first instar larva to adults of males and females of the giant ladybird.

Keywords: *Anisolemnia dilatata*, bamboo habitat, differential haemocyte count, giant coccinellid predator, woolly aphids.

HAEMOLYMPH of insects consists of fluid plasma and haemocytes. Numbers and sizes of circulating haemocytes vary in relation to age and life cycle stage of insects. Haemolymph constitutes 5–40% of the total body weight[1] and performs several functions such as metabolic, endocrine, reproductive, phagocytosis, encapsulation of parasites and pathogens, detoxification, immunological and in transport of essential materials between cells, tissues and organs[2,3]. Profound biochemical changes occur in the haemolymph during metamorphosis. Haemocyte count of insects is a good indicator of their physiological preparations during growth and adulthood[4] because pathogens are important factor of mortality in all developmental stages[5]. A majority of the existing studies on haemolymph are confined to the insects of families Lepidoptera, Orthoptera, Diptera, Hemiptera and Hymenoptera[6–9], and a few studies exist on coleopteran insects[5,10–13] including the only study on the beetle of Coccinellidae, *Coccinella septempunctata* L., which is a generalist predator of aphids[13]. The aim of this study was to document the

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differential counts of cellular population of haemolymph and physical characteristics of different kinds of haemocytes by light microscopic analysis in different life stages of giant ladybird predator *Anisolemnia dilatata* (F.) to augment data on this beetle species adapted to specialized insect food [woolly aphids (Homoptera: Aphididae)] in bamboo habitats of South East and Far East Asia[14].

Males and females of the *A. dilatata*, collected from the forest at Ishanchandranagar (23°45.669′N and 91°15.967′E, 34 m amsl) near Tripura University Campus, were used to establish the stock culture in an environmental chamber maintained at 22 ± 1°C temperature, 65 ± 2% RH and 16 : 8 h L : D photoperiod. Ten pairs of adult beetles were kept in a ventilated plastic container (10 cm width × 15 cm height), and these were offered live aphids of *Ceratovacuna silvestrii* (Takahashi) collected from infested bamboo plants. Beetles were sexed by their copulation behaviour and confirmed following the morphological characters described by Majerus[15]. Beetles readily ate woolly aphids in laboratory, and females laid batches of eggs on corrugated papers[16] kept in the container. Eggs from these females were initially transferred to 9 cm diameter paired petri dishes (one egg batch per dish) and these were separated into individuals by the tip of a fine '00' camel hair brush. On hatching, 30 larvae were kept singly in 5 cm diameter petri dishes lined on bottom with slightly moistened filter paper and reared to pupation on a surplus supply of aphid prey. Unfed aphids left at the end of 24 h intervals were replaced by the fresh ones when petri dishes were cleaned. Developing larvae were observed twice a day, at 11 am and at 5 pm, until pupation. Pupae were kept undisturbed in the respective petri dishes till the emergence of adults. Within 48 h of pupal eclosion, 10 adult females was paired with similar aged males collected from the stock culture, and each pair was placed in ventilated culture dishes (15 cm diameter × 1.5 cm height). Ten such dishes were maintained for two generations in order to acclimatize the beetles to the laboratory conditions (22 ± 1°C temperature, 65 ± 2% RH and 16 : 8 h L : D photoperiod). Progeny obtained from the second generation females was used in the experiments.

Twenty larvae, five each of first, second, third and fourth instars, five pupae, five adult females and five adult males of *A. dilatata*, all of 1-day-old approximately, were used from the stock culture (Figure 1). In cases of mobile larvae and adults, haemolymphs were collected in 10 µl capillary tubes by severing the legs of individual larva and adult but in cases of immobile pupae, it was done from the tips of abdomen by making incisions using a sharp entomological scissor. The resultant samples were transferred to clean slides for each life stages and on each sample a drop of phosphate buffer (pH 7.2) was added. Thereafter, a thin film was drawn with the edge of another slide, air dried for about 1–2 min, and fixed in absolute methanol for 6 min. Haemolymph film, thus prepared, was stained in 25% Giemsa (MERCK made) for 1 min followed by the addition of 2 drops of double distilled water on the slide, and kept for 5–8 min after which the slides were gently washed in distilled water and air dried at room temperature. Dried slides were dehydrated with absolute ethanol (1 min), cleared in xylene (6 min) and mounted in DPX medium using a round glass cover slip. This method was repeated five times for each of the four larval instars, pupal and adult stages. Mounted slides were studied under light microscope (Leica DM1000) for differential counts from 150 cells selected at random from the seven life cycle stages and computed by the formula: DHC = ∑ number of cells of each haemocyte type/total haemocytes counted. Diameters of cells and nuclei of different types of haemocytes were recorded to the nearest 0.01 µm using the microscopy software LEICA application suit, and ratios of nucleus diameter to cell diameter for each cell type ($n = 15$ for each haemocyte type) were determined. Photomicrographs of the stained haemocytes were taken using digital photographic facility (Leica DFC295 camera).

Data of different haemocyte counts recorded from the life cycle stages were subjected to one-way analysis of variance and their mean values were compared using Scheffe's multiple comparison test[17]. Sample variance (s^2) was used as a measure of scatter of distance of individual observations from the mean in the ratios of cell to nuclei diameters of each haemocyte type from life cycle stages. Incomplete or dividing haemocytes were excluded from the statistical analysis. A significance level of 0.05 was used to reject the null hypothesis. Data analyses were performed using Origin 7 software[18].

Microscopic images of haemolymph smear recorded from the seven life cycle stages of *A. dilatata* are presented in Figure 2. Studies revealed five morpho-types of haemocytes present in all the seven life cycle stages.

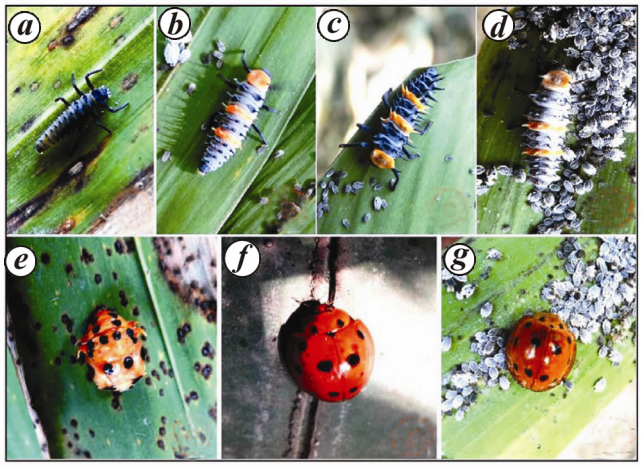

Figure 1. Life cycle stages of *Anisolemnia dilatata*. ***a***, First instar larva; ***b***, Second instar larva; ***c***, Third instar larva; ***d***, Fourth instar larva; ***e***, Pupa; ***f***, Adult female; ***g***, Adult male.

Table 1. Mean ± SE values (µm) of diameters of cells and nuclei of different haemocyte types (n = 15) including ratio and sample variance of nucleus to cell diameter recorded in the haemolymph of *A. dilatata*. Abbreviations of haemocyte types used in the table are the same as in the text

Haemocyte types	Cell diameter	Range	Nucleus diameter	Range	Ratio of nucleus to cell diameter	
					Mean ± SE	Variance (%)
PR	11.59 ± 0.37	9.2–13.4	6.26 ± 0.36	4.3–9.2	0.54 ± 0.02	0.0052 (0.96%)
PL	18.46 ± 1.24	10.2–26.7	7.92 ± 0.51	4.6–11.3	0.43 ± 0.01	0.0023 (0.53%)
GR	15.16 ± 0.45	12.8–18.5	7.03 ± 0.27	4.8–8.9	0.47 ± 0.01	0.0033 (2.13%)
SP	21.59 ± 0.57	17.2–24.7	9.51 ± 0.20	8.3–11.2	0.45 ± 0.01	0.0022 (0.44%)
OE	26.21 ± 1.11	20.2–32.7	12.29 ± 0.39	9.3–14.1	0.47 ± 0.01	0.0029 (0.62%)

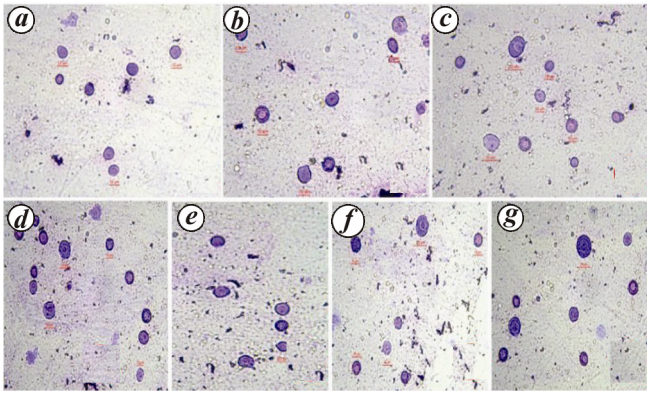

Figure 2. Microscopic images of haemolymph smear from different life cycle stages of *A. dilatata*: ***a***, First instar larva; ***b***, Second instar larva; ***c***, Third instar larva; ***d***, Fourth instar larva; ***e***, Pupa; ***f***, Adult female; ***g***, Adult male.

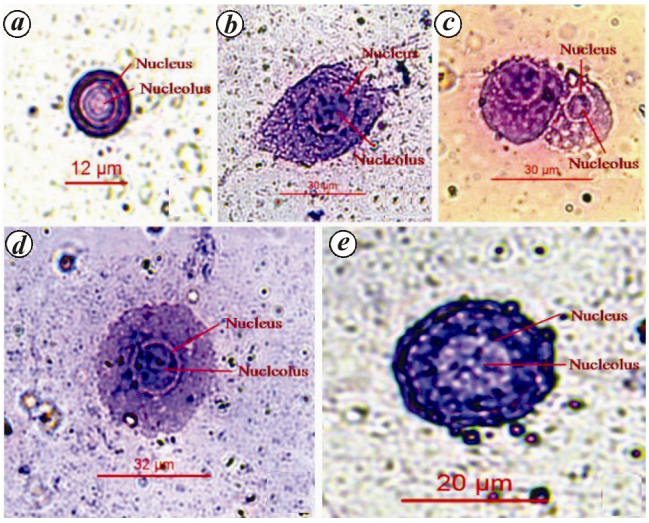

Figure 3. Haemocyte types recorded in larval, pupal, adult females and males of *A. dilatata*: ***a***, Prohaemocyte; ***b***, Plasmatocyte; ***c***, Granulocyte; ***d***, Spherulocyte; ***e***, Oenocyte.

These were identified as prohaemocytes (PR), plasmatocytes (PL), granulocytes (GR), spherulocytes (SP) and oenocytoids (OE) based on differences in diameters of nucleus, cell, shape of the cell, and intensity of staining (Table 1, Figure 3). In general, each type of haemocyte showed small variance in sizes of cells and nuclei and, more so, in the ratio of nucleus to cell diameter across different life cycle stages. Minimum and maximum variances from the means recorded as percentage were recorded in the ratios of SP (0.44%) and GR (2.13%) respectively (Table 1). Prohaemocytes (Figure 3 *a*) were smallest (mean ± SE = 11.59 ± 0.37 µm), round or oval in shape and each cell showed intensely stained central nucleus with a prominent nucleolus. Mean ± SE ratio of nucleus diameter to cell surface diameter of PR was recorded to be 0.54 ± 0.02. Plasmatocytes (Figure 3 *b*) were most variable in shapes and sizes. These were either round, oval or irregular in shape with a mean ± SE cell diameter of 18.46 ± 1.24 µm. These cells contained large euchromatin nucleus with prominent large spherical nucleolus and cell surface protrusions, and a mean ± SE nucleus to cell diameter ratio was 0.43 ± 0.01. Granulocytes (Figure 3 *c*) were round, oval or irregularly rounded cells with a mean ± SE diameter of 15.16 ± 0.45 µm and nucleus to cell diameter ratio was 0.47 ± 0.01. Spherulocytes (Figure 3 *d*) were rounded with a mean ± SE diameter of 21.59 ± 0.57 µm, nucleus contained deeply stained nucleolus, and mean ± SE nucleus to cell diameter ratio was 0.044 ± 0.01. Oenocytes (Figure 3 *e*) were larger than SP, roundish, with a mean ± SE diameter of 26.21 ± 1.11 µm, cytoplasm showed homogeneous granular appearance, strongly basophilic in nature, and mean ± SE nucleus to cell diameter ratio was 12.29 ± 0.39 (Table 1).

Data on differential counts recorded in larval, pupal and adult stages are provided in Figure 4. First and second instars larvae showed nearly similar pattern of differential counts and did not show significant difference for any of the five types of haemocytes between the two early stages of development. Third and fourth instars larvae, however, showed significantly smaller and higher numbers of PR and SP respectively, in comparison to those present in early instars larvae but did not show significant difference in the numbers of PL, GR and OE. Pupae showed higher number of PR in comparison to that of third and fourth instars larvae but the number and the relative abundance of SP were recorded to be highest in this stage of life cycle. Differential counts recorded in adult females

and males did not show difference between the two sexes and, interestingly, these were nearly similar to those recorded in the first and the second instars larvae (Figure 4). Among five types of haemocytes, plasmatocytes showed dominance followed by granulocytes, prohaemocytes, spherulocytes and oenocytes respectively, in that order, in all the life cycle stages.

The laboratory study revealed five morpho-types of circulating haemocytes, viz. prohaemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytes in all the post-embryonic stages of *A. dilatata*. Similar results were obtained by Suhail *et al.*[13] in the haemolymph of larvae of another ladybird beetle, *C. septempunctata*, but Giglio *et al.*[5] recorded four morpho-types of haemocytes in *Carabus* (*Chaetocarabus*) *lefebvrei* (Coleoptera: Carabidae). However, the classification of insect haemocytes is somewhat controversial; different workers[19–22] use different terminology to classify each cellular type. Haemocytes also change in number and morphology accompanying metamorphosis[22,23]. Among the studied haemocytes, GR and OE showed maximum nucleus to cell diameter ratio which possibly indicate the greater role of these haemocytes in defense mechanism of giant ladybird against parasites and pathogens which are largely unknown so far[24] compared to other haemocytes[25].

In this study, PL dominated the differential counts followed by GR, PR, SP and OE in that order. Higher counts of PL could be attributed to their being highly polymorphic, and ability to convert into other types of haemocytes, particularly into GR[26]. The same is also true of PR which acts as basic stem cell that gives rise to PL and subsequently other types of haemocytes[25]. Total number of GR showed no significant differences among the seven life cycle stages of *A. dilatata* presumably because each of the different life stages is exposed to similar risks of attacks from parasites and pathogens and, therefore, maintained a definite number of GR to counteract the attacks. Granulocytes are known for their role in primary defence response by encapsulation of parasites or pathogens[26]. Spherulocytes and OE were represented by minimum numbers in comparison to other haemocytes in the present study. Oenocytes are known to be involved in the production of prophenoloxidase enzyme which actively participates in the mechanism of humoral defence in insects[19,21] as well as in the lipid synthesis and their storage[25] depending on the insect's physiological requirements. Higher numbers of SP and OE in the pupal stage vindicate this hypothesis, because insect pupae are comparatively less vulnerable to parasites and pathogens and undergo active process of synthesis and biochemical transformation during metamorphosis. Recent study has revealed that the main function of SP is their involvement in the coagulation process and these cells may also be involved in lipid synthesis and energy storage[27].

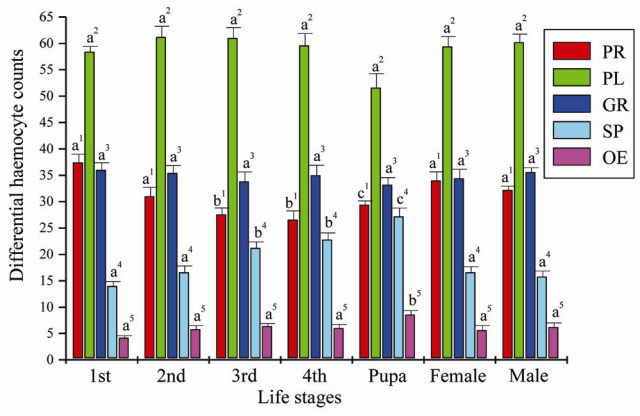

Figure 4. Bar diagram showing mean ± SE values of differential counts of different haemocytes in life cycle stages of *A. dilatata*. Similar alphanumerals accompanying bars of the same colour denote no difference in differential counts in respective life cycle stages by Schiffe's multiple comparison tests at 5% significance level.

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Comparative blood cell morphometry and differential leukocyte count of two breeds of turkey, *Meleagris gallopavo* (Linnaeus, 1758)

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Morphometry of erythrocytes and leukocytes and differential leukocyte count of two breeds of adult turkey (24 birds) were performed with respect to sexual dimorphism. Except nuclear length of erythrocytes, other parameters show highly significant difference at $P < 0.01$. Leukocytes reflected significant difference at $P < 0.01$ among and between breeds with respect to their dimensions. In case of DLC, except eosinophils, all leukocytes show significant difference ($P < 0.01$) among and between breeds. Morphometry of blood cells of two breeds of turkey is within the range mentioned for avian species, but the differential count revealed some abnormalities which might be due to stress or infection.

Keywords: Blood cell, differential leukocyte count, turkey, *Meleagris gallopavo*, morphometry.

CYTOMORPHOMETRY of blood cells, an important aspect of hematology, can reveal the physiological condition of organisms. In some birds, cytomorphometry of erythrocytes has only been reported[1–5]. The morphology and morphometry of both erythrocytes and leukocytes were earlier discussed in adult male ostrich[6]. But studies on nuclear morphometry of blood cells are inadequate in birds[7]. Measurements of both cellular and nuclear length and breadth of erythrocytes, lymphocytes and monocytes, cellular diameter of granulocytes and cellular dimensions of thrombocytes were earlier reported in different chickens[8]. But comparison of these parameters between those birds, especially with respect to breed and sex, is not reflected in their studies. In case of turkeys, data on blood cell morphometry are scanty. Many birds do not express clinical signs until late stages of the disease and the signs that they do exhibit may be subtle and non-specific[9] where DLC can be used as a valuable tool to determine the health status, genetic disease resistance, meat quality, stresses due to environment, nutritional, and pathological factors. The present study is an attempt to report breed-wise differences in morphometry of blood cells and DLC of turkey.

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